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THE ACTION OF TETRAPHENYLBORON AS A SYSTEM II ELECTRON DONOR AND ITS EFFECT ON THE DECAY OF THE ELECTRICAL FIELD ACROSS THE THYLAKOID MEMBRANE

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SUMMARY

The effect of tetraphenylboron on the O_2 evolution, the electron transport in normal and in Tris-washed chloroplasts, and on the decay of the electrical field across the thylakoid membrane has been investigated in spinach chloroplasts. It was found that:

1. The average O_2 yield per flash as a function of the tetraphenylboron concentration is dependent on the number of excitation flashes. With increasing flash number, the tetraphenylboron concentration which is required for 50 % suppression of the average O_2 yield per flash shifts toward higher values.

2. After the irreversible consumption of tetraphenylboron by System II oxidizing equivalents, the O_2 evolution reappears.

3. The electron flow from System II to System I, as indicated by the amplitude of the reduction kinetics of the 703 nm absorption change, remains unaffected in the tetraphenylboron concentration range and at flash numbers where the O_2 evolution is totally suppressed.

4. Tetraphenylboron restores electron transport in Tris-washed chloroplasts. This tetraphenylboron-mediated electron transport is completely blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

5. Preliminary titration experiments favor the assumption that tetraphenylboron acts in chloroplasts rather as a 1-electron donor than as a 2-electron donor.

6. Tetraphenylboron accelerates the decay of the electrical field across the thylakoid membrane. Because of its ability to act as a System II electron donor, the accelerating effect on the electrical field is transitory if System II is active. By contrast, in DCMU-blocked chloroplasts with active System I electron transport [mediated by the 2,6-dichlorophenolindophenol (DCIP) cycle], a permanent accelerating effect on the electrical field is observed.

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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From these results it has been concluded, that tetraphenylboron acts as an efficient 1-electron donor for System II. The functional integrity of the water-splitting enzyme system Y is not required for this donation. Tetraphenylboron itself does not destroy the O_2 evolution capability of chloroplasts, it rather acts as a competitive electron donor. However, an inhibitory effect arises probably from the oxidation products of tetraphenylboron.

The possible modes of action of tetraphenylboron are discussed.

INTRODUCTION

Within the photosynthetic electron transport of algae and higher plants, System II plays a central role since it allows the use of water as the natural electron source for the reduction of $NADP^+$. The nature of this System II, including the light-induced generation of primary electrons and holes and the oxidation of water to molecular O_2 performed within the water-splitting enzyme system Y, still remains to be elucidated, but in the last years some insight into this problem has been achieved. One way to attack the problem is the modification of functional elements of System II by chemicals. In this respect, 3 different types of effectors can be generally distinguished: (1) Inhibitors which specifically block the electron transport of System II (DCMU-type inhibition, see refs 1–5) or selectively destroy the water-splitting enzyme system Y (Tris-washing type inhibition, see refs 6–8). (2) Acceleration of the deactivation reactions of the water-splitting enzyme system Y (ADRY) agents catalyzing the discharge of the higher-trapped-hole accumulation states S_2 and S_3 within the water-splitting enzyme system Y [9–12]. (3) Artificial electron donors which are able to donate electrons to System II in the absence of the functional integrity of the water-splitting enzyme system Y [13–17].

Most of the effectors exert simultaneous effects, e.g. DCMU was found to act as an inhibitor and as an ADRY agent [18, 19], hydroxylamine acts as an electron donor of System II as well as an inhibitor of the water-splitting enzyme system Y [16].

