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ACTIONS OF TETRAPHENYLBORON ON THE ELECTRON FLOW IN PHOTOSYSTEM II OF ISOLATED CHLOROPLASTS

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SUMMARY

Isolated chloroplasts from pokeweed (*Phytolacca americana*) were used in studies of the actions of tetraphenylboron on photosynthetic electron transport. Any effects on sites which couple electron transport to photophosphorylation, could be eliminated by the use of uncoupled chloroplasts.

Very low concentrations of tetraphenylboron were found to strongly inhibit the reoxidation in the dark of the reduced primary acceptor Q in chloroplasts poisoned with 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Apparently, the tetraphenylboron anion has a high affinity to the oxygen evolving reaction complex where it is readily photoxidized.

Slightly higher concentrations of tetraphenylboron inhibited steady-state rates of oxygen evolution measurably, and induced rapid and irreversible photodestructions of Photosystem II. System I mediated electron transport was not affected by these photoinhibitory processes.

In many respects, the effects were similar to those observed in the presence of desaspidin or carbonylcyanide *m*-chlorophenylhydrazone. However, much lower concentrations of tetraphenylboron were required, and its actions were more restricted to Photosystem II. These properties may make tetraphenylboron a valuable tool in studies on the reaction mechanism of the oxygen-evolving apparatus.

INTRODUCTION

Tetraphenylboron is widely used as analytical agent in determinations of potassium in aqueous solutions, because it binds this alkali ion rather specifically to form a salt with a very low solubility[1,2]. In biological systems, tetraphenylboron has also been used in attempts to selectively affect K^+-dependent processes. PACKER and co-workers[3,4] have studied its effect on energy-transfer reactions in chloroplasts and mitochondria. They observed that tetraphenylboron was a powerful inhibitor of electron-transport-dependent phosphorylation. In chloroplasts, very small concentra-

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; EGTA, ethylenebis(oxyethylenenitrilo) tetraacetate; F_o, the invariable fluorescence, F_{DCMU} and F_{red}, the fluorescence emission in the presence of DCMU or hydrosulfite, respectively; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; Q, the primary electron acceptor in Photosystem II, the "quencher".

tions of this agent prevented light-induced volume changes, and destroyed proton gradients across the thylakoid membranes. Since an addition of K^+ did not reverse the effects of tetraphenylboron, the investigators concluded that it did not act by preventing K^+ from participating in some process essential to phosphorylation. They suggested[4] that tetraphenylboron might have disturbed the normal function of the organelle membranes by binding to exposed positive charges as provided, for example, by protonated amino groups of structural proteins.

Recent research on the problem of photosynthetic oxygen evolution has further supported the contention that this process, like phosphorylation, is very intimately dependent on the structure in which it occurs (see ref. 5 for review). Consequently, it was of interest whether tetraphenylboron would affect the photosynthetic oxidation of water in isolated chloroplasts.

It will be shown in this paper that low concentrations of tetraphenylboron rather specifically interfered with electron transport in the oxygen-evolving photosystem of isolated chloroplasts. The site and mode of action appeared to be quite similar to those of certain electron donors to System II, and of the two uncouplers carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and desaspidin[6]. Tetraphenylboron, however, was effective at concentrations lower than those required of any other agent.

MATERIALS AND METHODS

Chloroplast preparations

Chloroplasts were prepared from pokeweed (*Phytolacca americana*) leaves. Normal coupled chloroplasts, and chloroplasts uncoupled by washing with 0.5 mM EDTA, were prepared as previously described[6]. For reasons unrelated to the subject of this present work, we often replaced EDTA by ethylenebis(oxyethylenenitrilo) tetraacetate (EGTA). No difference was noted in the uncoupling action of these two chelators.

"Cl^--deficient" chloroplasts were obtained by the method of IZAWA *et al.*[7]. Artificial inactivations of Photosystem II were achieved by either washing with 0.8 M Tris–HCl buffer (pH 8.0), or by a washing with 5 mM Tricine in the absence or presence of 0.5 mM EGTA at pH 9.3, or, in some cases, by incubating the chloroplasts for 6 min in the presence of 1 mM hydroxylamine as described by CHENIAE AND MARTIN[8]. A partial restoration of the oxygen evolving activity by ascorbate and 2,6-dichlorophenolindophenol (DCIP)[9] was possible only with Tris washed, but not with hydroxylamine-treated[8] or pH-shocked chloroplasts.

