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The nanosecond decay of variable chlorophyll fluorescence in leaves of higher plants

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The influence of strong continuous wave illumination on the yield of chlorophyll fluorescence was investigated using a two-beam method. This method is based on subnanosecond pulses to excite fluorescence and strong continuous wave light to induce variations of fluorescence yield. The application of a boxcar integrator (gate duration, 0.22 ns) enables us to measure the light-induced variations of fluorescence yield at different times during the fluorescence pulse and, thus, to investigate the relation of fluorescence induction to the different components of the multiexponential fluorescence decay. The experiments were carried out at room temperature using leaves of maize and spruce. We have determined the decay of continuous wave light-induced variable fluorescence to be single exponential with a lifetime of 2.0 ± 0.2 ns. The variable fluorescence is attributed to the recombination of separated charges in the reaction center of Photosystem-II.

Introduction

The light-induced variation of chlorophyll *a* fluorescence yield (fluorescence induction) is a widely used method for investigation of primary processes of photosynthesis [1–3]. This variation of fluorescence in the 10^{-2}–10^{3} s range (also known as the Kautsky effect [4]) is strongly connected with photosynthetic efficiency evident by its correlation to the time-courses of O_2 evolution [1,4,5], ΔpH about the membrane [5] and ATP concentration [6]. Because of the increasing application of this phenomenon as an analytical tool in plant physiology it is of interest to know the origin of fluorescence and to understand the mechanism of its light-induced variations. In this connection it is a substantial question, whether chlorophyll fluorescence of green plants is of homogeneous or heterogeneous origin. Beside the known spectral heterogenity of fluorescence (preferably visible at low temperatures) the analysis of fluorescence induction suggests an additional heterogeneity of F-685, the dominating fluorescence band at room temperature, which is mainly emitted from PS II. If a homogeneous origin of F-685 and the complementarity between photochemistry and fluorescence are assumed, the variation of fluorescence $F_{max}/F_{min} \approx 5$ is inconsistent with the variation of the photochemical yield (0–0.95 [7]). This contradiction can be solved, assuming that F-685 consists of a constant (independent of the state of the reaction center) and a variable component.

The assumption of a constant fluorescence is supported by the fact that the initial level of fluorescence during the induction period F_0 (minimum value of fluorescence yield) is relative insensitive to the photochemical state of the pho-

Abbreviations: PS, Photosystem; LHC, light-harvesting chlorophyll-protein complex; Ph, pheophytin.

tosynthetic apparatus [2,3]. A powerful method to explore the heterogenity of fluorescence is the measurement of fluorescence decay kinetics in the 10^{-10}–10^{-9} s range (reviewed in Refs. 8 and 9). Recently triple exponential fluorescence decay was reported for chloroplasts [10–12] and green algae [10,13] at room temperature.

To investigate the relationship between fluorescence induction and fluorescence decay kinetics we have developed a new experimental method which connects these phenomena. This method is based on the boxcar technique which transforms a series of nanosecond fluorescence pulses into a signal output in the seconds range. The output signal of the boxcar integrator reflects the shape of the fluorescence pulse (if the gate is scanned through the pulse) or the intensity of fluorescence in a definite time interval during the fluorescence pulse (if the gate is fixed). The latter method of signal processing enables us to measure the variations of fluorescence yield, induced by strong continuous illumination, at different times during the fluorescence pulse. Using this method we have determined the nanosecond life-time of variable fluorescence.

Materials and Methods

The measurements were carried out with leaves of maize (*Zea mays*) and spruce (*Picea abies* Karsten). The samples were second or third leaves of 14–20-days-old maize plants or 1-year-old needles of 4-years-old spruce plants, respectively. Both were grown under natural light conditions.

