The Polyphasic Rise of Chlorophyll Fluorescence upon Onset of Strong Continuous Illumination: I. Saturation Characteristics and Partial Control by the Photosystem II Acceptor Side

Christian Neubauer and Ulrich Schreiber

Lehrstuhl Botanik I, Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg, Bundesrepublik Deutschland

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Applying a rapid modulation system for measurement of chlorophyll fluorescence yield (U. Schreiber, Photosynth. Res. **9**, 261–272 (1986)) the induction kinetics upon onset of strong actinic illumination previously studied by Delosme (Biochim. Biophys. Acta **143**, 108–128 (1967)) are reinvestigated. With increasing actinic intensity the fluorescence rise is changed from the typical O-I-P characteristic to a more complex rise curve with two intermediary levels I_1 and I_2, both of which show saturation at high intensity. The typical kinetics at saturating light intensity ($O\text{-}I_1\text{-}D\text{-}I_2\text{-}P$) are observed in a variety of plant species. The properties of the kinetics with respect to light intensity, temperature, electron acceptors and PS II inhibitors suggest that the $O\text{-}I_1$ phase is controlled by photochemical charge separation (photochemical phase), while the $I_1\text{-}D\text{-}I_2\text{-}P$ transients are limited by dark reactions (thermal phases). Dichlorophenyl-dimethylurea (DCMU) eliminates the thermal phases by raising I_1 to the original I_2 level. While in principal the previous findings by Delosme are confirmed, there, is the new aspect of two distinct components in the thermal part of the rise curve, which display different properties. Electron acceptors suppress only the $I_2\text{-}P$ phase, which appears to parallel the reduction of the plastoquinone pool, which is a fluorescence quencher when oxidized. While the DCMU effect suggests quenching control during $I_1\text{-}I_2$ by reoxidation of PS II acceptors, this suggestion is contradicted by the observed saturation of I_1 with light intensity and at low temperatures. The relevance of these results with respect to quenching analysis of chlorophyll fluorescence by the saturation pulse method is discussed.

Introduction

Chlorophyll fluorescence is a complex indicator of the photosynthetic reactions in chloroplasts (for reviews, see ref. [1–3]). The complexity arises from the existence of various quenching mechanisms by which the fluorescence yield of chlorophyll *in vivo* is affected. In principal, one may distinguish between photochemical and non-photochemical types of fluorescence quenching. Bradbury and Baker [4] have suggested an experimental approach by which it is possible to differentiate between these two types of quenching: Applying a second, strong actinic illumination on top of any given fluorescence state, all photochemical quenching may be temporarily removed, allowing the determination of photochemical and non-photochemical components of total quenching (light-doubling method). For practical applications this method has been further developed by the use of modulation techniques [5–8]. Recently, a particularly selective modulation fluorometer was introduced, which allows the application of very intense actinic light at a very low level of measuring light, which by itself does not induce any variable fluorescence [8, 9]. With this system quenching analysis by the so-called "saturation pulse method" has become a practical tool in plant physiological work [10–12]. It has been shown, that under appropriate conditions the photochemical quenching determined by this method correlates well with assimilation rate [10]. However, recent work has also revealed that at high levels of membrane energization a correction has to be applied: Weis *et al.* [11] showed that there is a kind of non-photochemical quenching which lowers the quantum yield of open PS II centers. Furthermore, when calculating rate from photochemical quenching it also has to be considered that cyclic electron flow around PS II may cause "non-linear

Abbreviations: PS, photosystem; PQ, Plastoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q_A, primary stable acceptor of photosystem II; Q_B, secondary acceptor of photosystem II.

Reprint requests to Dr. U. Schreiber.

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photochemical quenching" [13]. These aspects call for more information on the differentiation between photochemical and non-photochemical quenching components by saturating light. While the saturation pulse method, as it is normally applied, considers only the peak fluorescence level reached after several hundred milliseconds, there is detailed kinetic information available from the rise kinetics during the pulse [9].

