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# CHLOROPHYLL *a* FLUORESCENCE AS A MONITOR OF NANOSECOND REDUCTION OF THE PHOTOOXIDIZED PRIMARY DONOR *P*-680$^+$ OF PHOTOSYSTEM II *

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## Summary

1. Changes in the fluorescence yield of aerobic *Chlorella vulgaris* have been measured in laser flashes of 15 ns, 30 ns and 350 ns half time. The kinetics after the first flash given after a 3 min dark period could be simulated on a computer using the hypothesis that the oxidized acceptor Q and primary donor $P^+$ are fluorescence quenchers, and $Q^-$ is a weak quencher, and that the reduction time for $P^+$ is 20—35 ns.
2. The $P^+$ reduction time for at least an appreciable part of the reaction centers was found to be longer after the second and subsequent flashes. In the first 5 flashes an oscillation was observed. Under steady state conditions, with a pulse separation of 3 s, a reduction time for $P^+$ of about 400 ns for all reaction centers gave the best correspondence between computed and experimental fluorescence kinetics.

## Introduction and theoretical considerations

### *Chlorophyll $a_2$ fluorescence quenching*

In algae the primary photoreaction of Photosystem II (PS II) is the formation of $P^+Q^-$ (see list of symbols at the end of this paper) in the reaction center. In 1963 Duysens and Sweers [1] discovered that the redox state of Q was an important parameter controlling the chlorophyll $a_2$ fluorescence yield. This could be explained by assuming that P only efficiently traps the excitation

* Dedicated to Professor Dr. E. Havinga on the occasion of his 70th birthday and retirement from the chair of Organic Chemistry of the State University at Leiden.

energy from the antenna chlorophyll $a_2$ molecules if the reaction center is in the state PQ (Q oxidized). In this state the chlorophyll $a_2$ fluorescence is quenched and the yield is low. On the other hand a maximum fluorescence yield is found if the reaction center is in the state $PQ^-$ (Q reduced) and less or no trapping of excitation energy occurs. More recently evidence has been obtained, indicating that also the redox state of P controls the chlorophyll $a_2$ fluorescence yield. It was concluded that the oxidized reaction center chlorophyll $P^+$ acts as an efficient fluorescence quencher [2—8]. All data so far obtained point to approximately equal fluorescence yield for the reaction centers in state PQ or $P^+Q^{(-)}$ [6—8], which suggests that PQ and $P^+Q^{(-)}$ are equally efficient in energy trapping and fluorescence quenching; only in the state $PQ^-$ the fluorescence yield is high.

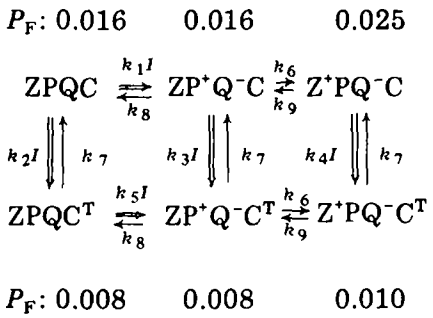
Besides $P^+$ and Q there has been found a third chlorophyll $a_2$ fluorescence quencher which is formed with a high probability upon excitation of a photosynthetic unit with its reaction center in the state $PQ^-$ before the flash [4,9,10]. The quenching can be described by the reaction $PQ^-C \xrightarrow{h\nu} PQ^-C^T$, in which $C^T$ is a strongly and C is a weakly quenching substance. C is a carotenoid which in the triplet state $C^T$ quenches the chlorophyll $a_2$ fluorescence. The dark reaction $C^T \rightarrow C$ occurs in a few microseconds [4,6,9—11].

*Aim of the measurements*

Very recently van Best and Mathis concluded from kinetic absorption measurements that $P^+$, formed in a 2 ns laser flash given in the state PQ, was reduced by the secondary donor of PS II in 25—45 ns [12]. From the point of view of the given $P^+$ fluorescence quenching hypothesis this means that there should be a 25—45 ns rise in the fluorescence induction during or after a short laser flash reflecting the $P^+$ reduction (i). Furthermore, we were interested in the $P^+$ reduction as reflected in the fluorescence kinetics after one or more preceding flashes (ii). In order to investigate (i) and (ii) we have measured the fluorescence kinetics in the nanosecond and submicrosecond range in *Chlorella vulgaris* without and with preilluminating flashes, using three laser flashes ($t_{1/2}$ = 15, 30 and 350 ns) as both actinic and measuring flashes.

*Theoretical considerations*

For the computer simulation of the fluorescence yield changes we have used a model of a pigment system with limited energy transfer between photosynthetic units which is an extension of models used by Den Haan [9] and Otten [13]. Recently we have found that the matrix or lake model [15] can also be used for the simulations of the measured fluorescence kinetics with approximately similar values of the rate constants with a somewhat different description of the carotene triplets. For understanding the section Results and Interpretation it is not necessary to study the following detailed derivation of the differential equations used in the computer simulations. The photochemical and dark reactions in a PS II unit are represented in the scheme of Fig. 1. In this scheme the probabilities of a (particular) unit for fluorescence emission ($P_F$), carotenoid triplet formation ($P_{ISC}$) and for charge separation in the reaction center ($P_T$) are given also. These parameters were calculated following the procedure of Duysens [14] and Otten [13]. The probability for immediate




$k_1 = P_T(ZPQC) = 0.479$
$k_2 = P_{ISC}(ZPQC) = 0.032$
$k_3 = P_{ISC}(ZP^+Q^-C) = 0.032$
$k_4 = P_{ISC}(Z^+PQ^-C) = 0.050$
$k_5 = P_T(ZPQC^T) = 0.245$
$k_7 = 0.33\ \mu s^{-1}$
$k_8 = 0.005\ \mu s^{-1}$
$k_9 \simeq 0\ \mu s^{-1}$
$k_6 = ?\ \mu s^{-1}$

