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CHARGE ACCUMULATION AT THE REDUCING SIDE OF SYSTEM 2 OF PHOTOSYNTHESIS

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SUMMARY

A study was made of the reactions between the primary and secondary electron acceptors of Photosystem 2 by measurements of the increase of chlorophyll fluorescence induced in darkness by dithionite or by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The experiments were done either with chloroplasts to which hydroxylamine or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was added, or with chloroplasts treated with tris(hydroxymethyl)aminomethane (Tris) to which phenylenediamine and ascorbate were added as donor system. Under these conditions the fluorescence increase induced by dithionite or DCMU added after illumination with short light flashes was dependent on the flash number with a periodicity of two; it was large after an uneven number of flashes, and small after a long darktime or after an even number of flashes. The results are interpreted in terms of a model which involves a hypothetical electron carrier situated between Q and plastoquinone; this electron carrier is thought to equilibrate with plastoquinone in a two-electron transfer reaction; the results obtained with DCMU are explained by assuming that its midpoint potential is lowered by this inhibitor.

INTRODUCTION

The mechanism of interaction of Q and A, the primary and secondary electron acceptor pools of Photosystem 2, respectively, is not well understood. Q is apparently a one-electron acceptor: in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which inhibits between Q and A (ref. 1), it can be almost completely reduced in a single short saturating flash [2, 3]; A mainly consists of the two-electron acceptor plastoquinone [4]; in a series of flashes each flash reduces one plastoquinone per two chains [5]. Any model intended to describe the interaction of Q and A will have to accommodate this; it has to explain how two-electron carriers are reduced by a one-electron carrier. At present, the only model that has this quality appears to be the one originally proposed by Stiehl and Witt [5], which was

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

somewhat modified more recently [6]. It was postulated that the reaction centers of System 2 are coupled in pairs [5, 6]; both electron acceptors of a pair would donate one electron to two neighbouring plastoquinone molecules [6]. The twin plastoquinone$^-$–plastoquinone$^-$ then would rapidly dismutate into plastoquinone^{2-} and plastoquinone [5, 6].

Another point that has to be accommodated concerns the variability of the apparent equilibrium constant between Q and A. In continuous light of not too low intensity, the apparent equilibrium constant is low, near 1 (refs 7, 2, 8). In the dark it is much higher [8, 9]. The model of Stiehl and Witt [5, 6] cannot readily explain this phenomenon. It was tentatively explained in a different model, proposed by Joliot and Joliot [10], which involves two different Q molecules per reaction center of Photosystem 2. However, recent evidence [9] argues against this model.

In a previous paper [9] the interaction between Q and A was studied after an addition of dithionite in the dark. It appeared that dithionite reduces the secondary electron acceptor pool A, and that Q is reduced indirectly, via A. It was also shown [9] that when the chloroplasts were illuminated before the addition of dithionite part of the Q reduced by dithionite via the A pool was reoxidized by what appeared to be an oxidized product of Photosystem 2. Because of this reoxidation of Q^- the kinetics of the dithionite-induced fluorescence increase after preillumination were rather complicated [9].

The present paper reports the effect of preilluminating flashes upon the interaction of Q and A in chloroplasts treated in such a way that reoxidation of Q^- by oxidized photoproduct was inhibited. Under these circumstances the dithionite-induced fluorescence increase was dependent on flash number with a periodicity of two. From the results obtained it is concluded that reaction centers of System 2 do not cooperate in the reduction of plastoquinone; electron transport between Q and plastoquinone appears to involve an additional electron transport component, situated between Q and plastoquinone, which is reduced by Q^- in two one-electron transfer reactions and oxidized by plastoquinone in a two-electron transfer reaction.

MATERIALS AND METHODS

Chloroplasts were prepared from spinach leaves as described in ref. 11. To obtain Tris-washed chloroplasts freshly isolated chloroplasts were suspended in 0.2 M Tris–HCl (pH 10.0) to about 0.25 mM chlorophyll. For some experiments (Fig. 4) the incubation medium also contained 0.1 M sucrose. After standing for about 10 min (at 0 °C) the suspension was centrifuged for 2 min at $12\,000 \times g$; the precipitate was resuspended in a small volume of isolation buffer. Other conditions and procedures as described in Material and Methods of ref. 9.