The kinetics of the fluorescence induction curve at extreme light intensities were already studied 20 years ago by Delosme [14]. This author described the separation of the rise kinetics at saturating light intensity into two phases with largely differing properties. Only the first phase was found to parallel the photochemical charge separation at PS II reaction centers (photochemical phase), while the second phase was shown to be limited by dark reactions (thermal phase). From the properties of the thermal phase, Delosme concluded that it reflects the reduction of a secondary acceptor R, closely associated with the PQ-pool. In later work, Vernotte *et al.* [15] demonstrated that non-primary PQ can quench chlorophyll fluorescence in a non-photochemical way. However, the widespread opinion reflected in the literature that R-quenching can be explained by this finding is not in agreement with Delosmes data [14], who actually showed that inhibitors such as DCMU increase the photochemical phase at the expense of most of the thermal phases. Furthermore, in a subsequent study, Delosme discovered that the fluorescence yield, observed during application of a μsec-flash, depends on the S-states of the watersplitting enzyme system [16]. Until to date it has not become clear in which way this S-state dependent quenching and R-quenching are related. The issue has been further complicated by the discovery of a secondary PS II acceptor, which also is a PQ-molecule and was called "R" [17], and by the characterization of various types of PS II heterogeneities (for a review, see ref. [18]). It may be stated that there is considerable confusion in the literature with respect to the significance of various forms of fluorescence quenching, controlled by different types of centers, photochemically or non-photochemically, by donor or acceptor side properties.

Since the development of the saturation pulse method [4–9], and in view of its great potential for practical use in plant physiology, there is renewed interest in the question of what quenching mechanisms govern fluorescence yield in saturating light. If there is non-photochemical quenching, corresponding to Delosme's R-quenching, removed during a saturation pulse, this should be considered when determining photochemical quenching. Also the aspects of donor side dependent quenching and non-linear photochemical quenching deserve to be further investigated. Therefore, a reinvestigation and extension of Delosme's original work [14, 16] under the particular conditions of the saturation pulse method appears to be of great interest.

In the present study, the fluorescence rise kinetics upon onset of continuous illumination were measured under a variety of conditions, causing changes in the properties of photochemical and thermal phases. While we can confirm the essential findings of Delosme [14, 16], additional information will be presented which may lead to a better understanding of the mechanism of non-photochemical quenching. It will be shown that what has been referred to as "thermal phase", is in fact a multitude of rise components with largely differing properties, which are controlled by PS II acceptor and donor sides. The results were devided in to two parts: This communication deals primarily with the general phenomenology of the polyphasic fluorescence rise kinetics and with aspects of the PS II acceptor side. In the accompanying contribution [19] the control of the rise kinetics by the donor side is investigated and a general evaluation of the results is presented.

Materials and Methods

Spinach (*Spinacia oleracea* L., Yates Hybrid 102) was grown in the greenhouse. Intact spinach chloroplasts were isolated following standard procedures [20]. Usually, about 80% of the chloroplasts had intact envelopes, as estimated by the ferricyanide method [21]. Chloroplasts were suspended in an isotonic medium containing 330 mM sorbitol, 50 mM HEPES-KOH at pH 7.6, 5 mM $MgCl_2$, 0.5 mM NaH_2PO_4. Chlorophyll concentration was 50 μg/ml. Chlorophyll fluorescence was measured with a modulation fluorometer (PAM chlorophyll fluorometer, H. Walz, Effeltrich, FRG) [8, 9]. The fluorometer was equipped with four-armed fiberoptics connecting a suspension cuvette with a light-emitting diode (pulsed excitation light), a photodiode detector, a source for continuous actinic light (150 W halogen lamp, Osram Xenophot HLX) and a source for

