# Modification of Histidine Residues of Photosystem II by Diethyl Pyrocarbonate Inhibits the Electron Transfer between the Primary ($Q_A$) and Secondary ($Q_B$) Quinone Acceptors

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The effect of diethyl pyrocarbonate (DEPC) on the photosynthetic electron transport was investigated in isolated spinach thylakoids by partial electron transport rate and thermoluminescence measurements. Incubation of thylakoids at pH 6.5 with 5 mM DEPC for 15 min resulted in a considerable inhibition of electron transport from water to dichlorophenolindophenol. The inhibition was only partially releaved by addition of the donor, diphenylcarbazide indicating the effect of DEPC both on the donor and acceptor sides of PS II. In the thermoluminescence glow curve DEPC-treatment abolished the B band ($S_2Q_B^-$ radiative charge recombination) at 30 °C with a concomitant appearance of the Q band ($S_2Q_A^-$ charge recombination) at 10 °C. This suggests that in isolated thylakoids possessing an active water-splitting system DEPC affects the electron transfer from $Q_A$ to $Q_B$ but does not inhibit the electron transport from manganese to $Q_A$ during the $S_1 \rightarrow S_2$ transition of the water-splitting system. At the acceptor side of PS II the targets of DEPC are probably the histidines which are coordinated to the non-heme iron. Illumination of thylakoids at −80 °C following DEPC addition after two preflashes at 5 °C resulted in the replacement of the A($A_T$) thermoluminescence band at −30 °C with a band appearing at −15 °C. This observation can be explained by the effect of DEPC on a donor side histidine component participating in the generation of the A($A_T$) band. Consequently, in the interpretation of results obtained by DEPC treatment of PS II, both the donor and acceptor side effects of DEPC should be considered.

## Introduction

Several amino acid side chains of the D1 and D2 proteins or photosystem II (PS II) function as ligands toward transition metal ions or participate in oxidation-reduction redox reactions of the photosynthetic electron transport chain without additional cofactors. It has been demonstrated that histidine [1], carboxyl [2] and glutamate [3] residues provide ligands to the manganese atoms of the water-splitting system. Moreover, electron transfer between the oxygen evolving complex and the reaction center chlorophyll, P 680 is mediated by a tyrosine residue, $Y_Z$ (Tyr-161 of D1) [4–5]. In addition to $Y_Z$ there is a second redox-active tyrosine in PS II designated as $Y_D$ (Tyr-161 of D2) [6].

The D1 and D2 reaction center proteins of PS II contain ten and eight histidine residues, respectively [7]. Several histidine residues participate in the ligation of the four manganeses of the water-splitting system [1, 8–9]. Both $Y_Z$ and $Y_D$ are suggested to form a hydrogen bond with histidine 190 in the D1 and D2 protein, respectively [10]. Histidine 198 of both D1 and D2 are proposed to ligate P 680 [11]. Thermoluminescence (TL) [12–13] and EPR [14–15] observations support the earlier proposal [16–17] that a histidine residue also partici-

*Abbreviations:* D1 and D2, reaction center proteins of PS II; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; $DQH_2$, durohydroquinone (tetramethyl-p-hydroquinone); DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; MV, methyl viologen; P680, reaction center chlorophyll of PS II; $Q_A$, primary quinone acceptor of PS II; $Q_B$, secondary quinone acceptor of PS II; Q band, TL band associated with $S_2Q_A^-$ charge recombination; B band, TL band associated with $S_2Q_B^-$ charge recombination; PS II, photosystem II; $S_1$ and $S_2$, oxidation states of the water-splitting system; TL, thermoluminescence; TMPD, N,N,N′,N′-tetramethylphenylenediamine; $Y_D$, redox active tyrosine-161 of D2 protein; $Y_Z$, redox active tyrosine-161 of D1 protein.

