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REACTIONS BETWEEN PRIMARY AND SECONDARY ACCEPTORS OF PHOTOSYSTEM II IN *CHLORELLA PYRENOIDOSA* UNDER ANAEROBIC CONDITIONS AS STUDIED BY CHLOROPHYLL *a* FLUORESCENCE

J. A. VAN BEST and L. N. M. DUYSENS

Department of Biophysics, Huygens Laboratory of the State University, Wassenaarseweg 78, Leiden (The Netherlands)

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SUMMARY

The kinetics of the fluorescence yield Φ of chlorophyll *a* in *Chlorella pyrenoidosa* were studied under anaerobic conditions in the time range from 50 μs to several minutes after short ($t_{\frac{1}{2}}$ = 30 ns or 5 μs) saturating flashes. The fluorescence yield "in the dark" increased from $\Phi = 1$ at the beginning to $\Phi \approx 5$ in about 3 h when single flashes separated by dark intervals of about 3 min were given.

After one saturating flash, Φ increased to a maximum value (4–5) at 50 μs, then Φ decreased to about 3 with a half time of about 10 ms and to the initial value with a half time of about 2 s. When two flashes separated by 0.2 s were given, the first phase of the decrease after the second flash occurred within 2 ms. After one flash given at high initial fluorescence yield, the 10-ms decay was followed by a 10 s increase to the initial value. After the two flashes 0.2 s apart, the rapid decay was not followed by a slow increase.

These and other experiments provided additional evidence for and extend an earlier hypothesis concerning the acceptor complex of Photosystem II (Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256; Velthuys, B. R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94): reaction center 2 contains an acceptor complex QR consisting of an electron-transferring primary acceptor molecule Q, and a secondary electron acceptor R, which can accept two electrons in succession, but transfers two electrons simultaneously to a molecule of the tertiary acceptor pool, containing plastoquinone (A). Furthermore, the kinetics indicate that 2 reactions centers of System I, excited by a short flash, cooperate directly or indirectly in oxidizing a plastohydroquinone molecule (A^{2-}). If initially all components between photoreaction 1 and 2 are in the reduced state the following sequence of reactions occurs after a flash has oxidised A^{2-} via System I: $Q^-R^{2-}+A \rightarrow Q^-R+A^{2-} \rightarrow QR^-+A^{2-}$. During anaerobiosis two slow reactions manifest themselves: the reduction of R (and A) within 1 s, presumably by an endogenous electron donor D_1, and the reduction of Q in about 10 s when R is in the state R^- and A in the state A^{2-}. An endogenous electron donor, D_2, and Q^- compete in reducing the photooxidized donor complex of System II in reactions with half times of the order of 1 s.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

INTRODUCTION

Changes in the fluorescence yield of chlorophyll *a* in *Chlorella* occurring 50 μs to 0.1 s after a short flash are mainly caused by the reduction or oxidation of the primary acceptor Q of the oxygen-evolving System II of photosynthesis: oxidized Q quenches the fluorescence, reduced Q, Q^-, does not [1]. The absorption changes caused by the $Q \rightarrow Q^-$ transition were discovered by Stiehl and Witt [2] and identified by van Gorkom [3] as caused by the reduction of a (plasto)quinone to a semi (plasto)quinone.

We have studied the fluorescence kinetics after laser ($t_{\frac{1}{2}} = 30$ ns) or xenon ($t_{\frac{1}{2}} = 5$ μs) flashes, under anaerobic conditions. These kinetics provide independent evidence for the occurrence of an acceptor complex QR [4, 5] consisting of a one-electron-transferring, primary acceptor molecule Q, and a molecule R which mediates electron transfer to the plastoquinone pool A [6]. R probably has the same redox potential as A and thus may be a plastoquinone [7]. Furthermore, kinetics and other details of various reactions in which the complex QR can participate are given.

