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Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer

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Abstract. A newly developed modulation fluorometer is described which operates with 1 μsec light pulses from a light-emitting diode (LED) at 100 KHz. Special amplification circuits assure a highly selective recording of pulse fluorescence signals against a vast background of non-modulated light. The system tolerates ratios of up to $1:10^7$ between measuring light and actinic light. Thus it is possible to measure the "dark fluorescence yield" and record the kinetics of light-induced changes. A high time resolution allows the recording of the rapid relaxation kinetic following a saturating single turnover flash. Examples of system performance are given. It is shown that following a flash the reoxidation kinetics of photosystem II acceptors are slowed down not only by the inhibitor DCMU, but by a number of other treatments as well. From a light intensity dependency of the induction kinetics the existence of two saturated intermediate levels (I_1 and I_2) is apparent, which indicates the removal of three distinct types of fluorescence quenching in the overall fluorescence rise from F_0 to F_{max}.

Abbreviations

Q_A and Q_B, consecutive electron acceptors of photosystem II; PS II, photosystem II; P 680, reaction center chlorophyll of photosystem II; F_0, minimum fluorescence yield following dark adaptation; F_{max}, maximum fluorescence yield; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; DCCD, N,N′-dicyclohexylcarbodiimide; PQ, plastoquinone; DAD, diaminodurene.

Introduction

Chlorophyll fluorescence has found numerous applications as a sensitive indicator of photosynthetic reactions (for reviews, see refs. [21, 25, 27, 29]). It was Duysens and Sweers [12] who introduced the concept of the fluorescence quencher Q controlling fluorescence yield. These authors recognized that variable fluorescence originates primarily from the pigments serving photosystem II (PS II) and that fluorescence quenching is closely correlated with the availability of a PS II acceptor molecule Q_A in the oxidized

Dedicated to Prof. L.N.M. Duysens on the occasion of his retirement.

state. The Q-concept has been extremely helpful for the interpretation of fluorescence changes during the past 20 years. However, Duysens and Sweers [12] also clearly demonstrated a second type of fluorescence quenching which does not depend on oxidized Q_A. Recently, there has been renewed intensive interest in the differentiation between the two main types of fluorescence quenching, the photochemical quenching (Q-quenching) and the non-photochemical quenching [5, 6, 20, 28].

The discoveries made by Duysens and Sweers largely depended on the development of a special type of modulated fluorescence measuring system, which made it possible to study the effects of light preferentially absorbed by pigment systems I or II on the fluorescence yield. The fluorescence excitation beam was chopped at 50 Hz by means of a disc mounted on a synchronous motor, and only the 50 Hz component of the signal was amplified by a phase and frequency sensitive amplifier. Hence, when continuous actinic light of various spectral compositions was applied, no direct signal due to stray light components or fluorescence of this illumination was detected. The modulated signal recorded only the indirect effect of this light on the redox state of the quencher Q_A.

In recent years, substantial progress has been made in the characterization of the detailed reaction mechanisms in the vicinity of PS II (for reviews, see refs. [1, 8, 40, 42]). In particular, the existence of a primary electron acceptor between the reaction center chlorophyll P 680 and Q_A has been recognized [19, 38]; P 680^+ was found to cause fluorescence quenching [7, 13] and a two-electron gate mechanism at the secondary PS II acceptor Q_B was discovered [3, 39].

So far, fundamental research on photosynthetic primary reactions, mostly carried out by biophysicists, has had little impact on the applied photosynthesis studies of plant physiologists. This has been mostly due to the requirements of very specialized experimental equipment to analyse the rapid primary electron transport steps. It was the intention of the present work to design a fluorometer which is sufficiently flexible to serve the sophisticated requirements for analysing the primary reactions and to be used as a tool in plant physiological studies.