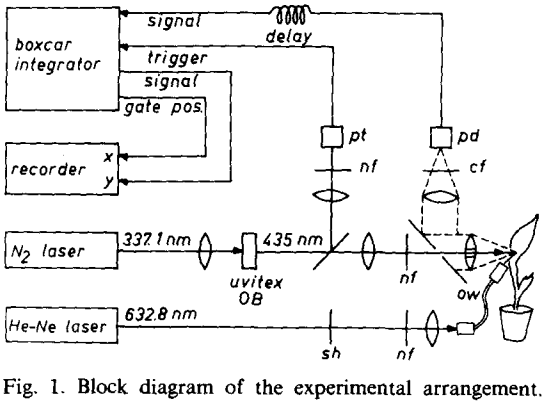

Fig. 1. Block diagram of the experimental arrangement. nf, neutral filter; cf, cut-off filter; sh, shutter; pt, phototransistor; pd, photodiode; ow, optical waveguide.

Fluorescence was measured with the laser pulse fluorimeter LIF 200 consisting of a nitrogen laser IGT 50, a broad band dye laser, a silicon photodiode SP 102 and a boxcar integrator BCI 280 connected with an *X-Y* recorder (block diagram in Fig. 1). To excite fluorescence we have used nitrogen-laser-excited superluminescence pulses from different dyes (uvitex OB and rhodamine 6 G with 435 and 573 nm peak wavelengths, respectively). The halfwidth of the pulses was 0.4 ns and the repetition rate was 40 Hz. The time-resolution of the LIF 200 allows to determine fluorescence lifetimes down to 0.1 ns by application of a deconvolution procedure according to

$$F(t) = \int_0^t E(t') \sum_{i=1}^{n} \alpha_i \exp\left(\frac{t-t'}{\tau_i}\right) dt' \qquad (1)$$

($F(t)$ is the fluorescence pulse, $E(t')$ is the excitation pulse, τ_i is the lifetime and α_i is the relative amplitude). Because of the relative low sensitivity of the photodiode (compared to a photomultiplier) we had to use relatively strong excitation pulses (about 10^{15} photons per cm^2). Pulse intensity and repetition rate yield an average light intensity of about $4 \cdot 10^{16}$ photons per cm^2 per s. This light intensity causes a state F_{med} (Fig. 2a) between F_0 (all PS II reaction centers are open) and F_{max} (all PS II reaction centers are closed). Fluorescence was measured in a backward geometry (180° to the exciting beam, Fig. 1) without spectral decomposition. The scattered excitation light was suppressed by a cut-off filter.

To measure fluorescence induction we used a He-Ne laser ($\lambda = 632.8$ nm) as the source of additional actinic continuous wave light. Its power was varied with neutral filters and is given in connection with the measured curves. The actinic continuous wave light excites continuous wave fluorescence and closes a number of PS II reaction centers. The latter effect is visible as a variation of the fluorescence yield of the nanosecond pulses, whereas the continuous wave fluorescence signal is not registered by the boxcar integrator. The fluorescence induction experiments were carried out in three steps.

(1) The excitation pulse and the fluorescence pulse were recorded by scanning the gate of the

boxcar integrator (duration 0.22 ns) through the pulses.

(2) To measure continuous wave light induced fluorescence induction at different times during the fluorescence pulse the gate was fixed at a definite time, denoted in relation to the maximum of the fluorescence pulse (zero point). A scheme of fluorescence induction measurements is given in Fig. 2a. The numbered arrows have the following meaning: 1, start of excitation pulses causing a weak induction effect; 2, the steady-state level of fluorescence F_{med} has been reached and the time-base of the recorder (slow time axis) is started; 3, the shutter in front of the photodiode is opened and F_{med} is recorded; 4, the continuous wave light shutter is opened and an additional fluorescence appears; 5, the continuous wave light shutter is closed and the plant adapts to the F_{med} state; 6, the shutter in front of the photodiode is closed. When the cycle described was over, the gate of the boxcar integrator was shifted within the fluorescence pulse and the cycle was repeated at another (dark-adapted) position on the leaf. The time-constant (rise-time) for measuring the induction curve depends on the averaging procedure chosen in the boxcar integrator as well as on the repetition rate of the excitation pulses and it was about 4 s.