MATERIALS AND METHODS

Algae (*Chlorella pyrenoidosa* Chick, strain Emerson 3) were grown as described previously [8], then centrifuged, suspended in fresh growth medium and brought to an extinction at 680 nm (corrected for scattering at 720 nm) of 0.2 in a layer of 5 mm thickness. Before measurement, the algae were bubbled for several hours with air and 5 % CO_2 in light, then for 20–30 min by nitrogen with 5 % CO_2 in the dark, and hereafter transferred quickly by means of a syringe into a $30 \times 30 \times 5$ mm cuvette which was subsequently kept closed during the whole experiment. Care was taken to minimize contact with air. The algae sedimented in a few minutes. All experiments were carried out at room temperature.

For actinic excitation a flash from a Q-switched ruby laser (Impuls Physik GmbH) and/or xenon flashes were used. The laser (which is no longer commercially available) was modified in order to suppress small spurious laser pulses preceding and following the giant pulse. A pulse shaper which differentiated the step voltage for the Kerr cell laser switch was used and a passive dye (cryptocyanine) was placed in the cavity. The maximum energy density on the cuvette was 1.5 mJ/cm^2, the half time of the flash was 30 ns. The xenon flash tube was a General Electric FT 230; $C = 10$ μF, $V = 1800$ V; half time = 5 μs. The xenon-lamp current oscillations were damped by two back-biased diodes (AEG D42/1600 B) and a resistor (0.1 Ω) in series across the capacitor. Each diode was bypassed by two 8.2 MΩ resistors in series for overvoltage protection. They reduced the tail of the flash by a factor of more than 10. The xenon flash light passed the filter set: Schott KG3/2, Calflex-C, Corning CS4/96, which transmitted a band between 400 nm and 550 nm. The maximum energy density on the cuvette was about 80 μJ/cm^2. Unless otherwise indicated, the time between flashes of a series was 0.2 s.

For fluorescence measurement, a second non-saturating flash lamp mounted in a coaxial house was used (GE FT 230) fed by a 0.1 μF capacitor at 1.8 kV. The filter combination was: Schott KG3/2, Calflex-C, Schott AL433, Schott BG18/2 which transmitted a band with a maximum at 433 nm and a half width of 20 nm. The half

time was 0.5 μs and the energy density on the cuvette was about 0.1 $\mu J/cm^2$. This flash could be repeated every 3 ms. Continuous far-red illumination could be given with a 24 V, 240 W tungsten iodide lamp with the filter set: Schott KG3/3, Calflex-C and Schott AL740. The maximum intensity on the cuvette was $6.2 \cdot 10^{-4}$ W/cm^2.

Fluorescence was measured at $\lambda = 677$ nm with the filter combination: Schott DAL677, Schott RG1/1, Schott KV550/3. The photomultiplier was a S-20 type (Philips XP1002) which was gated by a negative voltage between the cathode and the first and third dynode during all actinic flashes. A fraction of the weak measuring flash was measured by means of four photodiodes (Monsanto MD1) as a reference.

Without preceding actinic excitation, the relative energies of both signal and reference beams were recorded after "integration" by RC filtering on peak voltmeters. The first weak fluorescence signal flash occurring 50 μs after the last actinic flash was recorded without integration on a fast storage oscilloscope (Tektronix 7623) in order to avoid disturbances by the actinic flash. The amplitude of the reference was also recorded without integration on a peak voltmeter. The time of 50 μs was chosen because Φ at that time is nearly independent of the number of preceding flashes and is about equal to the maximum value [9]; at 50 μs there is no marked S-state dependency, as was observed by Delosme a few μs after a flash [10]. The subsequent weak signal and reference flashes were recorded simultaneously after integration in the chopped mode on two slower storage oscilloscopes (Tektronix 549 and R564B) with different time bases. By means of a manually operated pointer connected to a potentiometer, the data were read from polaroid photographs of the oscilloscope screens, put on papertape by means of an analog-to-digital converter, and analyzed by a computer.

RESULTS AND INTERPRETATION

All experiments we know of are in agreement with the hypothesis that the primary acceptor of System II quenches the chlorophyll *a* fluorescence in the oxidized but not in the reduced state [1]. Other substances and factors have been shown to affect the fluorescence yield, but unless stated otherwise, in this discussion we will assume that the fluorescence decrease and increase after one or more flashes are caused by the oxidation and reduction of the primary acceptor Q.