This paper describes a new type of modulated chlorophyll fluorometer, which may be considered a modern version of the fluorometer introduced by Duysens and Sweers [12], which allows detection of the rapid fluorescence changes reflecting PS II primary reactions. Outstanding properties of the new fluorometer are a high time resolution, extreme insensitivity to non-modulated signals, great compactness and easy operation. The new measuring system is based on a high frequency light emitting diode (LED), substituting for a mechanically chopped excitation light source, and a photodiode replacing the photomultiplier. In some examples of experimental applications the performance of the new fluorometer is demonstrated in particular with respect to the resolution of rapid fluorescence kinetics. It

will be shown that the system is capable of resolving the fluorescence decay kinetics corresponding to the reoxidation of the primary PS II acceptor Q_A by the secondary acceptor Q_B. From the light intensity dependence of the light-on induction kinetics the existence of three distinct components in variable fluorescence from F_0 to F_{max} is demonstrated.

Materials and Methods

Spinach (*Spinacia oleracea* L., Yates Hybrid 102) was grown in the greenhouse at day/night cycles of 13/11 h. Intact spinach chloroplasts were isolated following standard procedures [15, 16]. Intactness of the outer membrane was estimated by the ferricyanide method [14]. Usually, about 80% of the chloroplasts had intact envelopes. Class D chloroplasts were obtained from intact chloroplasts by mild osmotic shock and isotonic resuspension, as described previously [34].

Chlorophyll fluorescence was measured with a newly developed "pulse-amplitude-modulation" fluorometer, features of which are detailed in the following section. This fluorometer is the prototype of an instrument which has become commercially available (PAM 101, 102, 103 chlorophyll fluorometer, H. Walz, Effeltrich, Germany). The fluorometer is equipped with four-armed fiber-optics connecting a suspension cuvette with an LED emitter, a photodiode detector, a Xenon flash lamp (EG & G FX 6A, flash duration about 5 μsec) and a source for continuous actinic light (150 W halogen lamp, Osram Xenophot HLX). Actinic light intensity was varied by use of neutral density filters (Schott NG series). Flash-triggering as well as opening and closing of electro-magnetic shutters (Compur electronic-m) were controlled by trigger and timer circuits provided by the fluorometer control unit. Full shutter opening occurred within 0.8 msec. Kinetic traces were recorded on a Digital Storage Oscilloscope (Nicolet Explorer III) from where they were photographed.

The measuring system

Most chlorophyll fluorometers employed in past photosynthesis studies make use of the same light both for fluorescence excitation and for driving photosynthetic reactions (actinic light) (for a technical review, see ref. [33]). With such fluorometers, separation between incident light and fluorescence depends on optical filters only – any light which can pass the red cut-off filter in front of the photodetector must be eliminated from the incident light (normally by a blue filter). Another feature of such fluorometers is the linear dependence of the fluorescence signal on incident light intensity, i.e. any change in actinic light intensity will cause corresponding fluorescence changes, irrespective of any true changes in fluorescence yield (fluorescence intensity/light intensity).

In modulation fluorometers, as e.g. introduced by Duysens and Sweers

[12], a modulated fluorescence measuring beam is used in addition to the non-modulated actinic light. As the amplifier system selects the modulated signal, actinic illumination can be varied within wide ranges without corresponding signal changes. With constant measuring light intensity the signal reflects relative fluorescence yield, which in green plants may vary by a factor of up to 5, depending on the redox state of the quencher Q_A and the extent of non-photochemical fluorescence quenching.

Conventional measuring systems for modulated fluorescence employ a mechanically chopped measuring beam and a lock-in amplifier for phase and frequency sensitive signal amplification. Such systems are normally limited with respect to frequency, response time, sensitivity and selectivity. To monitor the rapid fluorescence relaxation kinetics following a single turnover flash a time resolution of less than 150 μsec is required and over-saturation of the amplifier system must be avoided. Furthermore, the intensity of the measuring light should be low enough as not to have any actinic effect on the sample. In the following, a new type of modulated fluorescence measuring system is described which combines selectivity with speed and sensitivity.

Figure 1 shows a schematic diagram of the new measuring system. A master pulse generator controls the current pulses which drive emission from a light emitting diode (LED). The LED light pulses, after passing an optical short-pass filter ($\lambda < 680$ nm), will excite pulsed fluorescence in a sample. An optical long-pass filter ($\lambda > 700$ nm) rejects all stray excitation light and lets the long wavelength fluorescence pass to a photodiode detector, together with any long wavelength component of stray actinic light and of fluorescence excited by such light. Separation of the latter signals from the pulsed fluorescence signal is achieved in two steps, first, by an AC-coupled pulse amplifier and second, by a selective amplifier which is synchronized with the master pulse generator which also controls the LED driver.