RESULTS

It was shown in a previous paper [9] that the kinetics of the rise of chlorophyll-*a* fluorescence upon addition of dithionite to chloroplasts are apparently biphasic, and dependent on the number of preilluminating flashes.

The apparent biphasity of the kinetics was explained by assuming that Q, the

primary acceptor of Photosystem 2, and quencher of fluorescence when oxidized, is reduced by dithionite via the pool of secondary acceptors (A). The effect of preillumination appeared to be mainly due to reoxidation of reduced Q by some photooxidized product, present in high concentration in the S_2 and S_3 states. This reoxidation was correlated with luminescence [9]. Addition of a high concentration of hydroxylamine, after the preillumination, but prior to the addition of dithionite abolishes the dithionite-induced luminescence, presumably because of reduction of S_2 and S_3 by the hydroxylamine [9, 12]. This indicates that reoxidation of Q^- does not occur (in the presence of hydroxylamine), and thus cannot affect the dithionite-induced fluorescence increase in these circumstances. Therefore, one might expect that the fluorescence increase would now be independent of preillumination. The results of an experiment performed under these conditions are given in Fig. 1A (broken line). Remarkably, not all of the flash dependence has disappeared. After one and three flashes the amplitude of the fluorescence increase is significantly larger than after zero, two or four flashes. Fig. 2 shows the result when carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), another substance which is known to accelerate the deactivation of S_2 and S_3 (ref. 13), was added prior to the dithionite addition. Again some flash dependence for the dithionite-induced fluorescence increase remained, qualitatively the same as with hydroxylamine.

According to Bouges-Bocquet [14] incubation of chloroplasts with 2,6-di-

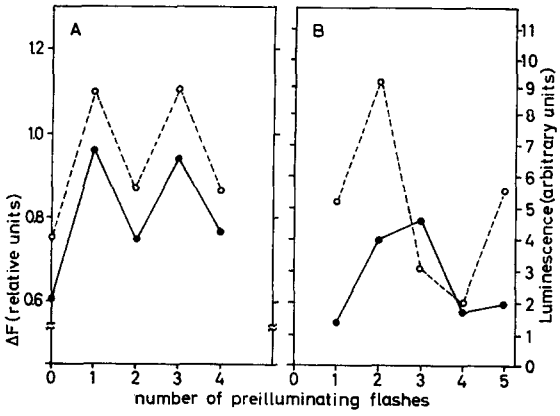


Fig. 1. (A) Dithionite-induced fluorescence increase in the presence of hydroxylamine, after a series of short saturating flashes. ●–●, dark-adapted chloroplasts were incubated with 0.025 mM DCIP and 0.25 mM ascorbate for 30–60 min. Illumination was given as a series of short saturating flashes. 3 s after the last flash an equal volume of 20 mM hydroxylamine was added; 7 s later dithionite was added to a final concentration of 10 mM. ΔF is the increase in fluorescence, excited by a band around 472 nm, observed 22 s after the addition of dithionite. ΔF is plotted in relative units, taking 1 for the fluorescence yield of dark-adapted chloroplasts to which buffer instead of dithionite was added. In the same units the maximal ΔF obtained by illuminating a dark-adapted sample with strong blue actinic light (2 mW/cm^2) was 4.2. Chlorophyll concentration, $1.25 \cdot 10^{-5}$ M; pH, 7.8; temperature, 16 °C. ○- - -○, as ●–●, but without preincubation with DCIP and ascorbate. (B) Effect of incubation with DCIP and ascorbate on dithionite-induced luminescence. ●–●, chloroplasts, preincubated with DCIP and ascorbate as for Fig. 1A, were illuminated by a series of short saturating flashes. 10 s after the last flash an equal volume of 20 mM dithionite was added. L is the peak intensity of the luminescence obtained after the dithionite addition (see ref. 9). ○- - -○, as ●–●, except that DCIP and ascorbate were now added 3 s after instead of 30–60 min before the illumination.

