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# Photoreduction of pheophytin as a result of electron donation from the water-splitting system to Photosystem-II reaction centers

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**Reversible photoreduction of pheophytin (Pheo) accompanied by a decrease of chlorophyll-fluorescence yield is observed in subchloroplast oxygen-evolving preparations of Photosystem II (PS II) under anaerobic conditions. This photoreaction is activated at addition of CCCP, inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and reactivated upon subsequent addition of ascorbate. Benzyl viologen as well as methyl viologen accelerates dark oxidation of reduced pheophytin, indicating that they are able to accept an electron from $Pheo^-$. The data on both the photoreduction of pheophytin in the absence of exogenous reductants – when electron donation to reaction centers of PS II occurs only from water – and the inhibition of this photoreaction by DCMU show that the pheophytin photoreduction is sensitized by reaction centers of PS II, and it probably occurs as a result of electron donation from the water-splitting system being in the sate $S_3$ to $P^+$-680$Pheo^-Q^-$, producing the long-lived state $S_0$ P-680$Pheo^-Q^-$ and $O_2$. Photoreduction of pheophytin in the presence of ascorbate (and dithionite) evidently occurs as a result of donation of its electrons to $P^+$-680$Pheo^-Q^-$ by means of the S-states of the water-oxidizing system. It is shown that the photoinduced decrease of fluorescence in chloroplasts under anaerobic conditions is due to two processes: photoreduction of pheophytin in Photosystem II and photooxidation of $Q^-$ by Photosystem I. It is suggested that photoreduction of pheophytin takes also place under aerobic conditions when Q is reduced. It may contribute to the P–S fluorescence decrease during fluorescence induction in leaves.**

## Introduction

During recent years much experimental evidence has become available [1–9] showing that pheophytin '*a*' (Pheo) participates in the primary photosynthetic reactions acting as an intermediary electron acceptor in reaction centers of Photosystem II (PS II) between the primary electron donor, chlorophyll P-680, and the primary ('stable') electron acceptor Q (a complex of plastoquinone with Fe). If Q is prereduced in the dark (with dithionite), reversible photoreduction of pheophytin is observed [1,2]. Its relation to the phototransfer of electron in the reaction center of PS II is indicated by many observations. This photoreaction (1) is observed only in preparations containing active PS II and is not seen in PS-I preparations or in the light-harvesting complex [1–3], (2) has a sensitivity to heating (40–50°C) similar to that of the photooxidation of P-680 [3], and (3) is inhibited upon extraction of Mn from PS-II preparations and is reactivated (together with other PS-II photoreactions) upon subsequent addition of

Abbreviations: PS II, Photosystem II; PS I, Photosystem I; *F*, fluorescence; $\Delta F$, change of fluorescence yield; $F_{max}$, maximal level of *F*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; Pheo, pheophytin.

$Mn^{2+}$ [3–5]. When the state P-680$Pheo^-Q^-$ is accumulated the radicals $Pheo^-$ and $Q^-$ are in exchange interaction [5,6,8,9] (the distance between them is 0.8–0.11 nm [5]). In the nanosecond timescale absorbance changes are detected in PS-II preparation where the spectrum shows photoseparation of charges between P-680 and pheophytin [7]; photoreduction of pheophytin is observed at temperatures as low as 100 K [1] or 5 K [8,9]. Work on pheophytin photoreduction has been done under strongly reducing conditions [1–9]. In the absence of reductant, photoaccumulation of $Pheo^-$ could not be observed so far, because the stable primary electron acceptor Q either efficiently oxidizes reduced pheophytin or permits recombination between $Pheo^-$ and $P^+$-680.

Reduction of Q is known to be accompanied by a (3–4)-fold rise of the fluorescence yield ($F$) of PS-II chlorophyll (so-called 'variable fluorescence' or $\Delta F$) [5,10]. This fluorescence increase is actually an increase of the luminescence arising from charge recombination in the pair $P^+$-680$Pheo^-$ formed in the primary photoreaction [1–7]. Variable fluorescence disappears when $Pheo^-$ accumulates, a result which is interpreted in terms of inability of reaction center in the state P-680$Phe^-Q^-$ to catalyse photoseparation of charges, and hence to allow charge recombination [1–7]. In chloroplasts the photoinduced decrease of chlorophyll emission related to the pheophytin photoreduction interferes with the fluorescence decrease induced by photooxidation of $Q^-$ by PS I [3,5,11]. The latter effect is completely eliminated by DCMU which blocks electron transfer between PS I and PS II [1,3,11].

