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THE EFFECTS OF TETRAPHENYLBORON ON ENERGY-LINKED REACTIONS IN SPINACH CHLOROPLASTS

JAN W. T. FIOLET and KAREL VAN DAM

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)

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SUMMARY

1. The inhibitory action of tetraphenylboron, a lipid-soluble anion, on the proton uptake, the photophosphorylation and the light-induced quenching of the fluorescence of 9-aminoacridine by spinach chloroplasts was studied.

2. The inhibition of the three processes by tetraphenylboron was transient; the proton uptake was affected to a much smaller extent than either the photophosphorylation or the fluorescence quenching.

3. The inhibitory effects of tetraphenylboron on the proton uptake and the fluorescence quenching of 9-aminoacridine were qualitatively the same in CF_1-depleted chloroplasts, that were recoupled with *N,N'*-dicyclohexylcarbodiimide (DCCD).

4. The reversal of the fluorescence quenching of 9-aminoacridine upon addition of tetraphenylboron in the light was found to be very fast, being completed within the response time of the apparatus.

5. The presence of tetraalkylammonium salts in the incubation medium prevented the inhibitory effect of tetraphenylboron.

6. Tetraphenylboron disappeared from the chloroplast suspension in a light-dependent irreversible way; in the dark no 'uptake' of tetraphenylboron could be detected.

7. The effects of tetraphenylboron may be explained by the presence of groups with a high affinity for tetraphenylboron in the membrane; these groups become protonated upon illumination of the chloroplasts.

INTRODUCTION

Tetraphenylboron has long been known as a relatively specific analytical reagent for potassium[1,2]. For this reason it was thought to be useful as a tool for the study of ion movements across biological membranes. On the other hand tetraphenylboron is a lipid-soluble anion. Its energy-dependent uptake in submitochondrial particles and bacterial chromatophores was described and attributed to a mechanism

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; S_{13}, 5-chloro,3-*tert*-butyl,2'-chloro,4'-nitrosalicylanilide; FCCP, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone; MOPS, 2-(*N*-morpholino)propane sulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

in which the lipid-soluble anion migrates across the membrane in response to an electrical field generated by a primary H^+ pump[3–5].

In an attempt to elucidate the role of K^+ in oxidative phosphorylation, it was found that tetraphenylboron exhibited uncoupling activity in submitochondrial particles[6]. In illuminated chloroplasts it was shown, that addition of tetraphenylboron caused a collapse of monovalent cation gradients, in particular the pH gradient[7].

Because it was known that tetraphenylboron was able to bind to mitochondrial membranes resulting in extensive structural changes[8], it was suggested that tetraphenylboron reacted with free positively charged groups in the membrane, presumably amino groups[7]. This would result in changes of the permeability characteristics.

Recently it was reported that tetraphenylboron interfered with the oxygen evolving system in chloroplasts[9]. It was shown that tetraphenylboron acted as an electron donor for Photosystem II. At the same time it inhibited oxygen evolution. System I-mediated electron transport was reported to be unaffected by tetraphenylboron.

It has long been known that the light-induced proton uptake in chloroplasts is intimately connected with the energy conserving mechanism[10–12]. The energized state of chloroplasts can be read out by the fluorescence response of some acridine derivatives[13–16]. In this paper the relationship between the proton uptake and the energized state as monitored by the fluorescence quenching of 9-aminoacridine is described. It will be shown that both processes are affected by addition of tetraphenylboron, the proton uptake, however, to a much lesser extent.

MATERIALS AND METHODS

Intact chloroplasts were prepared in a short-time procedure: 25 g of deribbed spinach leaves were homogenized for 5 s in a Brown blender at maximal speed in 40 ml of an isolation medium consisting of 300 mM sucrose, 30 mM 2-(*N*-morpholino) propanesulphonic acid (MOPS), 3 mM $MgCl_2$, brought to pH 7.2 with NaOH. After filtration through four layers of perlon net (mesh width, 56 μm) the homogenate was centrifuged in an MSE centrifuge at $3000 \times g$ for 15 s. The pellet was resuspended in 1–2 ml of the isolation medium. EDTA–chloroplasts were prepared as described by McCarty[17]. The chlorophyll content was determined according to Whatley and Arnon[18]. The incubation medium contained 100 mM sucrose, 20 mM NaCl, 3 mM $MgCl_2$ and 3 mM Na_2HPO_4 unless otherwise stated; *N*-tris(hydroxymethyl)methyl glycine (Tricine) buffer (pH 8.0) was present at 1 mM in the experiments where proton uptake was measured or at 5 mM where phosphorylation was carried out. Phosphorylation and ATPase were continuously monitored as pH changes of the incubation medium; the electrode responses were calibrated with standard solutions of oxalic acid.