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pates in physiological electron transfer reaction from the manganese cluster to P 680, either *via* the redox active $Y_Z$ or on an alternative pathway. It has been suggested that the $S_2$ to $S_3$ transition occurring in $Ca^{2+}$ depleted PS II corresponds to the oxidation of a histidine residue [14]. Light-induced formation of this histidine radical can also occur in functional PS II [18]. It has been also reported that the so-called $A_T$ TL band which appears at about −30 °C in the glow curve of manganese-depleted PS II particles is abolished by the histidine modifier, diethyl pyrocarbonate (DEPC) [12, 13] in a manner parallel with the loss of photoactivation capability [19]. It was suggested that a redox active histidine residue is mediating electrons from the manganese cluster to $Y_Z$, and the $A_T$ TL band is generated by charge recombination between this photooxidized histidine and the reduced primary quinone, $Q_A^-$ [12]. Recently Tamura *et al.* [1] found by DEPC treatment of wheat PS II particles that a histidine residue on the D1 protein is involved in binding Mn during photoactivation. Inhibition of manganese binding by DEPC in the high-affinity Mn-binding assay also suggests that histidine residues are involved in binding Mn functional in the $O_2$-evolving process [2, 9]. It might happen that the same putative electron-mediating histidine is providing a hydrogen bond to $Y_Z$ [10, 19] and is essential for photoligation of exogenous Mn (II) atoms during photoactivation [1, 2, 12, 19].

Histidine residues also participate in the structural arrangement of the acceptor side of PS II. By analogy with the bacterial reaction center [20] four histidines on helices IV and V of both the D1 and D2 subunits were suggested to be involved in binding of non-heme iron [7]. Histidine 214 of the D2 protein is involved in binding of $Q_A$ [21] and histidine 215 of the D1 protein is a ligand to $Q_B$ [22, 23]. Consequently, it should be considered in the interpretation of results obtained by DEPC treatment of thylakoids, that DEPC can modify not only the donor – but the acceptor – side histidines, as well.

The present study demonstrates that modification of histidine residues by DEPC treatment inhibits electron transfer not only on the donor side of PS II but also on the acceptor side, between $Q_A$ and $Q_B$. Contrary to the expectation, electron transfer between the manganese cluster and $Q_A$ during the $S_1$ to $S_2$ transition of the water-splitting complex is not inhibited by DEPC treatment in manganese-containing spinach thylakoids.

## Materials and Methods

Thylakoid membranes (broken chloroplasts) were isolated from 1–2 month old spinach grown in a green house as described earlier [24] and suspended in a medium containing 0.4 M sorbitol/10 mM NaCl/1 mM $MnCl_2$/5 mM $MgCl_2$/2 mM EDTA and 50 mM phosphate buffer of pH 6.5 unless otherwise mentioned. In order to modify the histidine residues of the D1 and D2 proteins of PS II freshly prepared thylakoids were incubated with different concentrations of the histidine modifier, DEPC (from Sigma) for different intervals of time at 20 °C. DEPC treatment was carried out at pH 6.5 which is optimal for modification of histidine residues. The reaction was stopped at a given time by addition of 20 mM histidine containing suspending medium, washed twice and resuspended in the suspending medium. Due care was exercised in not allowing ethanol concentration to exceed 0.025% in any of the measurements.

The rate of photosynthetic oxygen evolution and uptake was measured at saturating light intensity by using a Clark-type electrode in a temperature controlled cell at 25 °C. 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 thylakoids carrying 50 μg chlorophyll in a final volume of 3.0 ml. Different parts of the electron transport chain were studied by addition of electron acceptors and donors: 100 μM MV (PS I + PS II), 2 mM ascorbate/500 μM TMPD and 100 μM MV (PS I). The DCIP-Hill activity was measured with the help of an Aminco spectrophotometer used in the split-beam mode. The DCIP photoreduction was assayed by recording absorbance changes at 590 nm. In the assay, 40 μM DCIP (for water to DCIP) or 500 μM DPC and 40 μM DCIP (for DPC to DCIP) were added. The intensity of the illuminating red actinic light (Kombinat VEB NARVA, TGL 10619) was 300 W $m^{-2}$.

TL profiles were measured in an apparatus similar to that described by Tatake *et al.* [25] and the conditions of measurements have been described elsewhere [24]. No extra care was taken to avoid the distortion of TL bands at 0 °C during the ice-water transition.