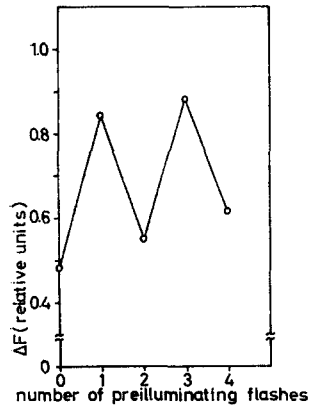


Fig. 2. Dithionite-induced fluorescence increase in the presence of FCCP, after a series of short saturating flashes. FCCP was added to a final concentration of 10 μM, 10 s after the last flash. 10 s later dithionite was added to a final concentration of 10 mM. ΔF as for Fig. 1A. The maximal ΔF, measured as for Fig. 1A, was equal to 3.6.

chlorophenolindophenol (DCIP) and ascorbate converts most of S_1 into S_0. This is confirmed by the result of an experiment in which dithionite-induced luminescence was measured as function of number of preilluminating flashes (Fig. 1B). With chloroplasts incubated with DCIP and ascorbate the highest dithionite-induced luminescence was observed after the third flash (Fig. 1B, solid line); without such treatment S_1 predominates as initial state [15, 16] and the highest dithionite-induced lumines-

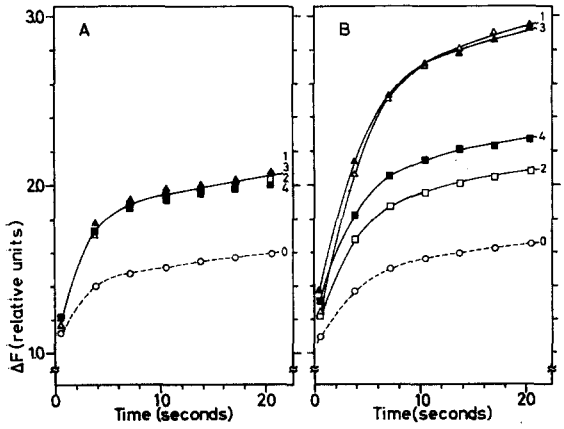


Fig. 3. Fluorescence yield of dark-adapted and preilluminated Tris-treated chloroplasts as a function of time after the addition of dithionite to a final concentration of 10 mM. (A) Dark-adapted Tris-treated chloroplasts were preilluminated by a series of short saturating flashes. At $t = 0$, 10 s after the last flash, an equal volume of 20 mM dithionite was added. Fluorescence was excited as for Fig. 1A, 0.3 s for each measuring point. The fluorescence yield is plotted in relative units, taking 1 for the fluorescence yield of chloroplasts to which buffer instead of dithionite solution was added. Number of preilluminating flashes: △–△, 1 flash; □–□, 2 flashes; ▲–▲, 3 flashes; ■–■, 4 flashes; ○- - -○, no preillumination given. (B) As for (A), except that now before illumination 50 μM p-phenylenediamine and 1 mM ascorbate was added to the chloroplast suspension.

cence is observed after the second flash (Fig. 1B, broken line) [9]. The incubation with DCIP and ascobate did not have a significant effect on the oscillation in fluorescence increase induced by dithionite in the presence of hydroxylamine (Fig. 1A, solid line). Obviously, the initial amount of S_1 was of no importance for the flash number-dependent phenomenon studied here.

Treatment of chloroplasts with a high concentration of the unionized form of Tris results in inhibition of the water splitting system [17, 18]. Water can no longer act as electron donor to Photosystem 2. Electron transfer by Photosystem 2 is possible, however, in the presence of artificial electron donors, like *p*-phenylenediamine (plus ascobate) [17, 18]. Luminescence, after addition of dithionite 10 s after a series of one to four flashes, was too weak to be measured with our apparatus, both in the absence and in the presence of *p*-phenylenediamine plus ascorbate (not shown). The fluorescence kinetics of Tris-treated chloroplasts, without electron donor, are shown in Fig. 3A. The first flash has an effect similar to the effect of the first flash given to untreated chloroplasts (plus hydroxylamine). Additional flashes, given shortly after the first, had no influence; the chloroplast behaved as if Photosystem 2 could turn over only once. Addition of *p*-phenylenediamine (plus ascorbate) to Tris-treated chloroplasts restored the dependence on the flash number of the dithionite-induced fluorescence increase. This is shown in Fig. 3B. The flash number dependence was qualitatively the same as with normal chloroplasts (plus hydroxylamine), but more pronounced. We did measure the dithionite-induced fluoresence increase for a series of up to nine flashes (not shown). As for up to four flashes (Fig. 3B) a damped oscillation with a period of two was seen.