single turnover saturating flashes (XST 103, Walz). Actinic illumination was controlled by an electromagnetic shutter (Compur-electronic-m). Heat filtered white actinic light ($\lambda < 700$ nm, Balzers DT Cyan) was used, the intensity of which was varied by neutral density filters (Schott NG series). The selectivity of the fluorometer for the pulsed fluorescence signal was not disturbed at the highest light intensities applied. Kinetic traces were recorded on a Digital Storage Oscilloscope (Nicolet 2090) from which they were plotted. If not stated otherwise, leaf samples or chloroplasts were dark adapted for at least 2 h before use. The measuring light was turned on briefly before actinic illumination. Simultaneously with onset of illumination, modulation frequency was increased from 1.6 to 100 kHz by intrinsic triggering circuitry provided with the fluorometer (PAM 103, Walz). The time resolution of the measuring system was limited by the shutter opening time, which was determined with a PIN-photodiode receiving continuous light *via* shutter and fiberoptics. Full shutter opening was within 1 msec. At 400 and 700 μsec, 50% and 90% opening was observed, respectively.

Results and Discussion

At moderate actinic light intensities a predarkened sample displays the well-known two-step fluorescence rise kinetics, denoted with O-I-P in the literature [1–3]. Despite a great number of studies dealing with this O-I-P rise, there is still uncertainty about the detailed interpretation of the two rise phases. Relevant information may be obtained from the light intensity dependence of the rise kinetics. This can be favorably studied with the given modulated measuring system (see Materials and Methods), which allows application of a large range of actinic light intensities at low, constant measuring beam intensity.

Fig. 1 shows a series of induction curves at increasing actinic light intensities for intact spinach chloro-

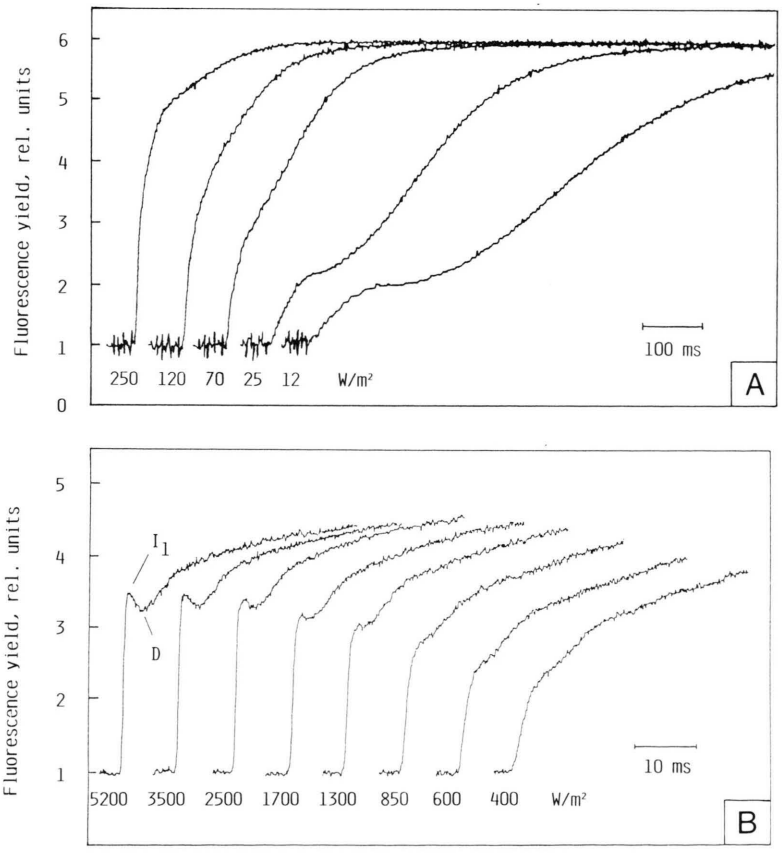

Fig. 1. Light-induced increase of fluorescence yield at varying light intensities. A) Light intensities ranging from 12 W/m^2 to 250 W/m^2. B) Light intensities ranging from 400 W/m^2 to 5200 W/m^2. Note the difference in time scales between (A) and (B). In (B) the first intermediate level, I_1, and the dip, D, are indicated: only the first part of the following rise to I_2 is shown which displays two subphases. Intact spinach chloroplasts; 20 °C.

plasts. With increasing intensity the time course is not only speeded up, but there is a remarkable change in curve characteristics, as additional phases and sub-phases may be distinguished.