## Results

The effect of the histidine modifier, DEPC on the photosynthetic electron transport chain was investigated by partial electron transport rate measurements. In agreement with earlier observations [1, 26] the extent of inhibition was dependent on the concentration and incubation time. Fig. 1 shows the inhibition of various partial electron transport reactions after 15 min incubation of isolated spinach thylakoid membranes with increasing concentrations of DEPC. While the whole chain electron transport from water to methylviologen was completely inhibited by 1 mM DEPC the photosystem I reaction from TMPD, which bypasses the oxidation site of plastohydroquinone [27], to methylviologen was little affected even by 5 mM DEPC. Thus PS II is more sensitive to DEPC than the PS I electron transport. Fig. 1 also shows that the inhibition of the $H_2O \rightarrow$ DCIP Hill reaction is considerably higher than that of the DPC→DCIP reaction. Since DPC is directly donating electrons to $Y_Z$ [28–29] this observation suggests that one of the action sites of DEPC is located between the manganese cluster and $Y_Z$. Addition of DPC to the DEPC treated thylakoids can only partially releave the inhibition of the water to DCIP reaction, indicating a DEPC-induced modification of the acceptor side of PS II before the acceptory site of DCIP. Another inhibitory site may exist between the plastoquinone pool and PS I as suggested by comparison of the inhibition of $DQH_2 \rightarrow$MV and TMPD→MV reactions (Fig. 1).

The TL technique proved to be a very sensitive method to follow small changes in the redox states of the Mn cluster and the primary ($Q_A$) and secondary ($Q_B$) quinone acceptors [30–32]. Fig. 2, curve A shows the TL glow curve of untreated spinach thylakoids excited at −80 °C. In the glow curve the so called B band originating from $S_2Q_B^-$ charge recombination [32, 33] appeared at around 30 °C. A small satellite band could also be observed at about −30 °C. This band probably corresponds to the A band [34] and is suggested to be

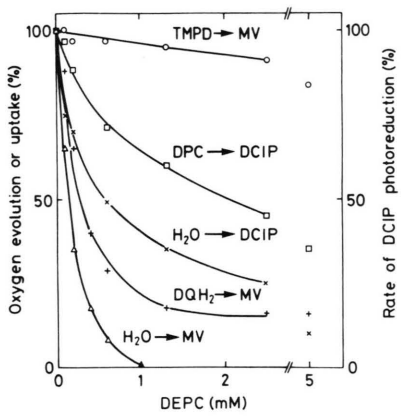


Fig. 1. Effect of DEPC on various partial electron transport reactions. The assay medium contained 0.1 M D-sorbitol, 10 mM $K_2HPO_4$, 20 mM NaCl, 4 mM $MgCl_2$, 2 mM EDTA, 50 mM HEPES, pH 7.5 and thylakoids carrying 50 μM chlorophyll in 3 ml. Concentrations of the acceptors and donors were: 100 μM MV, 500 μM TMPD, 40 μM DCIP, 500 μM DPC, 120 μM $DQH_2$. 100% rates in the $H_2O \rightarrow$MV, $DQH_2 \rightarrow$MV and TMPD→MV reactions were 125, 162 and 180 μmol $O_2$ consumed per mg chlorophyll per h, respectively. Control rates in the $H_2O \rightarrow$DCIP and DPC→DCIP photoreductions were 170 and 190 μmol of DCIP (mg of Chl)$^{-1}$ h$^{-1}$, respectively.

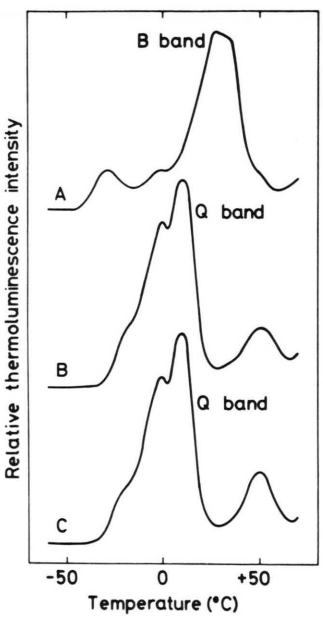


Fig. 2. Effect of DEPC on the thermoluminescence of spinach thylakoids. The samples contained 0.4 M sorbitol, 10 mM NaCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM phosphate buffer pH 6.5 and 50 μM chlorophyll. Thermoluminescence was excited at −80 °C for 1 min with white light of 50 W m$^{-2}$. (A) Control; (B) Incubated in the presence of 5 mM DEPC for 15 min; (C) Treated with 10 μM DCMU.