Fig. 1. Working scheme representing the photochemical and dark reactions in a PS II unit and indirectly the corresponding fluorescence yield kinetics in the 0—1 $\mu$s time range. Six different states are used to describe the fluorescence yield kinetics. The reactions start in the aerobic dark adapted state ZPQC (Q oxidized and no carotenoid triplet present). By the light-driven reactions $k_1 I$ and $k_5 I$ charge separation occurs. The possibility for charge separation is given by $P_T(ZPQC)$ and $P_T(ZPQC^T)$, respectively. The light reactions $k_2 I$, $k_3 I$ and $k_4 I$ cause the formation of the fluorescence quencher $C^T$ (carotenoid triplet), which is formed with the probabilities $P_{ISC}(ZPQC)$, $P_{ISC}(ZP^+Q^-C)$ and $P_{ISC}(Z^+PQ^-C)$, respectively; $k_7$ is the rate constant for the dark back reaction $C^T \rightarrow C$; $k_7 \simeq 0.33\ \mu s^{-1}$ [9]. The probability for fluorescence emission of a unit in a state $i$ is given by $(P_F)_i$. The given $P_T$, $P_{ISC}$ and $P_F$ values in the scheme are calculated as explained in the text using the parameters of Table I. $k_1$—$k_5$ are quantum efficiencies and I means the absorption frequency in quanta per s per unit. The rate for the back reaction $k_8$ is about 5 $ms^{-1}$ [3] and the back reaction $k_9$ is assumed to be negligible. The rate for the reduction of $P^+$, $k_6$, is the subject of this work. The kinetics of the various states of the reaction centers, the $C_i$'s ($i = 1, 6$), are obtained by numerically solving the differential equations of Table II. The fluorescence kinetics can then be calculated from Eqn. 8.

emission of excitation energy as fluorescence by an excited chlorophyll *a* molecule is $p_f$, and $p_h$ is the probability for energy transfer from one chlorophyll molecule to another. For the reaction center chlorophyll we take $p_f^t$ and $p_h^t$ because of the additional trapping probability $p_t$ in the state ZPQ. We call the fractional concentration of traps $T$ ($T \simeq 0.005$); then the fractional concentration of antenna chlorophyll is $(1 - T)$. We can now write an equation, which describes the probability $P_F$ for an excitation to be finally emitted by a unit as fluorescence after a photon is absorbed by that unit

$$P_F = (1 - T)p_f + (1 - T)p_h P'_F + Tp_f^t + Tp_h^t P''_F \qquad (1)$$

where $P'_F$ is the probability after one transfer step of being finally emitted as fluorescence by a unit and $P''_F$ is the probability of being finally emitted as fluorescence after returning from the trap. If it is supposed that $P_F \simeq P'_F \simeq P''_F$ which is the case if $p_h > p_t$ [13,15,16]

$$P_F = [(1 - T)p_f + Tp_f^t]/[1 - (1 - T)p_h - Tp_h^t] \qquad (2)$$

Similar equations can be derived for the probability of triplet formation (via intersystem crossing) in a unit, $P_{ISC}$, for the probability of losses by internal conversion, $P_{IC}$, and for the probability of energy transfer from one unit to

another unit, $P_{TR}$. The equation for the trapping probability $P_T$ in a unit for an excitation which has been absorbed by that unit is given by

$$P_T = \frac{p_t T}{1-(1-T)p_h - p_h^t T} \tag{3}$$

In order to describe the fluorescence quenching by the carotenoid triplet we introduce an additional trap different from the reaction center. This will change Eqn. 2 into:

$$P_F = \frac{(1-T-A)p_f + Tp_f^t + Ap_f^q}{1-(1-T-A)p_h - Tp_h^t - Ap_h^q} \tag{4}$$

where $p_f^q$ is the probability for immediate fluorescence by an excited chlorophyll molecule which is quenched with a rate constant $k_q$ by the carotenoid triplet; $A$ is the fraction of chlorophyll molecules quenched in a unit. When deexcitation of an excited chlorophyll *a* molecule can take place by fluorescence ($k_f$), internal conversion ($k_{ic}$), intersystem crossing ($k_{isc}$), energy transfer to another molecule in the unit ($k_h$) and energy transfer to a chlorophyll molecule in another unit, 'averaged' over all chlorophyll molecules of a unit, ($k_{tr}$) we can write $p_f$ as:

$$p_f = \frac{k_f}{k_f + k_{ic} + k_{isc} + k_h + k_{tr}} \tag{5a}$$

The equations for $p_{isc}$, $p_{ic}$, $p_{tr}$ and $p_h$ are similar. For the probability of energy trapping upon excitation of the reaction center Z PQ we can write:

$$p_t = \frac{k_t}{k_f + k_{ic} + k_{isc} + k_h + k_{tr} + k_t} \tag{5b}$$

where $k_t$ is the rate constant for energy trapping in the state ZPQ. Using the in vitro parameters of chlorophyll *a*, $k_{isc} \simeq 2\,k_f$ and $k_{ic} << k_f$ [17] we find:

$$P_{ISC} = 2P_F \text{ and } P_{IC} \simeq 0 \tag{6}$$

The expression for the total fluorescence yield $\Phi$ of a system of units in different states is analogous to Eqn. 1 and given by:

$$\Phi = \sum_i C_i \cdot (P_F)_i + [\sum_i C_i \cdot (P_{TR})_i]\Phi' \tag{7}$$

$C_i$ is the fractional concentration of units in the state $i$ and $\Sigma_i C_i = 1$. If we make the assumption that $\Phi \simeq \Phi'$ (see Otten [13]), then

$$\Phi = \frac{\sum_i C_i \cdot (P_F)_i}{1 - \sum_i C_i \cdot (P_{TR})_i} \tag{8}$$

The fluorescence yield $\Phi(ZPQ^-C)$ of a system with all Q reduced is a special case of Eqn. 8 and can be written as:

$$\Phi(ZPQ^-C) = \frac{P_F(ZPQ^-C)}{1 - P_{TR}(ZPQ^-C)} \tag{9}$$

TABLE I

THE RELATIVE VALUES OF THE DIFFERENT RATES FOR DEEXCITATION OF A (REACTION CENTER) CHLOROPHYLL MOLECULE IN THE LOWEST EXCITED SINGLET STATE WHICH ARE USED FOR THE CALCULATION OF THE PARAMETERS $P_T$, $P_{ISC}$ AND $P_F$ OF FIG. 1

For further explanation see text. In order to obtain an idea of the real rate constants: $k_f = 6.7 \cdot 10^7$/s.

| | | |
|---|---|---|
| $k_f = 1$ | $k_t(ZPQC) = 8.7 \cdot 10^3$ | $k_Q(=k_t(ZPQC)) = 8.7 \cdot 10^3$ |
| $k_{ic} = 0$ | $k_t(Z^{(+)}PQ^-C) = 1.5 \cdot 10^3$ | $k_t(ZP^+Q^-C) = 8.7 \cdot 10^3$ |
| $k_{isc} = 2$ | | |
| $k_{tr} = 30$ | | |
| $k_h = 2 \cdot 10^4$ | | |

Assuming that the fluorescence yield $\Phi$ of PS II in the state $ZPQ^-C = 0.1$ and $P_{TR}(ZPQ^-C) = 0.75$ (see also [18] and [19]) it follows that $P_F(ZPQ^-C) = 0.025$. This means that

$$P_{TR} = 30P_F \tag{10}$$

Assuming [18] the values $\Phi(ZPQC) = 0.03$ and $\Phi(ZPQ^-C) = 0.10$ and using

TABLE II

DIFFERENTIAL EQUATIONS

From the differential equations given in this table the time dependence of the fractions of the various states of the photosynthetic units in Fig. 1 can be calculated. $I$ is proportional to the flash intensity, which is a function of time.

