# Tribromotoluquinone Induced Modifications of the Oscillation Pattern of Oxygen Evolution and of Herbicide Binding in Thylakoids and PS II Membrane Fragments from Spinach

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*Dedicated to Professor Achim Trebst on the occasion of his 60th birthday*

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In the present study the effect of TBTQ on PS II and its mutual interaction with DCMU was analyzed by measurements of the oxygen yield oscillation pattern and of DCMU binding. It was found:

1) TBTQ in its reduced form is able to induce the reduction of $D^{ox}$ which gives rise to an accelerated decay of $S_2$ and $S_3$ of the wateroxidizing complex.
2) Triton X-100 treatment used for isolation of PS II membrane fragments does not significantly affect the lateral mobility of plastoquinone within the membrane. TBTQ bound to the thylakoid membrane does not enhance the electron pool capacity in PS II membrane fragments.
3) Preincubation of thylakoids with TBTQ diminishes the blockage of $O_2$-evolution by DCMU significantly. In correspondence with previous findings [18, 19] the effect strongly depends on the order of addition of TBTQ and DCMU.
4) Excitation with a single saturating flash causes enhanced DCMU binding in TBTQ pretreated samples leading to the inhibition of flash induced oxygen evolution. The rate of the latter process depends on the DCMU concentration.
5) In thylakoids pretreated in the dark with TBTQ the oxygen yield of the 3rd flash slowly declines as a function of dark incubation time at constant DCMU concentration.

Based on the above mentioned findings it is inferred that a mutual interaction between TBTQ and DCMU takes place at the PS II acceptor side. Two alternative mechanisms are discussed: i) TBTQ tightly (covalently?) bound at the $Q_B$-site (or very close to it) is modified in its function by DCMU *via* structural effects (allosteric type), or ii) there occurs a TBTQ/DCMU exchange that is fast in the light and slow in the dark.

## Introduction

The key steps of photosynthetic water cleavage take place in a thylakoid membrane bound polypeptide complex referred to as system II (see ref. [1]). A first rough structural model was proposed [2] on experiments obtained with trypsin treated thylakoids. It was postulated that the plastoquinone molecules $Q_A$ and $Q_B$ at the PS II acceptor side are incorporated into a protein matrix of multifunctional relevance: (a) it regulates the electron transfer from $Q_A$ to $Q_B$ ($Q_B^-$), (b) it acts as a shield of $Q_A$ ($Q_A^-$) to rapid interaction with exogenous redox components and (c) it provides the binding site (s) for herbicides that block $Q_A^-$ reoxidation by $Q_B$ ($Q_B^-$). Later it was postulated that plastoquinone is tightly bound to the $Q_B$-site only in its semiquinone form [3, 4] at a polypeptide identified as a 32 kDa protein [5] referred to as D-1 [6]. Recently, different lines of evidence led to the conclusion that polypeptide D-1 together with another polypeptide D-2 forms a complex which acts as apoprotein of PS II carrying not only the plastoquinone molecules of the acceptor side but also all the other essential prosthetic groups P680, Pheo, Z (Tyr 161 of D-1, see ref. [7]) of the PS II reaction center and even the catalytic site of water oxidation (for reviews, see ref. [8, 9]). Despite the huge progress in our understanding of the structural organization of system II during the last years [8, 9] the possi-

*Abbreviations:* ADRY, acceleration of the deactivation reactions of the water splitting enzyme system Y; D, redox active component giving rise to EPR-signal $II_s$; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-[N-morpholino]ethanesulfonic acid; PS II, photosystem II; $Q_A$,$Q_B$, primary and secondary plastoquinone acceptor of photosystem II; $S_2$,$S_3$, redox states of the catalytic site of water oxidation; TBTQ, tribromotoluquinone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; $Y_n$, oxygen yield of the *n*-th flash.

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ble role of other polypeptides (CP 47, Cyt *b* 559) intimately associated with the D-1/D-2 complex for the functional integrity of PS II is not clarified (for review see ref. [10]). The structure of system II is obviously even more complex because latest studies revealed that at least one additional low molecular weight polypeptide is very likely an essential constituent of the reaction center and other integral subunits of 4–5 kDa are important for an intact oxygen evolving core complex [11].

