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A ONE MICROSECOND COMPONENT OF CHLOROPHYLL LUMINESCENCE SUGGESTING A PRIMARY ACCEPTOR OF SYSTEM II OF PHOTOSYNTHESIS DIFFERENT FROM Q

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 luminescence of chlorophyll *a* in *Chlorella vulgaris* were studied in the time range from 0.2 μs to 20 μs after a short saturating flash ($t_{\frac{1}{2}}$ = 25 ns) under various pretreatment including anaerobiosis, flashes, continuous illumination and various additions. A 1 μs luminescence component probably originating from System II was found of which the relative amplitude was maximum under anaerobic conditions for reaction centers in the state SPQ^- before the flash, about one third for centers in the state S^+PQ^- or SPQ before the flash, and about one tenth for centers in the state S^+PQ before the flash. S is the secondary donor complex with zero charge; S^+ is the secondary donor complex with 1 to 3 positive charges; P, the primary donor, is the photoactive chlorophyll *a*, *P*-680, of reaction center 2; Q^- is the reduced acceptor of System II, Q. Under aerobic conditions, where an endogenous quencher presumably was active, the luminescence was reduced by a factor two.

The 1 μs decay of the luminescence is probably caused by the disappearance of P^+ formed in the laser flash according to the reaction $ZP^+ \rightarrow Z^+P$ in which Z is the molecule which donates an electron to P^+ and which is part of S. After addition of hydroxylamine, the 1 μs luminescence component changed with the incubation time exponentially (τ = 27 s) into a 30 μs component; during the same time, the variable fluorescence yield, measured 9 μs after the laser flash, decreased by a factor 2 with the same time constant. Hereafter in a second much slower phase the fluorescence yield decreased as an exponential function of the incubation time to about the dark value; meanwhile the 30 μs luminescence increased about 50 % with the same time constant (τ = 7 min). Heat treatment abolished both luminescence components.

The 1 μs luminescence component saturated at about the same energy as the System II fluorescence yield 60 μs after the laser flash and as the slower luminescence components. From the observation that the amplitude is maximum if the laser flash is given when the fluorescence yield is high after prolonged anaerobic conditions (state SQ^-), we conclude that the 1 μs luminescence is probably caused by the reaction

$$PWQ^- + h\nu \rightarrow P^*WQ^- \rightarrow P^+W^-Q^- \rightarrow P^*WQ^- \rightarrow PWQ^- + h\nu$$

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

in which W is an acceptor different from Q. The presence of S^+ reduced the luminescence amplitude to about one third. Two models are discussed, one with W as an intermediate between P and Q and another, which gives the best interpretation, with W on a side path.

GLOSSARY OF TERMS

A	Amplitude of luminescence components.
A_A	Amplitude of the ultra fast luminescence component ($\tau < 0.3$ μs), mainly due to an artifact.
A_F	Amplitude of the fast luminescence component ($\tau = 0.7$ to 1.4 μs)
A'_F	Theoretical amplitude of the fast luminescence component, computed via Eqn. 2 (see text).
A_S	Amplitude of the slow luminescence component ($\tau = 11$ to 30 μs).
A_τ	Amplitude of the luminescence component with a decay time of τ μs.
A_{pool}	Plastoquinone pool between the two photosystems.
D	Endogenous donor which can give an electron to the oxidized primary donor of System II, P^+, in about 11 to 30 μs in competition with the secondary donor Z [8].
D_1	Endogenous donor which reduces the A_{pool} under anaerobic conditions [10].
D_2	Endogenous donor which reduces the S-states under anaerobic conditions [10].
DCMU	3-(3',4'-dichlorophenyl)-1,1-dimethylurea which stops the electron flow between the primary and secondary acceptor of System II, Q and R, respectively.
NH_2OH	Hydroxylamine, which stops the electron flow between Z and P^+ and which donates electrons to *D* in a few seconds [8].
LUM	Relative luminescence amplitude.
N	Number of actinic flashes before measurement.
P,P^+	The primary donor of System II, *P*-680.
Q,Q^-	The "primary" acceptor of System II.
$(Q),(Q^-)$	Fraction of the reaction centers in the state Q and Q^- respectively.
R,R^-,R^{2-}	Secondary acceptor of System II.
S	Secondary donor complex of System II, including the secondary donor Z.
S^0,S^{1+},S^{2+},S^{3+}	S with zero, 1, 2 or 3 extra positive charges.
$(S),(S^+)$	Fraction of the reaction centers in the state S^0 and S^{n+}, respectively (with $n = 1$, 2 or 3).
T	Carotenoid triplet, quenches the fluorescence of chlorophyll *a* [8].
W,W^-	A primary acceptor of System II different from Q.
Z,Z^+	Secondary donor of System II.
a.u.	Arbitrary units; a.u. are in general comparable with each other.
P	Probability of transfer of an energy quantum from a unit with the reaction center in the state Q^- to another unit.
q^-	(Q^-)
t_i	Incubation time.

β	Factor in Eqn. 2 probably due to a quencher which disappears under anaerobic conditions.
Φ	Relative fluorescence yield; $\Phi = 1$ for dark-adapted aerobic algae without additions.
Φ_0	Minimum Φ under given conditions.
Φ_{dark}	Φ of dark-adapted algae under given conditions.
Φ_{max}	Maximum Φ under given conditions.
Φ_B	Φ just before a flash series.
Φ_L	Φ just before the (last) laser flash.
Φ_9 or Φ_{60}	Φ measured 9 μs or 60 μs after the laser flash.
τ	1/e — rise or decay time.

INTRODUCTION

The delayed light emission (luminescence) of higher plants may be attributed to the back reaction between the oxidized primary donor, $P\text{-}680^+$, and the reduced primary acceptor, Q^-, of Photosystem II [1]. On account of measurements in the region from 65 μs to 800 μs, Zankel [2] concluded that three exponential decays were present in the luminescence of isolated chloroplasts after a saturating flash, with a half time of about 10 μs, 35 μs and 200 μs respectively. Haug et al. [3] concluded from measurements on *Scenedesmus obliquus* (wild type, mutant 8 and II) in the range from 4 ns to several seconds with chopped continuous laser light, that most delayed light originates from Photosystem II. With a He-Ne laser phosphoroscope Lumpkin and Hillel [4] measured the steady state luminescence of *Chlorella* and found it spectrally identical with chlorophyll *a* fluorescence; the decay half time was about 110 μs and the luminescence was suppressed by DCMU. Haveman and Lavorel [5] found a 110 to 150 μs component in spinach chloroplasts and sub-chloroplast particles after a 500 μs laser flash, correlated with EPR signal II_f measured by Babcock and Sauer [6] and concluded that a time shorter than about 1 μs for the reaction $P^+Z \rightarrow PZ^+$ was reasonable, where Z is the secondary donor of Photosystem II. Lavorel [7] found three kinetic components in the range from 6 to 600 μs following a microsecond flash and studied the effect of DCMU and hydroxylamine. Duysens et al. [8] measured a 130 μs and a 10 to 20 μs luminescence component after saturating flashes in intact *Chlorella* which showed an oscillation of four in amplitude, saturated like System II fluorescence, and were eliminated by addition of DCMU. We will describe a new luminescence component of about 1 μs which obviously originates from System II and which has a maximum yield when the reaction centers were closed before the flash, unless a positive charge was present in the secondary donor complex of System II. From our measurements we conclude that a primary acceptor of System II different from Q is present.