Recently it has been shown by Homann [20] that tetraphenylboron, first introduced by Packer and co-workers [21, 22] as an inhibitor of electron transport-dependent volume changes and proton gradients, specifically interacts with the electron transport of System II. From the effect of tetraphenylboron on the dark regeneration of the fluorescence rise in DCMU-poisoned chloroplasts, it has been concluded that the site and mode of action of tetraphenylboron resembles those of ADRY agent-type uncouplers like carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) or certain System II electron donors. However, tetraphenylboron seems to act as an electron donor, whereas for stoichiometrical reasons it was inferred, that ADRY agents like CCCP do not act as System II electron donors [23]. Therefore, it is improbable, that tetraphenylboron acts as an ADRY agent. Furthermore, an inhibition of the O_2evolution in continuous light has been reported for tetraphenylboron. For this suppression two different modes of action have to be discussed: (a) Tetraphenylboron exerts an inhibitory effect either of DCMU or of the Tris-washing type. (b) Tetraphenylboron acts either as an electron donor simultaneously destroying the function of the water-splitting enzyme system Y and donating electrons to System II, or as an ideal competitive electron donor which donates electrons in preference to

water into System II without damaging the water-splitting enzyme system Y.

Hence, the question arises, whether the different effects of tetraphenylboron can be explained exclusively on the basis of its ability to donate electrons to System II.

In the present paper it will be shown, that tetraphenylboron acts as an efficient electron donor to the holes produced by Photosystem II, thereby suppressing the O_2 evolution without damaging the water-splitting enzyme system Y. The functional integrity of the water-splitting enzyme system Y is not required for the electron donation by tetraphenylboron. Tetraphenylboron was not found to be an inhibitor per se. However, an inhibitory effect on System II is exerted probably by the oxidation products of tetraphenylboron.

Furthermore, it will be shown, that tetraphenylboron accelerates the decay of the electrical field across the thylakoid membrane, probably by a fast transport of the tetraphenylboron anion.

MATERIALS AND METHODS

Preparation of the chloroplasts

The chloroplasts were prepared from market spinach according to the method of Winget et al. [24], except that 10 mM ascorbate was present during the grinding of the spinach. For the storage in liquid N_2, 5 % dimethylsulfoxide was added. After thawing, the activity (Hill reaction rate and average O_2 yield per flash) of the stored chloroplasts was nearly the same as for freshly prepared chloroplasts.

Tris–washed chloroplasts were obtained by the method of Yamashita and Butler [25] using 0.8 M Tris buffer, pH = 8.0. The ability to evolve O_2 by flash-light excitation was completely lost by the Tris–washing procedure.

Reaction mixture

The standard reaction mixture contained chloroplasts (50 μM chlorophyll for O_2 measurements and 80 μM for measurements of absorption changes), 0.25 mM $Na_3[Fe(CN)_6]$, 20 mM NaCl, 5 mM $MgCl_2$ and 50 mM *N*-tris(hydroxymethyl)-methylglycine (Tricine)–NaOH, pH = 7.2. Other reaction mixtures are indicated in the legends of the figures.

Reagents were of the highest purity commercially available, except for Na_3-$[Fe(CN)_6]$ which was prepared from $K_3[Fe(CN)_6]$ by passing through a column of Dowex 50 in its Na^+ form.

Measurements

The O_2 measurements were performed with a Clark-type electrode (IL 125 B Instrumentation Laboratory Inc., Watertown) by a repetitive technique as is described in ref. 11. For the details of the flashlamp device, see ref. 26.

The absorption changes at 520 nm and at 703 nm were measured with a repetitive-flash spectroscopic technique similar to that published in ref. 27. The signals were averaged in a Fabri-Tek. The electrical bandwidth ranged from 0–5 kHz, except for the measurements of the fast 520 decay kinetics, where a range of 0–33 kHz was used.

The optical pathlength was 1 mm, the bandwidth of the monitoring light (λ = 520 nm or 703 nm, respectively) was 5 nm. The exciting Xenon-lamp flashes were passed through a Schott filter RG 1, 2 mm, for the 520 nm measurements and through

a Schott filter BG 28, 4 mm, for the 703 nm measurements. The flash duration was approx. 20 μs.

All measurements were carried out at room temperature.

RESULTS

The effect of tetraphenylboron on the average O_2 yield per flash

The question whether the suppression of the O_2 evolution by tetraphenylboron is caused by a competitive electron donation or by a true inhibitory effect, can be answered by measuring the average O_2 yield per flash as a function of the tetraphenylboron concentration at different numbers of short excitation flashes.