In recent papers (Ref. 12; see also Heber, U., Kobayashi, Y., Leegood, L.C. and Walker, D.L., unpublished results), observations on a photoinduced decrease of chlorophyll fluorescence in anaerobic chloroplasts have been described which are difficult to interprete. Fluorescence quenching in such chloroplasts may be caused by a combination of the effects described above.

In the present paper, the possibility for a photoreduction of pheophytin in PS II is investigated under anaerobic conditions when the water-splitting system is the sole source of electrons for a PS-II reaction center. The data show that the photoreduction of pheophytin in oxygen-evolving preparations of PS II can be observed in the absence of exogenous electron donors, and that it is closely related to the process of water oxidation. This is confirmed in particular by inhibition of the pheophytin photoreduction by DCMU.

## Materials and Methods

Oxygen-evolving subchloroplast particle enriched in PS II were prepared from pea chloroplast using detergents digitonin (0.4%) and Triton X-100 (0.1%) and centrifugation at $20000 \times g$ (DT-20 particles) as in Refs. 1–4, and from spinach chloroplasts using triton X-100 (referred to as T-20 particles) as in Ref. 13. The rate of oxygen evolution under illumination of the particles in the presence of 1 mM ferricyanide and 0.5 mM 2,5-dimethyl-*p*-benzoquinone in 50 mM Hepes (pH 6.7) was about 120 and 180 $\mu$mol $O_2$ per mg Chl per h, respectively. During the experiments the particles and chloroplasts (isolated as in Ref. 13) were suspended in a medium containing 50 mM Hepes (pH 6.7)/35 mM NaCl/5 mM $MgCl_2$/5 mM KCl. Anaerobic conditions were created by the addition of glucose (10 mM), glucose oxidase (approx. 50 U/ml) and catalase (approx. 1000 U/ml) to a tightly closed 1 cm cuvette as described earlier (Heber, U., Kobayashi, Y., Leegood, R.C. and Walker, D.L., unpublished results).

Photoinduced absorbance changes ($\Delta A$) and changes of chlorophyll fluorescence yield ($\Delta F$) with $\lambda > 660$ nm were measured with the phosphoroscopic set-up described earlier [1–4].

## Results and Discussion

*Photosystem-II particles*

Under aerobic conditions the yield of chlorophyll fluorescence ($F$) excited in oxygen-evolving PS-II particles (T-20 and DT-20) by a low-intensity measuring light is increased from the level $F_0$ to the level $F_{max}$ equal to $F_0 + \Delta F$ when actinic light II which excites mainly PS II) is added (Fig. 1A). The fluorescence increase reflects photoreduction of Q. After switching off the actinic light the fluorescence yield returns to the level $F_0$ showing that the weak measuring light is not able to keep Q in state $Q^-$ under aerobic conditions.