The fluorescence of 9-aminoacridine was measured with a modified Eppendorf fluorimeter. The exciting wavelength was 366 nm, the emission wavelength 430–460 nm.

Fluorescence and pH were measured simultaneously under continuous stirring in a cuvette with a volume of 1.5 ml, thermostatted at 25 °C and recorded on a multi-channel recorder (Rikadenki Kogyo). Side illumination was provided by a

quartz–iodine lamp, the light of which was passed through a 5-cm water filter and a red cut-off filter (Schott RG 1).

The pH was determined with a sensitive pH-measuring system (Electronic Instruments Ltd) including a micro glass electrode (GM 23/B), a calomel reference electrode *plus* remote junction (R.S.M. 23), a pH measuring unit (C 33 B-2) and a Vibron electrometer (33 B-2).

The 'uptake' of tetraphenylboron was determined as a change in electrical potential across an artificial black lipid membrane as described by Skulachev and co-

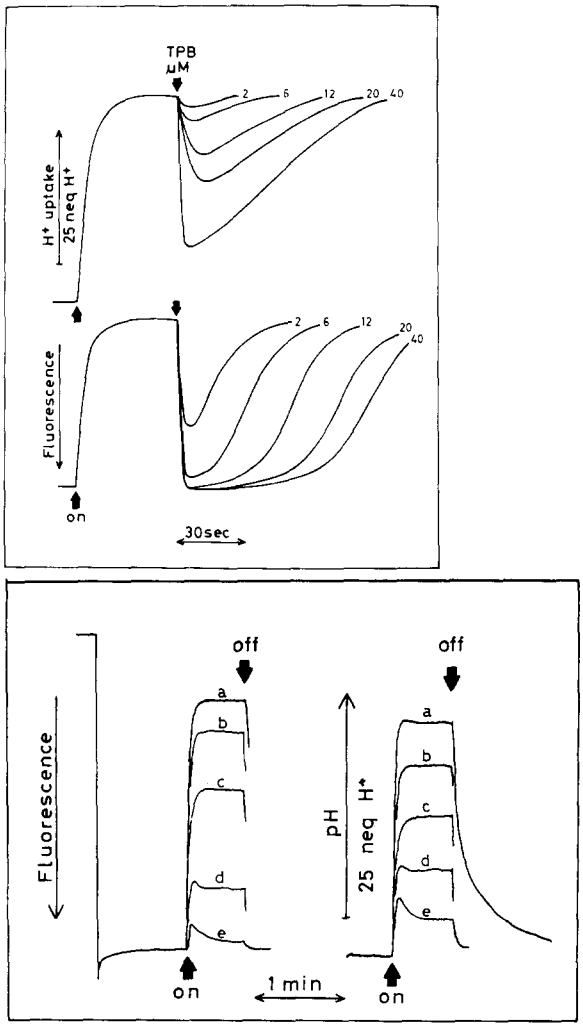

Fig. 1. (A) The effect of tetraphenylboron (TPB—) added in the light on the proton uptake and the fluorescence of 9-aminoacridine. The reaction mixture was as described under Materials and Methods. 10 μM pyocyanin was present. The chlorophyll content was 95 μg in a final volume of 1 ml. The concentration of 9-aminoacridine was 3 μM and tetraphenylboron was added at concentrations as indicated in the figure. (B) The effect of S_{13} on the proton uptake and the fluorescence of 9-aminoacridine, conditions as described in (A). The chlorophyll content was 100 μg in a final volume of 2.5 ml. a, no addition; b, 0.1 μM S_{13}; c, 0.2 μM S_{13}; d, 0.3 μM S_{13}; e, 0.4 μM S_{13}.

workers[3,4]. The artificial membrane was made from a 1% solution of egg lecithin *plus* 0.5% cholesterol in *n*-decane.