A. Fluorescence changes up to 0.2 s

Since under aerobic conditions the fluorescence yield decays after a flash to a low value in less than 3 ms ($t_{\frac{1}{2}} \approx 0.2$ ms [9]), we conclude that the reaction of Q^- with an oxidized secondary acceptor, R, occurs within 3 ms. If the fluorescence decay after a flash is slower, we assume that at the end of the flash the secondary acceptor R is in the reduced state.

A few seconds after the algae had been put in the cuvette, the fluorescence yield was about the same as under aerobic conditions indicating that traces of oxygen were still present. The initial fluorescence yield then increased in a few minutes by about 50 %, and the fluorescence yield after one flash decayed with a half time of about 10 ms (Fig. 1). It follows that at the moment of the flash R was in the reduced state; the 10 ms oxidation of Q^- then occurred by photoreaction 1. Oxidized donor

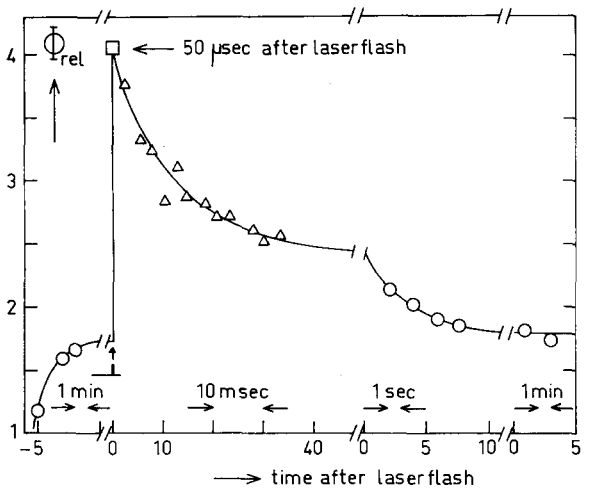

Fig. 1. Φ before and after one single saturating laser flash which occurs 6 min after insertion of the cuvette in the apparatus. The following legend also applies to Figs 2–6. Time curve of the fluorescence yield Φ of *Chlorella pyrenoidosa* in a closed cuvette after being bubbled by $N_2+5\,\%\ CO_2$ in the dark for 20 min. The fluorescence yield of aerobic *Chlorella* is about 1.0. 0, Δ: Φ in weak "measuring" xenon flash ($\lambda = 430$ nm; $t_{1/2} = 0.5$ µs; $E(0) = 0.1$ µJ/cm², $E(\Delta) = 0.025$ µJ/cm²). □: Φ at 50 µs after a saturating flash, sometimes preceded by another with an interval time of 0.2 s. A vertical bar indicates a saturating laser flash ($\lambda = 694.3$ nm; $t_{1/2} = 30$ ns; $E = 1$ mJ/cm²), a triangle indicates a saturating xenon flash ($\lambda = 430 \ldots 500$ nm; $t_{1/2} = 5$ µs; $E = 80$ µJ/cm²). Note the different scales for the time axis.

molecules of system 1, P^+-700, are generated which oxidize Q^- via the plastoquinone pool A [6] and R.

Recent experiments on methylviologen reduction in a series of light flashes [11] in spinach chloroplasts suggest that the number of reaction centers of Systems I and II are about equal. The observation (Duysens, L. N. M and van den Berg, R. J., unpublished) that the amplitude of oxgyen pulses in a series of light flashes is increased by only about 10 % when a background of far-red light is given indicates that the same is true for *Chlorella*. The observation that 15 ms after one flash the variable

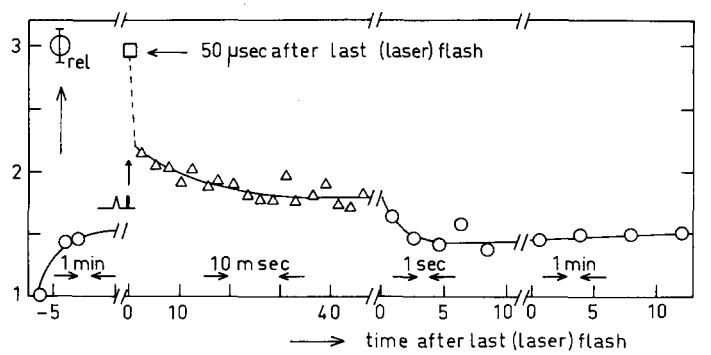

Fig. 2. Φ before the first and after the second flash, 6 min after insertion. See legend of Fig. 1.