Creation of anaerobic conditions leads even in

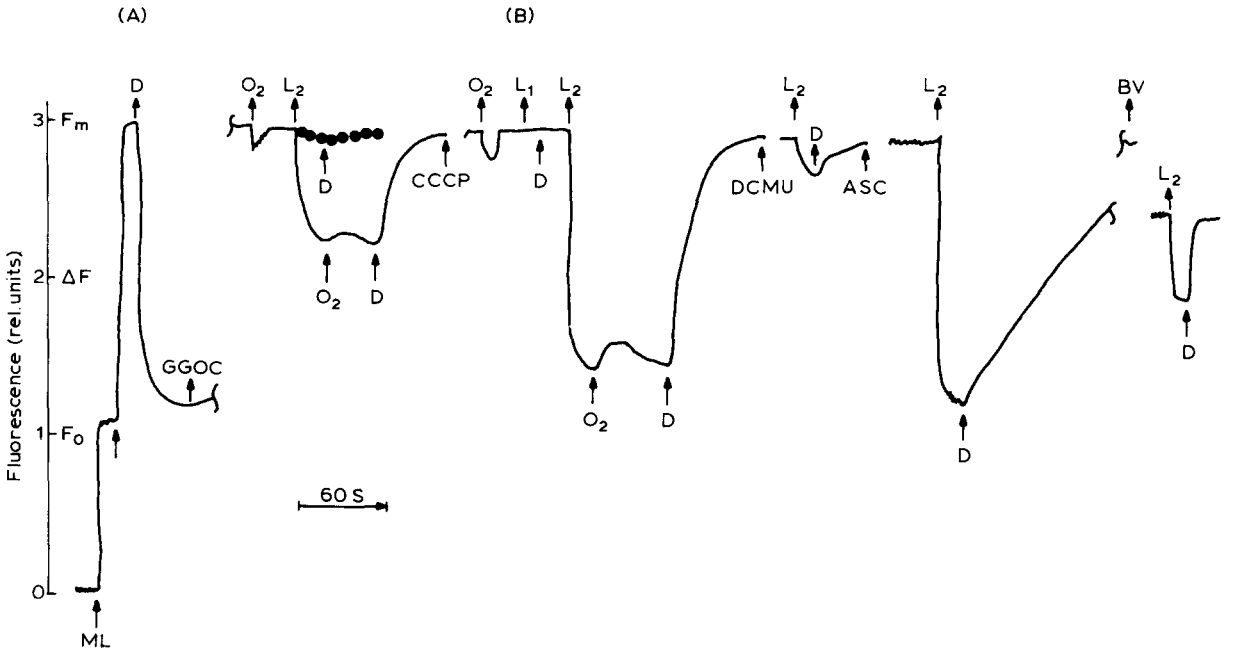


Fig. 1. Kinetics of photoinduced changes of chlorophyll fluorescence yield ($\Delta F$) in subchloroplast oxygen-evolving photosystem-II preparations (T-20 particles) under aerobic (A) and anaerobic (B) conditions; Chl concentration is 20 $\mu$g/ml. Designations: ↑, the moment of switching on (L, light) and off (D, dark) or addition of agents: ML, the measuring light ($\lambda = 480$ nm; approx. 0.1 $J \cdot m^{-2} \cdot s^{-1}$) exciting chlorophyll fluorescence; L2, actinic light 2 exciting mainly PS II ($\lambda > 600$ nm, $10^2$ $J \cdot m^{-2} \cdot s^{-1}$); L1, actinic light I exciting predominantly PS I ($\lambda > 710$ nm, 30 $J \cdot m^{-2} \cdot s^{-1}$); GGOC, glucose (10 mM), glucose oxidase (50 U/ml) and catalase (approx. 1000 U/ml); $O_2$, a fast air bubbling (1 $cm^2$) through the sample; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone (1 $\mu$M); DCMU, 3-(3,4-dichlorophenyl-1,1-dimethylurea) (10 $\mu$M); asc, sodium ascorbate (5 mM); BV, benzyl viologen (3 mM); -//-, dark period for 10–30 min. The dashed line shows the effect of light II in the presence of DCMU in a separate experiment.

the absence of intensive actinic light to slow increase of the fluorescence up to $F_{max}$ (Fig. 1B), indicating photoreduction of Q by the measuring light. This is confirmed by the fact that a pulse of $O_2$ (bubbling the sample with air) lowers the fluorescence yield temporarily (Fig. 1B). Oxygen added to the sample is subsequently removed by the enzymic $O_2$ trap.

In the absence of $O_2$ actinic light II induces a significant decrease of $F$, which is due to a reductive photoreaction, since oxygen added during the illumination results in an increase of fluorescence (Fig. 1B). The value of the photoinduced decrease of fluorescence ($-\Delta F$) is diminished by a factor of 8 when 10 $\mu$M DCMU is added to the sample (Fig. 1B). Actinic light I (exciting predominantly PS I) does not have any effect on $F$ of PS-II particles in the absence (Fig. 1B) as well as in the presence of DCMU.

Rates and extent of the light-induced fluorescence decrease under anaerobic conditions are enhanced upon addition of 1 $\mu$M CCCP (Fig. 1B). Again the fluorescence decrease is related to photoreduction of some substance, since the air bubbling during illumination leads to increase of $F$ (Fig. 1B). After addition of DCMU, the negative $\Delta F$ induced by light II in the presence of CCCP is significantly diminished, and it is strongly activated by the subsequent addition of ascorbate (Fig. 1). In the latter case, the rate of dark relaxation of the negative $\Delta F$ is much slower, but it is remarkably increased upon addition of 3 mM benzyl viologen which also lowers the dark level of fluorescence (Fig. 1). Similar effects were seen upon addition of 3 mM methyl viologen.