A true System II inhibitor leads to the blockage of the electron transport at a specific site (or sites) within System II, so that for a given inhibitor concentration c_I, a definite fraction $p(c_I)$ of all System II electron-transport chains does not operate. Hence, the average O_2 yield per flash as a function of the inhibitor concentration and of the number n_f of the excitation flashes, $M_1(c_I, n_f)$, should be independent of the flash number n_f.

If on the other hand, an efficient electron donor competes with water very effectively for the holes produced by Photosystem II, the average O_2 yield per flash is also suppressed, but the donor is irreversibly consumed by the oxidation process, so that the average O_2 yield per flash, $M_1(c_D, n_f)$, is dependent on the donor concentration c_D as well as on the flash number n_f. An ideal competitive electron donor for the positive charges produced by System II is characterized by two properties: (a) high affinity for the positive charges of System II, so that no water is oxidized until the donor is stoichiometrically consumed. (b) The functional and structural integrity of the water-splitting enzyme system Y is not influenced by the donor molecules.

If each donor molecule provides m_e electrons for the discharge of positive charges produced by System II, the reaction is given by:

$$m_e \oplus + \text{donor}_{red} \longrightarrow m_e \bigcirc + \text{donor}_{ox} \qquad (1)$$

where $\oplus$ represents one oxidizing equivalent of System II, and $\bigcirc$ its discharged form.

Assuming c_{II} to be the concentration of the active System II centers and supposing for the sake of simplicity an ideal competitive electron donor with an equilibrium constant for Reaction 1 of $K_{eq} \to \infty$, one obtains for the average O_2 yield per flash, $M_1(c_D, n_f)$:

$$M_1(c_D, n_f) = \frac{1}{n_f} [n_f \cdot c_{II} - m_e \cdot c_D] \qquad (2)$$

Eqn 2 is valid only if the deficiency of trapped holes of dark-adapted chloroplasts which was found to be approx. 1 trapped hole per System Y can be neglected. In the present experiments, n_f was large enough to justify this approximation.

By introducing the normalization factor $[M_1(c_D = 0, n_f)]^{-1}$, the relative average O_2 yield per flash $\varphi(c_D, n_f)$ as a function of the donor concentration and of the flash number n_f gives:

$$\varphi(c_D, n_f) = 1 - \frac{m_e \cdot c_D}{n_f \cdot c_{II}} \qquad (3)$$

In contrast with a true System II inhibitor, in the presence of an ideal competitive electron donor, the relative average O_2 yield $\varphi(c_D, n_f)$, should depend strongly

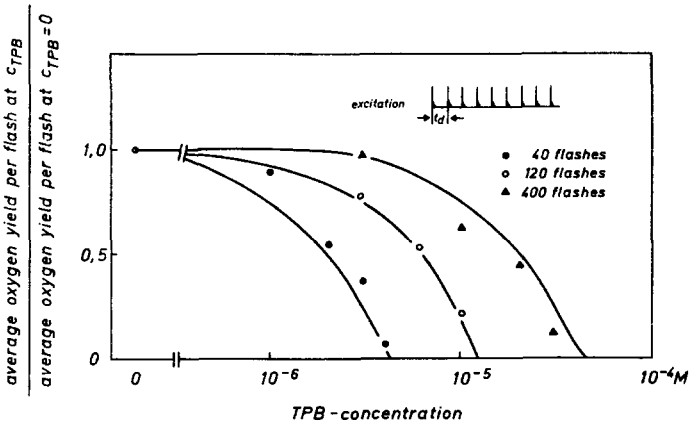

Fig. 1. Relative average O_2 yield per flash as a function of the tetraphenylboron (TPB) concentration and of the number of excitation flashes in normal chloroplasts. Experimental conditions as in Materials and Methods. The number of excitation flashes is indicated in the figure. The theoretical curves are computed according to Eqn 3 with $m_e = 1$.