To reach a high amplitude pulse signal, without inducing actinic effects by the measuring beam, the LED pulse was chosen to be only 1 μsec wide, at frequencies of 1.6 KHz or 100 KHz. Currents of up to 150 mA during single pulses were allowed for the given LED (Stanley, USBR 2000). A PIN photodiode (Hamamatsu, S 1723) with 6 V negative bias was sufficiently fast to resolve the single pulse signals. The combination of short-pass (Balzers DT Cyan) and long-pass (1 mm Schott RG 9) filters effectively blocked stray measuring light to reach the detector. Highly selective amplification of modulated fluorescence is favored by the special character of the pulse signal, which is extremely short and rapid.

Figure 2 shows the signal at the output of the AC-coupled pulse amplifier, which is determined by the loading (positive peak) and unloading (negative peak) of the coupling capacitor. By appropriate electronic circuits it is possible to amplify the difference between the "loading peak" and the

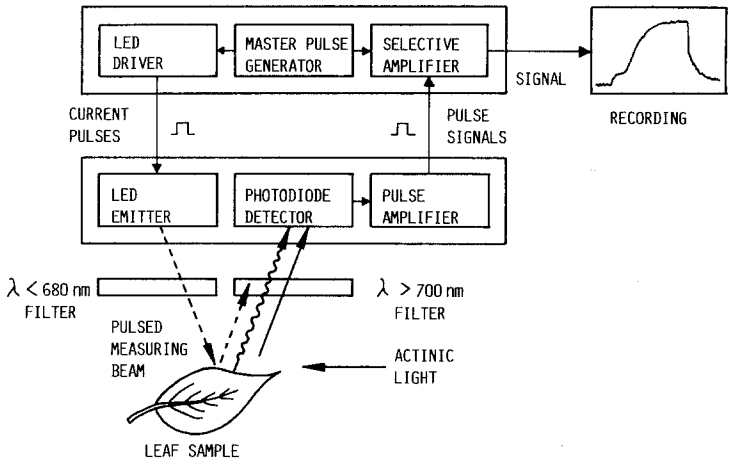

Figure 1. Schematic diagram of the pulse modulation chlorophyll fluorometer. A master pulse generator controls LED pulse emission and selective amplification of the pulse fluorescence signals. Optical short-pass and long-pass filters between the LED, sample and photodiode detector assure that no stray LED light can reach the detector. The modulated fluorescence yield is modified by the action of non-modulated actinic light, any direct signal of which is rejected by the combination of an AC-coupled pulse amplifier and the selective amplifier. In practice, the various light paths are connected via flexible, multibranched fiberoptics.

"unloading peak", which is unaffected by any overlapping signals, provided these do not show significant changes within the 2.5 μsec between the two sampling periods. Rapid electronic switches (CMOS) which are controlled by the same pulse generator which controls the LED driver, in combination with suitable decimal timers, assure that signal amplitudes are sampled at the correct times with respect to the LED pulse.

Due to the very large linearity range of the photodiode and to the highly selective amplification system, ratios of up to $1:10^7$ between measuring light and actinic light are tolerated by the measuring system. This feature makes it possible to record fluorescence at low measuring light intensity (e.g. 10 mW/m^2) and still drive photosynthetic reactions at saturating intensities of unfiltered white light (e.g. 1000 W/m^2).

When applying saturating single turnover flashes (about 10^6 W/m^2) even a dynamic range of $1:10^7$ is not sufficient to prevent amplifier overloading, which would require about 300 μsec to recover, spoiling resolution of the rapid relaxation kinetics. To avoid such overloading, a gating circuit was designed which short-circuits the photodiode output and prevents signal sampling at the selective amplifier for the duration of the flash.