MATERIALS AND METHODS

Algae (*Chlorella vulgaris*, var. *viridis*) were grown as described previously [9]. After centrifugation, they were suspended in fresh growth medium and brought to an extinction of 0.2 at 680 nm (corrected for scattering at 720 nm) in a layer of 5 mm thickness. Pretreatment to obtain anaerobic conditions was done as described pre-

viously [10]. For aerobic measurements, the algae were illuminated at about 20 °C during several hours while being bubbled with air enriched with 5 percent CO_2 and subsequently transferred quickly by means of a syringe into a $30 \times 30 \times 5$ mm cuvette which was kept closed during the experiment. The algae sedimented in a few minutes in the horizontally placed cuvette. If the relative fluorescence yield in the dark, measured by means of a weak non-saturating xenon flash after a dark period of a few minutes, started to increase by about 5 percent, indicating the onset of anaerobicity, the cuvette was shaken with an air bubble in the light in order to restore aerobicity.

For actinic excitation, a flash from a Q-switched ruby laser ($t_{\frac{1}{2}} = 25$ ns; $\lambda = 694.3$ nm) was used, sometimes preceded by saturating xenon flashes. The apparatus, described previously [10], was slightly modified. In order to obtain a better cut off of the laser flash, the Kerrcell was replaced by a $\lambda/4$ wave plate in combination with a Pockelscell (Electro Optic Developments LTD 01.PC 12 KD; longitudinal KD*P crystal) which was driven by a fast step generator (Electro Optic Developments LTD Q-switch driver 02.PCD; risetime < 0.7 ns). Fast closing (< 1 ns) of the cell was obtained by the use of a delay line consisting of 28 m coaxial 50 Ohm cable between the electrodes of the Pockelscell in order to generate a square wave of 140 ns, which was required for building up a laser flash with a halftime of 25 ns. In order to enhance the accuracy of measurement, the anode of the gated photomultiplier was connected to an oscilloscope preamplifier (Tektronix 7 A13) by a cable terminated by 50 Ohm. The output of the oscilloscope ($R = 1$ k Ω) (Tektronix 7623) was connected via a 10 μF capacitor shunted by a 10 nF ceramic disk capacitor to the 50 Ohm input of a transient recorder (Biomation model 8100). The stored data (2048 8-bit numbers equally spaced over 25 μs) were scanned at a slow rate via the Y-output on the vertical amplifier (7A9) of an oscilloscope (Tektronix R 564 B). The bandwidth, the amplification factor, the time base and time base delay of this oscilloscope could be set to an appropriate value, after the measurement had taken place.

The sensitivity of the apparatus was normalized before each experiment by means of the measurement of the relative fluorescence yield of rhodamine B "dissolved" in plastic. The data from the oscilloscope screen were analyzed as described previously [10], or the digital data from the transient recorder were transferred directly to a magnetic tape recorder and afterwards analyzed by a computer (Digital Equipment, PDP 9).

The distortion of the measuring system after a laser flash was checked by monitoring either the signal of a light emitting diode placed directly in front of the photomultiplier, or the fluorescence yield of a chemical compound (cresyl violet) excited by the low intensity xenon flash; the total distortion was less than 5 %.

In order to prevent interference by luminescence from other sources than the algae, the luminescence was isolated by means of Schott interference filters DAL 677 and AL 673 in front of the photomultiplier; the second filter was selected for minimum luminescence from a number of similar filters available in the laboratory. The peak transmission of the filter set was 20 % at 674 nm and the halfwidth was 14 nm. The transmittance at 694.3 nm was less than 0.05 %. The luminescence signal of the algae was corrected for the remaining false luminescence from filter, cuvette and lens by subtracting the relatively small "luminescence" of algae heated at 75 °C for 5 min. The "luminescence" of heated algae was found to be proportional to the laser energy.

RESULTS AND INTERPRETATION

Luminescence under aerobic conditions at low Φ_L

The luminescence decay curve of *Chlorella vulgaris* between 0.2 μs and 25 μs after the last (laser) flash of a series of one or more flashes, can be described as the sum of three exponentially decaying components with a decay time of $\tau_1 < 0.35\,\mu$s, $\tau_2 = 0.7$ to 1.4 μs and $\tau_3 = 10$ to 30 μs and with an amplitude A_A, A_F and A_S respectively. A_A did not saturate with increasing leaser energy at intensities several times higher than needed for saturation of reaction center 2 and will not be discussed here in detail. This component is mainly an artifact.

A typical luminescence decay curve of aerobic *Chlorella vulgaris* after an oversaturating laser flash is shown in Fig. 1. The drawn line corresponds to a fit achieved with the use of a programmable calculator (HP 65). It was not possible to obtain a reasonable fit with the use of less than 3 exponential decays. Later measurements with improved apparatus resulting in a better time resolution and better curve fitting are given in Fig. 8 (curve $t_i = 0$). The dependence of the amplitudes of these decays on the laser flash energy is shown in Fig. 2. A_F and A_S and the relative fluorescence yield 60 μs after the laser flash given after a dark time of 3 min, Φ_{60}, saturated with increasing laser energy at about the same actinic energy density. The relative fluorescence yield of the algae under aerobic conditions after a dark time of 3 min is defined in all experiments as $\Phi_0 = 1.00$.

A_S is known to originate from System II and shows an S-state dependency [8]. A_F at saturating laser energy is about 12 a.u. (= arbitrary luminescence unit). All a.u. can be compared with each other; however, a maximum fluctuation of the luminescence amplitude by a factor of 2, which is not due to the apparatus, occurs between different batches. A_A appears to be linear with the laser energy in this range.

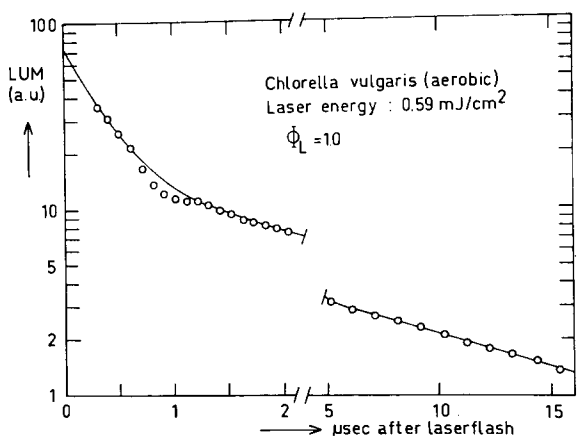

Fig. 1. Kinetics of the luminescence (LUM) of aerobic *Chlorella vulgaris* at $\lambda = 674$ nm after an oversaturating laser flash ($E = 0.59$ mJ/cm^2; $\lambda = 694.3$ nm; $t_{\frac{1}{2}} = 25$ ns) preceded by a dark period of 3 minutes. The luminescence is expressed in arbitrary units (a.u.) but 1 a.u. represents approximately the same intensity in all figures. The drawn curve corresponds to the equation:

$L(t) = 4.5 \exp(-t/13.5) + 15 \exp(-t/1.44) + 55 \exp(-t/0.3)$.

Note the different time scales.