Concomitantly with the reduction of Q, dithionite reduces the secondary elec-

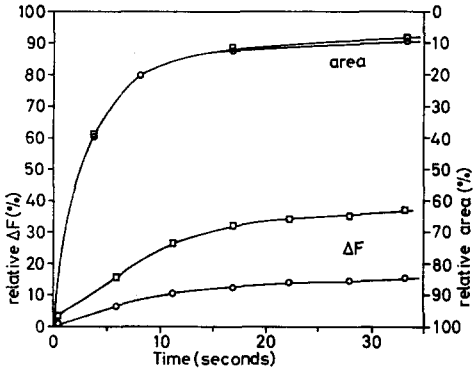


Fig. 4. Tris-treated chloroplasts (*p*-phenylenediamine and ascorbate added), dark-adapted or preilluminated by one flash. Dithionite was added, at $t = 0$, to a final concentration of 2 mM. Lower curves (ΔF): time courses of dithionite-induced fluorescence yield increase. The fluorescence yield increase was measured in relative units; the fluorescence yield increase obtained by dithionite plus illumination by strong blue actinic light was taken as 100 %. Upper curves (area): time courses of dithionite-induced decrease of area over fluorescence rise curve. Fluorescence rise curves were measured in strong blue actinic light (2 mW/cm^2), given at various times after the addition of dithionite. The measured values for the area over the rise curves were normalized with respect to the area over the fluorescence rise curve obtained with chloroplasts to which buffer was added instead of dithionite solution. ○–○, no preillumination; □–□, preillumination by one flash, 10 s before the addition of dithionite.

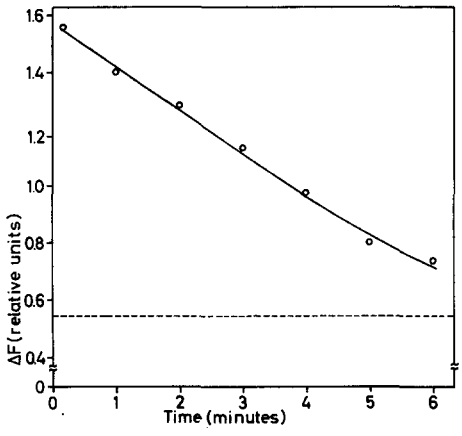


Fig. 5. Dithionite-induced fluorescence increase of Tris-treated chloroplasts (with *p*-phenelenediamine and ascorbate), preilluminated by one flash, as function of time between preillumination and dithionite addition. Further conditions as for Fig. 3B. ΔF is the increase in fluorescence observed 11 s after the addition of dithionite; it is expressed in the same units as for Fig. 3B. The broken line indicates the ΔF obtained with dark-adapted chloroplasts. The maximal ΔF, obtained by dithionite plus illumination by strong blue light, was equal to 3.9.

tron acceptor pool of Photosystem 2 (ref. 9). In Fig. 4 the kinetics of reduction of the total acceptor pool upon addition of dithionite are given for Tris-treated chloroplasts (with *p*-phenylenediamine and ascorbate) which were dark-adapted or preilluminated by one flash. In contrast with the reduction of Q the reduction of the total acceptor pool appeared to be very little dependent on flash number. It seems allowed to conclude (but see Discussion) that the apparent equilibrium constant between Q and A depends on the number of flashes; it is relatively low in the "F_u state", i.e. shortly after an uneven number of flashes; it is relatively high in the "F_e state", i.e. after dark adaptation or after an even number of flashes. Fig. 5 shows that the F_u

TABLE I

THE EFFECT OF *o*-PHENANTHROLINE ON THE DECAY OF THE F_u STATE

Conditions as for Fig. 5. Preillumination was given by one flash, at $t = 0$. Where indicated before the preillumination 0.2 mM *o*-phenanthroline was added to the chloroplast suspension. To take away the *o*-phenanthroline 0.3 mM $ZnSO_4$ was also added, 30 s before the addition of dithionite. ΔF was measured 11 s after addition of dithionite. Controls without *o*-phenanthroline and $ZnSO_4$ are also given. The maximal ΔF, measured as for Fig. 5, was equal to 3.6.