fluorescence decays to about half of its maximum value, then indicates that only about half or perhaps less of the oxidizing equivalents produced by System I are used for the oxidation of Q^-. Presumably each plastoquinone molecule oxidizes one molecule of Q^-. A plausible hypothesis [4, 5] is that completely reduced R is a two-electron donor (R^{2-}) and reacts with plastoquinone to give R. Subsequently, Q^- reduces R to R^-.

This hypothesis is also consistent with and strongly supported by the kinetics of fluorescence decay in the ms region after a second flash given 0.2 s after the first one. About half of the variable fluorescence then decays in less than 2 ms (Fig. 2), indicating that about half of the reaction centers Q^- transfers an electron to R^- or R. This is in accordance with the conclusion from the preceding paragraph that about half of the reaction centers are in the state QR^- and about half in the state Q^-R^{2-} after the first flash. Part of the reaction centers in the state Q^-R^{2-} then reacts with plastoquinone (A) which had been oxidized by System I in 10 ms (see also Fig. 7):

$$Q^-R^{2-}+A \rightarrow Q^-R+A^{2-} \rightarrow QR^-+A^{2-} \quad (1)$$

The small 10 ms decay after the second flash can be explained by these reactions. The third decay phase with a 2 s half time is discussed below.

After a period of anaerobiosis and suitable flash regime (see below) the steady-state fluorescence yield is high (4–5), and most reaction centers are in the state Q^-R^{2-}. The kinetics after one and two flashes starting from a high initial fluorescence yield are shown in Figs 3 and 4. The rapid phases <3 and 10 ms are the same as for initial low fluorescence as predicted by the hypothesis given above (see also Fig. 7). The same is true for an intermediate initial yield (Fig. 5). A 10 ms decay after one flash at high initial fluorescence yield was found very recently in anaerobic *Scenedesmus* [12]. This was measured with continuous measuring light of relatively strong intensity which made it impossible to measure the kinetics which we observed at longer times (see below).

The above discussion also implies that two reaction centers of System I excited by one flash must cooperate to oxidize one plastohydroquinone molecule (which requires 2 equivalents) either directly or via intermediates such as plastoquinone,

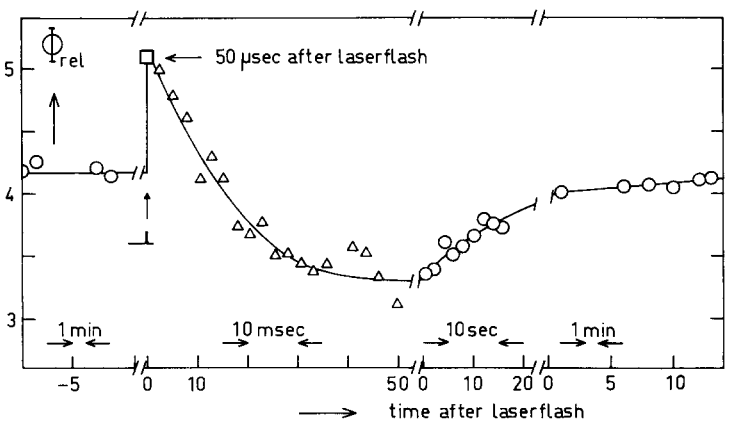

Fig. 3. Φ before and after a single saturating laser flash which occurred at high initial fluorescence yield ($\Phi = 4.2$). See legend of Fig. 1.