For further explanation see text and the legend of Fig. 1.

$$\frac{dC_1}{dt} = -\left(\frac{k_1 I + k_2 I}{X}\right) C_1 + k_7 C_4 + k_8 C_2$$

$$\frac{dC_2}{dt} = \frac{k_1 I}{X} \cdot C_1 - \left(\frac{k_3 I}{X} + k_6 + k_8\right) C_2 + k_7 C_5 + k_9 C_3$$

$$\frac{dC_3}{dt} = -\left(\frac{k_4 I}{X} + k_9\right) C_3 + k_6 C_2 + k_7 C_6$$

$$\frac{dC_4}{dt} = \frac{k_2 I}{X} \cdot C_1 - \left(\frac{k_5 I}{X} + k_7\right) C_4 + k_8 C_5$$

$$\frac{dC_5}{dt} = \frac{k_3 I}{X} \cdot C_2 + \frac{k_5 I}{X} \cdot C_4 - (k_6 + k_7 + k_8) C_5 + k_9 C_6$$

$$\frac{dC_6}{dt} = \frac{k_4 I}{X} \cdot C_3 + k_6 C_5 - (k_7 + k_9) C_6$$

$$X = 1 - \sum_i (P_{TR})_i \cdot C_i \qquad (i = 1, 6)$$

$$\sum_i C_i = 1 \qquad (i = 1, 6)$$

$C_1 = [ZPQC]$, $C_2 = [ZP^+Q^-C]$, $C_3 = [Z^+PQ^-C]$, $C_4 = [ZPQC^T]$, $C_5 = [ZP^+Q^-C^T]$ and $C_6 = [Z^+PQ^-C^T]$.

the Eqns. 1—10 we are able to calculate $k_t(ZPQC)$ and $k_t(ZPQ^-C)$, if we know $k_h$. In Table I the relative values for $k_f, k_{ic}, k_{isc}, k_{tr}$, $k_t(ZPQC)$, $k_t(ZP^+Q^-C)$, $k_t(ZPQ^-C)$ and $k_q$ are shown taking $k_f = 1$ and $k_h = 20\,000$. This value for $k_h$ is obtained from [15]; it is assumed that the trapping rates of reaction centers, in which $P^+$ and/or Q are present, and the rate of quenching of excitation energy by the carotenoid triplet are equal [$k_t(ZPQC) = k_t(ZP^+Q^-C) = k_q$]. Knowing the parameters of Table I one is able to calculate the probabilities mentioned in Fig. 1 for energy trapping ($P_T$), fluorescence ($P_F$) and carotenoid triplet formation ($P_{ISC}$) for any state of a unit. For the formation of carotenoid triplets it is assumed that this occurs via the chlorophyll triplet (probability $P_{ISC}$ in a unit to be formed); it is supposed that the quenching properties of the chlorophyll and carotenoid triplets do not differ.

Using Fig. 1 and describing the time dependence of the various states of the photosynthetic units on basis of the foregoing assumptions with simple differential equations, mentioned in Table II, we are able to compute the fluorescence kinetics and/or integrated fluorescence of a system of units during or after a short laser flash of variable intensity.

We already mention here that good simulation kinetics were obtained with $A = 2\,T$ (see Eqn. 4). In this model processes like singlet-singlet and/or triplet-triplet annihilation were neglected because of the relatively low photondensity, for the 15 and 30 ns flashes $<10^{15}$ and for the 350 ns flash $<3 \cdot 10^{16}$ quanta/cm$^2$, we used for our measurements. Because of the short experimental time range ($<1$ μs) the influence of the PS II donor side upon the fluorescence yield is negligible during the experiment.

## Materials and Methods

### *Materials*

Algae (*Chlorella vulgaris*) were grown as described previously [20]. After centrifugation they were suspended in fresh growth medium and brought to an extinction of 0.4 at 680 nm in a 1 cm cell after subtracting the absorbance at 750 nm as a correction for scattering. All measurements were done at room temperature with a closed horizontal $30 \times 30 \times 5$ mm$^3$ cuvette. A dark adaptation period of at least 3 min preceded each experiment.

In some experiments a solution of hydroxylamine (of the same pH as that of the growth medium) was added to dark adapted samples and incubated for 10 min prior to the measurements.

### *Apparatus and procedure of fluorescence yield determinations*

In order to measure the fluorescence yield of the algae in the 0—70 ns range a ruby laser flash ($t_{1/2} = 30$ ns and $\lambda = 694.3$ nm) was used both as actinic and measuring light. The fluorescence was measured with a Philips XP 1002 photomultiplier (S-20 type kathode) and the anode of the photomultiplier was connected to a 7A13 Tektronix oscilloscope preamplifier by a cable terminated with 50 ohm. After amplification and integration ($RC \simeq 1$ μs) the signal was stored in a Biomation 8100 transient recorder, which was connected to a Digital Equipment PDP 11/45 computer, which processed the data. The relative fluorescence yield, $\Phi$, was defined as $\Phi = F_{int}/E$, where $F_{int}$ is the integrated

fluorescence during the flash at the laser energy $E$. The energy was varied by placing Schott neutral density filters in the laser beam. The maximum energy density of the laser flash on the cuvette was 1 mJ/cm$^2$; a combination of Schott AL 722, RGN 9/3, NG 3/4 and NG 9/1 filters was placed in front of the photomultiplier. The peak transmission occurred at 727 nm and amounted to $10^{-5}$; the half width was 17 nm. A detailed description of the experimental set up for the experiments done with the ruby laser is given in [6].

The integrated fluorescence yield of the algae in the 0—35 ns range was measured in a similar way with a flash of a frequency doubled Nd-YAG-laser ($t_{1/2}$ = 15 ns and $\lambda$ = 530 nm). In this case a Schott filter combination DAL 681, NG 1/1 and NG 4/2 was placed in front of the photomultiplier. The peak transmission was at 681 nm and was about $10^{-4}$; the half width was 17 nm.