Different chemicals were found to affect specifically the PS II reaction pattern either by blocking the $Q_A^-$ reoxidation (for review, see [12, 13]) or by selective interaction with different redox states of the catalytic site of water oxidation, *e.g.* as ADRY type reagents [14, 15]. Among the great variety of compounds the effects of 1,4-benzoquinone derivatives are of special interest because of their similarities with the natural plastoquinones. Therefore, 1,4-benzoquinones are expected to interact with the $Q_B$-site thereby functioning either as artifical acceptor or as inhibitor of $Q_A^-$-reoxidation. In addition to that, another possibility is mechanistically very interesting. If exogenous quinones are able to bind in the PS II complex [16, 17] further redox groups could be incorporated that might effect the reaction sequence of water cleavage. Recently, halogenated 1,4-benzoquinones were shown to affect specifically the DCMU sensitivity of the PS II acceptor side [18, 19]. On the other hand, DCMU was previously found to affect indirectly also the lifetime of $S_2$ and $S_3$ [20, 21]. Therefore, it appeared worthwhile to study the effect of tribromotoluquinone and its mutual interaction with DCMU at the donor and acceptor side of PS II in more detail.

## Materials and Methods

Thylakoids and PS II membrane fragments were prepared from spinach according to Winget *et al.* [22] and a modified procedure of Berthold *et al.* [23] as described in ref. [24].

Flash induced oxygen yield measurements were performed with an unmodulated Joliot type electrode [25] as outlined in ref. [26]. The sample contained thylakoids or PS II membrane fragments (1 mg/ml chlorophyll) suspended in the dark with TBTQ (oxidized or reduced with $NaBH_4$) and/or DCMU at concentrations and incubation times indicated in the figure legends. The control contained the same amount of methanol as the treated sample. After centrifugation the thylakoids were resuspended in a solution containing 20 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tricine/NaOH, pH = 7.6. The same solution was used as flow buffer in the Joliot-type electrode. In the case of PS II membrane fragments $MgCl_2$ was omitted and MES/NaOH, pH = 6.5, used instead of Tricine/NaOH. The time between the flashes was 300 ms under standard conditions. Other excitation conditions are indicated in the figures. Before starting the measurements the samples sedimented for 3 min (PS II membrane fragments) or 5 min (thylakoids) on the platinum cathode. All steps were carried out at the dim light of a yellow/green lamp, Osram 4543.

Herbicide binding was measured by a method similar so that of ref. 27 as described in detail in ref. [28].

Error bars characterizing the experimental data are given in the figure.

## Results

### *Effects of TBTQ on the PS II donor side*

In order to study possible effects of TBTQ on the donor side of PS II the oscillation pattern of oxygen evolution induced by a flash train in dark adapted thylakoids was measured in the absence and presence of TBTQ. As only the effect of membrane bound TBTQ should be detected thylakoid suspensions (3 mg chlorophyll/ml) were incubated in the dark on ice with either methanol or 300 μM TBTQ dissolved in methanol so that the final concentration of this solvent in the samples was 15%. After 3–4-fold dilution the samples were centrifuged and resuspended in buffer to remove soluble TBTQ. The oscillation patterns obtained with these samples (1 mg chlorophyll/ml) are depicted in Fig. 1. The top traces reveal two interesting TBTQ induced phenomena: a) the oxygen yield of the second flash is markedly reduced indicative of a diminished probability of double hits (β) due to retardation of the $Q_A^-$ reoxidation [19], and b) the probability of misses (α) becomes higher and especially the ratio of the oxygen yield caused by the 3rd and 4th flash, respectively, ($Y_3/Y_4$), becomes smaller after TBTQ pretreatment. This could be either due to a decrease of the equilibrium constants of the acceptor side reactions $Q_A^-Q_B \rightleftharpoons Q_AQ_B^-$ and $Q_A^-Q_B^- \rightleftharpoons Q_A\,Q_B^{2-}$ (it is assumed that TBTQ is able to bind to the $Q_B$-site) or caused by an