For the recording of rapid kinetics a high modulation frequency is essential. On the other hand, high frequency pulse illumination may have an actinic effect on the sample. To allow high frequency measurements without significant preillumination effects, a special circuit was developed

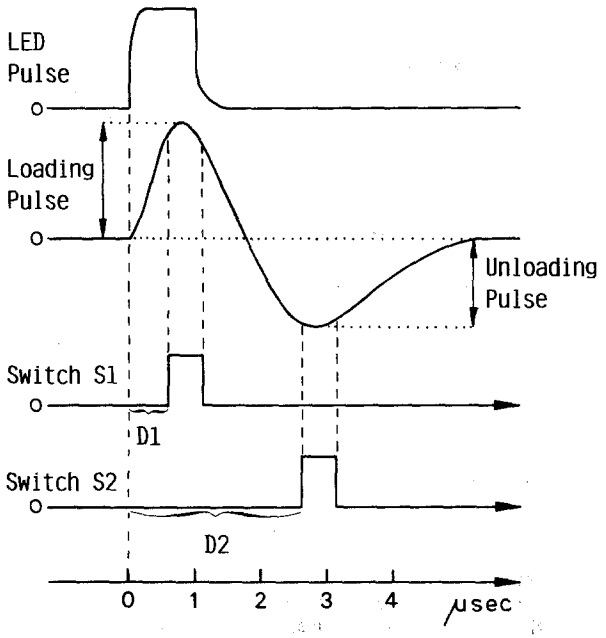

Figure 2. Time correlation between an LED measuring pulse, the signal at the output of the pulse amplifier and the sample-hold switches of the selective amplifier. The square LED pulse produces a corresponding fluorescence pulse which is transformed by the photodiode detector and the AC-coupled preamplifier into a characteristic signal, consisting of a loading pulse and an unloading pulse. The selective amplifier contains logical elements which enable it to sample the signal amplitudes at the peaks of the loading and unloading pulses and to store these amplitudes in capacitors at the two entrances of a differential amplifier until the next pulse signal arrives. The switches S_1 and S_2 are operated in synchrony with the LED pulses and are appropriately delayed, D1 and D2, to coincide with the peaks of the pulse signals.

by which modulation frequency is switched from 1.6 KHz to 100 KHz 3 msec before triggering of a flash or of continuous actinic illumination.

System performance and experimental results

Rapid decay kinetics following a flash. Figure 3 shows oscilloscope traces of the fluorescence relaxation kinetics following a saturating single turnover xenon flash in spinach leaves (A, B) and isolated chloroplasts (C, D). In Figure 3A the penetration of the PS II inhibitor DCMU into a spinach leaf is demonstrated. With increasing penetration times there was a small increase in F_0, a marked increase in the maximal yield induced by the flash and progressive retardation of the decay kinetics. These curves reflect the well-known effect of DCMU on the PS II acceptor complex: The inhibitor competes with PQ for the binding site at the B-protein [23, 24, 41] and thus blocks Q_A^- reoxidation by Q_B. Due to the 130 μsec gating period, the very