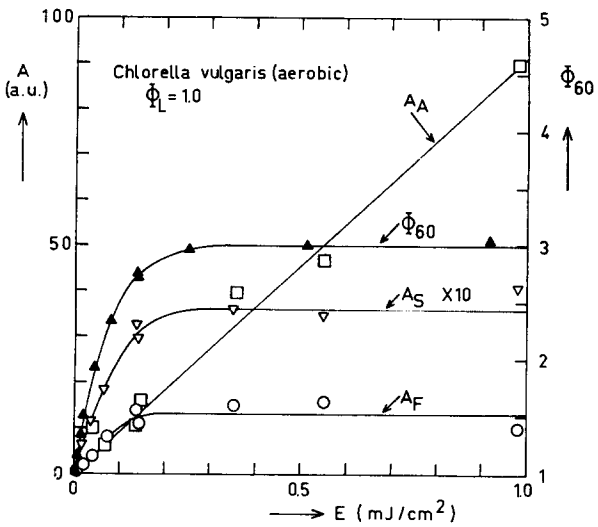

Fig. 2. Relative luminescence amplitudes of the 11 μs (A_S), 1.4 μs (A_F) and 0.33 μs (A_A) components and the relative fluorescence yield 60 μs after the laser flash (Φ_{60}) as function of the laser energy density (E) for *Chlorella vulgaris* after a dark time of 3 min.

Luminescence under anaerobic conditions at different Φ_L

Under anaerobic conditions, the fluorescence yield in the dark was first stepwise increased to a maximum value (5.0) by saturating flashes with an interval time of 3 min. [10]. Then the fluorescence was decreased by far red illumination* and increased again with flashes to about the desired value. After a saturating laser flash given at high Φ_L, the value of A_F was surprisingly high (about 200 a.u.); the decay was monophasic and somewhat faster (0.9 μs) than under aerobic conditions (Fig. 3, curve A). A_F appeared also to be an increasing function of Φ_L (Fig. 4, circles). In order to explain the relation between Φ_L and A_F by means of energy transfer between units, a simple expression between the variable fluorescence yield and the fraction of non quenching states, q^-, was derived from [11]:

$$(\Phi_L-\Phi_0)/(\Phi_{max}-\Phi_0) = q^-(1-P)/(1-Pq^-) \qquad (1)$$

where Φ_L is the relative fluorescence yield before the flash; Φ_0 is the minimum fluorescence yield under these anaerobic conditions (= 1.5); $\Phi_{max} = 5$; and the adjustable parameter P is the probability of energy transfer to another unit of a quantum absorbed by a unit in the non quenching state (Q^-). If the fraction of closed centers, q^-, is supposed to be proportional with A_F in this range, the experimental points give also a relation between q^- and Φ_L which corresponds with the theoretical curve (1) for $P = 0.35$ (Fig. 4, drawn curve). The meaning of this is explained as follows. The values of P for fluorescence reported in the literature [11, 12, 13] vary from 0.5 to 0.7. If P for luminescence would be zero, a value of $P = 0.5$ to 0.7 would be expected for the curve drawn through the open circles (Fig. 4). The intermediate experimental value of $P = 0.35$ suggests that the probability of energy transfer from a reaction

* 1 mW/cm^2 at λ = 740 nm for 10 s.

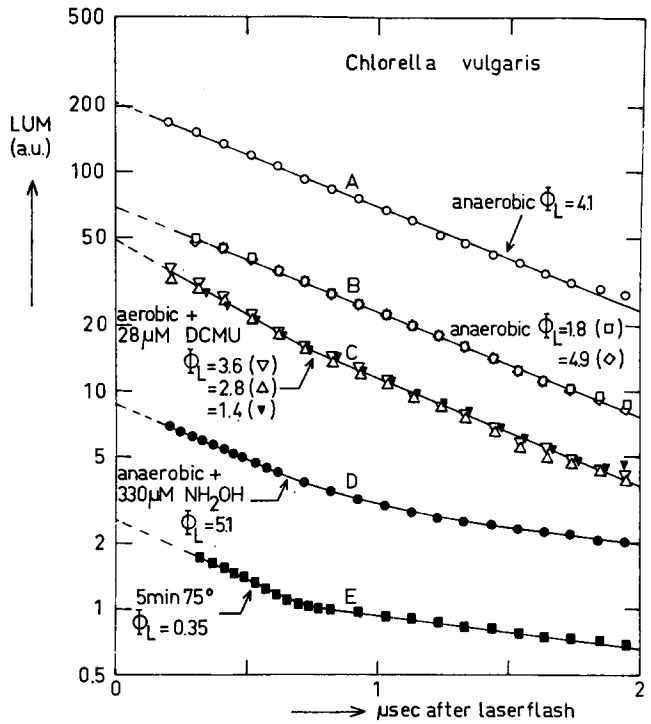

Fig. 3. Luminescence kinetics of *Chlorella vulgaris* after the approximately saturating (last) flash. Pretreatments and conditions are given in Table I. In all these cases, the amplitude of the 10 to 30 μs luminescence component is lower than 2 a.u.. Φ_L = relative fluorescence yield before the (last) flash.

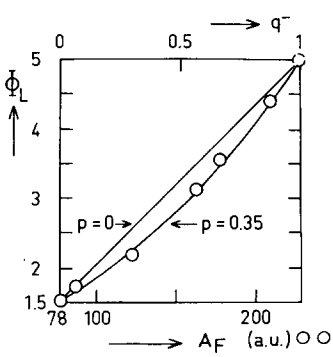

Fig. 4. The relative fluorescence yield before the approximately saturating laser flash, Φ_L (○), as function of the amplitude A_F of the 0.9 μs luminescence component of anaerobic *Chlorella*. The upper broken circle (right) was calculated by extrapolation of the curve to the maximum observed relative fluorescence yield in the dark of this algal batch (Φ = 5.0). The two drawn curves are calculated by means of the theoretical expression (1) giving Φ_L as function of q^- for $P = 0$ and $P = 0.35$, respectively. For further comment, see text.

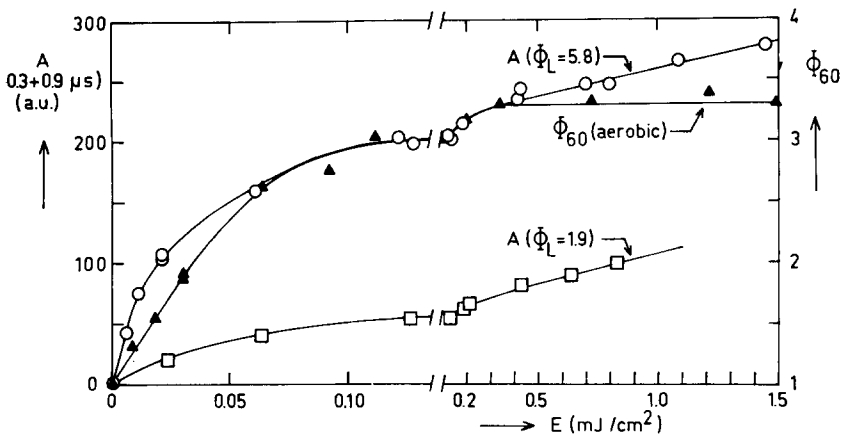

Fig. 5. Sum of the 0.3 μs and 0.9 μs luminescence component (A) of anaerobic *Chlorella vulgaris* at high (Φ_L = 5.8, circles) and at low (Φ_L = 1.9, squares) initial fluorescence yield before the laser flash, and the relative fluorescence yield 60 μs after the laser flash (ϕ_{60}, triangles) as function of the laser energy density (E). Note the different energy scales.

center, presumably located in the "middle" of a unit, to another unit is smaller than the average probability upon excitation of antenna chlorophyll. The independence of the luminescence for the carotenoid triplet quencher T [8] might be explained by the assumption that this quencher is not situated in the vicinity of the reaction center.