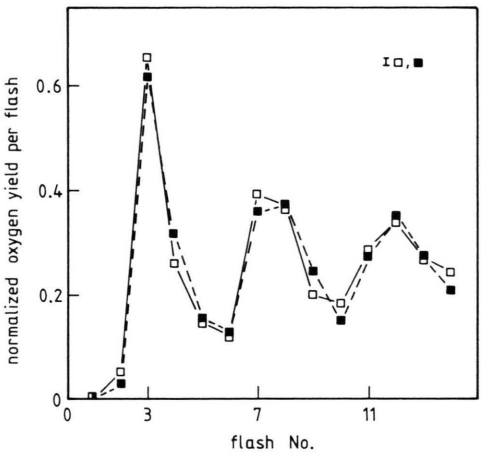


Fig. 1. Normalized oxygen yield per flash as a function of flash No., n, in dark adapted spinach thylakoids pretreated in the absence (□) or presence (■) of 300 μM TBTQ.

acceleration of the $S_2$ and/or $S_3$ decay. To check this point the lifetime of $S_2$ was determined by varying the time between the first and second flash of the sequence. The results obtained are shown in Fig. 2. In control samples the expected two-phasic decay is observed. In these samples the extent of the fast kinetics is higher than usually observed. In the TBTQ pretreated samples the fast kinetics are even more pronounced. This effect could explained by a higher extent of reduced component D which causes rapid $S_2(S_3)$ decay [29]. As D, recently identified as Tyr 160 of polypeptide D-2 in Synechocystis sp. PCCC 6803 (30), is a one electron donor its effect on $S_2$ and $S_3$ should be eliminated by a preflash leading to $D^{ox}$-formation. To check this idea samples were preilluminated with one flash and after 5 min dark time the $S_2$ decay was measured. In both samples (control and TBTQ pretreated thylakoids) the fast kinetics disappeared. Therefore, they are entirely due to a rapid reduction of $S_2$ by a redox component that is exhausted in its donor capacity by a single flash and does not regenerate during a five minute dark period. This component is D as confirmed by comparative room temperature EPR-measurements which show that signal $II_s$ reflecting $D^{ox}$ is decreased by TBTQ pretreatment (data not shown). Based on the above mentioned findings TBTQ is inferred to interact not only with the acceptor side of PS II but also with its donor side at the level of D. The reactive species is probably the reduced form of TBTQ. Experiments with TBTQ reduced by using $NaBH_4$ strongly support this assumption (data not shown).

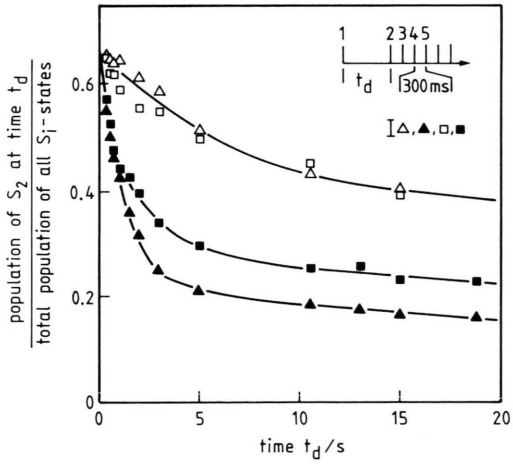


Fig. 2. Decay kinetics of $S_2$ in spinach thylakoids pretreated in absence (squares) or presence (triangles) 300 μM TBTQ. After the treatment the samples were either dark adapted (closed symbols) or preilluminated by a saturating flash 5 min before excitation with the flash train (open symbols).

*Lateral mobility of plastoquinone in PS II membrane fragments and effects of TBTQ on the pool size*

Beyond its reaction with D at the donor side bound TBTQ as a redox active quinone might also act as an electron acceptor thereby increasing the total pool size. To address this problem experiments were performed with PS II membrane fragments. In these samples the pool capacity is limited because the reduced $PQH_2$ cannot be rapidly reoxidized between the flashes due to lack of PS I. Therefore, the oxygen yield rapidly declines during the flash sequence. In normal thylakoids the plastoquinone molecules are mobile so that the effective pool size per active PS II becomes large if the degree of DCMU blockage increases [31, 32]. This technique can be used to answer the question whether the Triton X-100 treatment step applied for isolation of PS II membrane fragments restricts the lateral mobility of the plastoquinone molecules. Experiments were performed at different DCMU concentrations. The results obtained are summarized in Fig. 3. The blockage of PS II by DCMU is reflected by the decrease of the oxygen yield induced in the 3rd flash ($Y_3$) of the sequence. If the plastoquinone molecules are laterally mobile within PS II membrane fragments the oscilla-