A dye laser flash was used for measuring the fluorescence yield kinetics in the time interval 0—650 ns. $F(t)$, the fluorescence variation of the algae as a function of time was measured during the flash. The intensity time course $I(t)$ of this flash was measured from the fluorescence of a highly fluorescent dye (Nile blue A perchlorate) at the same laser energy with the same experimental set up as used for the measurement of $F(t)$. The photomultiplier signal was fed directly to a transient digitizer (Biomation 8100). The relative fluorescence yield as a function of time is given by $\Phi(t) = F(t)/I(t)$. The flashlamp pumped dye laser (Lambda Physik FL 3B) had a maximum energy density of about 5 mJ/cm$^2$ at 600 nm and $t_{1/2} \simeq 350$ ns. The energy was varied by placing Schott neutral density filters in the laser beam; the fluorescence was detected through Schott RG 665/3 and AL 685 filters in combination with suitable Schott neutral density filters. The maximum transmission was at $\lambda$ = 685 nm and the half width was 21 nm.

## Results and Interpretation

The integrated fluorescence $F_{int}$ during a 30 ns ruby laser flash was measured in aerobic *Chlorella*. The aerobic algae were in the dark adapted state ZPQC before the flash. The laser energy density E varied from $10^{-7}$—$10^{-3}$ J/cm$^2$. Fig. 2 shows the experimental points (o) of the relative fluorescence yield $\Phi = F_{int}/E$ as a function of $E$. At very low energies the aerobic algae have a fluorescence yield $\Phi_0$ and at an energy which is about saturating for the variable fluorescence one observes a maximum in the $\Phi$-$E$ plot. This maximum is lower than the normal $\Phi_{max}$ ($\simeq 3.3\ \Phi_0$), which is found when all Q is reduced, which we attribute to the presence of the fluorescence quenchers $P^+$ and $C^T$. The reactions occurring are shown in Fig. 1. For energies much higher than saturating, the quenching by the carotenoid triplets predominates and $\Phi$ is lower than $\Phi_0$ for $E \gtrsim 0.5$ mJ/cm$^2$. As already mentioned in the Introduction we have used a model of a pigment system with limited energy transfer between the photosynthetic units. Using this model we have simulated the $\Phi$-$E$ relationship for the experimental points o of Fig. 2. This is shown by curve A in this figure which has been simulated for a $P^+$ reduction time of 20 ns; the curves B and C show the simulated $\Phi$-$E$ curves for $P^+$ reduction times of 10 and 50 ns respectively. It can be seen from the curves A-C that the best fit of the experimental points o is obtained for a $P^+$ reduction time of about