In Fig. 5, the amplitude of the luminescence at time zero (obtained by extrapolation) is shown as function of the laser flash energy. This amplitude is equivalent to the sum of A_F and A_A (an analysis of the curve in two components had not been carried out at the time of the experiment). Two luminescence amplitude curves are given, one at high initial fluorescence yield before the flash (Φ_L = 5.8, circles) and one at low initial yield (Φ_L = 1.9 squares). In addition the relative fluorescence yield of aerobic algae 60 μs after the laser flash is given as function of the flash energy. The increase at energies higher than about 0.4 mJ/cm^2 is linear with the energy and is probably caused by A_A. The higher value of the relative luminescence at flash energies lower than 0.07 mJ/cm^2 compared to the value of the relative fluorescence yield (both were normalized at a just saturating laser energy) may be caused by the energy transfer between units. A_F of anaerobic algae at high initial fluorescence yield before the flashes was decreased by roughly a factor of 2 or 3 when the laser flash was preceded by one or two saturating xenon flash(es) with an interval time of 0.2 s. As shown in ref. 10, the relative fluorescence yield just before the second or third flash is decreased more than a factor of 2 or 3.

Luminescence under aerobic conditions at high Φ_L

The low 1 μs luminescence of *Chlorella* under aerobic conditions could be increased about 3 to 4 times by the pretreatment consisting of continuous illumination*, which reduced the primary acceptor almost completely (Φ = 4.2), followed within 1 ms by a saturating laser flash (Φ_L = 4.0) after which A_F was measured.

* 11 mW/cm^2 at λ = 400 to 550 nm for 0.28 s.

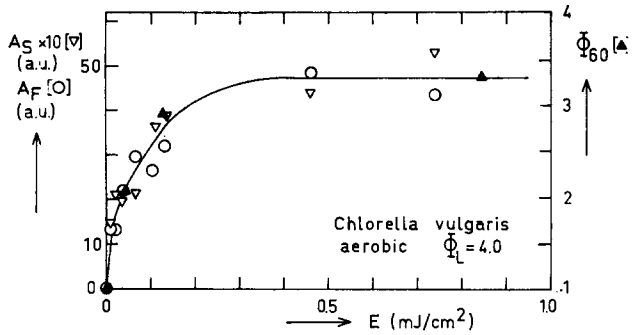

Fig. 6. Amplitude of the 1.0 μs (A_F, circles) and 17 μs (A_S, $\times 10$) (open triangles) luminescence component of aerobic algae, after a laser flash given 1 ms after the completition of a 0.28 s pulse of continuous blue light illumination (11 mW/cm^2; λ = 400 to 550 nm) and the relative fluorescence yield of aerobic algae 60 μs after the laser flash (Φ_{60}, closed triangles) without preceding illumination as function of the laser density (E). Φ_L = relative fluorescence yield before the laser flash = 4.0.

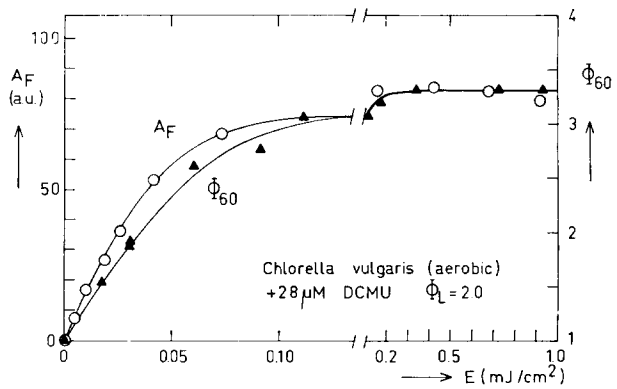

Fig. 7. The amplitude of the 0.8 μs component of the luminescence of aerobic *Chlorella vulgaris* (A_F, open circles) in the presence of 28 μM DCMU after a laser flash preceded by a dark time of 3 minutes, and the relative fluorescence yield 60 μs after the laser flash (Φ_{60}, triangles) of aerobic *Chlorella* without DCMU, as function of the laser energy density (E).

Again A_F saturated at increasing laser energy like the slower component A_S (20 to 30 μs) and like the fluorescence yield 60 μs after the laser flash recorded after a dark period of 3 min (Fig. 6). The lower amplitude of A_F (about 45 a.u.) compared to that of anaerobic algae at high initial fluorescence yield will be discussed below. A_A is left out of consideration.

A_F of aerobic algae could also be increased by a factor of about 5 by addition in the dark of 28 μM DCMU (Fig. 3, closed triangles). DCMU addition increased the fluorescence yield in the dark from 1.0 to between 1.4 and 2.0, due to the reduction of Q by R^- induced by DCMU [14]. This luminescence saturated again like System II fluorescence (Fig. 7). The difference between the two curves at lower energies can again be explained by the energy transfer between units.

Effect of hydroxylamine and heat treatment

A_F was eliminated by addition of 0.3 or 10 mM hydroxylamine (pH 7) after an incubation time of 40 min even under anaerobic conditions at high initial fluorescence yield ($\Phi_L = 5.1$) (Fig. 3, curve D). In aerobic algae, after incubation for 30 min with 10 mM hydroxylamine at pH 7, there was after one flash instead of a 1 μs luminescence component, a component of 20 to 30 μs. This component, A_S, saturated at about the same laser energy density as the relative fluorescence yield of algae without addition of hydroxylamine, measured 60 μs after the laser flash. The luminescence kinetics after one laser flash, given under aerobic conditions, upon addition of 0.36 mM hydroxylamine at pH 7, are shown in Fig. 8, with the incubation time t_i as parameter. The fast luminescence component of about 0.7 μs changed gradually in about 1 min in a slow component of about 30 μs. Without addition of hydroxylamine a slow component of only about 15 μs was present ($t_i = 0$). The amplitudes of the fast and the slow luminescence components, A_F and A_S respectively, and the relative fluorescence yield measured 9 μs after the laser flash, Φ_9, were measured simultaneously as a function of the incubation time (Fig. 9). This was done by measuring the luminescence from 0.2 to 4 μs after the laser flash, and by measuring the relative fluorescence yield on a separate oscilloscope by means of a weak xenon flash, given 9 μs after the same laser flash. To prevent spreading of the measurements due to fluctuation of the laser energy, an oversaturating laser flash with an energy density of about 0.45 mJ/cm^2 was

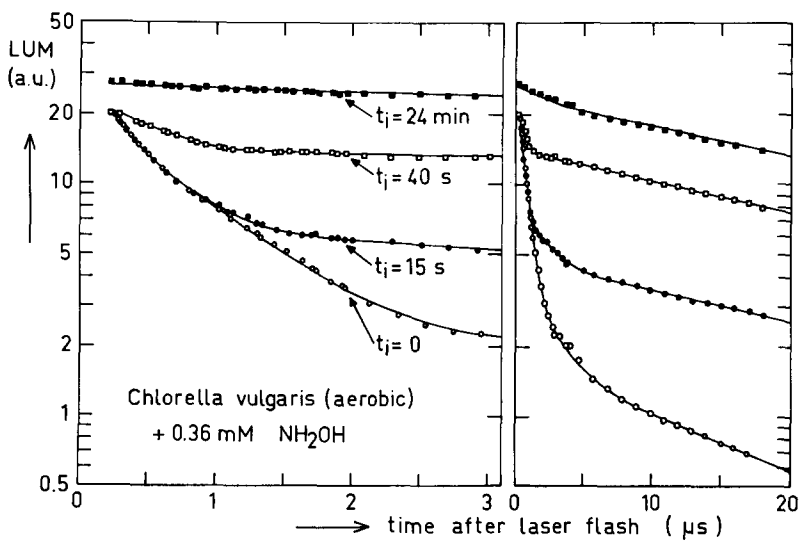

Fig. 8. Luminescence kinetics of *Chlorella vulgaris* after one oversaturating laser flash ($E = 0.45$ mJ/cm^2) given under aerobic conditions after addition of 0.36 mM hydroxylamine (pH 7) in the time range from 0.2 to 3 μs and from 0.2 to 20 μs respectively. The parameter t_i indicates the incubation time with hydroxylamine. The drawn curve for $t_i = 0$, i.e. without addition of hydroxylamine, corresponds with:

$$\mathrm{LUM}(t) = 2.23 \exp -(t/14) + 22.0 \exp -(t/0.75) + 84 \exp -(t/0.078) \quad (t \text{ in } \mu s)$$

The 78 ns component is probably due to the fluorescence excited by the 30 ns laser flash, which is somewhat distorted by the 25 MHz bandwidth of the apparatus. Part of the points of the curves were omitted for the sake of clarity.