Preillumination	Additions		ΔF
	o-Phenanthroline	Dithionite (time of addition (s))	
+	+	33	1.65
+	+	270	1.3
−	+	33	0.3
+	−	33	1.5
+	−	270	0.9
−	−		0.5

state, after one flash, decays with a half-time of about 3.5 min (at a temperature of about 16 °C). This decay is inhibited by *o*-phenanthroline, added before the flash (Table I). In this experiment *o*-phenanthroline was removed before the addition of dithionite, by the addition of $ZnSO_4$ (refs 19 and 20). The inhibition by *o*-phenanthroline of the decay of the F_u state is probably related to its well-known inhibition of the oxidation of Q^- by secondary acceptors.

The "F states" are not only observed with dithionite. Fig. 6 shows that they also manifest themselves upon addition of DCMU, in the dark. Similar results were obtained with *o*-phenanthroline (not shown). An enlarged fluorescence increase induced by these inhibitors with preilluminated as compared to dark-adapted chloroplasts was previously observed by Bennoun and Li [20].

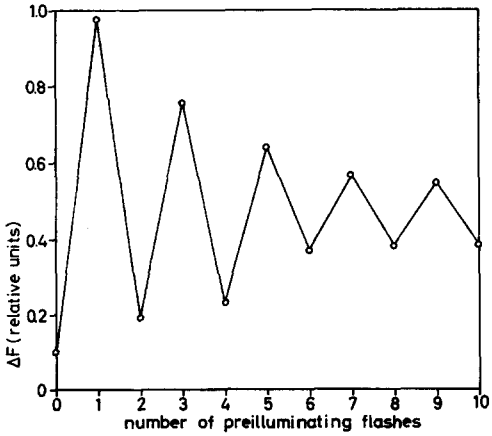
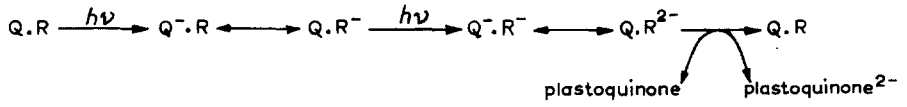


Fig. 6. DCMU-induced fluorescence increase of Tris-treated chloroplasts (with *p*-phenelenediamine and ascorbate), after a series of short saturating flashes. Same conditions as for Fig. 5, except that DCMU was added, to a final concentration of 3 μM, instead of dithionite. ΔF is the increase in fluorescence observed 22 s after the addition of DCMU. Same units as for Fig. 5.

DISCUSSION

As periodicity of four in the evolution of oxygen by System 2 as function of the number of flashes, first observed by Joliot et al. [21], was explained by Kok et al. [15] in terms of a linear four-step mechanism. In the oxidation of water the reaction centers of System 2 do not cooperate. The positive charge generated in the light in the photocenters, one per absorbed quantum, i.e. one per short saturating flash, is stored in each center until four charges have been accumulated. Only then the charges are used up in a reaction with water, the electron donor of System 2. On basis of the results reported in this paper we propose that something similar to what occurs at the oxidizing side of Photosystem 2, i.e. accumulation of charges, also occurs at the reducing side of Photosystem 2. We propose that the reaction centers do not cooperate in the reduction of secondary electron acceptor, and that the pool of plastoquinone, which is a two-electron carrier, is only reduced when two negative charges have been accumulated by a reaction center. If Q has been photoreduced only once, oxidation of Q^- in the dark results in the reduction of a secondary electron acceptor, R, which

does not belong to the plastoquinone pool. After a subsequent photoreduction of Q one molecule of plastoquinone becomes reduced, and R^- reoxidized. Light-induced electron transport can then be schematically written as:



The model implies that there is no real equilibrium between Q and the rest of the electron acceptor pool in or shortly after illumination (see also ref. 8; it was concluded there that after illumination no direct reaction exists between Q and the rest of the pool). Q can only equilibrate with R. No rapid equilibration is possible between Q molecules and R molecules of different chains; the Q molecules and R molecules of different chains do not, like plastoquinone does [22], form a common pool. R can contain at least two electrons (see below) and can only equilibrate with the plastoquinone pool in a two-electron transfer reaction. This model explains why the "apparent equilibrium constant" between Q and A is different in illuminated as compared to dark-adapted chloroplasts, and why this constant fluctuates with a periodicity of two. The damping of the oscillations with increasing number of flashes can be explained by assuming that with every flash a certain fraction of the centers get out of phase; this may be caused by "misses" and by "double hits". A similar explanation was given for the damping of the fluctuation in amounts of oxygen evolved by short saturating flashes [15, 23].