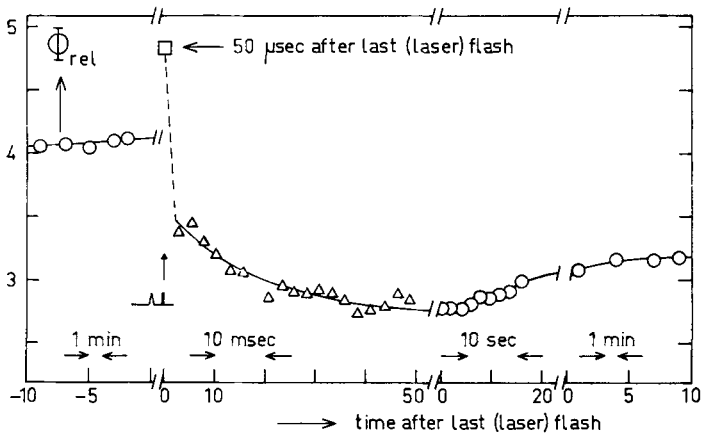

Fig. 4. Φ before and after two saturating flashes which occurred at high initial fluorescence yield (Φ = 4.1). See legend of Fig. 1.

plastocyanine or cytochrome molecules, unless one assumes that oxidized equivalents formed by System I in an earlier flash have even under anaerobic conditions a lifetime of hours, since the 10 ms decay after the first flash is observed after a dark period of hours.

The fluorescence yield 50 μs after a flash is indicated by squares in the figures. During a flash the yield increases rapidly and reaches a maximum Φ_{50} at about 50 μs after a flash [9]. The primary acceptor is then predominantly in the state Q^-. We observed that both the initial fluorescence yield and Φ_{50} after one flash increased relatively rapidly by about 50 % in a few minutes after the moment the suspension was put in the cuvette. It is possible that plastoquinone quenches somewhat the chlorophyll fluorescence. Upon reduction of plastoquinone the fluorescence yield of System II then somewhat increases. Similar suggestions have been made by Delosme [13] in order to explain a second non-photochemical phase in the rise of the fluorescence

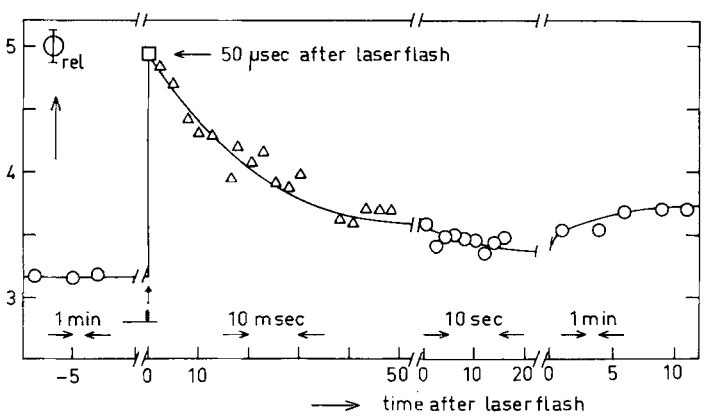

Fig. 5. Φ before and after one single laser flash which occurred at moderate initial fluorescence yield (Φ = 3.16). See legend of Fig. 1.

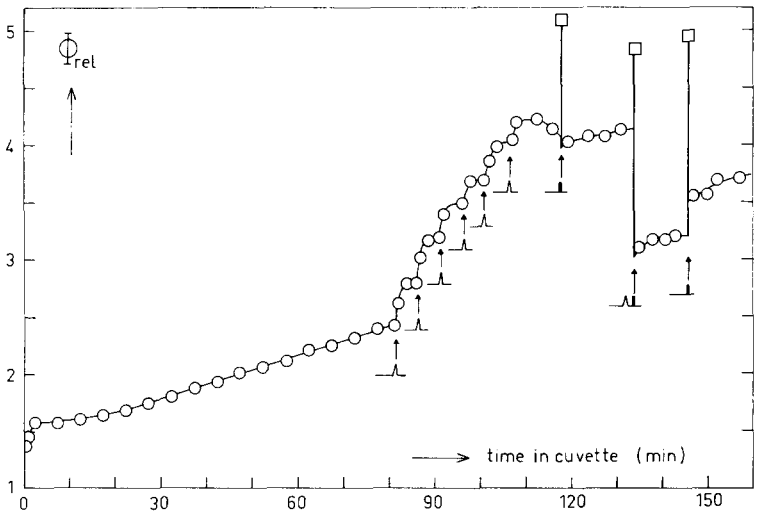

Fig. 6. The relative fluorescence yield during the experiment. The symbols are the same as in Fig. 1. Twice as many weak flashes were given as indicated by the circles. The fast components of the kinetics after the laser flashes are given in Figs 3, 4 and 5.

yield in strong continuous light. After a stationary phase of the order of 10 min, the fluorescence yield further rises, provided flashes are given (Fig. 6, see below for further discussion).