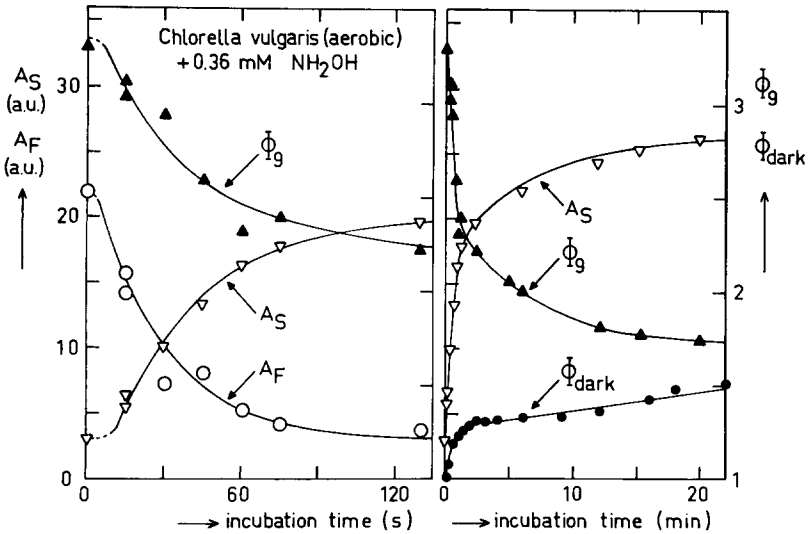

Fig. 9. The following quantities are plotted as function of the incubation time with 0.36 mM hydroxylamine at pH 7: (1) A_F: The amplitude of the fast luminescence component ($\tau = 0.7$ μs) after one oversaturating laser flash ($E = 0.45$ mJ/cm^2). (2) A_S: The amplitude of the slow luminescence component ($\tau = 15$ μs without additions and $\tau = 30$ μs after addition of hydroxylamine) after the same flash. (3) Φ_9: The relative fluorescence yield measured 9 μs after the same flash. (4) Φ_{dark}: The relative fluorescence yield after a dark time of three minutes. The drawn curves for A_F, A_S and ϕ_9 represent a computed exponential fit with two time constants of 27.1 s and 6.9 min, respectively (see text).

given. The decay of A_F and Φ_9, and the increase of A_S had the same time constant ($\tau = 27$ s$\pm$10 %) during the first two minutes if a delay of about 5 s was taken into account. This delay may be due to the diffusion time of hydroxylamine to the reaction centers. The increase of A_S and the decrease of Φ_9 were biphasic with the same time constants ($\tau = 27$ s and $\tau = 6.9$ min); the latter time constant is two to three times shorter than determined by fluorescence experiments on *Chlorella pyrenoidosa* [15]. Whether this difference is due to the use of different species or to the rather different experimental conditions has not yet been clarified. The drawn curves in Fig. 9 represent the functions:

$$A_F(t) = 3.0 + 18 \exp-[(t-5)/27.1]$$
$$A_S(t) = 26.6 - 9.2 \exp-[(t-5)/414] - 17.4 \exp-[(t-5)/27.1]$$
$$\Phi_9(t) = 1.7 + 0.74 \exp-[(t-5)/414] + 0.89 \exp-[(t-5)/27.1]$$

with t = incubation time in s.

For incubation times of more than about 130 seconds, measurement of the amplitude of the fast component (A_F) became impossible because A_F was small and the accessory luminescence was obscured by the noise of the slower component with the large amplitude A_S. The relative fluorescence yield in the dark, Φ_{dark}, increased in a biphasic manner with roughly the same two time constants as Φ_9, A_F an A_S (Fig. 9)*.

* These experiments were made several months after the first experiments with another batch of algae, which is responsible for the doubled fast luminescence amplitude at incubation time $t_1 = 0$. Also the precision of the measurements and the time resolution were improved.

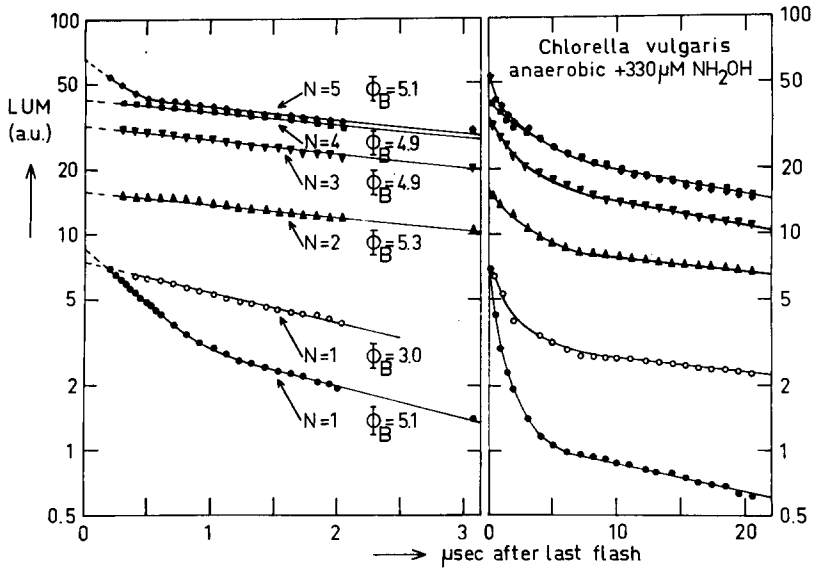

Fig. 10. Luminescence of *Chlorella vulgaris* under anaerobic conditions after addition of 0.33 mM hydroxylamine (at pH 7) in the dark and after an incubation time of 40 min, as function of the time in the range from 0.2 to 3 μs and from 0.2 to 20 μs after a number (N) of saturating flashes given with an interval time of 0.2 s. Φ_B = fluorescence yield in the dark before the flash series.

In anaerobic algae, the acceptor side of System II is unaffected by 10 mM or less hydroxylamine: the relative fluorescence yield in the dark can be increased even easier than for algae without hydroxylamine by single sufficiently spaced flashes to the maximum value of 5; the yield is also decreased by far red light to the minimum value of about 1.5 observed under these conditions for algae without hydroxylamine. The 1 μs luminescence component was absent under anaerobic conditions after addition of hydroxylamine* even at high Φ_L (Fig. 3, curve D). However the amplitude of the remaining slow luminescence after one laser flash was much lower than that of the 1 μs component at low Φ_L without addition of hydroxylamine, and increased subsequently to about the original value after 5 flashes (Fig. 10). Further comments on the hydroxylamine experiments will be given in the discussion.