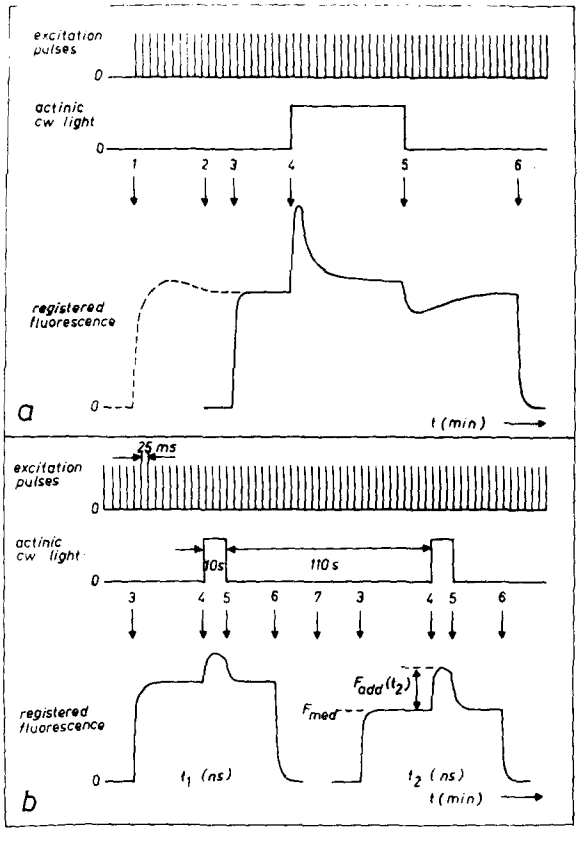

Fig. 2. Scheme of measuring continuous wave light induced variations of fluorescence yield at different times during a nanosecond pulse. (a) Fluorescence induction at a definite time during the fluorescence pulse; the dashed part of the curve is not registered in Figs. 4 and 5. (b) Maximum of the additional fluorescence at different times during the fluorescence pulse. The meaning of the numbered arrows is given in the text.

(3) To determine the lifetime of the additional fluorescence, its peak value F_{add} (Fig. 2b) was determined at different times during the fluorescence pulse. Because of the quantitative analysis of these values they were measured at the same position on the leaf to exclude fluctuations in fluorescence intensity due to the heterogenity of the leaf. Long-time actinic illuminations of plants induce long-time alterations of the plants state which are evident by the difference between the first and second induction period (Fig. 4). Therefore we used short periods (10 s) of actinic continuous wave illumination separated by 110 s periods with only pulse illumination as shown in Fig. 2b (the arrows 3–6 have the same meaning as described above: the arrow 7 has the meaning that the gate of the boxcar integrator is shifted within the fluorescence pulse to another time t_i (in ns)).

This time-course of measurements prevents a long-time decrease of F_{add} at successive illuminations. Furthermore, the gate was shifted to later times and back to eliminate long-time effects.

Results

Firstly we have measured the excitation pulse and the fluorescence pulse (Fig. 3). An exact deconvolution of the fluorescence pulse was not possible because its slow decay component was superimposed by some signal reflections. Nevertheless, a fit of the measured fluorescence pulse was possible only with more than one lifetime and a dominating component of (100 ± 30) ps lifetime and a relative amplitude of more than 0.9.