B. Fluorescence changes in the range 0.2 s to several minutes

Bennoun [14] has shown that in the presence of DCMU, which prevents reoxidation of Q^- by secondary acceptors [1], the reaction $S^+Q^- \rightarrow SQ$ occurs with a half time of about 1 s. S^+ is an oxidized state of the donor complex of System II, formed in the flash. Presumably this reaction is also responsible for the 2 s decay following the 10 ms decays in fluorescence yield (see Figs 1 and 2).

When the initial fluorescence yield is high (Fig. 3), no 2 s decay occurs, as predicted, after a flash, since only little S^+ state was formed because electrons cannot be transferred to the primary acceptor which was reduced already before the flash. Instead, a slow increase with a half time of 10 s occurs after the 10 ms decrease. As discussed, within 50 ms of the flash, half of the reaction centers were in the state QR^-. After one flash, the fluorescence yield rises to the initial high yield in 1 min, but after two flashes, the yield remains lower than the initial yield (Figs 3 and 4). We conclude that the reaction center in the state QR^-, present after one flash, is reduced presumably by a reversal of Reaction 1: Apparently when A becomes increasingly reduced by the endogenous donor, the equilibrium shifts in 10 s to the state Q^-R^{2-}. The 10 s increase in fluorescence yield does not occur if one flash is given at low initial fluorescence yield because the S^+ formed in this flash recombines with Q^- in 2 s. Since an initial, low fluorescence yield remains almost stationary in the dark, Q in the complex QR^{2-} is not reduced or reduced much more slowly. This is because the equilibrium of the reaction $Q^-R^- \rightarrow QR^{2-}$ is to the right and because there is no other reductant than Q^- able to reduce R^- at a measurable rate. When the initial fluorescence is

intermediate, the fluorescence yield kinetics following one or two flashes are intermediate between those for initial high and low yields (Fig. 5).

The stationary fluorescence yield after two flashes is lower than the initial yield before the flashes were given, which is in accordance with the preceding hypotheses. These hypotheses also predict that after one flash, the fluorescence after a temporary rapid decrease rises to the initial level within 1 min. It is observed, however, (Fig. 6) that after one flash an increase a little higher than this level occurs. This can be explained by the additional assumption that under anaerobic conditions part of the S^+ formed is reduced by an endogenous donor, D_2, in competition with the reaction $S^+Q^- \rightarrow SQ$.

The slow rise (Fig. 6) between 20 and 40 min is caused by the weak measuring flashes given. If no flashes were given at all, the fluorescence yield did not increase markedly for hours.

Far-red light mainly excites Photosystem I [1]. We observed that in light of 740 nm and intensity 0.6 mW/cm^2 the fluorescence yield decreased from 5.5 to 1.3 in less than a few seconds. When the far-red light was turned off, no increase in fluorescence yield took place for more than 1 h, when no flashes were given. Extensive experiments with *Scenedesmus* with far-red light have been published by Schreiber and Vidaver [12].

Immediately after 5 s of far-red light, the fluorescence excited by one flash after the light was turned off decreases in less than 2 ms. However, if the flash was given about 0.5 s after turning off the light, the 10 ms decay was half restored. We conclude from this that R and A are reduced by the endogenous donor D_1 with a half time of about 0.5 s.

One saturating xenon flash had about the same effect as one saturating laser flash and two saturating xenon flashes had about the same effect as one laser flash preceded by one xenon flash, indicating that also in the xenon flash only one turnover occurs for both photosystems.