The final suspension medium for the chloroplasts, and the reaction media, contained: 400 mM sucrose, 50 mM Tricine–NaOH (pH 7.4), 10 mM NaCl, and 5 mM $MgSO_4$. NaCl was omitted from all solutions used for Cl^--deficient chloroplasts.

Measurements

The methods used for measuring oxygen evolution, $NADP^+$ photoreductions, fluorescence characteristics, and chlorophyll concentrations, were those described earlier[6,10]. The absorbance decrease during DCIP photoreductions was recorded continuously using a setup in which actinic light from a 1000-W projection lamp was passed through a cell with running tap water, several glass lenses, a red plastic sheet

and, finally, a 650-nm long-wave pass filter (Optics Technology); our weak measuring illumination at 595 nm was selected with a Bausch and Lomb monochromator, detected by a Hamamatsu 1P21 phototube, and amplified by an Aminco microphotometer. All measurements were carried out at room temperature (25°).

For calculations of the concentration of DCIP in our reaction media we used an extinction coefficient of $1.9 \cdot 10^4$ $l \cdot cm^{-1} \cdot mole^{-1}$ at 595 nm. When saturated with air, our chloroplast suspensions were assumed to contain 0.26 μmole O_2 per ml at 25° (ref. 11).

Reagents

The chemicals were of reagent grade. From the available brands, only Tricine from Calbiochem was found to have a Cl^- content low enough for use in the preparations of Cl^--deficient chloroplasts. Tetraphenylboron was purchased as sodium salt, purissimum, from Aldrich Chemical Comp., CCCP and valinomycin were obtained from Calbiochem, and desaspidin, phloridzin, antimycin A, and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) were kindly provided by Dr. H. Gaffron. The Hoffmann–LaRoche antibiotic X464 was generously supplied by Dr. R. L. Harned from the Commercial Solvents Corp. Supposedly this compound is closely related, or even identical to nigericin[12,13]. Solutions of $Na_3Fe(CN)_6$ were prepared by passing solutions of the potassium salt through a column of Dowex 50 in its Na^+ form.

RESULTS

Effect of tetraphenylboron on electron transport in unpoisoned chloroplasts

After having read the statement by HORTON AND PACKER[1] that "under noncyclic electron flow conditions ferricyanide reduction is unaltered" by 0.07 to 13.3 μM

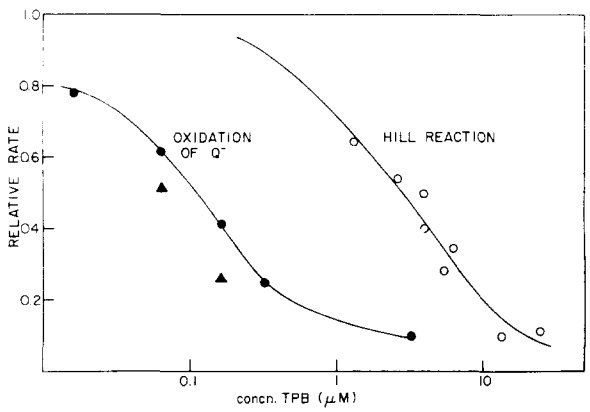

Fig. 1. Inhibition by tetraphenylboron (TPB) of the oxygen evolution during a Hill reaction, and of the reoxidation in the dark of Q^-. See MATERIALS AND METHODS for procedures and reaction mixtures. ○, oxygen evolution with 0.4 mM $Na_3Fe(CN)_6$ as Hill oxidant, and EGTA-washed chloroplasts (5 μM chlorophyll) in 120000 ergs·sec^{-1}·cm^{-2} red light (between 600 and 700 nm); rate in absence of tetraphenylboron (160 μmoles·mg^{-1} chlorophyll·h^{-1}) was set equal unity. ●, ▲, relative rate of reoxidation of Q^- given as restored area over the fluorescence induction curve (see Fig. 2) after 2 sec darkness relative to that measured with chloroplasts in the absence of tetraphenylboron; coupled (●) or EGTA-washed (▲) chloroplasts (7 μM chlorophyll) in the presence of 10 μM DCMU; fluorescence excited with 1 nEinstein·sec^{-1}·cm^{-2} green light (λ_{max} = 530 nm).