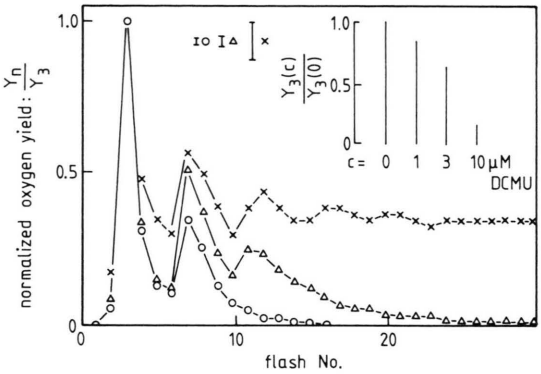


Fig. 3. Oxygen yield per flash as a function of flash No., n, normalized to that of the 3rd flash, $Y_n/Y_3$, in dark adapted PS II membrane fragments from spinach in the absence (○) or presence of 3 μM (△) or 10 μM (×) DCMU.

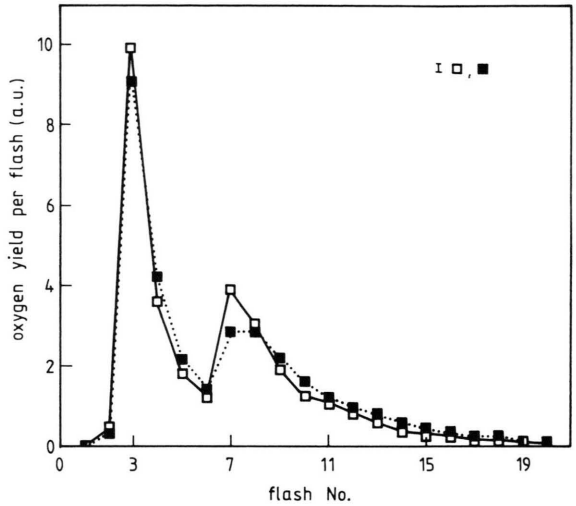


Fig. 4. Oxygen yield per flash as a function of flash No., n, in dark adapted PS II membrane fragments from spinach pretreated in the absence (□) or presence (■) of 300 μM TBTQ.

tion pattern is expected to change significantly. In order to illustrate this effect each sequence was normalized to the oxygen yield $Y_3$. The data of Fig. 3 show that in the control the oxygen yield rapidly drops down to a level close to zero after the 10th flash. If, however, about 30% of the PS II centers are blocked by addition of 3 μM DCMU the drop is shifted to a significantly higher flash No. whereas the integral oxygen evolution (sum of all absolute flash yields) remains constant. At a drastically higher inhibition degree of about 80%, the oscillation pattern reaches a steady state level that does not decline up to the 30th flash. These findings clearly indicate that in the PS II membrane fragments the plastoquinone molecules remain laterally diffusable within the time resolution (order of magnitude: 0.1 s) of this type of experiment. The same approach was used to analyze the effect of TBTQ on the total pool size. A comparison of the data depicted in Fig. 4 shows that PS II membrane fragments treated with 300 μM TBTQ in the dark for 15 min before centrifugation and resuspension exhibited only marginal differences to the control. The integral oxygen yield of the first twenty flashes is practically the same in both sample types. This results clearly shows that TBTQ bound to the thylakoid membrane does not increase the total pool size. Previous observations suggesting a slight increase [19] could not be confirmed.

*Mutual interaction of TBTQ and DCMU with system II*

It was recently shown that TBTQ does interfere with the action of DCMU at the PS II acceptor side and that the mode of action depends on the sequence of addition of these substances [18, 19]. The latter effect should also give rise to a different oscillation pattern of flash induced oxygen evolution. Fig. 5 shows the results obtained with intact thylakoids. At 20 μM DCMU oxygen evolution is almost completely blocked. If, however, samples are pretreated with

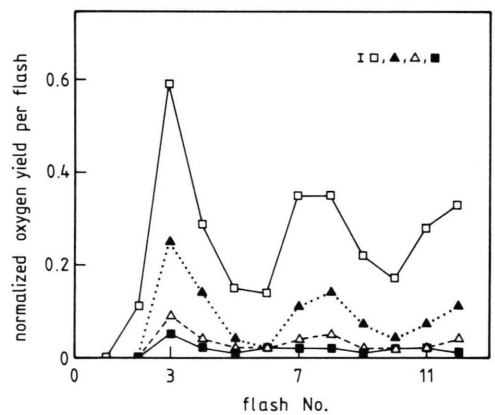


Fig. 5. Normalized oxygen yield per flash in dark adapted spinach thylakoids. □ control, ■ 20 μM DCMU, ▲ pretreatment with 100 μM TBTQ and addition of 20 μM DCMU after centrifugation and resuspension, △ 5 min incubation with 20 μM DCMU before addition of TBTQ. The sum of the $Y_n$-values for $n = 1-12$ obtained in the control was used for normalization of all data.