A_F was also eliminated by heating the algae between 70 °C and 80 °C for 5 minutes (Fig. 3, closed squares), after which treatment the relative fluorescence yield in the dark was about one third of the fluorescence yield in dark-adapted untreated aerobic algae, and the yield did not increase after a saturating laser flash indicating damage of Photosystem II. These observations are also consistent with the hypothesis that the luminescence originates from System II. Also Haug et al. [3] concluded from luminescence measurements on *Scenedesmus obliquus* (wild type, and mutant 8 and II) in the range from 4 ns to several seconds that most delayed light – if not all – originates from Photosystem II.

Interpretation of the results

From these results we can conclude that A_F probably originates from System II

* 0.33 mM at pH 7, incubation time 40 min.

and is in first approximation proportional to the number of closed centers before the flash. This can be explained by the simple hypothesis that, in addition to Q, a second acceptor is present, which we call W; W accepts an electron from the excited primary donor P when Q is reduced:

$$ZPWQ^- + h\nu \rightleftarrows ZP^*WQ^- \rightleftarrows ZP^+W^-Q^-$$

The 1 μs luminescence is attributed to the back reaction

$$P^+W^- \rightarrow P^*W \rightarrow PW + h\nu_L$$

Presumably the luminescence decays in 1 μs because P^+ is oxidized in this time by the secondary donor Z [8, 16]. Hydroxylamine blocks the rapid electron transfer between Z and P [15]. Instead of Z an endogenous donor D donates an electron to P^+ in about 11 μs or longer [8]. This explains the disappearance of A_F after addition of hydroxylamine under aerobic or anaerobic conditions.

Effect of a positive charge at the donor side

The high 1 μs luminescence was not always present when all Q was reduced before the flash. The relative fluorescence yield under anaerobic conditions 1 ms after a saturating flash given at low initial fluorescence ($\Phi_B = 1.8$) was about maximum ($\Phi = 4.9$), but A_F after a saturating laser flash given at that moment was exactly the same as if no second flash was given (Fig. 3, open squares and diamonds). The fluorescence yield under aerobic conditions after addition of 28 μM DCMU 1 ms (or 280 ms) after a saturating flash was about maximum: $\Phi = 3.6$ (or 2.8) but A_F after a saturating laser flash given at that moment was the same as if no second flash was given ($\Phi_L = 1.4$) (Fig. 3; triangles). The same was true for aerobic algae cooled till 2 °C; the fluorescence yield Φ_L 1 ms after the first flash was about 2.

These observations can be explained by the hypothesis that one or more positive charges at the donor side quench A_F about three times.

Survey of the experiments; empirical equation

A survey of typical experiments is given in Table I. The state of the reaction center is given by $S^{(+)}Q^{(-)}$. $S^{(+)}$ stands for S, with zero charge (S^0), or S^+ with 1 to 3 positive charges (S^{1+} to S^{3+}) in the donor complex; $Q^{(-)}$ stands for Q or Q^-. ($S^{(+)}Q^{(-)}$) is the fraction of centers in state $S^{(+)}Q^{(-)}$ before the last flash. These fractions were estimated as follows. Except for experiment No. 3, the fraction of the reaction centers of System II in the state $S^{(+)}Q^-$ before the (last) flash is derived from the relative fluorescence yield at that moment (Φ_L) by means of Eqn. 1; it was assumed that $P = 0.6$, $\Phi_0 = 1$ and $\Phi_{max} = 3.6$ under aerobic conditions and that $P = 0.6$, $\Phi_0 = 1.8$ and $\Phi_{max} = 5.0$ under anaerobic conditions. Under aerobic conditions, 20 % and under anaerobic conditions 100 % of the reaction centers of dark adapted algae were assumed to be in the uncharged state S.

Under anaerobic conditions S^+ is reduced in the dark by a reducing agent D_2 of which evidence was given in [10]. This was also concluded from luminescence experiments: it was shown [8] that under aerobic conditions after a dark time of a few minutes a strong 10 to 20 μs luminescence component became apparent after the second flash. This was attributed mainly to the occurrence of state S^{2+} before the second flash. Under anaerobic conditions a strong 10 to 20 μs luminescence compo-

TABLE I

SURVEY OF THE EXPERIMENTS

treatm.: treatment before insertion in the cuvette; ae = aerobic; an = anaerobic; 75° = 5 min 70 to 80 °C.
addit.: addition or pretreatment; CB = continuous blue light illumination (11 mW/cm² at λ = 400 to 500 nm during 0.3 s); DCMU = 28 μM DCMU (in dark); HA = 330 μM hydroxylamine at pH 7 (incubation time = 40 min).
Φ_B: relative fluorescence yield before the first flash of a series of saturating flashes, given after a dark period of three minutes.
N: number of saturating flashes before measurement.
t: time between saturating flashes (ms).
Φ_L: relative fluorescence yield at the moment of the last (laser) flash of a series of saturating flashes.
curve: corresponding luminescence kinetics in Fig. 3.
$(S^{(+)}Q^{(-)})$: estimated fraction of the reaction centers in the state $S^{(+)}Q^{(-)}$ before the (last) flash, S being the state with zero charges, S^+ the states with 1 to 3 positive charges in the donor complex (see text).
A'_F: $= \beta\,(200(SQ^-)+70\,(SQ)+70\,(S^+Q^-)+20\,(S^+Q))$; $\beta = 1$ after a short period of anaerobiosis and $\beta = 0.5$ under aerobic conditions (see text).
A_F measured amplitude of the 0.7 μs to 1.4 μs luminescence component after the last saturating flash (a.u.).

exp. no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
batch	1	1	1	2	2	2	3	3	3	4	4	5	5	5	3	3
treatm.	ae	ae	ae	ae	ae	ae	an	an	an	an	an	an	an	an	an	75°
addit.	–	–	CB	DCMU	DCMU	DCMU	–	–	–	–	–	–	–	–	HA	–
Φ_B	1.0	1.0	4.0	1.4	1.4	1.4	1.8	2.8	4.1	1.8	1.9	4.6	4.9	4.7	5.1	0.35
N	1	2	1	1	2	2	1	1	1	2	2	2	2	2	1	1
t	–	200	–	–	1	300	–	–	–	1	200	1	4	50	–	–
Φ_L	1.0	1.35	4.0	1.4	3.6	2.8	1.8	2.8	4.1	4.9	2.7	4.4	3.5	2.7	5.1	0.35
curve				C	C	C	B		A						D	E
(S^+Q)	0.8	0.72	0	0; 0.56	0; 0	0; 0	0	0	0	0.01	0.5	0	0	0	0	–
(S^+Q^-)	0	0.28	0.75	0; 0	0.7; 0.7	0.55; 0.55	0	0	0	0.99	0.5	0	0	0	0	–
(SQ)	0.2	0	0	0.7; 0.14	0; 0	0.15; 0.15	1	0.5	0.1	0	0	0.08	0.26	0.5	0	–
(SQ^-)	0	0	0.25	0.3; 0.3	0.3; 0.3	0.3; 0.3	0	0.5	0.9	0	0	0.92	0.74	0.5	1	–
A'_F	15	17	51	54; 41	54; 54	54; 54	70	135	190	70	45	190	166	135	–	–
A_F	12	12	45	40	40	40	70	150	210	70	45	170	154	80	3	1

nent became apparent only after the third flash. From this it follows that before the flashes mainly S^0 was present.

After addition of DCMU under aerobic conditions, no centers in the state S^+Q^- are present after a few seconds, due to the recombination reaction $Q^-S^+ \rightarrow QS$ [17]. In columns 4, 5 and 6 of Table I, two values for $(Q^{(-)}S^{(+)})$ are given; the first one was calculated with the assumption that with DCMU no S^+ was present after a dark time of a few minutes, and the second one with the assumption that 80 % of the centers with oxidized Q were in the state S^+Q and 20 % in the state SQ before the (first) flash.