The photoinduced decrease of fluorescence under anaerobic conditions described above is accompanied in all cases by absorbance changes (with the same kinetics), the spectrum of which is presented in Fig. 2 (points). It is quite similar to

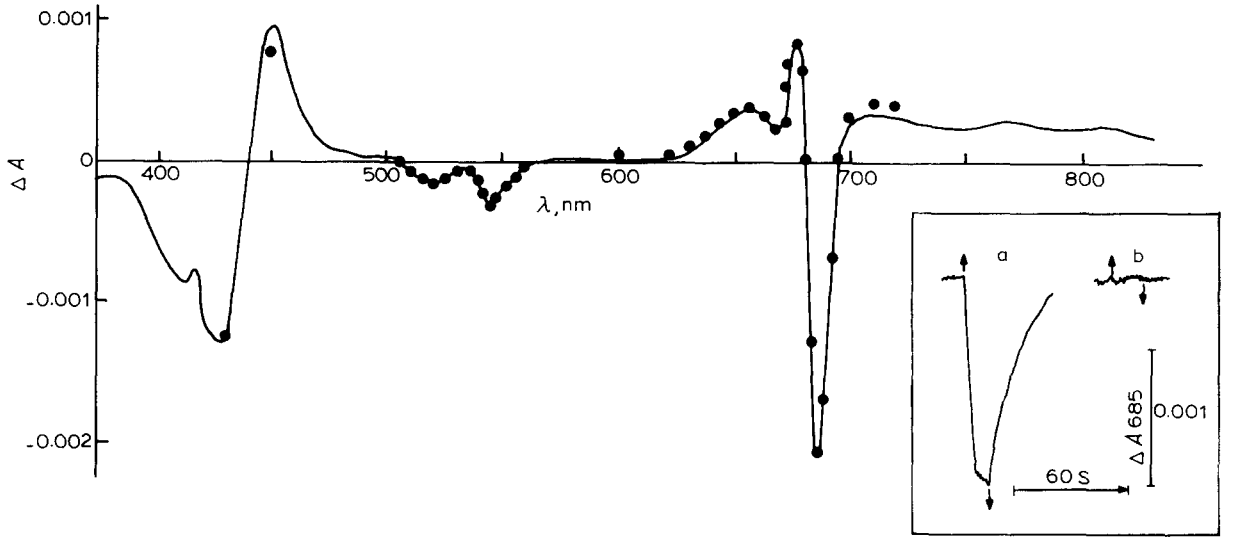


Fig. 2. The spectrum of light-induced absorbance changes ($\Delta A$) in oxygen-evolving PS-II particles (T-20) under anaerobic conditions (see Fig. 1) in the presence of 1 $\mu$M CCCP (closed circles); the solid line is the light-minus-dark difference absorption spectrum observed at photoreduction of pheophytin in PS-II preparation in the presence of dithionite (taken from Ref. 3). The spectra are normalized at 685 nm. Inset: kinetics of $\Delta A$ at 685 nm before (a) and after (b) addition of 10 $\mu$M DCMU. ↑ and ↓, the actinic light II is on and off, respectively. Chl concentration is 20 $\mu$g/ml.

the spectrum of photoreduction of pheophytin in PS-II particles at low redox potentials measured earlier [1–5] and shown in Fig. 2 by solid line. Photoinduced absorbance changes related to the reduction of pheophytin are inhibited (like $\Delta F$) upon addition of DCMU (Fig. 2, inset), and they are reactivated by ascorbate.

Thus, in PS-II particles under anaerobic conditions the weak measuring light keeps Q in the reduced state and strong illumination induces reduction of pheophytin which is accompanied by a decrease of the fluorescence yield of chlorophyll. This effect is quite similar to the photoreduction of pheophytin in PS II observed earlier in the presence of dithionite [1–5]. As suggested earlier [1–7], photoaccumulation of the state P-680Pheo$^-$Q$^-$ at low redox potentials is due to fast reduction of P$^+$-680 in the photoinduced state P$^+$-680Pheo$^-$Q$^-$ from a secondary electron donor (finally from dithionite). The data presented here show that the photoreduction of pheophytin can be seen in the absence of artificial electron donors for PS II, and that P$^+$-680 accepts an electron from the water-splitting system. This means that the pheophytin photoreduction is really sensitized by reaction centers of PS II, and that Pheo$^-$ photoaccumulation is permitted by electron donation from the water-oxidizing system to the state P$^+$-680Pheo$^-$Q$^-$. This conclusion is only at first sight questioned by the inhibition of the pheophytin photoreduction by DCMU which is known to block electron transfer between Q$^-$ and plastoquinone, and might therefore be expected to stimulate photoaccumulation of Pheo$^-$.