tetraphenylboron, it came as a surprise that in our experiments 10 μM tetraphenylboron almost completely eliminated oxygen evolution in EGTA-washed chloroplasts (Fig. 1). Furthermore, basal electron flow to ferricyanide in coupled chloroplasts was slowed down by 35% when 3 μM tetraphenylboron had been added. In this respect, the action of tetraphenylboron differed from that of compounds like CCCP which, in moderate concentrations, inhibit the Hill reaction in uncoupled, EDTA washed chloroplasts, but accelerate electron flow in coupled chloroplasts due to their uncoupling activity[6,14].

When the photoreduction of DCIP was followed in the presence of small amounts of tetraphenylboron, only a slight inhibition of the initial rate was noted. However, this inhibition became progressively more severe during the light period. No effect was observed on the System I-mediated photoreduction of $NADP^+$ by the ascorbate–DCIP couple. Methyl viologen could not be used as electron acceptor because it formed a precipitate with tetraphenylboron.

TABLE I

EFFECT OF TETRAPHENYLBORON ON SYSTEM I ACTIVITY AND ON FLUORESCENCE INTENSITY

For procedure see text; reaction mixtures: for DCIP reduction in 2.5 ml, EGTA-washed chloroplasts (7 μM chlorophyll), 40–80 μM DCIP, 70 mM phosphate buffer (pH 7.1), 1.7 μM plastocyanine; for $NADP^+$ reduction in 3.2 ml, further addition of saturating amounts of ferredoxin, ferredoxin–$NADP^+$ reductase (200 units), 5 mM ascorbate, 10 μM DCMU, DCIP to a final concn. of 80 μM, and N_2 in the gas phase; for fluorescence measurements as for DCIP reduction, but 2.5 mM ascorbate added, and for recording F_{red} also 10 μM DCMU and some $Na_2S_2O_4$ crystals. Illumination 140000 ergs·sec^{-1}·cm^{-2} red light (between 625 and 725 nm) in the photoreductions, and 10 nEinsteins·sec^{-1}·cm^{-2} green light in fluorescence measurements. Rates given as μmoles reduced per mg chlorophyll per h, fluorescence intensities as percent of an unilluminated control.

Addition	*DCIP reduction*		*$NADP^+$ reduction*	F_{red}–F_o
	Initial	*After 1.5 min*		
None	345	345	122	95
7 μM tetraphenylboron	275	40	116	26

Table I gives the results of another test for any sensitivity of Photosystem I to tetraphenylboron. Here, uncoupled chloroplasts were illuminated first in the presence of the Hill oxidant DCIP. In the sample containing tetrephenylboron, the rate of DCIP photoreduction had considerably decreased after 1.5 min, while it remained unchanged in the control. Now, ferredoxin and ferredoxin–$NADP^+$ reductase, ascorbate, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), $NADP^+$ and more DCIP were added, and the rate of $NADP^+$ photoreduction was measured. Alternately, the residual unreduced, blue DCIP was bleached by addition of some ascorbate so that the fluorescence of the chloroplasts could be recorded. Two main results are evident from the data in Table I: First, the light-dependent inhibition of the DCIP photoreduction in the presence of tetraphenylboron was accompanied by a significant loss of the "live"[15] chloroplast fluorescence (F_{red}–F_o). This indicated a considerable photodestruction of the reaction center complex in Photosystem II. Second, the rate of the $NADP^+$ photoreduction was not affected by this impairment of the electron-transport system.

Photoinhibitions of the kind just described occur in chloroplasts when the normal operation of the oxygen-evolving System II is disturbed by lack of Cl^- (ref. 7), by Tris washing[6], by a manganese deficiency[16], or by the presence of hydroxylamine derivatives[8], of CCCP[6], or of desaspidin (unpublished results).

In an earlier report[6] we have presented data which showed that the presence of CCCP in suspensions of uncoupled chloroplasts not only affected electron-transport events in Photosystem II, but also in System I. Most conspicuous after an addition of CCCP was the increased efficiency of far-red light in the reoxidation of Q^-, the reduced primary electron acceptor of System II. In analogous experiments we were unable to detect any such effect of tetraphenylboron, while the results with desaspidin showed certain similarities to those obtained with CCCP.