on the number of the excitation flashes. In Fig. 1 the experimental results are compared with the theoretical curves evaluated according to Eqn 3 with $m_e = 1$. Though the correspondence between the experimental data and the theoretical curves is not very close, the results clearly show, that tetraphenylboron acts rather as a competitive electron donor than as an inhibitor of System II. Furthermore, the results support evidence for the conclusion, that tetraphenylboron acts as a 1-electron donor rather than as a 2-electron donor in chloroplasts. Preliminary titration experiments on Tris-washed chloroplasts also indicate a 1-electron donor function of tetraphenylboron. However, in order to be able to draw unequivocal conclusions about m_e, further investigations are required.

If tetraphenylboron really does act as an ideal competitive electron donor, the linear electron transport should not be impaired by tetraphenylboron and the O_2 evolution should reappear after the consumption of the tetraphenylboron donor.

Appropriate experiments were performed in the following way: chloroplasts after a dark incubation of 4 min, were excited with 100 flashes (t_d = 250 ms) followed by 4 min darkness, then again 100 flashes (t_d = 250 ms), 4 min darkness, and so on (see Fig. 2). The average O_2 yield per flash of each illumination period was measured. The results obtained are depicted in Fig. 3.

In the absence of tetraphenylboron a continuous decrease of the average O_2 yield per flash is observed due to the slow thermal destruction of the water-splitting enzyme system Y.

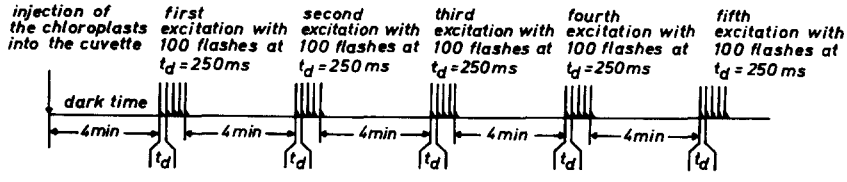

Fig. 2. Flash-light excitation conditions for the restoration experiments of the O_2 evolution.

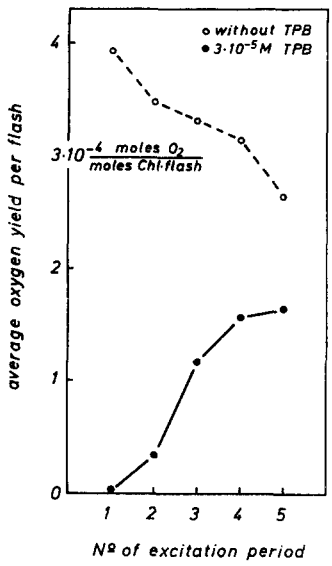

Fig. 3. Average O_2 yield per flash as a function of the number of the flash-light excitation period in the absence and in the presence of 30 μM tetraphenylboron (TPB) in normal chloroplasts. Experimental conditions as described in Materials and Methods. Excitation conditions as in Fig. 2.

By contrast, in the presence of 30 μM tetraphenylboron during the first illumination period, the O_2 evolution is totally suppressed, whereas in the following illuminations the O_2 production reappears.

The results unequivocally prove that tetraphenylboron acts as a competitive electron donor. However, tetraphenylboron does not act as an ideal competitive electron donor, because of the partial inhibition of the electron transport in System II as indicated by the nonperfect restoration of the O_2 evolution as compared with the control yield. This inhibitory effect may be due to the action of oxidation products of tetraphenylboron (see ref. 20).

The influence of tetraphenylboron on the non-cyclic electron flow as indicated by the 703 nm absorption change

The ability of tetraphenylboron to act as a System II electron donor assures that the number of electrons provided by Photosystem II under repetitive single-turnover flash light excitation conditions is not changed in the tetraphenylboron concentration range in which suppression of the O_2 evolution occurs. It has been discussed elsewhere [28, 29], that the amplitude of the 703 reduction kinetics after the oxidation of P700 by a single-turnover flash can be used as a measure of the number of electrons generated in System II and flowing through System I. Hence, the 703 nm amplitude should remain constant in the tetraphenylboron concentration range, where the O_2 evolution is significantly suppressed.