However, DCMU, by blocking the acceptor side of PS II, makes it impossible for the particles to reach the state $S_4$P-680Pheo$^-$Q$^-$ which produces $O_2$ and the long-lived state $S_0$P-680Pheo$^-$Q$^-$ *. The states $S_2$P-680Pheo$^-$Q$^-$ and (especially) $S_3$P-680Pheo$^-$Q$^-$ which can be formed by light in the presence of DCMU may be unstable due to fast charge recombination between Pheo$^-$ and the states $S_2$ and $S_3$. This can result in the decrease of the long-lived spectral changes related to photoreduction of pheophytin in our experiments. On the other hand, the well-known effect of acceleration of the decay rate of $S_3$ and $S_2$ states in the presence of CCCP (or other ADRY reagents) [14–16] can be responsible for the in-

* $S_0$–$S_4$ are the states of the water-oxidizing system [14–16].

crease of the rate of pheophytin photoreduction upon addition of CCCP (Fig. 1). In this case an additional path for formation of the long-lived $Pheo^-$ from the states $S_2P\text{-}680Pheo^-Q^-$ and $S_3P\text{-}680Pheo^-Q^-$ is opened.

The reactivation of photoreduction of pheophytin by ascorbate after its inhibition by DCMU can be explained by electron donation from ascorbate to the water-oxidizing system in the states $S_n$, where $\geq 2$, or directly to $P^+$-680. Photoreduction of pheophytin in the presence of dithionite is not eliminated by DCMU [1,5], but it is inhibited upon removal of Mn acting in the donor side of PS II [3,4] indicating reduction of $P^+$-680 (leading to accumulation of the state $P\text{-}680Pheo^-Q^-$) through the water-splitting system rather than as a result of direct electron donation to $P^+$-680 from dithionite.

The lowering of $F$ as well as acceleration of dark relaxation of the negative $\Delta F$ upon addition of benzyl viologen and methyl viologen which have midpoint redox potentials equal to $-320$ mV and $-440$ mV, respectively, show that both compounds are able to accept an electron from $Pheo^-$ (which has a redox potential equal to $-610$ mV [5]). The observations support the conclusion that the negative $\Delta F$ accompanies reduction of pheophytin.

*Chloroplasts*

In chloroplasts the creation of anaerobic conditions leads to the appearance of effects (Fig. 3) which are similar to those observed in PS-II particles. In the absence of $O_2$, the fluorescence yield is kept nearly the level $F_{max}$ by the measuring light and the actinic light II induces a decrease of fluorescence which is also activated by CCCP, and has a reductive nature (compare with Fig. 1 and its discussion). The effect is completely eliminated by DCMU and it is reactivated by subsequent addition of ascorbate (Fig. 3). These results show that the negative $\Delta F$ induced by light II in chloroplasts under anaerobic conditions may be also related to the photoreduction of pheophytin in PS