associated with $S_3Q_A^-$ charge recombination [35]. It is of note, that the $A_T$ band, which can be excited in manganese-depleted PS II particles, appears at the same temperature as the A band [35] and is assigned to charge recombination of an oxidized electron mediating histidine component ($His^+$) and $Q_A^-$ [12, 13]. At the descending side of the B band the appearance of a shoulder at about 50 °C indicated the presence of a small hidden C band [31, 36] under the envelope of the B band. The origin of the C band has not been clarified yet [31, 36]. We note that the distortion observable in the form of a shoulder or trough at 0 °C in all of the glow curves, is caused by the solid-liquid phase transition of water and should be neglected in the interpretation of results [37]. Incubation of thylakoids for 15 min in the presence of DEPC resulted in the loss of the B band with a concomitant appearance of a band at about 10 °C and also with the intensification of the band at 50 °C (Fig. 2, curve B). The same TL bands could also be observed in the glow curve of DCMU-treated thylakoids (Fig. 2, curve C). Therefore, the bands at +10 and 50 °C in the glow curve of DEPC-treated thylakoids can be considered as the Q and C bands, respectively, which are the characteristic TL bands in the glow curve of DCMU-treated chloroplasts [33, 38]. Thus, DEPC and DCMU have similar effects on the electron transport of thylakoids. Since the Q band in the glow curve of DCMU- and DEPC-treated thylakoids can be accounted for by the $S_2Q_A^-$ charge recombination, we suggest that DEPC, like DCMU, inhibits electron transfer between $Q_A$ and $Q_B$.

In agreement with the observation that the A band can not be charged in the presence of DCMU [35] both DCMU and DEPC abolished the $A(A_T)$ band at around −30 °C with a simultaneous appearance of a hidden band under the envelope of the Q band at about −15 °C (Fig. 2, curves B and C, respectively). While the band at −30 °C is probably associated with $S_3Q_A^-$ charge recombination [35] the origin of the band at −15 °C in the DCMU- and DEPC-treated thylakoids is not known.

Modification of the thylakoid membrane and inhibition of photosynthetic electron transport by DEPC treatment is a time dependent phenomenon as shown in Fig. 3. Correspondingly to the development of inhibition of electron transfer between

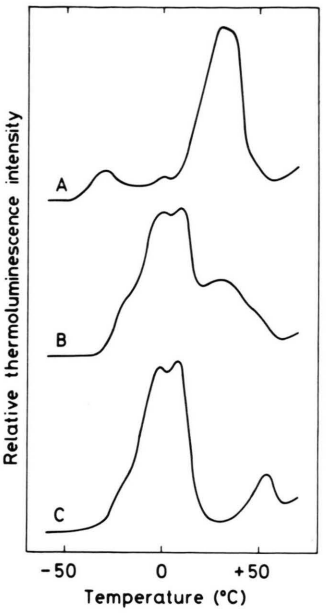


Fig. 3. Effect of DEPC on the thermoluminescence of spinach thylakoids as a function of incubation time. (A) Control; (B) Incubated for 5 min; (C) Incubated for 15 min. Incubation of thylakoids with 5 mM DEPC was carried out at room temperature in the dark. Other conditions as in Fig. 2.

$Q_A$ and $Q_B$ during DEPC treatment, the B band ($S_2Q_B^-$) is gradually abolished and replaced by the Q band ($S_2Q_A^-$). Under our experimental conditions, in the presence of 5 mM DEPC, complete establishment of the Q band and loss of the B band required incubation of 15 min (Fig. 3, curve C).

TL measurements were also carried out to determine the effect of DEPC treatment (histidine modification) on the $A(A_T)$ band which appears at −30 °C (Fig. 4, curve A). The $A(A_T)$ band was charged by two preflashes at 5 °C ($S_2 \rightarrow S_3$ transition) followed by quick DEPC treatment and cooling to −80 °C. At −80 °C an additional light excitation was applied. The two preflashes lifted the water-splitting system to the $S_3$ state, which is a requirement for the generation of the A band [35]. The low temperature illumination at −80 °C provided electrons for the $Q_A$ acceptor pool by including an additional electron transfer from the donor side to $Q_A$ (light-induced oxidation of an unidentified donor). Since the DEPC treatment was very short (not to allow the relaxation of the $S_3$ state after the two preflashes) the B band at 30 °C was