RESULTS

Horton and Packer[7] reported earlier that addition of tetraphenylboron to an illuminated chloroplast suspension causes a transient change in the pH of the suspending medium. The effect of tetraphenylboron, added in the light, on the proton uptake and the fluorescence quenching of 9-aminoacridine by spinach chloroplasts is depicted in Fig. 1A. The pH decreases transiently, confirming the observations of Horton and Packer[7], and also the fluorescence quenching is abolished transiently. The fluorescence quenching, however, is affected more extensively than the proton uptake: at 2 μM tetraphenylboron the reversal of the quenching is about 60%, whereas the proton uptake is hardly affected at this concentration. This is in contrast to the inhibitory effects of uncouplers such as 5-chloro-3-*tert*-butyl, 2'-chloro, 4'-nitro-salicylanilide (S_{13}) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCC CP), which inhibit both processes approximately to the same extent as is shown in Fig. 1B for S_{13}. Qualitatively similar effects of tetraphenylboron are observed when atebrin (2 μM) is used as the fluorescent probe, although the reversal of the quenching is less pronounced in this case.

It should be stressed that the reversal of the fluorescence quenching is a relatively fast process. The reversal upon addition of 6 μM tetraphenylboron was complete in less than 1.5 s, which is comparable to the rate of reversal of the quenching by addition of 1 μM S_{13}. This may reflect a high sensitivity of the energized state to tetraphenylboron, which is not seen by the proton uptake. More clearly the difference between the two processes in sensitivity to tetraphenylboron is shown in Fig. 2. 12 μM tetraphenylboron was added in the dark prior to illumination; proton uptake and fluorescence quenching were recorded at different light intensities. Onset of the light is followed immediately by a biphasic proton uptake. The extent of the fast

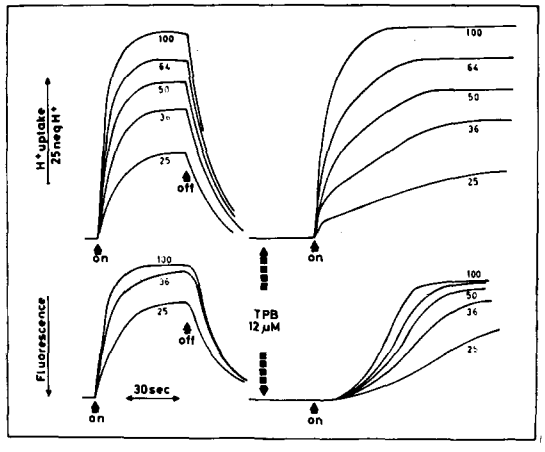

Fig. 2. Proton uptake and quenching of the fluorescence of 9-aminoacridine at different light intensities in the presence and absence of 12 μM tetraphenylboron (TPB—). Conditions as described under Fig. 1A. Saturating light intensity was set 100%.

phase is very much dependent on the light intensity. The quenching of the fluorescence of 9-aminoacridine only begins to appear when the fast phase of proton uptake is virtually complete. An apparent relationship between the slow phase of proton uptake and the time-course of the quenching may be seen. In the absence of tetraphenylboron (control curves) the extent of fluorescence quenching is not very sensitive to lowering the light intensity; at 36% of the initial intensity the fluorescence quenching is hardly affected, whereas at this intensity the proton uptake has been decreased considerably. Recently, a quantative relationship between the fluorescence quenching of 9-aminoacridine and the proton gradient in the steady state has been proposed by Schuldiner *et al.*[16]. The results of a calculation based on this model of the time-course of ΔpH, the proton gradient, is depicted in Fig. 3, assuming an osmotic volume of 20 μl per mg chlorophyll. The time-course of the proton uptake under these conditions is given as well. From the figure it is clear that the initial fast proton uptake in the presence of tetraphenylboron is not reflected in a proton gradient.

The relative insensitivity of the light-induced proton uptake to tetraphenylboron on the one hand and the high sensitivity of the energized state as read out by the fluorescence quenching, on the other hand, led us to investigate how tetraphenylboron affects other energy-dependent processes. The action of tetraphenylboron on phosphorylation and light-triggered ATPase is shown in Figs 4A and 4B, respectively. As can be seen the reversal of the quenching upon addition of tetraphenylboron in the light is paralleled by an immediate inhibition of ATP synthesis (Fig. 4A). The intimate relationship between the phosphorylation and the fluorescence quenching becomes clear if one considers the transient kinetics of the figure. At the lower tetraphenylboron concentrations the phosphorylation rate is ultimately completely restored. At the same time the fluorescence is quenched again to the