Eventually, at very high light intensities a saturated rise pattern is established. This pattern appears to represent basic properties of PS II, because it is observed as well in chloroplasts as in intact leaves of a great variety of plant species (see Fig. 2). To facilitate a discussion of the different rise components, it is necessary to introduce denotations for the various characteristic fluorescence levels (Fig. 3):

A rapid initial rise from the F_0-level to a first intermediate level, I_1, is followed by a dip, D. The following rise to a second intermediate level, I_2, displays two subphases. Eventually, fluorescence increases from I_2 to the peak-level, P (also denoted with F_m in previous work).

In Fig. 4 the light intensity dependencies of the I_1, I_2 and P levels are plotted. The measurements were carried out with intact chloroplasts without addition of an artificial electron acceptor. First the P level saturates, then the I_2 level, and only at considerably higher intensities there is saturation of the I_1 level.

In order to test, whether the I_1 level is influenced by thermal reactions causing PS II acceptor reoxidation, the rise kinetics were also measured at lower temperatures.

Fig. 5 shows a series of induction curves at maximal actinic light intensity with decreasing temperature. In Fig. 6 the temperature dependencies of the characteristic levels are depicted. Down to about −30 °C, the I_1 level is somewhat raised; at still lower temperatures I_1 is decreased again. The rates of the dip phase and of the following rise phases become slowed down and the I_2 and P levels are lowered with

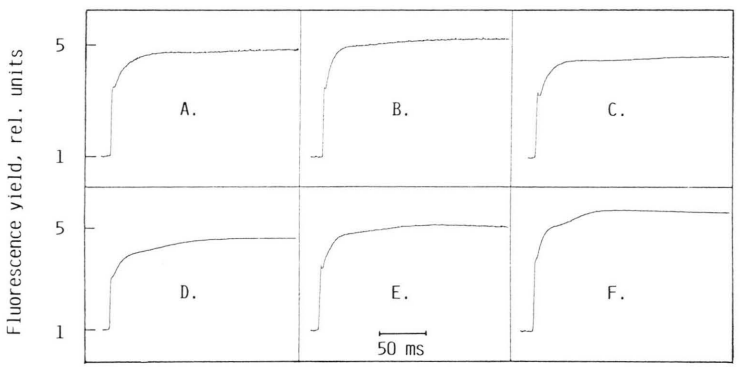

Fig. 2. Fluorescence rise kinetics at saturating light intensity of a variety of plant species. A. *Eremosphaera* spec.; B. *Marchantia polymorpha;* C. *Dryopteris filix-mas;* D. *Thuja occidentalis;* E. *Spinacea oleracea;* F. *Zea mais.* Light intensity, 3000 W/m^2. Temperature, 20 °C.

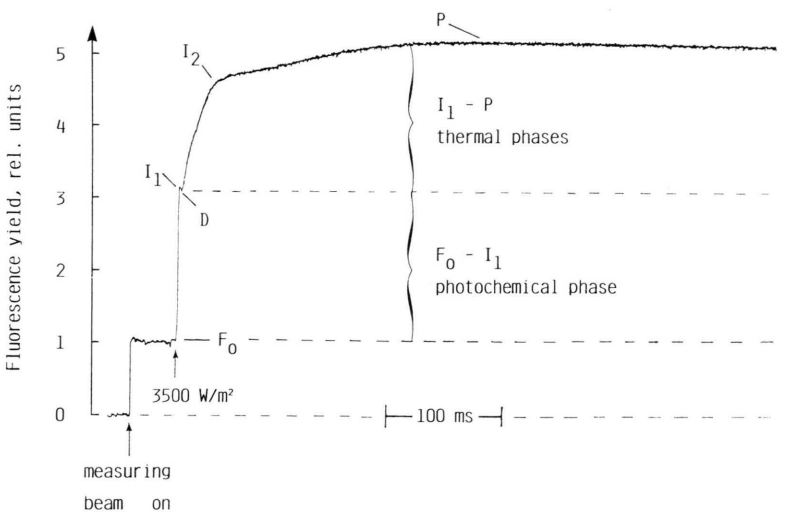

Fig. 3. Denotations of the various characteristic fluorescence levels in an induction curve at saturating light intensity. Intact spinach leaf. For explanation, see text.