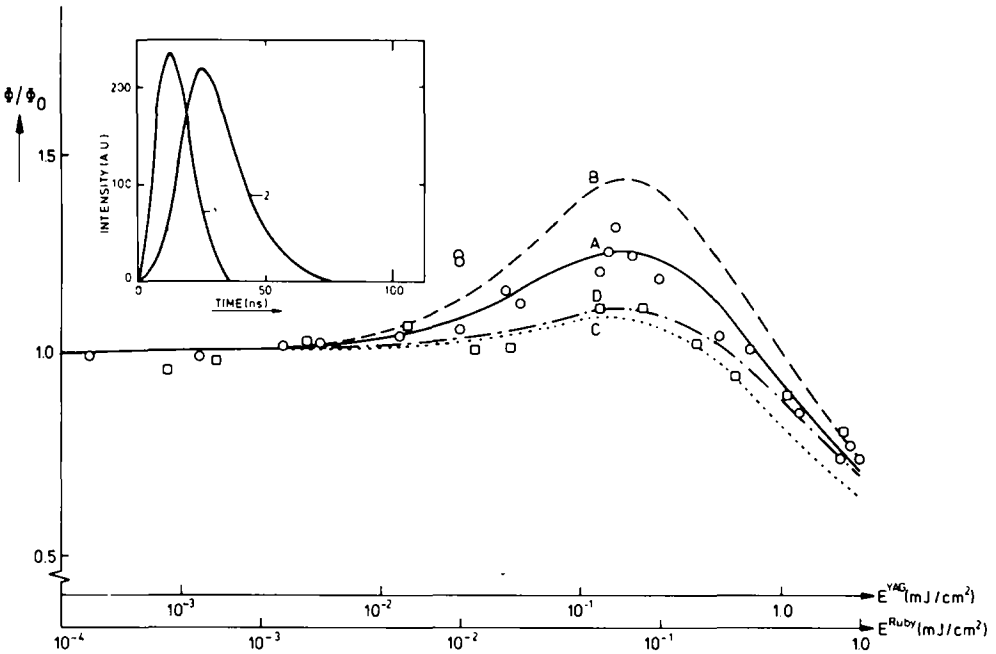


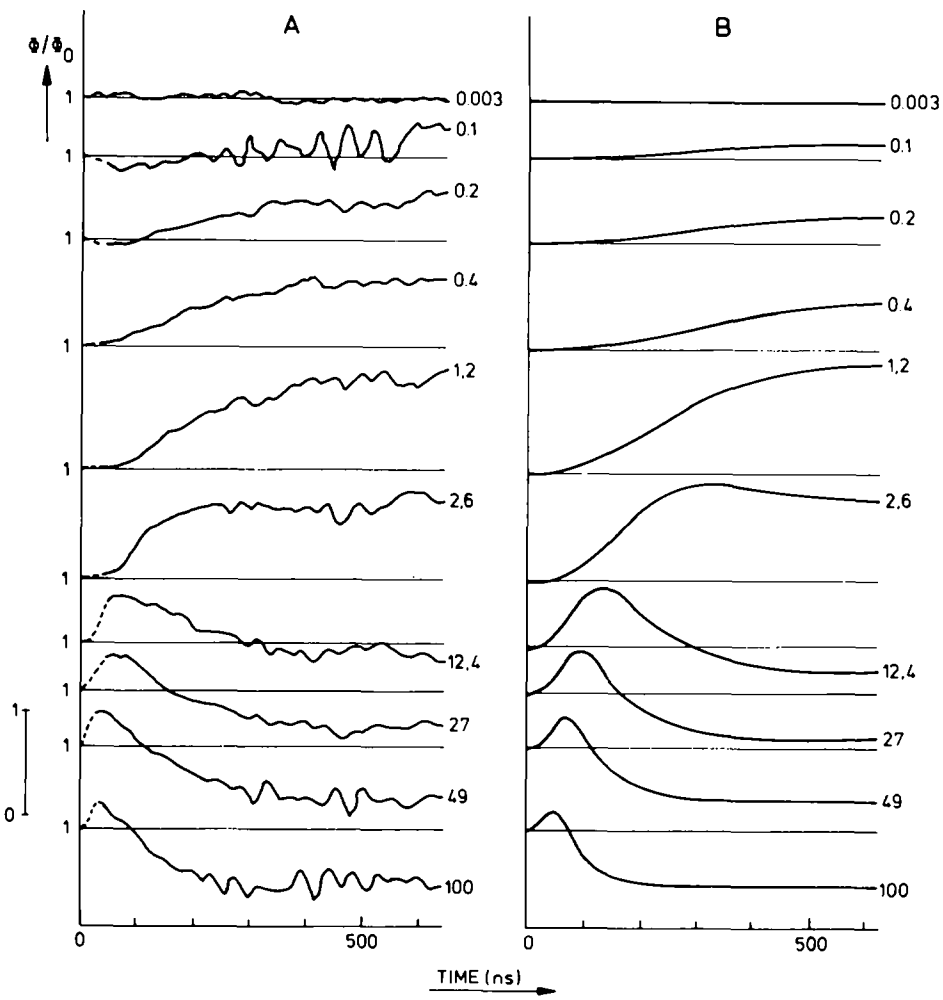

Fig. 2. The relative fluorescence yield Φ of aerobic adapted *Chlorella vulgaris* during a laser flash as a function of the laser energy density $E$. The points ○ are measured at λ = 725 nm using a 30 ns flash of a ruby laser (λ = 694.3 nm). The points □ (the average of about five measurements with five different batches of algae) are measured at 681 nm using a 15 ns flash of a Nd-YAG laser (λ = 530 nm). The curves A—D are computed with the simulation model presented in the Introduction. The best fit for ○ was obtained with a $P680^+$ reduction time of 20 ns and is represented by curve A (———). The curves B (- - - - -) and C (· · · · · ·) show the deviation from curve A when a $P680^+$ reduction time of 10 ns, respectively 50 ns, is used for the simulated Φ-$E$ relationship. The best fit for □ was obtained with a $P680^+$ reduction time of 25 ns, curve D (· — · — ·). The insert shows the time course of the intensity of the 15 ns flash of the Nd-YAG laser (1) and the 30 ns flash of the ruby laser (2).

20 ns. For the simulations it was assumed (for details see Introduction) that the fluorescence quenching by the open reaction center PQ is the same as for the reaction center in the state $P^+Q^{(-)}$. The fluorescence yield is only high in the state $PQ^-$ and $\Phi_{PQ^-} \simeq 3.3\,\Phi_{PQ}$. Furthermore, it was assumed that the fluorescence quenching by the carotenoid triplet is the same as for the open reaction center PQ. The carotenoid triplet is assumed to be formed via the chlorophyll triplet which has a probability of $P_{ISC} = 2\,P_F$ to be formed in a unit; $P_F$ is the probability for an excitation to be finally emitted by a unit as fluorescence after a photon is absorbed by that unit. Using these assumptions the best simulations were obtained when the fractional concentration of chlorophyll molecules quenched by a carotenoid triplet $A = 2\,T$, where $T$ is the fractional concentration of reaction center chlorophyll molecules in a unit.

The experimental points □ in Fig. 2 were measured with the 15 ns YAG-laser flash and one observes that the maximum in the Φ-$E$ plot for this flash is about 50% smaller than for the 30 ns ruby flash. This can be qualitatively explained by the relatively higher concentration of the fluorescence quencher $P^+$ during the 15 ns YAG-laser flash in comparison to the 30 ns ruby flash. From curve D of Fig. 2, which is the simulation of the Φ-$E$ relationship for the

□ points measured with the 15 ns flash for a $P^+$ reduction time of 25 ns, we conclude that a $P^+$ reduction time of 20—25 ns explains the experimental results as obtained with the two lasers.

Using a 350 ns dye laser we were able to measure the fluorescence kinetics of the algae during the flash. Fig. 3A shows the relative fluorescence yield $\Phi$ during the dye laser flash as a function of time for aerobic *Chlorella* in the dark adapted state for a number of different flash intensities. The intensity of the dye laser flash as a function of time, $I(t)$, is shown in Fig. 3G. Often there were found large variations in the experimental $\Phi(t)$ curves at times $<50$ ns. This is caused by some jitter along the time ordinate in the separately measured $F(t)$ and $I(t)$ curves. Therefore the ratio $F(t)/I(t) = \Phi(t)$ could not be determined accurately at the beginning of the flash where the slopes of the $I(t)$ and $F(t)$ curves are very steep. Because of this uncertainty we have dotted the measured $\Phi(t)$ at times $<50$ ns. The dotted part of the curves displays the more or less smoothed course of the much scattered experimental curves in this time