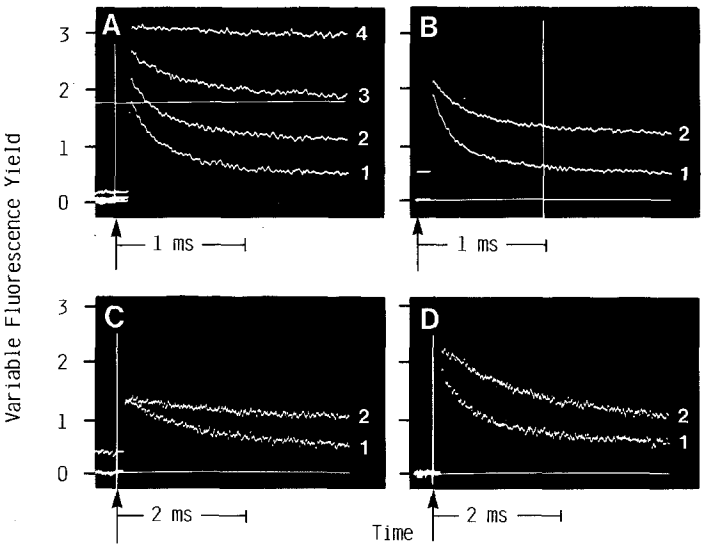

Figure 3. Fluorescence dark decay kinetics following application of a single turnover flash. Modification of the decay kinetics in spinach leaves (A, B) and class D chloroplasts (C, D) by various treatments. (A) Effect of 10^{-5} DCMU, penetrating via the upper epidermis of the leaf from which the cuticle was partially removed by gentle rubbing with carborundum powder; (1) control, (2) 5 min, (3) 10 min, (4) 20 min penetration time. (B) Effect of short heat-pretreatment; (1) control; (2) leaf exposed for 5 min to 46 °C hot water, then rapidly cooled back to 25 °C. (C) Effect of ATP-hydrolase inducing reverse electron flow. Following light activation, intact chloroplasts were osmotically shocked, isotonically resuspended and 0.5 mM ATP was added; (1) before addition of DAD, (2) 30 sec following addition of 0.2 mM of reduced DAD, which serves as donor for reverse electron flow. (D) Effect of 3×10^{-4} M DCCD on class D chloroplasts; (1) control, (2) after 5 min incubation of the inhibitor. In all experiments: The measuring beam of 50 mW/m^2 (integrated intensity) was switched on 1 sec before flash triggering. 3 msec before the flash pulse, frequency was increased from 1.6 to 100 kHz, with a corresponding increase of integrated measuring beam intensity. No signal is recorded during a 130 μsec gating period after the flash. Temperature: 25 °C with leaves (A, B); 12 °C with chloroplasts (C, D). One unit of variable fluorescence corresponds to yield at F_0.

first part of the decay kinetics is not recorded. However, from detailed previous work by Duysens and co-workers [10, 13] and other investigators [26, 45] it is known that after a saturating flash there is a biphasic fluorescence rise, correlated with the re-reduction of P^+_{680}, followed by a plateau, which lasts for about 100 μsec before the first decay phase sets in. Hence, the initial fluorescence values should be close to the true maximal amplitudes induced by the flash. An increase of flash-induced fluorescence by DCMU has been described before [37].

Other treatments which slow down the Q_A^- reoxidation kinetics are characterized by the data of Figures 3B, C, D. Figure 3B shows the effect of a short heat-treatment of a leaf. Phenomenologically this effect is similar to that of DCMU-treatment, but without the pronounced increase of

flash-induced fluorescence. Obviously, the heat treatment affects not only the PS II donor site [44] but the acceptor complex as well. In Figure 3C the effect of an active ATP-hydrolase in spinach chloroplasts is demonstrated. It has been shown before that under similar conditions ATP-hydrolysis can induce reverse electron flow and corresponding fluorescence stimulation [30, 32, 34]. It is apparent that the reverse electron pressure prevented rapid Q_A^- re-oxidation. Contrary to the action of DCMU, no increase of flash-induced fluorescence was seen. Finally, as shown in Figure 3D, there was also a marked inhibitory effect of DCCD on the decay rate. This substance is known to block proton channels [2, 36] in the CF_0 part of the ATP-ase complex, as well as in other membrane bound protein complexes. An inhibitory action of DCCD at the PS II acceptor side has been observed before [31]. Hence, one might consider the possibility of a proton channel serving Q_B^- protonation which may be essential for rapid Q_A^--reoxidation. So far, not much is known on protonation reactions at the PS II acceptor side.

These data demonstrate that the new modulation fluorometer is sufficiently fast and sensitive to record the rapid relaxation kinetics of PS II. In the past, these kinetics have been accessible only by point to point measurements, using weak detection flashes after variable delays with respect to an actinic flash [4, 10, 17, 26, 45]. The ease, with which such kinetics can now be measured, should stimulate its use as a tool to characterise the state of the PS II acceptor complex. This protein complex, which is known to be the site of PS II herbicide action [23, 24, 41] may also play a key role in the detrimental effects of environmental stress (see e.g. ref. [22]), which are expressed at physiological and biochemical levels.