Fig. 4 shows the influence of actinic continuous wave light on the fluorescence yield at different

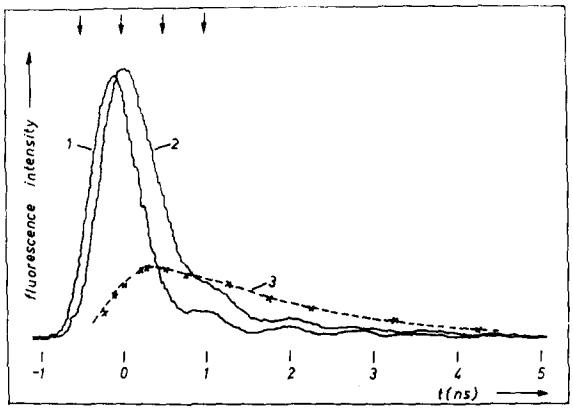

Fig. 3. 1, excitation pulse ($\lambda = 435$ nm); 2, fluorescence pulse from a leaf of maize without actinic continuous light; 3, continuous wave light (40 W/m^2) induced additional fluorescence. ×, experimental values, - - - - - -, calculated using Eqn. 1 with $\tau = 1.8$ ns; the arrows indicate the times at which the curves in Fig. 4 are measured.

times during the nanosecond fluorescence pulse. The continuous wave light induces an additional fluorescence, which shows a strong induction effect. The additional fluorescence increases in relation to the F_{med} level of fluorescence with increasing time on the nanosecond scale indicating different life-times of F_{med} and F_{add}. Within the limits of accuracy, determined by the intensity fluctuations of the excitation pulses, we found no significant differences in the shape of the induction curves at different times on the nanosecond scale. Contrary to that, the shape of the induction curve depends on the foregoing periods of light and darkness evident by the difference between the first and second induction period. The switch off of actinic continuous wave light leads to a fast ($t_{1/2} \leqslant 3$ s) decrease of fluorescence followed by a slow ($t_{1/2} = 40$–60 s) increase to the F_{med} level. The decrease of fluorescence to a level below F_{med} depends on the wavelength of the excitation pulses and is stronger at 573 nm excitation (Fig. 5) than at 435 nm excitation (Fig. 4).

To avoid long-time effects of actinic illumination the nanosecond decay of additional variable fluorescence was measured according to the third step described in Materials and Methods. A convolution of the measured excitation pulse with a single exponential decay according to Eqn. 1 gave a good fit of the values of F_{add} measured at leaves

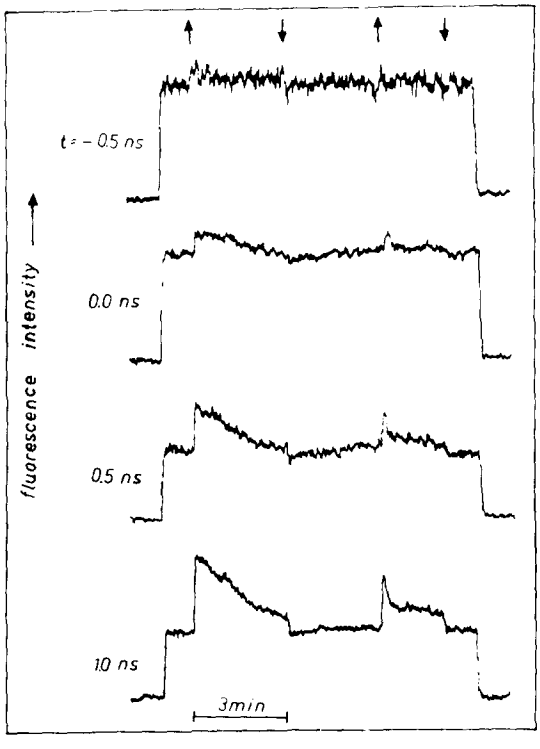

Fig. 4. Strong continuous wave light (400 W/m^2) induced variation of 435 nm excited fluorescence from a leaf of maize at different times during the fluorescence pulse; ↑, continuous wave light on, ↓, continuous wave light off.