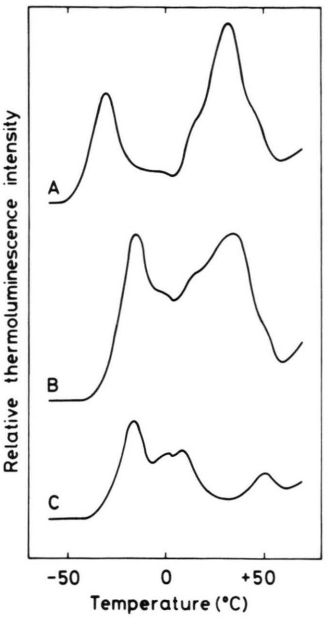


Fig. 4. Effect of DEPC on the thermoluminescence band appearing at −30 °C. Excitation of samples by two preflashes at +5 °C was followed either by the addition of 5 mM DEPC (B) or by 10 μM DCMU (C) and cooled quickly to −80 °C. At −80 °C the samples were illuminated with white light of 50 W $m^{-2}$ for 1 min. (A) Control; (B) Treated with 5 mM DEPC; (C) Treated with 10 μM DCMU.

only partially abolished in the glow curve (Fig. 4, curve B). This is understandable because complete DCMU-type effect can be achieved only by long-term DEPC incubation of thylakoids (Fig. 3, curve C). Treatment of the sample with DEPC after two preflashes and an additional illumination at −80 °C abolished the A($A_T$) band and a new band appeared at −15 °C (Fig. 4, curve B). The same phenomenon could be induced by using the same illumination procedure but replacing DEPC with DCMU (Fig. 4, curve C). The DEPC- or DCMU-induced replacement of the band at −30 °C with the band at −15 °C was also observed without preilluminating the sample by 2 flashes (see the shoulder at −15 °C in Fig. 2, curves B and C). However, this hidden band under the envelope of the Q band was less pronounced.

## Discussion

Diethyl pyrocarbonate (DEPC) is a specific reagent for the modification of histidine residues of proteins [39]. Since histidines provide binding sites for several electron transport components of PS II it can be expected that DEPC treatment influences the electron transport rate. However, Tamura *et al.* [1] observed only a very little effect of 500 μM DEPC on isolated PS II particles (TMF2) during 60 min incubation. They concluded that DEPC does not affect very much the intact manganese cluster in TMF2. In contrast, in manganese-depleted PS II particles DEPC severely affected the photoactivation capability of the preparation. The acceptor side effect of DEPC has not been considered.

In the present work, in agreement with the results of Singh *et al.* [26], the electron transport was considerably inhibited both at the donor and acceptor sides of PS II by incubating spinach thylakoids with 5 mM DEPC for 15 min.

The inhibition of electron transport at the donor side of PS II between the manganese cluster and the donation site of DPC (Fig. 1) can be explained by the DEPC-induced modification of the putative electron mediating histidine component which is bound to $Y_Z$ by a hydrogen bridge [10, 19] and participates in photoligation of exogenous manganese atoms during photoactivation [1, 2, 12, 19].

Our TL measurements demonstrate that DEPC also inhibits the electron transport at the acceptor side of PS II. The $Q_A$ binding histidine is not influenced by DEPC since the Q TL band ($S_2Q_A^-$) can be charged after DEPC treatment (Fig. 2, curve B). On the other hand, inhibition of electron transport between $Q_A$ and $Q_B$, as indicated by the abolishment of the B band in the glow curve, suggests that histidine residues participating in binding of non-heme iron and $Q_B$ are modified by DEPC. This conclusion is in agreement with earlier suggestions [7, 22], concerning the role of histidine residues in the structural arrangement of the acceptor side of PS II.

The inhibition of electron transport by DEPC at the acceptor side of PS II has several consequences. The effect of DEPC in the photoligation and photoactivation experiments [1, 40], and in the high affinity Mn-binding assay from DPC to DCIP [2, 40], should be interpreted with care in the light of our present results. The inhibition of electron transport between $Q_A$ and $Q_B$ influences the donor side reactions, as well. The water-splitting system can undergo only one transition affecting

the photoligation and photoactivation process of Mn, which probably requires the absorption of at least two light quanta and in turn, transfer of two electrons from the donor to the acceptor side [1]. However, this can not take place if the $Q_A \rightarrow Q_B$ electron transfer is inhibited and if the primary acceptor is already in the reduced state after the first photoact. Similarly, photoactivation which is facilitated in the presence of DCIP [41] might be slowed down or inhibited in the presence of DEPC, since electrons can not reach the exogenous acceptor bound at the $Q_B$ binding site. Partial inhibition of the $Q_A$ to $Q_B$ electron transfer by DEPC can also affect the high affinity Mn binding assay from DPC to DCPIP [2].