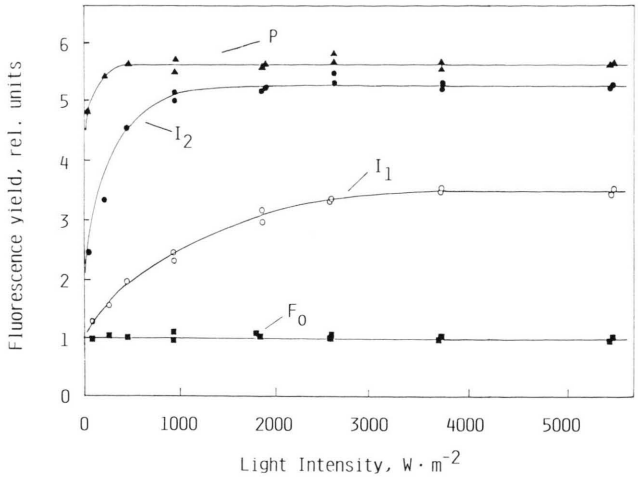

Fig. 4. Light-intensity dependencies of the characteristic fluorescence levels. Intact spinach chloroplasts; 20 °C.

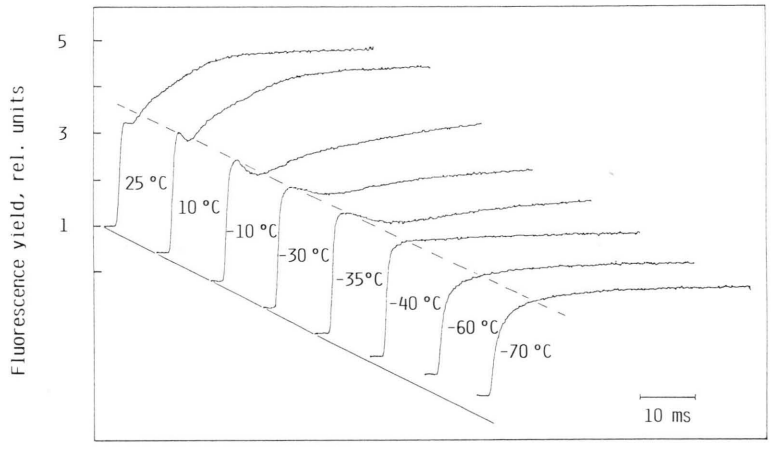

Fig. 5. Temperature dependency of induction kinetics at saturating light intensity. Spinach leaf discs. To avoid a preillumination effect, the measuring light was switched on about 1 sec before onset of actinic illumination (5200 W/m^2).

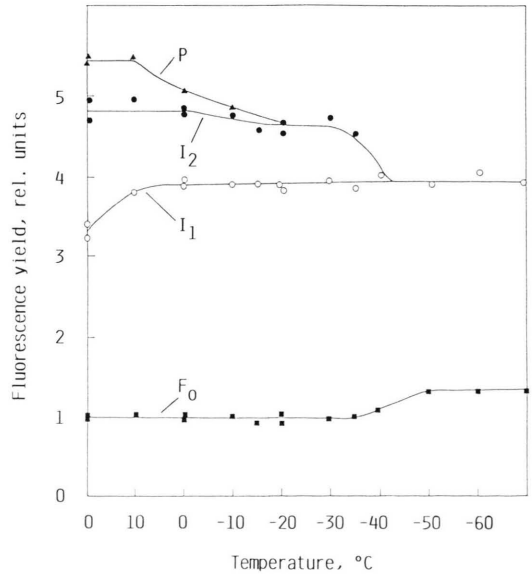

Fig. 6. Temperature dependency of the characteristic fluorescence levels observed in an induction curve at saturating light intensity. Conditions as in Fig. 5.