Under anaerobic conditions A_F is maximum (about 210 a.u.) if about all reaction centers are in the state SQ^- before the flash (exp. no 9). This explains the first term of the empirical Eqn. 2 below. The amplitude is 70 a.u. if all reaction centers are in the state SQ (exp. no 7) or S^+Q^- (exp. no 10) before the flash, which explains the second and the third term of Eqn. 2. The amplitude is about 45 a.u if 50 % of the centers are in the state S^+Q^- and 50 % in the state S^+Q (expt. no. 11) which suggests that the centers in the state S^+Q contribute to about 2(45–35) a.u. = 20 a.u., which explains the fourth term of Eqn 2.

The amplitude of the 1 μs luminescence component calculated from the states of the reaction center before the flash by means of the empirical equation

$$A'_F = \beta(200(SQ^-)+70(SQ)+70(S^+Q^-)+20(S^+Q)) \quad (2)$$

with $\beta = 1$ under anaerobic conditions and 0.5 under aerobic conditions, is also shown in Table I. When comparing the calculated (A'_F) and the measured (A_F) amplitudes of the 1 μs luminescence component, one has to bear in mind that some experiments were carried out for different algae batches and that the states of the reaction centers before the flash are only an estimate. A possible explanation for the dependency of A_F on the states of the reaction centers before the flash is given in the discussion. The factor β may be due to an extra quenching under aerobic conditions as explained below.

Removal of a quencher under anaerobic conditions

After insertion in the cuvette, the fluorescence yield in the dark of anaerobic algae, Φ_{dark}, increased in sufficiently spaced saturating flashes in an hour from the value of aerobic algae to almost the maximum value of the fluorescence yield 60 μs after a saturating flash, Φ_{60} (Fig. 11, left-hand side). This effect is explained in ref. 10. The ratio Φ_{60}/Φ_{dark} decreased from 2.7 to about 1.0 indicating an almost complete reduction of Q [18]. That the fluorescence increases about 60 % during the first 5–10 min is possibly not due to the reduction of Q but can be described by the disappearance of another quencher. This increase was also present when no flashes were given. During far red illumination* reduced Q is oxidized via system I but Φ_{dark} never decreased to a value lower than 1.6 to 2.0, which suggests that the quencher was still inactive. When air was admitted in the cuvette, Φ_{dark} decreased to a value of 1.0 and Φ_{60} to a value of 2.7, which can be explained by restoration of the quencher under aerobic conditions. Sometimes the fluorescence yield in the dark of anaerobic algae did not increase in this way upon flashes (Fig. 11, right-hand side). The donor D_2 [10]

* 0.8 mW at $\lambda = 740$ nm during 30 s.

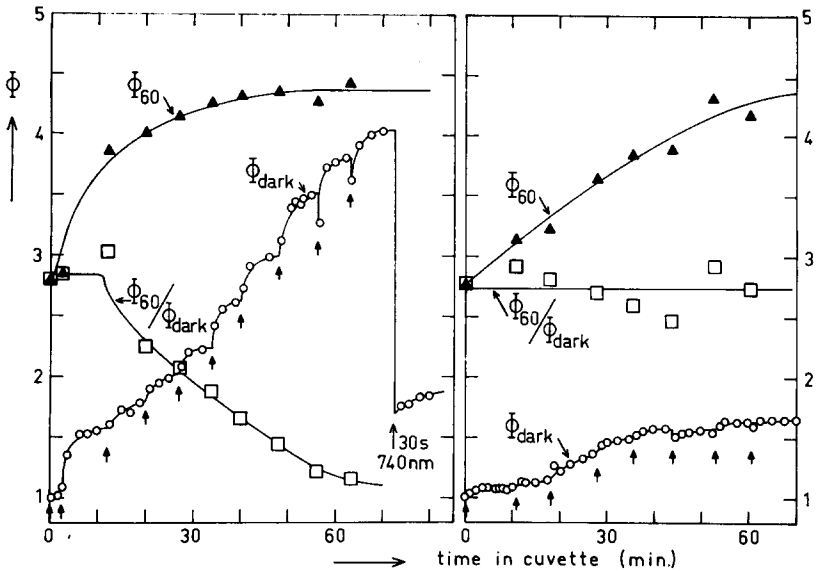

Fig. 11. Relative fluorescence yield in the dark (Φ_{dark}, circles), relative fluorescence yield 60 μs after a saturating flash (Φ_{60}, triangles) and the ratio Φ_{60}/Φ_{dark} (squares) versus time in a closed cuvette for anaerobic *Chlorella pyrenoidosa*. An arrow indicates a saturating flash. Left-hand side: normal conditions, all Q becomes reduced. Right-hand side: abnormal conditions (see text); no Q is reduced.

was apparently inactive, which may be due to incorrect pretreatment, and Φ_{dark} increased in an hour by only 50 to 80 % in the same way as Φ_{60}: the ratio Φ_{60}/Φ_{dark} remained constant. This suggests that no Q^- was present in the dark and that again a quencher was slowly removed under anaerobic conditions. Also Joliot and Joliot [19] suggested that a quencher, which they named Q_R, was removed if the A_{pool} was reduced, which is the case in anaerobic algae [10].

DISCUSSION

The 1 μs luminescence component

The amplitude of the 1 μs luminescence component, A_F, measured under a great variety of conditions (see Table I) can be described by the empirically derived Eqn. 2. In this discussion we will give some possible interpretations of this equation. The observation that A_F is high if Q^- is present before the laser flash, indicates that another acceptor, called W, functions under these conditions. We have given evidence that under these conditions the 1 μs luminescence is probably due to the back reaction of

$$SPWQ^- + h\nu \rightleftarrows SP^*WQ^- \rightleftarrows SP^+W^-Q^- \qquad (3)$$

and that the 1 μs decay is caused by the disappearance of P^+ formed in the laser flash according to the reaction $ZP^+ \rightarrow Z^+P$. S stands for the secondary donor complex, including Z. There are two possibilities:

(1) W is on a side path. The electron can be transferred directly from P* to W or to Q. For efficient photosynthesis the rate of the reaction $P^*Q \rightarrow P^+Q^-$ must be

higher than that of the reaction $P^*W \rightarrow P^+W^-$; the longer life time of P^* in the state WP^*Q^- than in the state WP^*Q explains the higher fluorescence yield when Q is reduced. Two back reactions with different luminescence yield are possible:

(a) $SWP^+Q^- \rightarrow SWPQ + h\nu_L$ $\quad A_F = 70$ a.u.
(b) $SW^-P^+Q^- \rightarrow SWPQ^- + h\nu_L$ $\quad A_F = 200$ a.u.

The presence of S^+ quenches reaction (a) to about one third (20/70) and reaction (b) presumably completely. The latter quenching may be due to the rapid ($\ll 1$ μs) non radiative transition $S^+W^- \rightarrow SW$. With these assumptions, Eqn. 2 and especially the remarkable coincidence of the squares and diamonds of curve B in Fig. 3 are explained; the luminescence yields after a laser flash given under anaerobic conditions in the state SWPQ or, when preceded by a second flash, in the state S^+WPQ^- are identical because they originate, according to this hypothesis, from the same back reaction:

squares: $SWPQ + h\nu \rightarrow SWP^+Q^- \rightarrow SWPQ + h\nu_L$
diamonds: $S^+WPQ^- + h\nu \rightarrow S^+W^-P^+Q^- \xrightarrow{\ll 1\,\mu s} SWP^+Q^- \rightarrow SWPQ + h\nu_L$

The coincidence of open and closed triangles of curve C in Fig. 3 is explained in the same way. The simplest assumption in order to interpret all the experiments is that W is an additional component of the donor complex S and that the reaction $WP^* \rightarrow W^-P^+$ is inhibited by hydroxylamine (see next section). A scheme according to this hypothesis is given in Fig. 12.