Reduction of Q by dithionite was shown in the previous paper to be correlated with the reduction of the pool of secondary electron acceptors [9]. The experimentally found relation between the reduction degree of Q and the reduction degree of the total acceptor pool, with dark-adapted chloroplasts, could be described by assuming that the total acceptor pool consists of three compounds: (1) Q, known to account for about 5 % of the total pool [2, 24], (2) the main secondary electron-acceptor pool, accounting for about 80 % of the total pool [9], and which we can assume to be plastoquinone, and (3) an additional secondary electron-acceptor pool [25, 9], accounting for the remaining 15 % of the total pool. This additional secondary electron-acceptor pool can now tentatively be identified with what we have called R in this paper. It was assumed in the previous paper that a one-electron-transfer equilibration could take place between all three components of the electron acceptor pool. This assumption is apparently contradicted by the results reported in the present paper. We concluded above that one-electron transfer could only take place between Q and R, and that between plastoquinone and R equilibration could only take place in two-electron-transfer reactions. The relatively large fluorescence increase upon addition of dithionite also after an odd number of flashes indicates that two-electron transfer can take place not only to Q.R. but to $Q.R^-$ too, and thus that R can contain more than one electron, as was assumed above. If not, one would have expected to see a relatively small dithionite-induced fluorescence increase in state F_u. The apparent biphasic character of the fluorescence increase induced by dithionite (see Figs 3 and 4) may in principle be explained in a similar way as done earlier [9], i.e. its cause may be that Q has a lower midpoint potential than the electron-transport chain components via which it is reduced by dithionite.

DCMU is known to inhibit the reoxidation of Q^- by secondary electron acceptors [1]. The model discussed above enables us to give a more precise identification of its site of action: between Q and R. It is also possible to give a tentative explanation of its mode of action: the effect of DCMU added after a series of flashes or to dark-adapted chloroplasts indicates that it lowers the midpoint potential of R, relative to that of Q. After an odd number of preilluminating flashes this will cause a reduction of Q by R^- and thus an increase of fluorescence. The relatively smaller increase after an even number of flashes is explained by the fact that R then is mainly in the oxidized state, and therefore less Q is reduced when DCMU is added. As can be seen from Fig. 6 only a very small increase in fluorescence occurs without preillumination. As photoreduced Q is oxidized via R, the lowering of the midpoint potential of R may also explain why DCMU inhibits this oxidation. It must be remarked, however, that Q is not completely reduced upon addition of DCMU even after one preilluminating flash, when one expects R to be almost completely in state R^-; this might indicate that the lowering of the midpoint potential of R by DCMU is not large enough to fully explain the inhibitory action of DCMU. The DCMU-induced change in the relative midpoint potentials of Q and R can be explained by assuming that DCMU causes a lowering of the midpoint potential of R as well as by assuming that it causes an increase of the midpoint potential of Q. We prefer the first hypothesis since this one, in contrast to the alternative one, also readily explains the increase in delayed fluorescence induced by DCMU added after preillumination (ref. 26 and Velthuys, B. R. and Amesz, J., unpublished), and the inhibition by DCMU of the reduction of Q by dithionite[27] via plastoquinone and R. *o*-Phenanthroline probably acts in the same way as DCMU. Reduction of Q under influence of DCMU may also be the cause of DCMU-induced increase in the rate of deactivation of oxidized intermediates on the pathway to water (ref. 28; see also ref. 29).

The presence of an electron acceptor different from Q and not belonging to the plastoquinone pool has also been concluded from measurements of fluorescence induction at −40 °C (ref. 11). The involvement of this electron acceptor in electron transport at −40 °C would be dependent on the S state [11], i.e. the number of oxidizing equivalents stored in the pathway to water. The presently available evidence does not enable to decide whether this electron acceptor is identical to R. As R is a secondary electron acceptor there is no reason to identify it with the compounds C550 (ref. 30) and X-320 (ref. 5). Although the relationship between these two compounds is not clear, both show the characteristics of a primary acceptor.

ADDENDUM

After submission of this manuscript we learned that Dr B. Bouges-Bocquet (personal communication), on the basis of measurements of methylviologen reduction similarly concluded that an acceptor B exists between Q and plastoquinone with the same properties as acceptor R.

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