Effect of tetraphenylboron on the reoxidation of Q^- in DCMU-poisoned chloroplasts

In normal, DCMU-poisoned chloroplasts, an actinic illumination of Photosystem II is capable of mediating a photoreduction of the primary electron acceptor Q with concomitant oxidation of some electron donor on the oxygen-evolving side of the system. This event is reflected by an increase in the fluorescence yield from the chloroplasts. A reoxidation of Q^-, and consequently a decay of this high fluorescence yield, may occur through a rather slow autoxidation, or through a cyclic flow of electrons back to oxidized electron carriers in the water-oxidizing reaction complex. Such back reactions in System II have been studied recently by several investigators[6,17–20]. Certain artificial electron donors to System II, and the uncoupler CCCP, retard the cyclic reoxidation of Q^- (refs 6, 18), presumably by competing successfully with Q^- in the reduction of stored oxidants.

Because of the observed similarities in the actions of CCCP and tetraphenylboron on Photosystem II, it was of interest whether the latter compound would also inhibit the reoxidation of Q^-. Fig. 2 shows that an addition of small amounts of tetraphenylboron indeed strongly decreased the decay rate of the high fluorescence yield in DCMU-poisoned chloroplasts: 0.10 μM tetraphenylboron retarded the reoxidation of Q^- in EGTA-washed chloroplasts by more than 50 % (Fig. 1). As in the

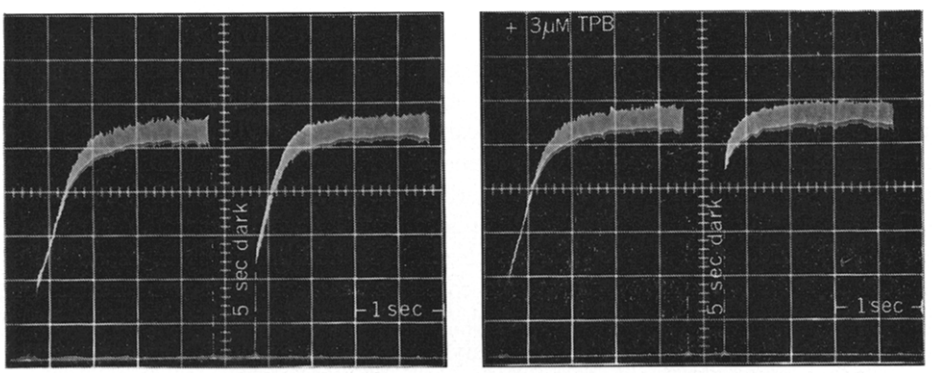

Fig. 2. Effect of 3 μM tetraphenylboron (TPB) on the dark restoration of the fluorescence induction in DCMU-poisoned chloroplasts. Reaction mixture as described in MATERIALS AND METHODS, coupled chloroplasts (8 μM chlorophyll); 5 sec darkness between the two recordings in each picture, time scale 0.5 sec per division. Illumination 1 nEinstein·sec $^{-1}$·cm $^{-2}$ green light.

experiments of UTSUMI AND PACKER[3], this inhibition could not be reversed by an addition of K^+.

Photoxidation of tetraphenylboron by chloroplasts

For some time we considered tetraphenylboron to be an unlikely electron donor to System II of the chloroplasts. The agent was not destroyed oxidatively by excess ferricyanide or manganipyrophosphate at pH 7. Furthermore, well established electron donors to Systems II such as 1,5-diphenylcarbazide, hydroxylamine and Mn^{2+} were much inferior to tetraphenylboron in their inhibitory activity on the re-oxidation of Q^- in DCMU poisoned chloroplasts (Table II).

TABLE II

EFFECTS OF VARIOUS COMPOUNDS ON THE DARK REGENERATION OF THE FLUORESCENCE RISE IN DCMU-POISONED CHLOROPLASTS

The regeneration of the fluorescence induction in DCMU-poisoned chloroplasts (7–8 μM chlorophyll) after 2 sec darkness was taken as a measure for the reoxidation of Q^- (see ref. 6). Experimental conditions see legend Fig. 2. The numbers in parentheses give the concentration of the added compound in μM. Inhibition given as percent relative to an appropriate control without addition.