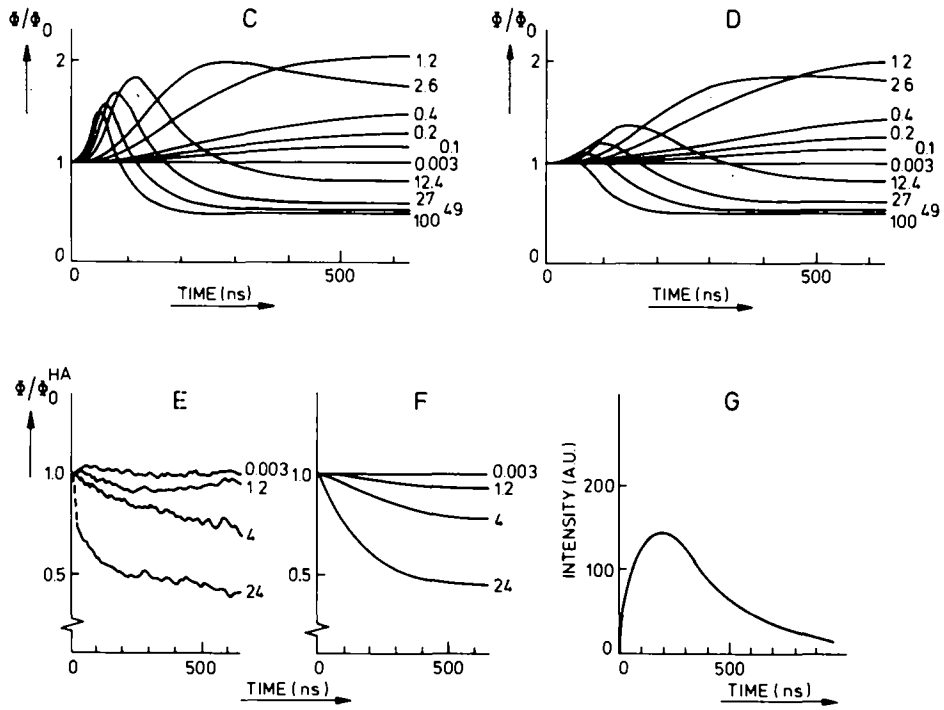



Fig. 3. Relative fluorescence yield Φ of dark adapted aerobic *Chlorella vulgaris* during a 350 ns dye laser flash (see Fig. 3G) of intensity as indicated in percents of the maximum intensity on the right. A: *Chlorella* with no addition; the average of three measurements is shown. B, C, D: Simulation of the kinetics of Fig. 3A with the model given in the Introduction for different $P^+$ reduction times: B is computed with a 35 ns reduction time of $P^+$ and C and D with a reduction time of 15 and 75 ns, respectively. E: *Chlorella* in the presence of 10 mM hydroxylamine (hydroxylamine makes the $P^+$ reduction time of the order of 35 μs); three measurements are averaged. $\Phi_0^{HA}$ means the $\Phi_0$ of hydroxylamine-treated *Chlorella*; $\Phi_0^{HA} \simeq 1.5$ the $\Phi_0$ of non-treated dark adapted *Chlorella*. F: The computed kinetics of Fig. 3E with the model presented in the Introduction; the $P^+$ reduction time is 35 μs (every reduction time $>>1$ μs yields a good fit of Fig. 3E). G: The intensity time course of the 350 ns flash of the dye laser.

range. At time zero all the curves of Fig. 3A start at the fluorescence level $\Phi_0$. Non-saturating flashes give rise to an increase of the fluorescence due to the transformation of the quencher Q into the weak quencher $Q^-$ during the flash. The kinetics in oversaturating flashes are different. After a fast increase of the fluorescence one observes a decrease. This decrease below $\Phi_0$ can be explained by the formation of a relatively high concentration of carotenoid triplets at these high energies. In Fig. 3B the curves are shown as simulated with the model for a $P^+$ reduction time of 35 ns. In order to show the influence of the $P^+$ reduction time of the simulated fluorescence kinetics Fig. 3C and 3D display the computer simulated $\Phi(t)$ curves for a $P^+$ reduction time of 15 and 75 ns respectively. The best fit is shown by Fig. 3B, indicating that the reduction time of $P^+$ after the first flash is about 35 ns.

It is possible that in Figs. 2 and 3A one does not observe the 20—35 ns $P^+$ reduction in the laser flashes of varying intensities, but another light generated quencher decaying in about 30 ns. To recognize that case we added 10 mM hydroxylamine to aerobic *Chlorella*. It is known that the $P^+$ reduction takes much longer (about 35 μs) under these conditions [3,4,6,9]. The results with

hydroxylamine treated dark adapted *Chlorella* are shown in Fig. 3E for different laser intensities. The fluorescence yield declines for each intensity below $\Phi_0$, apparently by the formation of carotenoid triplet quenchers, and no fluorescence increase is observed because the $P^+$ quenching remains present during the time scale of the experiment. Fig. 3F show a similar behavior for the model simulation kinetics if a $P^+$ reduction time $>>1$ μs is assumed. It should be noted that the initial fluorescence yield of *Chlorella* treated with 10 mM hydroxylamine $\Phi_0^{HA} \simeq 1.5\ \Phi_0^{N.A.}$ (untreated *Chlorella*) and therefore the simulation of the fluorescence kinetics in Fig. 3F is normalized to give this higher fluorescence yield at time zero. A change in the value of a rate constant, e.g. a decrease of $k_t$ (ZPQC) (Table I), which can also be used to bring the $\Phi_0$ at a higher level, approximately gives the same simulation curves at the same value for the rate of reduction of $P^+$ as obtained by the simple process of normalization.

After a dark period of 3 min a train of 350 ns dye laser flashes ($\Delta t$ = 3 s) was given. In Fig. 4 we present the fluorescence kinetics of aerobic *Chlorella* during a saturating flash (about 20% of the maximum intensity) as a function of the flashnumber. The results indicate a considerable attenuation of the maximum fluorescence yield at $t \simeq 75$ ns for the second and subsequent flashes. The fluorescence yield during the fourth flash stays even below $\Phi_0$.

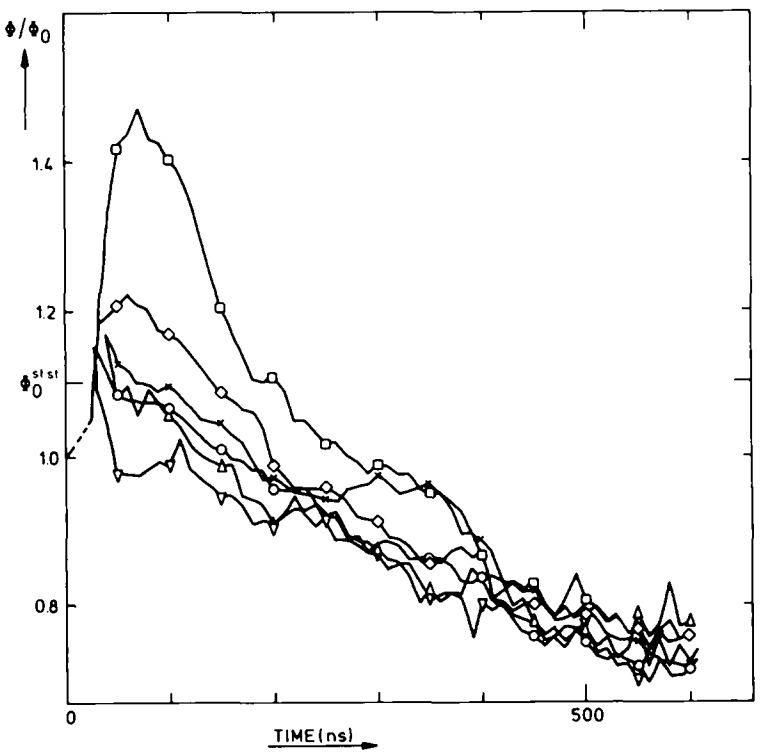



**Fig. 4. The relative fluorescence yield Φ of dark adapted *Chlorella vulgaris* during a flash (see Fig. 3G) as a function of the number of saturating dye laser flashes ($\Delta t$ = 3 s). $N$ = 1 (□), $N$ = 2 (◇), $N$ = 3 (△), $N$ = 4 (▽), $N$ = 5 (X) and $N$ = 6—10 (≃steady state) (○). $\Phi_0^{st.st}$ is the fluorescence yield at the beginning of the flashes under steady state conditions.**

These results can be explained by the assumption that the $P^+$ reduction time is considerably longer after one or more preilluminating flashes. Fig. 4 indicates further that there is an oscillation in the $P^+$ reduction time because after four preilluminating flashes, the fluorescence yield at $t \simeq 75$ ns is again higher than $\Phi_0$. After 5 preilluminating flashes the oscillation is practically damped. The fluorescence kinetics of the 6th—10th flash are the same as those for steady state conditions. These are intermediary between the first (□) and fourth flash (△).