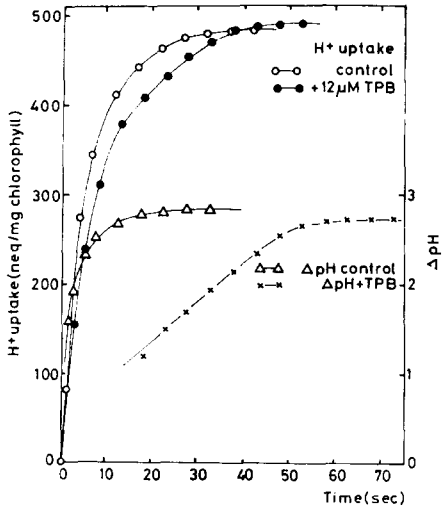

Fig. 3. Time-course of proton uptake and ΔpH in the presence and absence of 12 μM tetraphenylboron (TPB—) at saturating light intensity. Conditions as described under Fig. 1A. ΔpH was calculated according to Schuldiner *et al.*[16] assuming an osmotic volume of 20 μl per mg of chlorophyll.

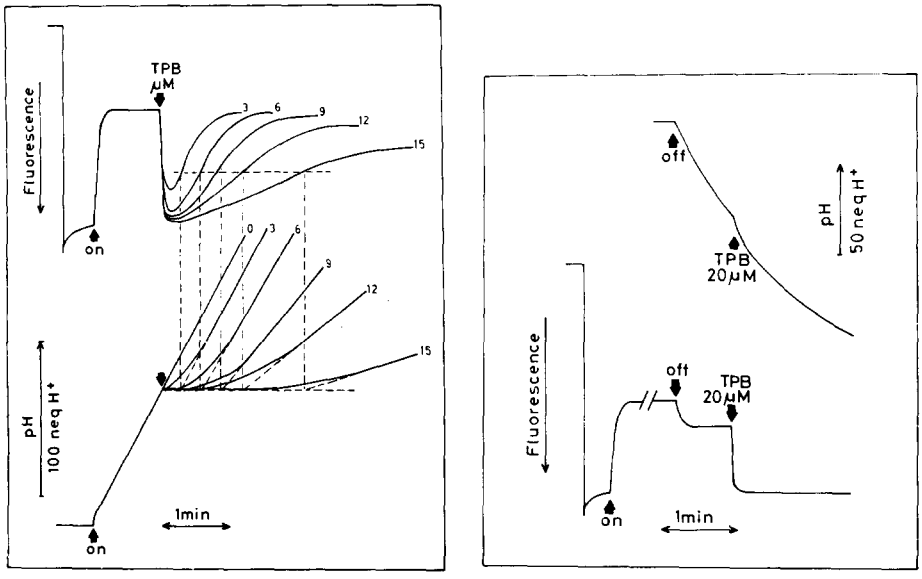

Fig. 4. (A) Transient uncoupling of cyclic phosphorylation by tetraphenylboron (TPB⁻). Conditions as described in Materials and Methods. 1 mM ADP was present. Tetraphenylboron was added at concentrations as indicated in the figure. (B) The effect of tetraphenylboron on the light-triggered ATPase. Conditions as described under (A), except that 2 mM dithioerythritol and 1 mM ATP were present instead of ADP.

original level. At the higher tetraphenylboron concentrations the final phosphorylation rate is lower, while the fluorescence is quenched to a smaller extent. Moreover, the level of fluorescence quenching is the same at the extrapolated times corresponding with the lag time in the phosphorylation at the different tetraphenylboron concentrations. It may be noted that the lag time is proportional to the concentration of tetraphenylboron added. Addition of tetraphenylboron in the dark under light-triggered ATPase conditions (Fig. 4B) results in complete reversal of the fluorescence quenching of 9-aminoacridine maintained in the dark. This reversal persists; no return of the quenching could be observed even after several minutes. The effect of addition of tetraphenylboron on the ATPase shows the relaxation curve, typical for uncouplers[19,20].