In Fig. 4, the relative amplitude of the 703 nm reduction kinetics as a function of the tetraphenylboron concentration is compared with the relative average O_2 yield per flash. The results clearly show, that the number of electrons produced by System II is not changed up to 0.1 mM tetraphenylboron, whereas the O_2 evolution

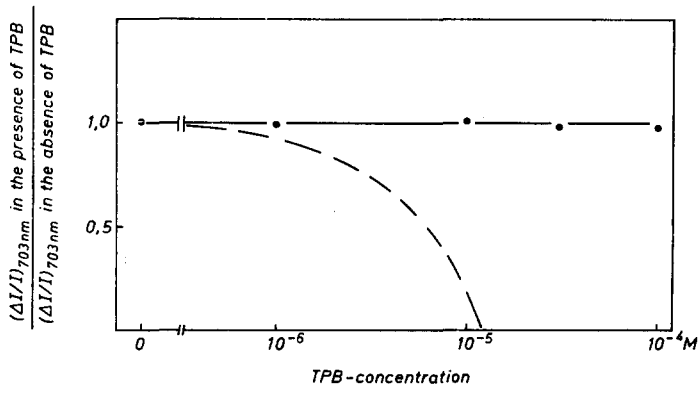

Fig. 4. Relative amplitude of the 703 nm reduction kinetics as a function of tetraphenylboron (TPB) concentration in normal chloroplasts. Experimental conditions as described in Materials and Methods. Excitation 128 flashes. The dotted curve is the relative average O_2 yield per flash for 120 flashes taken from Fig. 1.

is completely suppressed. In this way the donor function of tetraphenylboron in intact chloroplasts is proved.

The fact, that the electron donation by tetraphenylboron is totally inhibited by 2 μM DCMU, unequivocally proves that tetraphenylboron acts as a System II electron donor.

The next problem arising in respect to tetraphenylboron acting as an electron donor strongly competing with water, is the question of whether the functional integrity of the water-splitting enzyme system Y is required for the ability to donate electrons to System II.

To clarify this question, experiments were performed with Tris-washed chloroplasts characterized by a complete loss of the O_2-evolving capacity. In Fig. 5, the relative amplitude of the 703 nm reduction kinetics is depicted as a function of the tetraphenylboron concentration. It is seen, that the amplitude of the 703 nm absorp-

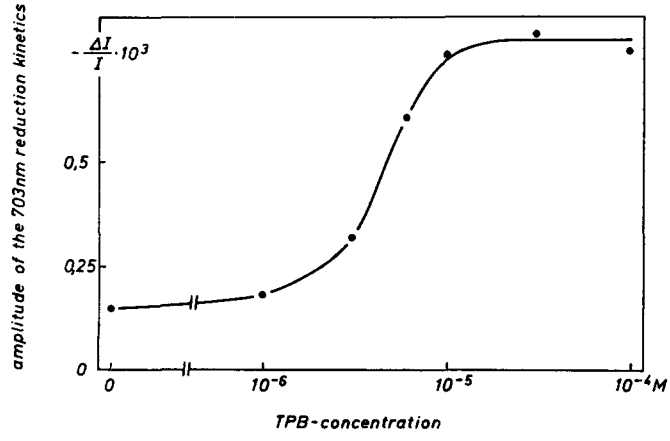

Fig. 5. Amplitude of the 703 nm absorption change as a function of the tetraphenylboron (TPB) concentration in Tris-washed chloroplasts. Excitation, 128 flashes. Other experimental conditions as described in Materials and Methods.

tion change under repetitive-flash light excitation increases with increasing tetraphenylboron concentrations, indicating an electron donation of tetraphenylboron in Tris-washed chloroplasts (the small amplitude of the 703 nm reduction kinetics at $c_{TPB} = 0$ is caused by intrinsic electron donors, see refs 30 and 31).