Reaction mixture	*Inhibition* (%)	*Reaction mixture*	*Inhibition* (%)
Coupled chloroplasts		*EGTA-washed chloroplasts*	
+ methylamine (16000)	0	+ CCCP (0.6)	50
+ phloridzin (500)	0	+ desaspidin (0.3)	50
+ atebrin (18)	0	+ tetraphenylboron (0.07)	50
+ HOQNO (15)	0	+ 1,5-diphenyl-carbazide (500)	$\leqslant$ 10
+ X464 (25), KCl (10000)	0	+ $MnSO_4$ (1000)	$\leqslant$ 10
+ valinomycin (25), KCl (10000)	0	*Tris-washed chloroplasts*	
+ antimycin A (18)	35	+ 1,5-diphenylcarbazide (500)	50
EGTA-washing	$\leqslant$ 10	+ $MnSO_4$ (1000)	40
+ hydroxylamine (10)	40		

Earlier[6] we had discussed the possibility that compounds like CCCP, desaspidin and tetraphenylboron might act on Photosystem II by interfering with membrane-dependent ion movements. However, our data in Table II do not reveal any obvious correlation between known actions of various phosphorylation inhibitors on ion transport, and their observed activity as inhibitors of the cyclic electron flow from Q^- to the oxidizing side of System II.

It was essential, therefore, to test whether tetraphenylboron was, after all, capable of serving as electron donor to Photosystem II. To our great surprise we could deduce from fluorescence measurements that chloroplast preparations with an inactivated oxygen-evolving system (pH shock, Tris washing, hydroxylamine treatment, Cl^- depletion) regained their ability to accumulate photoreduced Q after addition of tetraphenylboron. While this observation might be explained by the inhibited cyclic reoxidation of Q^- described above, a light-induced flow of electrons from tetraphenylboron to Q was at least as probable. Indeed, no inhibitory effects on chloroplasts were observed in suspension media from which another batch of

chloroplasts had been removed by centrifugation after a 3-min illumination in the presence of 20 μM tetraphenylboron. The inhibitor obviously had been photoinactivated.

Fig. 3 provides conclusive evidence for an oxidation of tetraphenylboron by chloroplasts in the light. In Fig. 3A the course of DCIP photoreduction by hydroxylamine-treated chloroplasts is shown before and after addition of tetraphenylboron or 1,5-diphenylcarbazide. In the presence of the latter electron donor one notices the well known regeneration of a relatively fast photoreduction of DCIP[21]. When tetraphenylboron was added instead, a high initial reduction rate was observed which declined rapidly and did not recover after further additions of either tetraphenylboron or 1,5-diphenylcarbazide. This observation demonstrated the electron donating, as well as the previously mentioned photodestructive activity of tetraphenylboron. Not included in Fig. 3A is a trace showing that this photoinhibition of System II was only slightly retarded when 1,5-diphenylcarbazide was also present in the reaction mixture.