Light intensity dependence of induction kinetics

An important practical advantage of a modulation fluorometer is the possibility of recording fluorescence yield over a wide range of intensities of actinic light using unchanging measuring beam intensity and amplifier gain. Figure 4 shows fluorescence induction kinetics of a spinach leaf at actinic intensities varied by a factor of 600. Whereas at moderate intensities the well-known induction pattern, O–I–P, is displayed (Figure 4A), at high light intensities the rise kinetics became considerably more complex and showed (Figure 4B, C, D) two characteristic intermediary levels, here called I_1 and I_2. With increasing intensities the I_1-level was raised, until at about 2000 W/m^2 it reached a saturation level, which amounted to 50–60% of F_{max}. At very high intensity there was a pronounced plateau or even a dip following I_1. The rise from I_1 to I_2 showed two steps which, depending on the leaf sample and the state of dark-adaptation, could be more or less pronounced. As the I_1-level, the I_2-level also approached a saturation value at very high light intensity. The last phase from I_2 to P, which was distinctly slower than the preceding phases, amounted to 10–15% of total variable fluorescence.

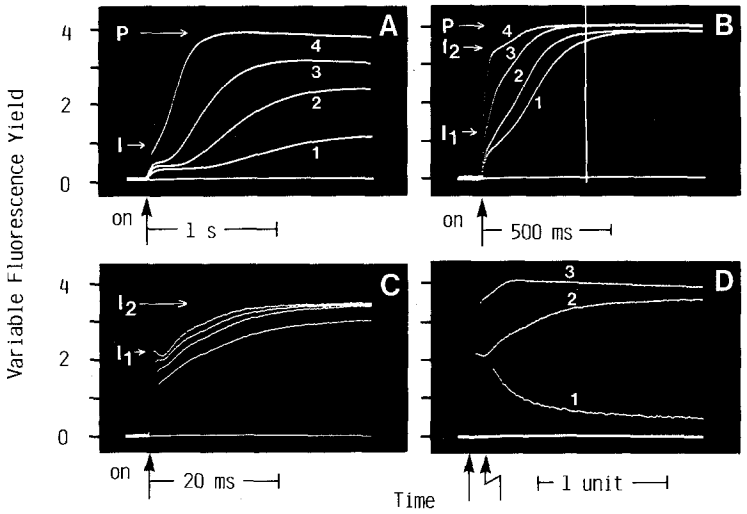

Figure 4. Fluorescence induction kinetics of spinach leaves at different actinic light intensities. (A–C) Increasing actinic intensities; recordings at different time scales. (A) (1) 5 W/m^2, (2) 10 W/m^2, (3) 20 W/m^2, (4) 40 W/m^2. (B) (1) 40 W/m^2, (2) 100 W/m^2, (3) 250 W/m^2, (4) 500 W/m^2. (C) curves from bottom to top, 500, 1000, 2000, 3000 W/m^2. (D) Comparative recordings of fluorescence increases induced by saturating continuous light (3000 W/m^2) and a saturating single turnover flash. (1) Flash illumination, 1 msec/time unit; (2) continuous illumination, 20 msec/time unit; (3) 40 msec/time unit. For other conditions, see Fig. 3.

A saturating single-turnover flash induced a fluorescence increase to a level which closely corresponded to the saturated I_1-level (Figure 4D). This feature is further elaborated by the data of Figure 5. By lowering the temperature to 0 °C, the I_1-level was increased, with a corresponding diminution of the I_1-I_2-phase (Figure 5A, C). At the same time also the flash-induced fluorescence rise was increased, and due to a slowing-down of the dark decay rate, any underestimation of flash-induced fluorescence (by the 130 μsec gating period) would have been smaller at the low temperature. When a flash was triggered at a moment during induction which corresponded to the I_1-level, this did not cause any appreciable fluorescence rise beyond the I_1-level, neither at 25 °C nor at 0 °C (Figure 5B, D).