Hence, it is concluded, that the functional integrity of the water-splitting enzyme system Y is not required for the electron donation of tetraphenylboron to System II. That means, the higher-trapped-hole accumulation state S_4 is not an indispensible precursor for the tetraphenylboron oxidation.

The tetraphenylboron-mediated electron transport in Tris-washed chloroplasts is DCMU sensitive in practically the same way as has been reported above for normal chloroplasts. This indicates that tetraphenylboron acts as a System II electron donor in Tris-washed chloroplasts.

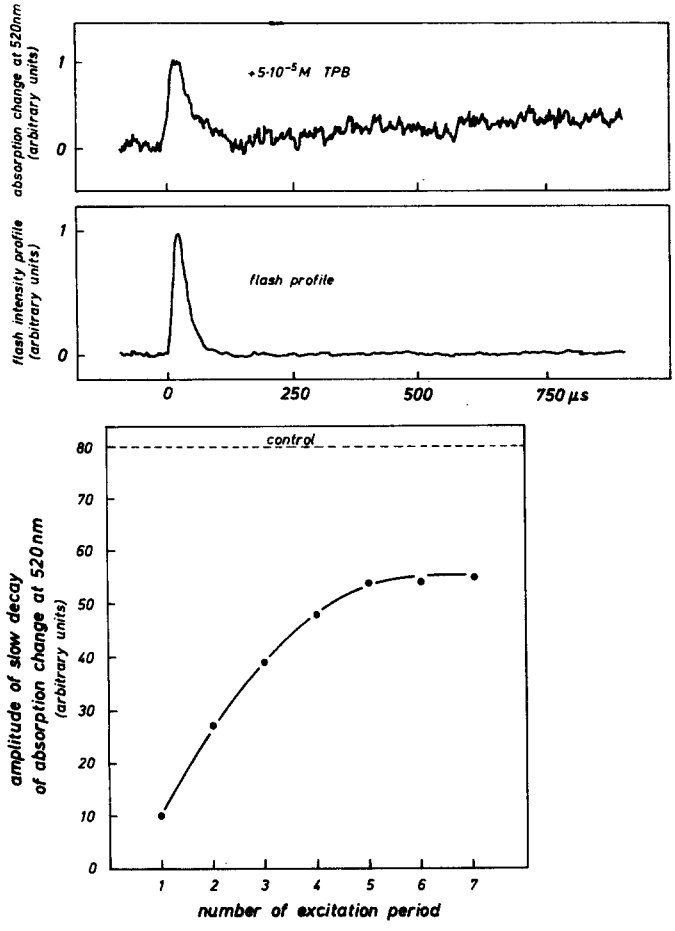

Fig. 6. The influence of tetraphenylboron (TPB) on the decay of the field indicating absorption change at 520 nm. (a) Comparison between the time course of the 520 nm absorption change in the presence of 50 μM tetraphenylboron and of the flash profile. (b) Amplitude of the slow decay of the absorption change at 520 nm as a function of the number of the flash-light excitation period in the presence of 20 μM tetraphenylboron in normal chloroplasts. Excitation, 64 flashes per illumination period. Other experimental conditions as described in Materials and Methods.

The action of tetraphenylboron as a permeable ion across the thylakoid membrane

Because the tetraphenylboron anion is reported to be permeable through lipid membranes [34–36]*, the ion should accelerate the electrical field decay across the thylakoid membrane as indicated by the 520 nm absorption change [37, 38]. Taking into account the ability of tetraphenylboron to act as a System II electron donor, in respect to a tetraphenylboron anion-induced electrical field decay, one would expect that tetraphenylboron exerts only a temporary effect, when Photosystem II is active.

Fig. 6a shows, that in the presence of 50 μM tetraphenylboron, the decay of the field indicating 520 nm absorption change becomes so fast, that a simple separation from the Type I decay, which has been attributed to an energy transfer indicating absorption change of carotenoids [39, 40] is impossible, i.e. under these conditions the field seems to decay in < 100 μs.