All the effects of tetraphenylboron reported so far, except the effect on light-triggered ATPase, exhibit a transient behaviour. It has been suggested that tetraphenylboron serves as an electron donor to Photosystem II (ref. 9). This would result in a decomposition of tetraphenylboron and presumably in the inactivation of the active uncoupling species. Therefore, the transient character of the tetraphenylboron effects should be completely light dependent and 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) sensitive. Fig. 5A shows that the inactivation is indeed light dependent; upon reillumination after a dark period during the inactivation process, the fluorescence of 9-aminoacridine is rapidly quenched up to the level reached before the dark period. After this fast phase the quenching proceeds with a rate identical to that in the control curve. In Fig. 5B it can be seen, that the in-

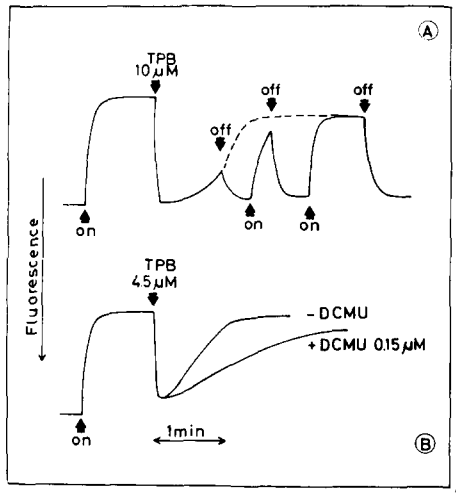

Fig. 5(A). Light dependency of the inactivation of tetraphenylboron (TPB—). Conditions as described under Fig. 1A. (B) DCMU sensitivity of the inactivation of tetraphenylboron. Conditions as described under Fig. 4A.

activation of tetraphenylboron is DCMU sensitive. At 0.15 μM DCMU the fluorescence becomes quenched again only after prolonged incubation in the light; at higher concentrations of DCMU (1 μM) the 9-aminoacridine fluorescence does no longer return to the quenched state, but under these conditions both the cyclic phosphorylation with pyocyanin as a cofactor and the fluorescence response of the probe are also severely inhibited by DCMU.

Decomposition of tetraphenylboron in the light should be detected as an irreversible 'uptake' of tetraphenylboron by illuminated chloroplasts. The 'uptake' of tetraphenylboron was determined by a technique essentially as described by Skulachev and coworkers[3,4], making use of an artificial black lipid membrane system, functioning as a tetraphenylboron electrode. It was found that hardly any 'uptake' occurred in the dark. Upon illumination up to about 95% of the tetraphenylboron disappeared slowly from the incubation medium. The 'uptake' was only slightly reversible upon darkening. It should be mentioned that no conclusions could be drawn from the kinetics of the 'uptake' because of the relatively high response time of the measuring system.

As to the mechanism of action of tetraphenylboron, it has been suggested that tetraphenylboron would bind with a high affinity to amino groups in the membrane[7]. To test this, we added tetraethylammonium chloride to the medium, to see if any competition for tetraphenylboron could be demonstrated. The results are given in Fig. 6. In the presence of tetraethylammonium chloride the inhibitory effect on the phosphorylation and the reversal of the fluorescence quenching upon addition of tetraphenylboron are largely prevented. Addition of tetraethylammonium chloride during the period of tetraphenylboron inactivation, however, had neither an effect on the time-course of the ATP synthesis nor on the time-course of the fluorescence quenching of 9-aminoacridine.

To test whether the uncoupling activity of tetraphenylboron was due to a

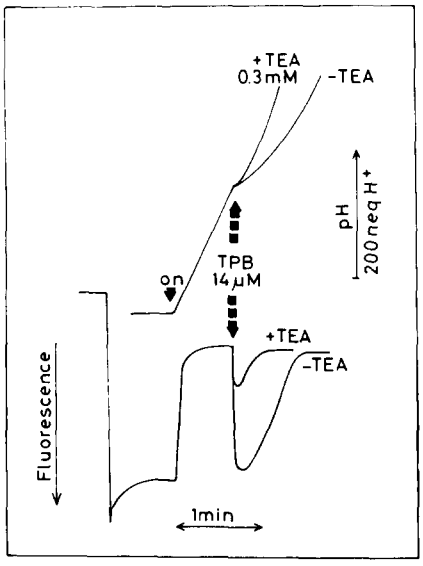

Fig. 6. The effect of tetraphenylboron (TPB^-) on phosphorylation and fluorescence of 9-aminoacridine in the presence and absence of tetraethylammonium chloride (TEA). Conditions as described under Fig. 4A.

direct interaction of the compound with the coupling factor CF_1, chloroplasts were prepared devoid of the coupling factor. The EDTA-treated chloroplasts were 'recoupled' with low concentrations of *N,N'*-dicyclohexylcarbodiimide (DCCD)[21] analogous to 'recoupling' of submitochondrial particles by oligomycin and DCCD[22,23] and of particles of *Escherichia coli* by DCCD[24]. It was found that EDTA-treated chloroplasts regained their proton uptake activity and the ability to quench the fluorescence of 9-aminoacridine after DCCD treatment. The effects of tetraphenylboron on the proton uptake and the fluorescence quenching of 9-aminoacridine in these 'recoupled' chloroplasts were found to be qualitatively the same as in intact chloroplasts.