100 μM TBTQ in the dark before centrifugation and resuspension, the inhibitory effect of DCMU is remarkably diminished. On the other hand, addition of DCMU prior to TBTQ almost completely abolishes this effect. These data are in correspondence with previous findings [18, 19] that were interpreted as an indication for tight (possibly covalent) binding of TBTQ at or close to the $Q_B$-site. In order to analyze this point more thoroughly experiments were performed at different DCMU concentrations. In Fig. 6 the oscillation patterns of oxygen evolution normalized to the yield in the third flash, $Y_3$, obtained in the presence of 10 μM and 50 μM DCMU are compared with that of the control. The most striking phenomenon of this experiment is the marked decline of the oxygen yield with increasing flash No. It could be explained by the assumption that some PS II centers contain tightly bound TBTQ molecule (s) acting as DCMU insensitive electron acceptor of very limited capacity. In order to check whether this capacity can be restored in the dark experiments were performed at longer times $t_d$ between the flashes. Surprisingly, in this case the oxygen yield $Y_3$ of the TBTQ treated samples in the presence of 50 μM DCMU rapidly decreased with increasing $t_d$. At a first glance this phenomenon could be due to an accelerated decay of $S_2$ and $S_3$. However, the drastic decrease of the integral oxygen evolution caused by the 3rd to 6th flash of the sequence with increasing

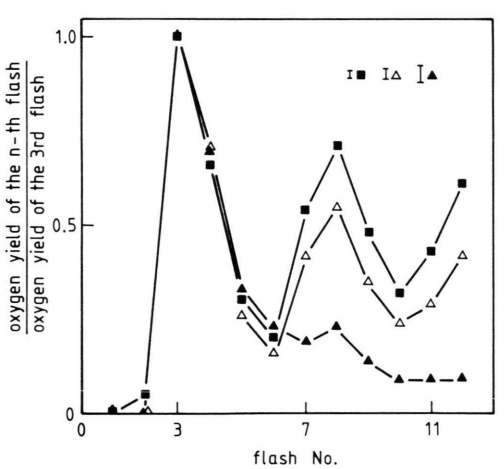


Fig. 6. Oxygen yield per flash as a function of flash No., n, normalized to that of the 3rd flash, $Y_n/Y_3$, in dark adapted spinach thylakoids pretreated with 100 μM TBTQ and subsequent addition of none (■) or 10 μM (△) or 50 μM (▲) DCMU.

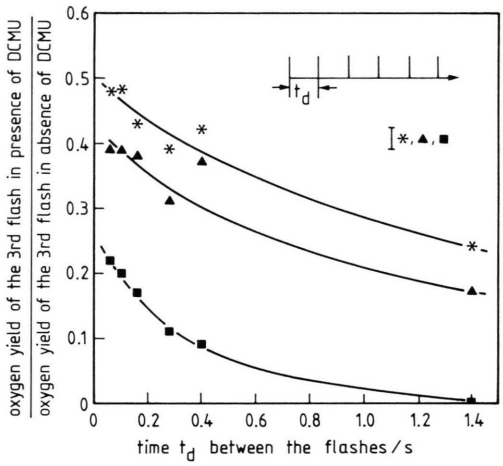


Fig. 7. Oxygen yield of the 3rd flash as a function of time $t_d$ between the flashes in TBTQ pretreated thylakoids in the presence of DCMU normalized to the corresponding values in the absence of DCMU. ✳ 5 μM DCMU, ▲ 10 DCMU, ■ 50 μM DCMU.

time between the first and second flash (data not shown) did not support this idea. So check the time dependence in detail, $Y_3$ was measured at different flash frequencies in TBTQ treated samples in the presence of 3 different DCMU concentrations. The data were normalized to the corresponding values measured at the same time $t_d$ in the control without DCMU. The results are summarized in Fig. 7. They exhibit two effects: a) the normalized $Y_3$ decreases with increasing $t_d$ at a rate that is DCMU dependent, and b) the normalized $Y_3$ becomes reduced with increasing DCMU concentrations even at short times $t_d$.