Fig. 5A shows the fluorescence kinetics of aerobic *Chlorella* during a 350 ns dye laser flash under steady state conditions for different laser intensities and the pulse separation time was 3 s. As is shown in Fig. 5B this situation can be simulated if one takes a time of about 400 ns for the $P^+$ reduction time of all reaction centers. It can be seen for the saturating intensities >9% that the fluorescence yield does not rise above the value $\Phi_0^{st.st} = 1$ unlike fluorescence

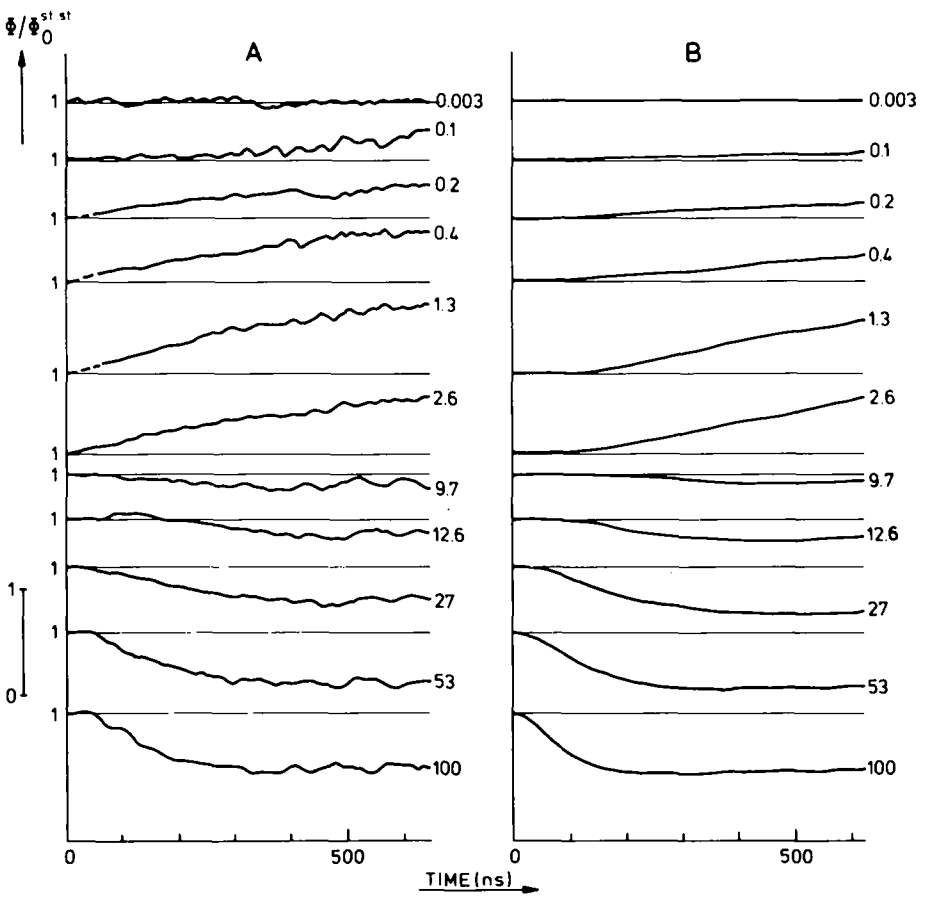



Fig. 5. A: The relative fluorescence yield $\Phi$ of *Chlorella vulgaris* during a flash (see Fig. 3G) in a train of dye laser flashes ($\Delta t$ = 3 s). For each curve the average intensity of the flashes is indicated in per cents of the maximum intensity at the right hand side. About 12 curves are averaged (flashes ca. 13—24). $\Phi_0^{st.st}$ is the fluorescence yield at the beginning of the flashes under steady state conditions. B: Simulation of the kinetics in Fig. 5A with the model described in the Introduction for a $P^+$ reduction time of 400 ns.

kinetics of the dark adapted *Chlorella* in Fig. 3A. However, for the nonsaturating intensities <2%, there is a small fraction of reaction centers which retain the 35 ns $P^+$ reduction because the experimental kinetics are steeper than the corresponding simulated kinetics of Fig. 5B. This is to be expected because for nonsaturating flashes there is a certain fraction of reaction centers which is not preilluminated by the train of flashes and which fraction therefore shows the 20—35 ns fluorescence kinetics of dark adapted algae upon excitation. It should be noted that for the steady state experiments with saturating flashes, with the pulses 3 s apart, the $\Phi_0$ was about 1.1 $\Phi_0$ of dark adapted *Chlorella*. Therefore the curves of Fig. 5B are normalized to give this higher fluorescence yield at time zero.

## Discussion

As already mentioned the results with hydroxylamine treated *Chlorella*, presented in Fig. 3E, confirm the hypothesis that $P^+$ is a fluorescence quencher since the fluorescence kinetics in this figure do not exhibit an increase for any light intensity. For the lowest intensity (1.2%) it can be observed that the fluorescence yield is almost equal to $\Phi_0$ under these conditions during the whole time scale. Under the same conditions non treated dark adapted *Chlorella* exhibit a marked fluorescence increase due to the formation of the highly fluorescent state $PQ^-$ (Fig. 3A). At this undersaturating light intensity only a small number of carotenoid triplets and more $PQ^-$ (no additions) or $P^+Q^-$ (with hydroxylamine) will be present during the dye laser flash. Therefore it can be concluded that the fluorescence yields have the same low values for the states $P^+Q$ and $P^+Q^-$ as for the dark adapted state PQ.

The simulation of the relative fluorescence yield as function of the ruby laser energy for dark-adapted aerobic *Chlorella* in Fig. 2 is in good agreement with the experimental points, if the $P^+$ reduction time in the model is assumed to be about 20—25 ns. The maximum height of the fluorescence yield at $E \simeq 60\ \mu J/cm^2$ is very sensitive to the chosen $P^+$ reduction time as is shown by the curves A, B and C in this figure. Qualitatively one might say from the results in Fig. 2 that the height of this maximum is dependent on the ratio of the half time of the laser flash and the reduction time of $P^+$ by the secondary donor Z. This means that one would not expect any maximum when the duration of the laser flash is short in comparison with the $P^+$ reduction time. This is shown nicely by the results of Mauzerall [21] who used a 7 ns (full width at half maximum intensity) laser flash. This $\Phi$-$E$ plot for aerobic *Chlorella* in the dark-adapted state does not show any increase or maximum over the whole energy range since the 7 ns laser flash is much shorter than the 20—35 ns reduction time of $P^+$ from our results.