DISCUSSION

Tetraphenylboron is a powerful inhibitor of cyclic photophosphorylation by spinach chloroplasts. Such an inhibition might be due either to an uncoupling effect of the compound or to inhibition of electron or energy transfer. The observation that tetraphenylboron stimulates the light-triggered ATPase as well as the fact that it abolishes the light-induced quenching of fluorescence of 9-aminoacridine strongly favours the first explanation. Furthermore neither $NADP^+$ reduction (ref. 9) nor reduction of diquat in the presence of dithioerythritol (not shown) was inhibited by tetraphenylboron.

The mechanism by which tetraphenylboron causes uncoupling, however, is not yet clear. One might speculate that tetraphenylboron, being a lipid-soluble anion, is accumulated by the chloroplasts *via* an electrogenic pump mechanism[3–5]. In accordance with such an explanation is the observation that the amount of energy spent

in other processes than ATP synthesis is proportional to the concentration of tetraphenylboron added (Fig. 4A). However, it should be expected also that prolonged incubation in the dark leads to release of the accumulated tetraphenylboron, so that in a second cycle of illumination the uncoupling activity is manifested again, which is not found experimentally.

Another possibility is that tetraphenylboron uncouples by increasing the permeability of the chloroplast membrane for protons[25]. The simplest mechanism by which this could occur, requires the movement of the undissociated acid across the membrane. However, the extremely low pK_a (ref. 26) of this acid makes the presence of significant concentrations of the undissociated form very unlikely. Furthermore, the undissociated acid is unstable and dissociates into triphenylboron and benzene[26]. In fact the protonation may be related to the apparent 'inactivation' of the tetraphenylboron, which is completely dependent on electron transport (Fig. 5B, ref. 9). On the other hand the 'inactivation' of tetraphenylboron may be the result of its light-dependent oxidation by the electron transport system.

The observations that the reaction of tetraphenylboron with the chloroplast membrane occurs only in the light, together with the fact that this reaction is prevented by tetraethylammonium ions, leads us to propose the following hypothesis. Illumination of the chloroplasts leads to proton uptake in the membrane itself, possibly by reaction with amino groups that become exposed due to the conformational change induced by energization[27]. The tetraphenylboron anion rapidly forms a complex with these protonated groups. This might explain on the one hand that tetraphenylboron addition has relatively little influence on the H^+ disappearance as measured by an external pH electrode and, on the other hand, that tetraphenylboron rapidly abolishes the energized state as read out by the quenching of 9-aminoacridine fluorescence, which may be a measure of the intramembrane protonation[28]. The presence of tetraphenylboron in the membrane results in uncoupling. Quaternary amines present in the suspending medium will compete with the membrane for binding of the tetraphenylboron.

In this context it is worth remembering the experiments of Hinkle[29] and Bakker *et al.*[30], who demonstrated that in phospholipid micelles the presence of tetraphenylboron strongly accentuates the effects of uncouplers of oxidative phosphorylation on the proton permeability. It was shown by others that NH_3 became strongly uncoupling in submitochondrial particles in the presence of very low concentrations of tetraphenylboron (7.3 μM) (ref.31). In our experiments with chloroplasts tetraphenylboron may analogously stimulate the back flow of protons taken up by the chloroplasts from the medium. The relatively slow 'inactivation' of tetraphenylboron could be a side-reaction in which the protons in the membrane react with the tetraphenylboron.

Finally we would like to draw attention to the fact that under some conditions (*cf.* Fig. 3) considerable amounts of protons are taken up by the chloroplasts without a concomitant large change in 9-aminoacridine fluorescence. Therefore the quantitative calculation of the ΔpH across the thylakoid membrane[16] from the quenching of 9-aminoacridine fluorescence should be interpreted with caution.

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