(2) W is an intermediate in the electron transfer path between P and Q. In

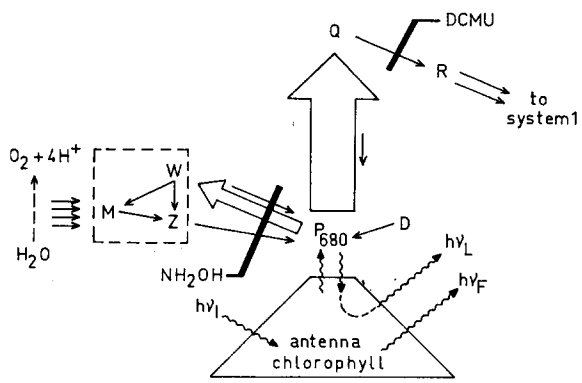

Fig. 12. Hypothetical electron pathways in the reaction center complex of system II with W as an additional component of the secondary donor complex S. M is a donor to Z, and S corresponds with the complex MZ. Arrows indicate the direction of electron transport; open arrows indicate transfer from excited P^*. Incident light: $h\nu_I$; fluorescence: $h\nu_F$; delayed fluorescence or luminescence: $h\nu_L$. The following reactions are postulated (see Discussion):

1. $SWPQ + h\nu \rightarrow SWP^+Q^- \rightarrow SWPQ + h\nu_L$ $\quad A_F = 70$ a.u.
2. $SWPQ^- + h\nu \rightarrow SW^-P^+Q^- \rightarrow SWPQ^- + h\nu_L$ $\quad A_F = 200$ a.u.
3. $S^+WPQ + h\nu \rightarrow S^+WP^+Q^- \rightarrow S^+WPQ + h\nu_L$ $\quad A_F = 20$ a.u.
4. $S^+WPQ^- + h\nu \rightarrow S^+W^-P^+Q^- \xrightarrow{\ll 1\,\mu s} SWP^+Q^- \rightarrow SWPQ + h\nu_L$ $\quad A_F = 70$ a.u.

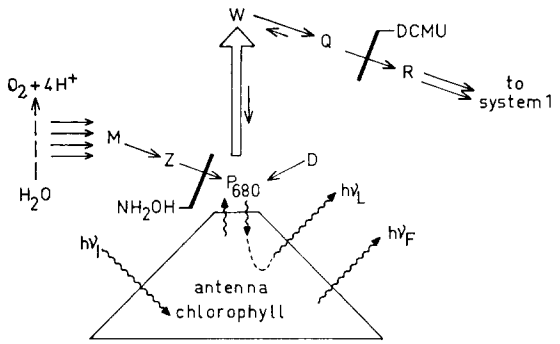

Fig. 13. Hypothetical electron transport pathways in the reaction center complex of System II with W as an intermediate between *P*-680 and Q. The symbols are the same as in Fig. 12.

order to explain the relatively low value of A_F in the state PQ before the flash, we can make two assumptions: (a) The rate of the reaction $W^-Q \rightarrow WQ^-$ is much higher than of the reaction $ZP^+ \rightarrow Z^+P$ and some W^- is continuously present due to a rapid back reaction $WQ^- \rightarrow W^-Q$; this W^- can recombine with P^+ under emission of luminescence. The relatively high amplitude of A_F in the state Q^- before the flash can be explained by the inhibited electron transfer from W^- to Q^-. (b) The rate of the reaction $W^-Q \rightarrow WQ^-$ is lower than that of the reaction $ZP^+ \rightarrow Z^+P$; after a flash following the states PWQ or PWQ^-, the states P^+W^-Q or $P^+W^-Q^-$ are present, which react back to the states $PWQ+h\nu_L$ or $PWQ^-+h\nu_L$, respectively. The delayed fluorescence (luminescence) of the second state is higher than of the first, because the fluorescence is not quenched by Q. This explains the relatively high amplitude of A_F in the state Q^- before the flash. The high chlorophyll fluorescence yield in the presence of Q^- can be explained by a less probable electron transfer from P* to W, due to the electrostatic field of the negative charge on Q^- or to a change in the conformation of the P, W and Q containing complex upon reduction of Q. Van Gorkom suggested that in DOC 2 particles* pheophytin** may possibly be an intermediate acceptor [20]. An intermediate acceptor, probably pheophytin, has been found in *Rps. sphaeroides* by picosecond spectrometroscopy [21] (see also [22]). The low 1 μs luminescence component measured when the reaction centers were in the state S^+PWQ (about 20 a.u.) or S^+PWQ^- (about 70 a.u.) before the flash might be attributed to a lower probability of recombination between P^+ and W^- due to the extra positive charge on the donor side, by which the luminescence yield is reduced to about one third. With these hypotheses, Eqn. 2 is made plausible. A scheme with W as intermediate is given in Fig. 13.

The effects of hydroxylamine

During the first two minutes of incubation with hydroxylamine under aerobic conditions, the fast component of the luminescence ($\tau = 0.7$ μs), A_F, disappeared almost completely while the fluorescence yield measured 9 μs after the laser flash, Φ_9,

* Membrane fragments of chloroplasts, treated by deoxycholate and isolated by centrifugation.
** Perhaps identified as C_{550}.

decreased from 3.3 to about 2.2 (Fig. 9). As the time constants of both decays were equal, we can conclude that at most 50 % of the reaction centers had a fast luminescence component, and that only these centers reacted with hydroxylamine in about one minute. It is also possible that only these centers were responsible for the fluctuation in the fluorescence yield, as measured in [23] and [24]. The fact that the increase of the slow luminescence component, A_S, and Φ_9 had the same biphasic course as function of the incubation time (Fig. 9) made it plausible that hydroxylamine blocks the electron transport between Z and P in both types of reaction centers [8]. The time of 9 μs after the flash was used because at that time the effect of the carotenoid triplet quenching was small and the contribution of the 25 to 30 μs fluorescence component was small compared to that of the fast fluorescence component [15]. We do not have an explanation of all effects of hydroxylamine: After a flash given under anaerobic conditions at high fluorescence yield in the dark before the flash, the luminescence decay time was increased, presumably because the reduction of P^+ occurred via the slow endogenous donor D instead of the inhibited secondary donor Z (Fig. 12). The decay became biphasic; the longest decay time was 30 to 80 μs which was similar to that of the back reaction $P^+Q^- \rightarrow PQ$, which was determined from fluorescence measurements [15, 24]. However the luminescence amplitude attained the expected value of 70 a.u. only after a few flashes (Fig. 10).

These experiments can be explained by the already mentioned hypothesis that W^- is an additional component of the secondary donor complex S. This hypothesis explains the suppression of A_F upon addition of hydroxylamine under aerobic or anaerobic conditions by the inhibition of electron transfer between P and S. The increase of the slow luminescence upon flashes given under anaerobic conditions at high initial fluorescence yield after addition of hydroxylamine (Fig. 10) is caused by the back reaction $P^+Q^- \rightarrow PQ + h\nu_L$; This reaction is enhanced upon flashes by the stepwise oxydation of Q^- via system I [10].

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