If an airbubble was admitted to a cuvette with high fluorescence yield, and the cuvette was turned upside down a few times, the fluorescence yield dropped to about 1.3 within a few seconds, and the decay of the fluorescence after a flash occurred in less than 3 ms, indicating that oxygen oxidized Q^- and R^{2-} presumably via plastoquinone.

The experiments were repeated several times and the results were all in accordance with the hypotheses given above. The slow increase of the fluorescence yield induced by single flashes during anaerobiosis (Fig. 6) did not occur for some cultures, or only after a period of five or more hours of anaerobiosis.

DISCUSSION

The scheme shown in Fig. 7 summarizes our conclusions on *Chlorella*. The 10-s reduction of Q after 1 flash at high initial fluorescence yield is given by the reactons:

$$QR^- \rightarrow Q^-R$$

$$Q^-R + A^{2-} \rightarrow Q^- R^{2-} + A$$

$$A + D_1 \rightarrow A^{2-} + D_1 \text{ ox}$$

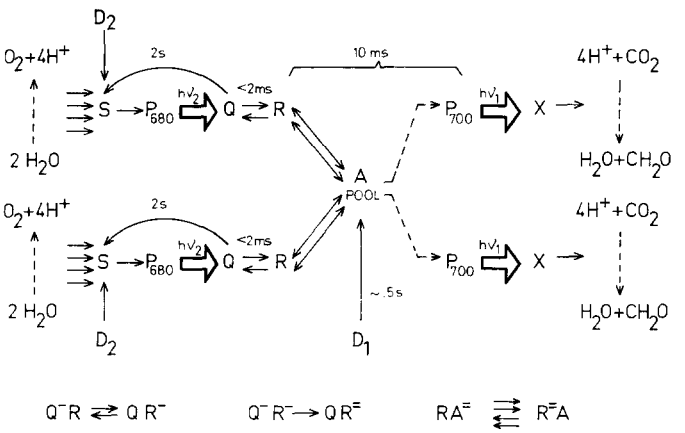

Fig. 7. Hypothetical scheme summarizing electron transfer reactions in *Chlorella* between molecules of the reaction center 2 complex and associated components. The number of parallel arrows indicates the number of electrons transported simultaneously. The dotted lines imply intermediate electron carriers. A is a pool common to a number of reaction centers 2 and 1 containing plastoquinone. D_1 and D_2 are unidentified electron donors. Q can be reduced by D_1 in 10 s (see Discussion). Presumably the number of reaction centers 1 is about equal to that of reaction centers 2.

These reactions are for technical reasons not included in the scheme. We found this the simplest interpretation of our and other experiments. However, it cannot be excluded that more complicated reactions cause the reduction of Q, e.g. by an endogenous two-electron donor, D_3:

$$D_3^{2-} + QR^- \rightarrow D_3 + Q^-R^{2-}.$$

In our interpretation of the Q^- oxidation by one flash at high inital fluorscence yield, it was assumed implicitly that the equilibrium of the reaction

$$Q^-R^{2-} + A \rightleftarrows QR^- + A^{2-}$$

is towards oxidized Q when at least 10 % of A had become oxidized by flash excitation of System I. If the ratio A/*P*-700 $\approx$ 5, as is the case in spinach chloroplasts [2], about 10 % of A is oxidized by one flash.

From the data in Fig. 3 we then estimate that the equilibrium constant is at least 5, certainly not 1 or smaller as concluded by Diner [7] from indirect evidence, among others amplitudes of oxygen pulses in short light flashes under anaerobic conditions. It is possible that under anaerobic conditions the donor sites of an appreciable fraction of the reactions centers 2 are inactive in oxygen evolution. On the other hand our results are not inconsistent with Diner's finding from more direct experiments that R and A have about the same redox potential. Our scheme, as far as the rapid reactions are concerned, is analogous to a scheme given by Bouges-Bocquet [15]. However, the equilibrium constant of the reaction

$$Q^- R^- \rightleftarrows QR^{2-}$$

is much larger than 1 as can be estimated from Fig. 4 and not equal to 0.5 as proposed by Bouges-Bocquet [15].

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