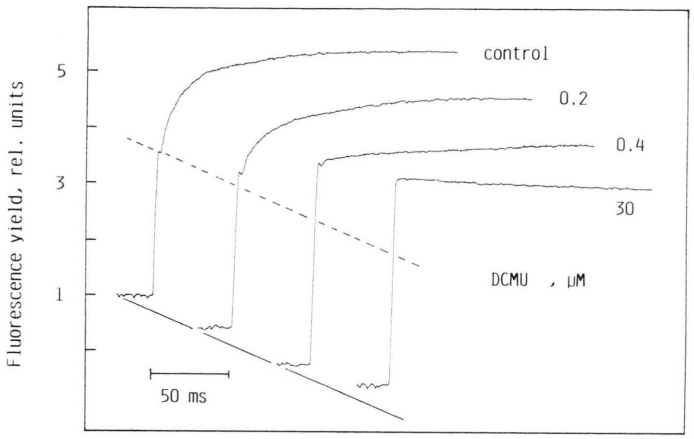

Fig. 7. Effect of the electron transport inhibitor DCMU on the induction kinetics in saturating light. Broken spinach chloroplasts, class D. Addition of DCMU 2 min before actinic illumination. Measuring beam switched on about 1 sec before actinic illumination.

decreasing temperatures. Below −35 °C the "thermal phases" (I_1-D, D-I_2 and I_2-P) are completely suppressed, resulting in an apparently monophasic rise curve. However, also the "photochemical phase" is slowed down, suggesting that the PS II quantum efficiency at very high light intensity is decreased at low temperature.

The findings that I_1 saturates at high light intensity (Fig. 4) and that lower temperatures do not lead to a substantial further increase of I_1 (Fig. 5), appears to rule out that the quenching at I_1 is caused by reoxidation of the primary PS II acceptor. Yet, it was already shown by Delosme [14] that PS II inhibitors like o-phenanthroline and DCMU eliminate the "thermal phase", at the same time increasing the amplitude of the photochemical phase. We have reinvestigated the effect of PS II inhibitors on the O-I_1-D-I_2-P kinetics in spinach chloroplasts. Fig. 7 shows the effect of DCMU on the rise kinetics. In Fig. 8 the DCMU concentration dependencies of the characteristic levels are plotted. It is apparent that with increasing concentration, DCMU causes first a decrease of P and I_2. Then I_1 is raised and the I_1-I_2 phase becomes eliminated. Eventually there is a rise of the O-level. Effects very similar to that of DCMU were observed with o-phenanthroline, ioxynil and phenylurethan (data not shown). A common observation for all of these inhibitors was that the dip phase was almost unaffected at concentrations where the I_1-level was already significantly raised; with o-phenanthroline it persisted at the highest concentrations applied.

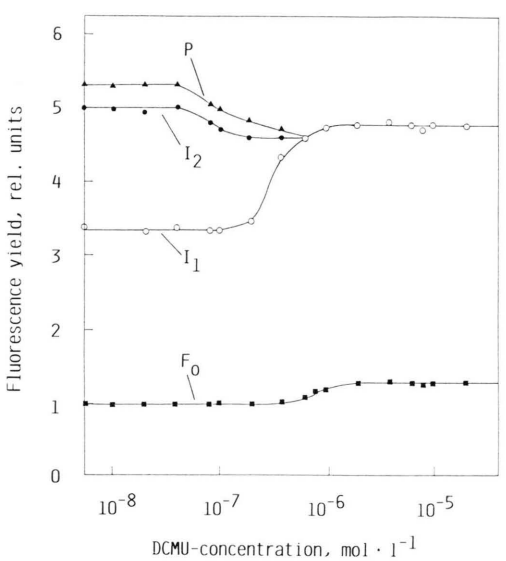

Fig. 8. DCMU-concentration dependency of characteristic fluorescence levels of induction kinetics in saturating light. Conditions as in Fig. 7.