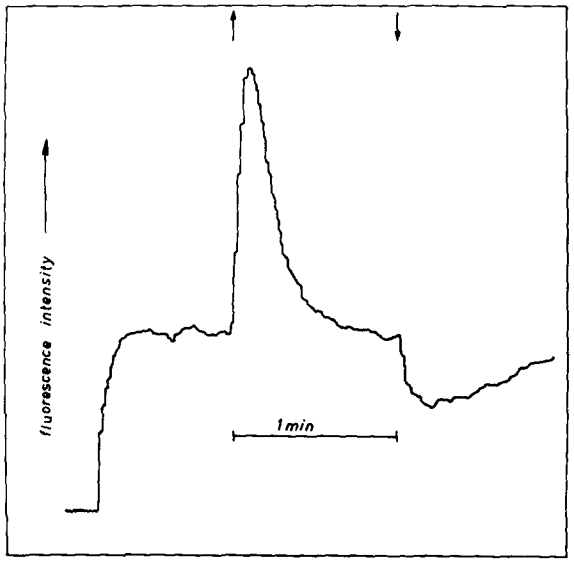

Fig. 5. Continuous wave light (20 W/m^2) induced variation of 573 nm excited fluorescence from a leaf of spruce at $t = 1.0$ ns; ↑, continuous wave light one, ↓, continuous wave light off.

of maize and spruce using a life-time $\tau = 2.0 \pm 0.2$ ns (Fig. 3).

Discussion

According to the experimental method used we have determined separately the life-time of the fluorescence without continuous wave illumination, and the life-time of the continuous wave light-induced additional fluorescence. A comparison of these values with those of other groups that found three fluorescence decay components using picosecond pulses and single-photon counting [10–12] leads to the following results.

(1) In contrast to a fast (40–130 ps) and a middle (200–500 ps) decay component measured at the F_0 level [10–12] we found a dominating ($\alpha \geqslant 0.9$) decay component of 100 ps life-time at the F_{med} level. This difference may be due to the higher excitation intensity used (about 10^{15} photons per cm^2 per pulse) which is above the threshold of exciton annihilation (the value 10^{12} photons per cm^2 per pulse [14] is related to 30 ps pulses, and may be a little higher for the 400 ps pulses used). Furthermore, the measured fluorescence decay time is a mixture of different decay kinetics arising from regions of different excitation intensities corresponding to the lateral intensity distribution within the excitation spot.

(2) The 2.0 ns life-time of additional fluorescence agrees with 2.0–2.4 ns life-times of the slow decay component measured at the F_{max} level [10,12] and is a little shorter than the 2–4 ns [15] and 4.3 ns [16] life-times of fluorescence measured under F_{max} conditions at chloroplasts and PS-II-enriched subchloroplast fragments, respectively.

From the facts that the decay of additional fluorescence is single exponential, whereas that of total fluorescence ($F_{med} + F_{add}$) is multiexponential, we conclude that the total fluorescence is composed of two (not necessarily homogeneous) parts: (1) a constant fluorescence (represented by the 0.1 ns life-time), which is independent of the state of the PS II reaction center; (2) an additional variable fluorescence (represented by the 2.0 ns life-time) caused by an additional recombination process, which is controlled by the state of the PS II reaction center.

This hypothesis is supported by the fact that the fast and middle decay components are little sensitive to the state of the PS II reaction centers, whereas the yield of the slow decay component increases drastically, if the PS II reaction centers are closed by DCMU + light [10,12,13] or by varying the redox potential [17,18].

Assuming a constant part of fluorescence the Q hypothesis by Duysens and Sweers [19], which relates the redox state of Q (the primary stable electron acceptor of PS II) to the fluorescence yield, is applicable to the variable part of the fluorescence only. Thus, the complementarity between total fluorescence and photochemistry is broken and the apparent inconsistency of fluorescence yield and photochemical yield during the induction period can be explained.