Further investigations are required in order to clarify the details of this effect.

If, however, by successive illumination periods (in the same way as is described for the O_2 evolution, see Figs 2 and 3), tetraphenylboron is consumed, the normal slow decay kinetics of the 520 nm absorption change reappear, as is shown in Fig. 6b. A complete restoration of the 520 nm amplitude was not observed due to the inhibitory effect probably caused by the oxidation products of tetraphenylboron, as has

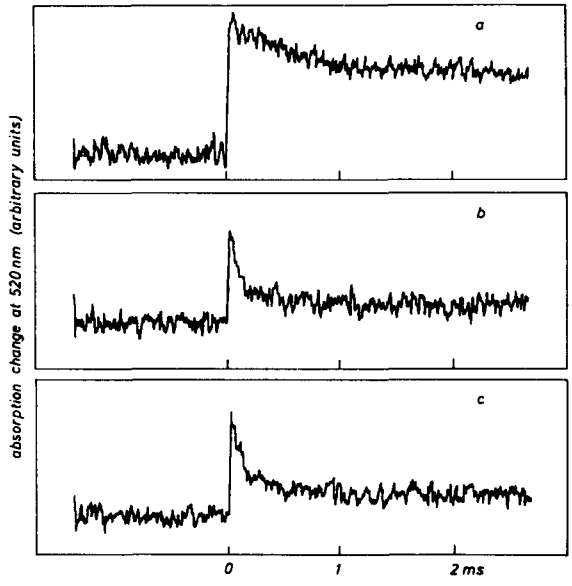

Fig. 7. Absorption change at 520 nm in DCMU-inhibited chloroplasts with DCIP-mediated cyclic electron transport around System I in the absence (a) and in the presence (b and c) of 4 μM tetraphenylboron. Reaction mixture: chloroplasts (10 μM chlorophyll), 27 μM DCIP, 20 mM NaCl, 5 mM $MgCl_2$ and 50 mM *N*-tris(hydroxymethyl)methylglycine (Tricine)–NaOH, pH = 7.2. Optical pathlength 20 mm, excitation, 32 flashes per illumination period. In Fig. 7c a preillumination of 320 flashes, corresponding to 10 illumination periods, was given, so that Fig. 7c represents the 520 nm absorption change of the 11th illumination period.

* See note added in proof.

been already shown for the O_2 restoration experiments (see Fig. 3). On the other hand, a totally different pattern should arise, if the System II activity is blocked, e.g. by DCMU, and the System I electron transport is supported by a cycle induced by 30 μM DCIP. Under these circumstances, tetraphenylboron should not be consumed and a permanent effect on the electrical field should be observed. The results obtained are shown in Fig. 7. It is clear that the tetraphenylboron-induced field decay remains constant during the different flash-light illumination periods.

DISCUSSION

The results obtained in the present study show, that tetraphenylboron acts as a System II electron donor, in agreement with earlier findings [20]. This electron donation was found to be responsible for the suppression of the O_2 evolution. The results of Fig. 3 unequivocally prove that tetraphenylboron acts as a competitive electron donor which does not destroy the water-splitting enzyme system Y. Since for the photosynthetic water oxidation the sequential accumulation of four positive charges (which is realized in a special storage device, see ref. 41) is required [32, 33], generally two different types of competitive electron donation by tetraphenylboron can be distinguished: (A) tetraphenylboron could directly compete with water for the trapped-hole accumulation state S_4 which is required for the water oxidation [32, 33]. (B) Tetraphenylboron could react either with a trapped-hole accumulation state of the water-splitting enzyme system Y lower than S_4, or directly with the primary electron donor of Photosystem II.