The fluorescence yield kinetics during a 350 ns dye laser flash, which are represented in Fig. 3A, indicate a $P^+$ reduction time of about 35 ns. This is shown by the simulation curves in Figs. 3B, C and D which display a best fit for all intensities when the reduction time of $P^+$ is taken to be 35 ns. The somewhat higher $P^+$ reduction time of 35 ns measured with the dye laser in comparison with the 20—25 ns $P^+$ reduction time obtained with the ruby- and YAG laser may well be caused by the less homogeneous laser beam of the first

laser: an inhomogeneous beam causes a smoothing of the curves which attenuates the curves at higher intensities in such a way that an apparent longer $P^+$ reduction time is found. The reduction of 20—35 ns is consistent with the results from absorption difference spectroscopy of $P^+$ in untreated dark-adapted spinach chloroplasts, where a phase with a half time of 25—45 ns was found in the $P^+$ absorption change at 820 nm [12]. Mauzerall [22] reported a 25 ns phase in the fluorescence induction of dark-adapted *Chlorella*. He described this phase to a 'primary reaction for the photosystem that makes oxygen'. This might be taken to indicate that the reduction of $P^+$ occurs with a half time of 25 ns. One should keep in mind, however, that the formation of carotenoid triplet fluorescence quenchers, which have a life time of several microseconds, and their effect on the kinetics was not taken into account by Mauzerall.

The much larger reduction time for $P^+$ after one or more saturating preilluminating flashes, which is displayed for a train of flashes in Fig. 5A, is simulated well for all saturating light intensities, if the $P^+$ reduction time is taken to be 0.4 $\mu$s (Fig. 5B). $P^+$ reduction times of the order of 1 $\mu$s or less were suggested earlier by Duysens et al. [4] and Den Haan [9]. Gläser et al. [23] obtained *indirect* evidence from absorption measurements that the reduction of the oxidized primary reaction center pigment occurred with a halftime $\leqslant 1$ $\mu$s in a series of repetitive flashes. Also Mauzerall [22] observed that in preilluminated organisms (four preceding flashes) the fluorescence yield increased in the $\mu$s-range. However, this $\mu$s-rise seems to be the result of the disappearance of the 3 $\mu$s living carotenoid triplet, which also quenches the fluorescence.

Before comparing the oscillations of the fluorescence yield in Fig. 4 around 75 ns in the 1st—5th flashes with those of the fluorescence yield in 2 [24] and 16 $\mu$s [15] flashes, which have a somewhat similar oscillatory pattern and are caused by the S-states, one should take into account oscillations of the baseline in the kinetics of Fig. 4, and the rather long separation of 3 s between the flashes. More experiments are needed to carry out such a comparison with sufficient precision. However, an important conclusion can be obtained by comparing the fluorescence yield kinetics in the first flash and that in the steady state flashes. Comparing the curves in Figs. 3A and 5A, for an intensity of 27%, one sees that the initial bump in Fig. 3A, indicating a 35 ns reduction of $P^+$ on the first flash, has disappeared completely in Fig. 5A, indicating a slow reduction of $P^+$ in most if not all reaction centers in the steady state. This shows that slow and rapid reductions are not specifically associated with different S-states of the reaction centers since in that case in the steady state at least one fourth of the centers, specifically the $S_1$ centers, should retain a rapid reduction of $P^+$.

Within the framework of our model we can conclude that for flash numbers $\geqslant 2$ at least an important fraction of the oxidized reaction center pigment $P^+$ is reduced in a time longer than 20—35 ns which is found for the first flash. For the 1st—5th flashes the time for $P^+$ reduction seems to be correlated with the S-states. After more than five preceding flashes a satisfactory correspondence between computed and measured curves is found for a 0.4 $\mu$s $P^+$ reduction time and at least the $S_1$ state does not manifest itself anymore in the 20—35 ns rate for $P^+$ reduction.

The reason for the marked decrease in the rate of $P^+$ reduction (by a factor of the order of 10) after a number of flashes may be that the rate of electron transfer from Z to $P^+$ is delayed, perhaps because of conformational changes, possibly due to the larger membrane potential upon illumination by several flashes, or Coulomb interactions, or that part of the centers is in the state $Z^+$ at the moment of formation of $P^+$. In the latter case $Z^+$, which is reduced in 0.4 $\mu$s in the presence of $P^+$, presumably is in a (dynamic) state of equilibrium with the next donor. A third possibility is that the rapid donor becomes blocked after the first flashes, and that another 0.4 $\mu$s donor can manifest itself. Additional experiments are required to establish which of these mechanisms occurs, and which is its physiological significance.

## List of symbols used

PS II, photosystem II

P, *P*-680 = reaction center chlorophyll *a* dimer of system II [25]; $P^+$ represents the oxidized state of P.

Q, the 'primary' electron acceptor of system II which is a plastoquinone molecule; $Q^-$ is the reduced state, the plastosemiquinone anion [25—27].

Z, chemically unknown secondary electron donor of system II; $Z^+$ is the oxidized state of Z.

C, carotenoid molecule in the singlet ground state.

$C^T$, carotenoid molecule in the lowest triplet state (see, however, next symbol).

$T$, fractional concentration of traps in a unit.

$A$, fractional concentration of chlorophyll molecules quenched by a carotenoid triplet quencher ($C^T$) in a unit.

$p_f$, the probability for immediate emission as fluorescence of excitation energy by an excited chlorophyll molecule.

$p_f^t$, $p_f$ for a reaction center chlorophyll molecule.

$p_{tr}$, probability, 'averaged' over all chlorophyll molecules of a unit, for energy transfer from an excited chlorophyll molecule in a unit to a chlorophyll molecule in another unit.

$p_{isc}$, probability for intersystem crossing in an excited chlorophyll molecule.

$p_{ic}$, probability for losses via internal conversion in an excited chlorophyll molecule.

$p_t$, probability for trapping of excitation energy by the excited reaction center chlorophyll molecule.

$p_h$, probability for energy transfer from an excited chlorophyll molecule in a unit to another chlorophyll molecule in that unit.

$p_h^t$, $p_h$ for a reaction center chlorophyll molecule.

$k_f$, $k_{tr}$, $k_{isc}$, $k_{ic}$, $k_t$ and $k_h$ are the corresponding *rate constants* for the deexcitation of an excited (reaction center) chlorophyll molecule.

$P_F$, $P_{TR}$, $P_{ISC}$, $P_{IC}$ and $P_T$ are the corresponding probabilities for a *unit*.

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