These findings are not in favour with the simple model of the existence of centers that contain covalently bound TBTQ acting as DCMU insensitive electron acceptor. Based on the time dependence a more likely interpretation is the assumption that a light induced turnover of PS II changes the TBTQ binding, so that it permits enhanced binding of DCMU which thereafter blocks the electron transfer. In order to check this hypothesis binding studies were performed with [$^{14}$C] DCMU in the PS II membrane fragments pretreated with or without 100 μM TBTQ before centrifugation and resuspension. The data of Fig. 8 show, that in the control samples without TBTQ the binding of DCMU remains almost unaffected by one flash. In the TBTQ pretreated sample without flash illumination the DCMU binding is markedly impaired. This effect indicates that

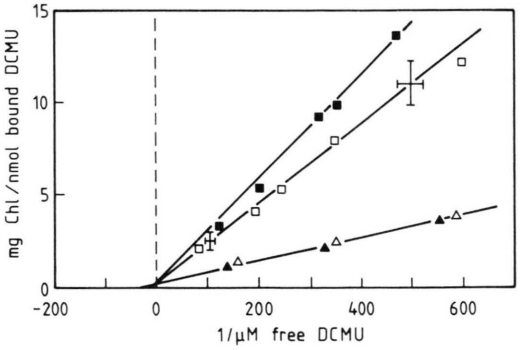


Fig. 8. Double reciprocal plot for binding of [$^{14}$C] DCMU to PS II membrane fragments pretreated in the absence or presence of 300 μM TBTQ. After dilution centrifugation and resuspension the samples were kept in dim light without (closed symbols) or illumination with one saturating flash (open symbols). △, ▲ control, □, ■ TBTQ pretreated.

TBTQ binding interferes with that of DCMU. Interestingly enough, 1 flash significantly enhances the DCMU binding in TBTQ pretreated PS II membrane fragments. This finding could be easily explained by the assumption of a light induced TBTQ/DCMU exchange. It furthermore shows that covalent TBTQ binding is very unlikely at a domain required for DCMU binding.

The latter conclusion implies another interesting question: What is the mutual interaction in the dark between TBTQ and DCMU and the PS II acceptor side? To address this point the effect of dark incubation with DCMU, $t_{inc}$ (DCMU), on $Y_3$ was analyzed in samples pretreated for 50 min with 100 μM TBTQ before centrifugation and resuspension. The data obtained are shown in Fig. 9. At a DCMU concentration of 20 μM and $t_{inc}$ (DCMU) = 8 min, $Y_3$ was about 50% of that in the control without DCMU. At prolonged $t_{inc}$ (DCMU) the value of $Y_3$ progressively declined and after 4 h a level of about 20% was obtained. The limited lifetime of the sample prevented measurements at much longer $t_{inc}$ (DCMU). A comparison of the time course with the control reveals a striking heterogeneity that can be interpreted by two alternative models: a) if one considers the value measured at $t_{inc}$ (DCMU) = 4 h as the final level of a presumed exchange process then extrapolation of the first order equilibration kinetics with a half lifetime of 25 min intersects the ordinate at a level corresponding with about 60% of all PS II centers. This assumption implies that the remaining 40% of PS II are blocked by DCMU in a similar way as a control without TBTQ pretreatment. b) If one assumes that all centers eventually bind DCMU at the expense of TBTQ, then another type of heterogeneity would arise for the TBTQ pretreated samples. In this case, fast and slow "exchanging" centers would exist, characterized by two very different DCMU/TBTQ dark exchange kinetics with half lifetimes of 8 min and 4 h, respectively. The present data do not permit an unambiguous distinction.