DEPC replaced the TL band peaking at −30 °C with a band appearing at about −15 °C (Fig. 4, curve B). This phenomenon can not be attributed to an inhibition of electron flow only at the acceptor side of PS II, because the B band ($S_2Q_B^-$) could be partially charged after a short DEPC treatment (Fig. 4, curve B). Thus the abolishment of the $A(A_T)$ band indicates an effect of DEPC on the donor side of PS II [1, 12] probably on a histidine residue which is suggested to be oxidized in the $S_2$ to $S_3$ transition [14, 18]. This putative photooxidizable histidine may also participate in the generation of the $A_T$ band [12, 13] which appears at the same temperature as the A band [35].

The donor side effect of DEPC develops faster than the electron transport inhibition at the acceptor side, because the replacement of the $A(A_T)$ band with the band at −15 °C preceds the disappearance of the B band (Fig. 4, curve B). Surprisingly, the formation of the $S_2$ state is not inhibited by DEPC as can be concluded from the appearance of the Q band ($S_2Q_A^-$) in the glow curve of DEPC-treated thylakoids (Fig. 2, curve B). This observation can hardly be reconciled with the existence of a putative intermediate histidine component between Mn and $Y_Z$. Modification of this histidine by DEPC should inhibit the formation of the $S_2$ state. The contradiction might be resolved by assuming the location of the electron-mediating histidine on an alternative pathway to P 680 as it was suggested in [13].

The band appearing at −15 °C simultaneously with the disappearance of the $A(A_T)$ band at −30 °C has not been reported earlier [31, 35, 36]. This band can not be attributed to charge recombination of the putative oxidized histidine component ($His^+$) with $Q_A^-$ [12, 13] because it appears at higher temperature than the $A(A_T)$ band. As it is reflected in the higher peak temperature [42] the midpoint redox potential of the donor responsible for the −15 °C band should be less positive than that of the donor ($S_3$ state or $His^+$) participating in the generation of the $A(A_T)$ band at −30 °C. We can assume that after two preflashes in the presence of DEPC or DCMU, an additional low temperature illumination at −80 °C results in the oxidation of an unidentified donor responsible for the −15 °C band. The identification of the donor responsible for the −15 °C band requires further investigation. Since the band at −15 °C can be charged in the presence of DCMU (Fig. 2 and 4) the acceptor participating in its generation is probably the reduced primary quinone acceptor, $Q_A^-$.

It is rather perplexing that DCMU, which is an inhibitor of electron transport at the acceptor side of PS II between $Q_A$ and $Q_B$, also resulted in the replacement of the −30 °C band by the −15 °C band (Fig. 4, curve C). As a possible interpretation we suggest, that in uninhibited thylakoids during illumination by continuous light at −80 °C following two preflashes at 5 °C some of the PS II reaction centers undergo two turnovers. After the first photoact the oxidized donor responsible for the −30 °C band ($S_3$ or $His^+$) is rereduced by a secondary donor of less positive potential which is responsible for the −15 °C band. In the second photoact the donor associated with the −30 °C band is oxidized, again. During TL measurement it undergoes charge recombination with $Q_A^-$ resulting in the appearance of the −30 °C band. In DCMU-treated thylakoids PS II can turn over only once and the −15 °C band appears in the glow curve. In the presence of DEPC the donor responsible for the −30 °C band (probably histidine) is inhibited and only the donor accounted for the −15 °C band can be oxidized. Consequently, in the presence of DEPC only the −15 °C band can be charged and observed. Inhibition of the donor responsible for the −30 °C band does not inhibit the appearance of the Q band ($S_2Q_A^-$) as shown in Fig. 2. Similarly, the appearance of the B band ($S_2Q_B^-$) is not influenced by replacement of the −30 °C band with the −15 °C band (Fig. 4, curve B). Subsequent development of the inhibition between $Q_A$ and $Q_B$ at the acceptor side histidines results in the disap-

pearance of the B band with a concomitant appearance of the Q band (Fig. 2, curve B).

In summary, we can say that in the interpretation of results obtained by DEPC-induced modifications of electron transport both the donor and acceptor side effects of DEPC should be taken into account. In addition to the well documented modification of an electron mediating histidine donor component at the donor side of PS II [1, 12, 13, 19], inhibition of electron transfer between $Q_A$ and $Q_B$ by DEPC indicates, in agreement with earlier suggestions [7, 22], the participation of histidine residues in the functioning of the acceptor side of PS II, as well. It was also found that in manganese-containing thylakoids DEPC does not inhibit electron transfer from manganese to $Q_A$ during the $S_1$ to $S_2$ transition of the water-splitting system.

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