These data suggest that variable fluorescence is composed of three parts with distinctly different properties, namely the 0-I_1, I_1-I_2 and I_2-P phases. The 0-I_1 rise probably corresponds to the "photochemical phase", first described by Delosme [9] who was investigating fluorescence induction at extreme light intensities in Chlorella. Delosme distinguished this "photochemical phase", corresponding to the reduction of the quencher Q_A [12, 18], from a slower "thermal phase", which he attributed to the removal of a "non-photochemical quencher R", closely related to the secondary PS II acceptor pool. Vernotte et al. [43] demonstrated fluorescence quenching

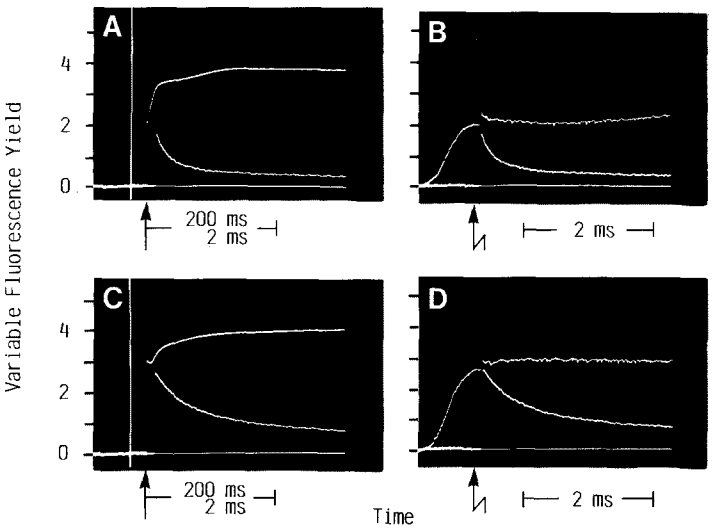

Figure 5. Effect of temperature lowering on fluorescence increases induced by saturating continuous light (3000 W/m^2) or a saturating single turnover flash. Spinach leaves were in contact with an aluminum block at 25 °C (A, B) or 0 °C (C, D). In (B) and (D) a saturating flash was triggered 1 msec following admission of continuous light, when the fluorescence rise has reached the saturated I_1-level. The two time scales in (A) and (C) apply to the curves with continuous illumination (200 msec) and flash illumination (2 msec). Other condition as in Figs. 3 and 4.

by the oxidized PQ-pool. While the extent of this quenching (about 10%) is too small to account for the I_1-I_2 phase, it could be responsible for the I_2-P phase.

Recently, there has been renewed interest in the differentiation between photochemical and non-photochemical quenching [5, 6, 11, 20, 28, 35], which is essential for practical applications of chlorophyll fluorescence methods in plant physiology [29]. Interpretation of fluorescence changes requires information on the relative contributions of the different quenching components to the overall changes. In practice, saturating light pulses are applied for complete reduction of PS II acceptors, with the assumption of complete removal of photochemical quenching. The remaining quenching has been considered to represent "non-photochemical quenching".

The above data on fluorescence changes at high light intensities demonstrate that it is not possible to reach fluorescence saturation by short light pulses, however intense, as the I_1-level cannot be surpassed. When longer pulses are applied, inducing a saturated fluorescence response (I_1-I_2-P phases) quenching which is likely to be of "non-photochemical" nature is also removed.

These considerations show that differentiation between photochemical and non-photochemical quenching by the saturation pulse method [5, 6, 11, 20, 28, 35] requires well defined saturation pulses, with respect to

pulse length and intensity. In principle the use of saturating single turnover flashes may provide the clearest results if true photochemical quenching is to be determined. On the other hand, with the use of more extended pulses, inducing the maximal P-level, photochemical as well as non-photochemical quenching appears to be eliminated, with the exception of the so-called "energy-quenching". In this latter way the differentiation is between "redox quenching" and "energy quenching", rather than between photochemical and non-photochemical quenching. As the $0\text{-}I_1$ rise, also the $I_1\text{-}I_2$ and $I_2\text{-}P$ rises are related to the reduction of the electron transport chain between the two photosystems. Hence, application of saturating "long flashes" is suitable for determination of the energy status of the thylakoid membrane, giving important information for the plant physiologist on the photosynthetic performance of leaves. Further investigations will have to clarify the detailed differences in information obtained with flashes inducing saturation at I_1, I_2 or P.

In conclusion, the newly developed pulse modulation fluorometer has been shown to be capable of measuring the rapid reoxidation of Q_A^- by Q_B, and to be unaffected even by very high actinic light intensities which were shown to be essential for complete removal of "redox quenching". By these features in combination with its compact design, the pulse modulation fluorometer opens new ways in basic photosynthesis research as well as in applied plant physiological studies.

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