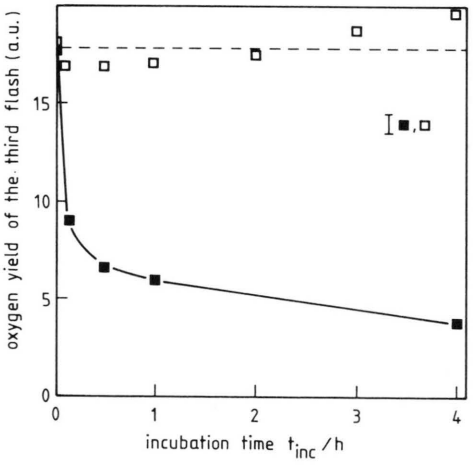


Fig. 9. Oxygen yield of the third flash as a function of incubation time with 20 μM DCMU in thylakoids preincubated for 50 min with 300 μM TBTQ before dilution, centrifugation and resuspension (■) and of control without DCMU (□).

## Discussion

The present study shows that the interaction of TBTQ with PS II gives rise to modifications of the donor and acceptor side. At the donor side incubation with TBTQ leads to an increased extent of reduced D (recently identified as Tyr 160 of polypeptide D-2 in *Synechocystis* sp. PCCC 6803 [30]). This effect causes accelerated $S_2$ ($S_3$) decay at the catalytic site of water oxidation. The fast decay was found to be eliminated by one preflash and subsequent 5 min dark incubation. Accordingly, $D^{ox}$ formed due to the preflash is not reduced during the 5 min dark period. This result indicates that the redox active Tyr of polypeptide D-2 is not easily accessible to TBTQ (either in a reduced form or *via* an oxidant induced reduction mechanism) attached to the thylakoid membrane.

The most interesting finding of this report is the discovery of a mutual dynamic TBTQ/DCMU interaction at or close to the $Q_B$ binding site. This reaction is fast in the light and slow in the dark. The observed phenomena can be explained by two alternative mechanisms: a) the functionally active TBTQ is not covalently bound and undergoes in the light a comparatively fast exchange reaction with DCMU, or b) TBTQ is tightly (covalently) bound and changes its orientation in the light (fast) or dark (slow) in a way that causes increased binding of DCMU and blockage of electron transport.

In the first mechanism the fast flash induced TBTQ/DCMU exchange can be most easily explained by the assumption that TBTQ becomes reduced and thereby its binding affinity is drastically reduced. After one flash in the absence of DCMU the reduced form of TBTQ could exchange with another quinone molecule, but in the presence of DCMU this inhibitor competes for the binding site. This simple mechanism, however, bears a problem. If one assumes that TBTQ binds in its oxidized form, then a single flash leads to formation of the semiquinone anion radical which is expected to be more stably bound than the quinone, provided that the relative binding affinities of the different redox states of TBTQ resemble those of PQ. Therefore, preillumination with a single flash should not enhance the binding affinity of DCMU, in contrast to the observation. This problem could be solved, if the stably bound species in the dark is the semiquinone form of TBTQ. After one flash reduction by $Q_A^-$ and subsequent protonation would give rise to $TBTQH_2$ which rapidly exchanges. The binding of a stable semiquinone form in the dark and its flash induced reaction is not in contradiction with recent measurements of UV-absorption changes [19]. The origin of the semiquinone form remains to be clarified. It could either arise by interaction with $Q_B^-$ that is present in a fraction of PS II centers or it can be formed with other reductants.

The second mechanism of a light induced change of orientation of tightly (covalently) bound TBTQ giving rise to higher DCMU binding affinity would be in line with the data of Fig. 8 which suggest an increase of the binding constant without affecting the total number of DCMU binding sites. However, the precision of these data does not permit an unambiguous conclusion. Therefore, further studies with radioactively labeled TBTQ are required to clarify this point.

Based on previous findings [20, 21] the steeper decline of normalized $Y_3$ at higher DCMU concentration could be interpreted by an indirect effect of this compound on the lifetime of $S_2$ and $S_3$. However, we do not favour this idea because the sum of $Y_3$ to $Y_6$ decreased markedly with increasing time between the first and second flash. This finding is more in line with the assumption of a light induced blockage due to enhanced DCMU binding.

An interesting implication from the slow DCMU binding in the dark in TBTQ pretreated thylakoids refers to the interpretation of experimental data obtained in the presence of different substances that interact with the same domain. In order to avoid misinterpretations one has to make sure that really a true steady state or equilibrium state is achieved.

The present results show that halogenated *p*-benzoquinones provide an interesting class of substances to study the binding properties of the $Q_B$-site of PS II and its environment.

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