The assumption of a constant and a variable part of fluorescence is consistent with the hypothesis of Klimov et al. [15,16] that variable fluorescence is caused by recombination of separated charges in the PS II reaction center. This hypothesis is based on a PS II model [20], which includes pheophytin Ph as the primary electron acceptor between the primary donor P-680 and the primary stable acceptor Q. If Q is in the oxidized state, trapping of an exciton by the reaction center leads to charge separation and charge stabilization, which is followed by an electron transport to PS I:

$$P\text{-}680^{*}\ Ph\ Q \rightarrow P\text{-}680^{+}\ Ph^{-}\ Q \rightarrow P\text{-}680^{+}\ Ph\ Q^{-}.$$

If Q is in the reduced state a back reaction of the primary charge separation followed by fluorescence takes place:

$$P\text{-}680^{*}\ Ph\ Q^{-} \rightarrow P\text{-}680^{+}\ Ph^{-}\ Q^{-} \rightarrow P\text{-}680^{*}\ Ph\ Q^{-}$$

$$\rightarrow P\text{-}680\ Ph\ Q^{-} + h\nu.$$

This recombination fluorescence (delayed fluorescence) is generated only if Q is in the reduced state and therefore its yield is proportional to the concentration of Q^{-}. Contrary to that the life-time and yield of fluorescence emitted from the PS II antenna directly (prompt fluorescence) are independent of the redox state of Q, but dependent on the redox state of Ph. Because of the short life-time of states including Ph^{-} ($P\text{-}680^{+}\ Ph^{-}\ Q$ with 0.14 ns [14] and $P\text{-}680^{+}\ Ph^{-}\ Q^{-}$ with 2.0 ns-lifetime of recombination fluorescence), their

concentration during continuous wave illumination is neglectable and the yield of prompt fluorescence is constant.

A further argument supporting the concept of recombination fluorescence in green plants is the well-investigated recombination fluorescence in purple bacteria [21–25]. This fluorescence has a lifetime of several nanoseconds and is generated by the decay of a radical pair consisting of bacteriochlorophyll (primary electron donor) and bacteriopheophytin (primary electron acceptor).

The observed variations of the additional fluorescence on the slow time axis (Figs. 4 and 5) correspond to the well-known OIDPSMT-kinetics of fluorescence induction (the letters denote the following characteristic points of the induction curve: O, initial level; I, inflection; D, dip; P, peak; S, steady state; M, second maximum; T, terminal level) [1–3]. The fast OIDP-kinetics is averaged to a OP-rise because of the 4 s rise-time of signal processing. Using 435 nm excitation pulses we found no significant differences between induction curves measured at different times during the fluorescence pulse (Fig. 3). This fact supports the homogeneous origin of variable fluorescence represented by its single exponential decay with a lifetime of 2.0 ns. Fluorescence induction, especially the period after switching off the continuous wave light (adaptation from strong continuous wave illumination to weak pulse illumination), depends on the wavelength of the excitation pulses. The fluorescence decrease during this period is more pronounced at 573 nm excitation than at 435 nm excitation (Figs. 4 and 5). We explain this difference on the basis of the State 1–State 2 transition [26–28], which is driven by a reversible phosphorylation of the LHC [29–31]. During this transition the energy transfer from the LHC to PS II, which is assumed to emit the dominating part of room temperature fluorescence, is decreasing. 573 nm light is mainly absorbed by the LHC, whereas 435 nm light is absorbed by chlorophyll *a* contained in all parts of the photosynthetic apparatus. Therefore the 573 nm excited fluorescence is more sensitive to the light-induced State 1–State 2 transition than the 435 nm excited fluorescence. In a forthcoming paper the State 1–State 2 transition will be investigated in more detail using different wavelengths of both pulse excitation and additional continuous wave light.

Conclusions

The described two-beam method enables to measure fluorescence induction at different times during a nanosecond fluorescence pulse. Using this method we have determined the decay of variable fluorescence to be single exponential with a lifetime of 2.0 ± 0.2 ns. This points to a superposition of a constant fluorescence and an additional variable fluorescence, and agrees with Klimov's hypothesis, which attributes variable fluorescence to the recombination of the ion-radical pair $P\text{-}680^+ Ph^-$.

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