For the realization of Mechanism A, the function of the intact water-splitting enzyme system Y is indispensible. However, the present study clearly shows, that tetraphenylboron is able to act as a System II electron donor in Tris-washed chloroplasts which have completely lost their O_2-evolution activity. Hence, tetraphenylboron probably does not interfere with the last step (or steps) of the water oxidation. It seems rather, that tetraphenylboron is reacting with the lower-trapped-hole accumulation state S_1 (which is characterized by different redox potential properties in comparison to S_2 and S_3, see ref. 42) or directly with the primary electron donor of Photosystem II. This mode of action of tetraphenylboron as a competitive electron donor according to Mechanism B, prevents the generation of S_4 also in intact water-splitting enzyme systems until tetraphenylboron is irreversibly consumed by the oxidation process.

It should be mentioned, that in contrast to the ADRY-agent-type catalyzed discharge of the States S_2 and S_3 by internal electron donors [29, 43], tetraphenylboron acts as an external competitive electron donor for the discharge of either free or trapped holes (see ref. 41) of System II. Preliminary flash group experiments indicate, that the discharge of System II holes by tetraphenylboron is fast (< 5 ms).

The action of tetraphenylboron as a competitive electron donor of System II, explains the differences found by Homann [20] for the dependencies of the fluorescence rise restoration in DCMU-blocked chloroplasts and of the Hill reaction rate, respectively, on the tetraphenylboron concentration. Whereas under ideal conditions the discharge of only one positive charge per electron-transport chain should be sufficient to prevent the reoxidation of the reduced primary electron acceptor by the oxidizing side of System II (which is reflected by the variable fluorescence), much more tetra-

phenylboron is required for the suppression of the O_2 evolution during a 1-min illumination period, which leads to the generation of numerous holes per System II.

The incomplete reappearance of the O_2-evolving capability, as well as of the slow phase of the 520 nm absorption change after the consumption of tetraphenylboron indicates, that an inhibitory effect is exerted on System II which is possibly caused by the oxidation products of tetraphenylboron. According to Homann [20], the inhibitory effect includes also the System II electron transport mediated by artificial electron donors. Hence, the oxidation products of tetraphenylboron seem to block close to the primary reactions of System II. The measurements of the 520 nm absorption change show, that tetraphenylboron strongly accelerates the decay of the electrical field across the thylakoid membrane which is generated by the primary events of photosynthesis [44]. This acceleration could be caused by tetraphenylboron, either in a direct way via the transport of the negatively charged tetraphenylboron anion through the thylakoid membrane, or in an indirect manner by a modification of the membrane structure thereby changing its permeability for other ions.

An irreversible enhancement of the membrane permeability by tetraphenylboron-induced structural changes can be excluded, because the effect on the electrical field decay is transitory if System II is active but permanent in System II-blocked chloroplasts. Hence, the field decay can be caused either by a tetraphenylboron-mediated transport of other ions, or by the transport of the tetraphenylboron anion itself. Since the tetraphenylboron anion is known to be permeable through artificial [32] and chloroplast [34] membranes, we interpret our results in terms of a field-induced rapid tetraphenylboron anion transport through the thylakoid membrane accompanied by a decay of the electrical field.

If one assumes, that the site of the generation and of the storage of System II holes is located near the inner side of the thylakoid membrane, the field-induced tetraphenylboron transport leads to an accumulation of tetraphenylboron at the inner side of the thylakoid. Hence, the facilitated transport of the reactant (tetraphenylboron) to the reactive centers of System II favors the oxidation rate of tetraphenylboron. In this way the powerful action of tetraphenylboron can be explained by three factors: (1) the permeability of the tetraphenylboron anion makes a field-induced transport to the reaction centers possible. (2) The ability of tetraphenylboron to be oxidizable, results in its donor function to System II. (3) The oxidation products irreversibly inhibit, possibly via a radical mechanism (phenyl radical?), the System II electron transport.

NOTE ADDED IN PROOF

Recently the diffusion coefficient for the transport of tetraphenyl-boron anion through artificial phospholipid membranes has been measured as to be $D = 4 \cdot 10^{-12}$ cm^2/s (Ref. 45).

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