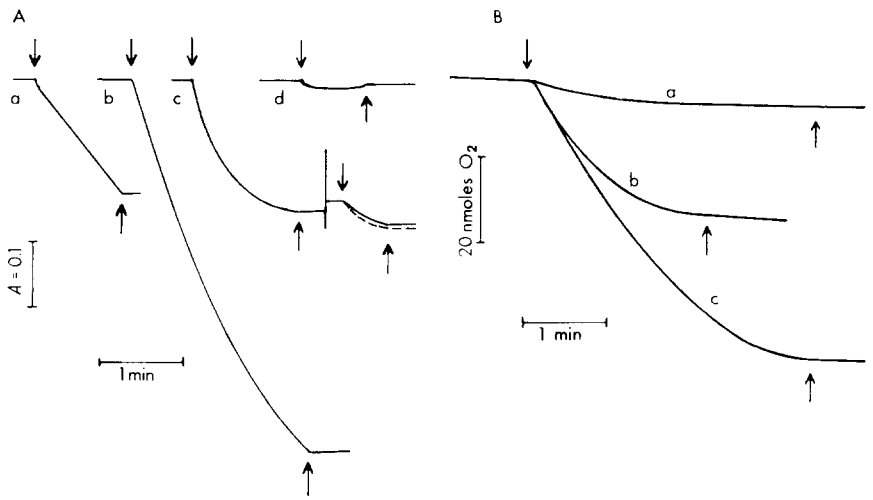

Fig. 3.A. Photoreduction of DCIP by hydroxylamine-treated chloroplasts. Procedure and reaction mixture see MATERIALS AND METHODS; 12.5 μM chlorophyll, 35 μM DCIP; 140 000 ergs·sec^{-1}·cm^{-2} red light (between 625 and 725 nm); downward arrows, light on; upward arrows, light off. a, no addition; b, + 500 μM 1,5-diphenylcarbazide; c, + 30 μM tetraphenylboron, then further addition of 30 μM tetraphenylboron or 500 μM 1,5-diphenylcarbazide (broken line) after first light period; d, + 30 μM tetraphenylboron + 10 μM DCMU. B. Oxygen uptake by illuminated chloroplasts in the presence of tetraphenylboron. Coupled chloroplasts (43 μM chlorophyll) in 150 mM sodium phosphate buffer (pH 7.1) + 0.5 mg catalase; illumination 160 000 ergs·sec^{-1}·cm^{-2} red light (between 600 and 700 nm). a, no addition; b, + 50 nmoles tetraphenylboron; c, + 100 nmoles tetraphenylboron.

From Fig. 3B, finally, one can deduce that, in the absence of any added electron acceptor, the oxidation of one molecule of tetraphenylboron by illuminated chloroplasts was accompanied by an uptake of about half a molecule of oxygen. DCMU (10 μM) inhibited both types of photoxidations of tetraphenylboron, that by DCIP (Fig. 3A), and that by oxygen (not shown).

DISCUSSION

Our experimental results leave no doubt that tetraphenylboron exerts its effects on Photosystem II primarily because of its ability to react with accumulated oxidants in the water-splitting reaction complex. Most probably, none of the observed inhibitions of the backreaction in System II (Table II) occur through mechanisms other than a direct electron donation[6,18]. This conclusion implies that phosphorylation inhibitors like CCCP, desaspidin and antimycin A are subject to photodestruction by illuminated chloroplasts. The marked difference in the effectiveness of the various agents can be explained in several ways. In most cases we do not know the concentration of the active species in aqueous buffers. Moreover, their affinity to the thylakoids and to the oxidation sites, as well as their ease of penetration to the oxygen-evolving center may vary widely. The rate of binding of NH_2OH to oxidants in Photosystem II, for example, has been found to be relatively slow[22]. Artificial electron donors may also differ in their preference for the available oxidation sites.

Rapid photoinactivations of Photosystem II by added electron donors have been observed not only for tetraphenylboron, CCCP and desaspidin[6,23], but also for certain derivatives of NH_2OH (ref. 8). Since DCMU retards the progress of such photoinhibitions, they must be the direct and indirect consequence of a continuous formation of hitherto unknown oxidation products (radicals?) from the added agents.

Among all of the known electron donors to System II, tetraphenylboron appears to be quite unique: It has an exceptionally high affinity to oxidants in Photosystem II; it has no detrimental effects in complete darkness, and can be easily removed by washing; it is photooxidized specifically and in a stoichiometric reaction by System II; because of its considerable size, tetraphenylboron must be assumed to bind to accessible oxidants on the surface of the thylakoid membrane, unless the components of the water-splitting reaction complex are located in rather large membrane cavities.

In addition to these biological implications, the ability of illuminated chloroplasts to oxidize tetraphenylboron is certainly fascinating in itself. Most of the published work on oxidations of tetraphenylboron deals with its mechanism in organic solvents[1,24–26]. Under such conditions, biphenyl- and diphenylboronium ion have been shown to be the reaction products of the two-electron process[25]. Preliminary studies have revealed that, in a photoxidation with rose bengal as sensitizer in an aqueous solution, one mole oxygen was consumed per mole tetraphenylboron. No significant amounts of H_2O_2 were formed. It appears, therefore, that the mechanism of the chloroplast reaction is more closely related to the electrooxidation in acetonitrile[25] than to the dye-sensitized photoxidation. Studies are in progress to elucidate further the mechanism of the surprisingly strong interaction between tetraphenylboron and components of the oxygen-evolving center on the thylakoids.

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