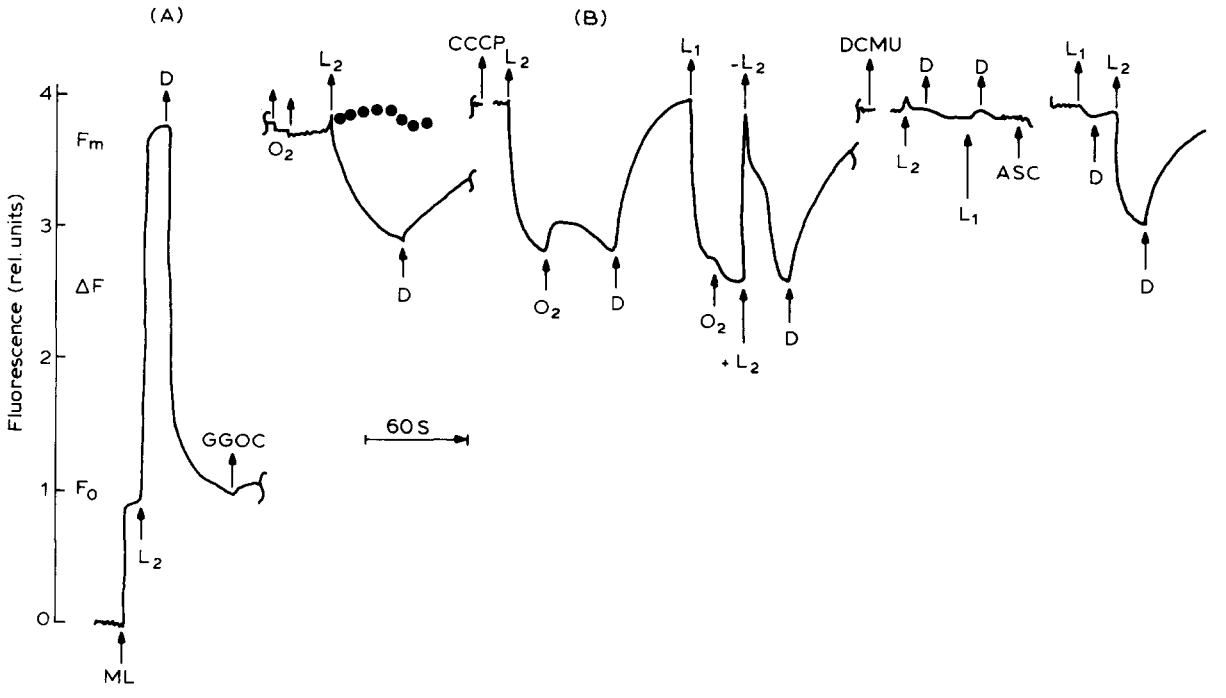


Fig. 3. Kinetics of photoinduced changes of chlorophyll fluorescence yield in spinach chloroplasts under aerobic (A) and anaerobic (B) conditions; Chl concentration is 20 μg/ml. Designations: +L2 and −L2 is switching light II on and off, respectively, during background illumination by light I. The other abbreviations as in Fig. 1.

II (see previous section).

In contrast to PS-II particles, however, in chloroplasts light I also induces a decrease of fluorescence, the nature of which is quite different from that produced by light II. The air bubbling during illumination by light I leads to a further decrease of fluorescence (Fig. 3), indicating that the fluorescence decrease induced by light I is an oxidative effect. Photooxidation of $Q^-$ to Q by PS I is responsible for the negative $\Delta F$ induced by light I, since switching on the light II during illumination by light I (Fig. 3) results in a very fast increase of $F$ to the level $F_{max}$ thus reflecting photoreduction of Q oxidized by light I. If the illumination by a light II is continued the fluorescence starts to decrease (due to the pheophytin photoreduction described above) resulting in the typical effect of 'induction of fluorescence' (Fig. 3).

The fluorescence decrease related to oxidation of $Q^-$ by light I is inhibited by DCMU and in the sample poisoned by DCMU the negative $\Delta F$ induced by light I is not reactivated by ascorbate (in contrast to the effect related to pheophytin photoreduction by light II (Fig. 3).

Thus, in chloroplasts two different types of photoinduced fluorescence decrease are observed under anaerobic conditions when Q is reduced before illumination by actinic light. One of them reflects photoreduction of pheophytin in PS II, while the other is due to oxidation of $Q^-$ by PS I. Both of these effects were observed earlier in chloroplasts in the presence of dithionite [3,11]. It is likely that the photoinduced decrease of fluorescence observed recently (Ref. 12, and see also Heber, U., Kobayashi, Y., Leegood, R.C. and Walker, D.L., unpublished results) in chloroplasts under anaerobic conditions is a combination of the two effects. The results reported here suggest that pheophytin photoreduction also may take place under aerobic conditions in leaves or whole algae cells when Q is reduced. It may contribute to the often observed decrease of fluorescence ('P–S') during illumination by strong light (Kautsky effect, see Ref. 17). In fact, in the presence of the active water-splitting system strong light is capable of photoreducing both Q and pheophytin, thereby producing a long-lived state with $Q^-$ and $Pheo^-$, since in intact PS II pheophytin should be efficiently isolated from possible oxidants except Q.

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