Electron acceptors, which suppress the I-P rise of fluorescence at moderate light intensities, selectively suppress the I_2-P rise at saturating light intensity. In Fig. 9 the effect of methylviologen on intact chloroplasts is depicted. Very similar results were obtained with Class D chloroplasts, using NADP + ferredoxin, methylpurple or low concentrations of ferricyanide (up to 5×10^{-4} M) as acceptors. At higher concentrations, ferricyanide also lowered the I_1-level (not in the figures).

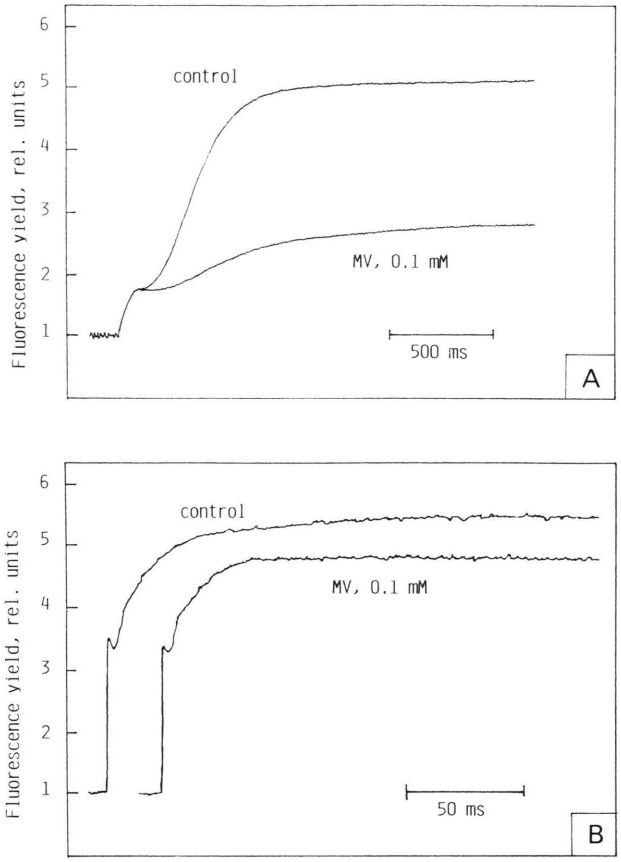

Fig. 9. Effect of the electron acceptor methylviologen on the induction kinetics. A. At moderate light intensity (25 W/m^2). B. At saturating light intensity (3000 W/m^2). Intact spinach chloroplasts. Temperature, 15 °C.

Discussion

By applying very high actinic light intensity, the fluorescence rise kinetics are clearly separated into a "photochemical" and a "thermal" part, as was already observed by Delosme [14]. While this author considered one homogenous "thermal phase", the present results allow to differentiate between I_1-D, D-I_2 (with two sub-phases) and I_2-P. This differentiation is essential, as the I_2-P phase displays properties which are distinctly different from those of the major "thermal phase" between I_1 and I_2 (see also discussion below). As has become obvious from the light saturation characteristics, it is not possible to reach the maximal fluorescence yield within less than about 200 msec, however high the applied actinic intensity. This findings is important for practical applications, like with the saturation pulse method [5–13], where a short light pulse is used to determine the maximal fluorescence yield. With application of saturating flashes, in the μsec to msec time range, the maximal fluorescence can not be induced, a point which was not considered in a recent report [22].

As was already noted by Delosme [14], the rate with which the high fluorescence state is formed, is remarkably independent of light intensity (see Fig. 1B). Hence, a thermal step appears to limit this reaction. However, it may be noted as well that cooling from 25 °C to 10 °C did not cause much slowing down of D-I_2, which is significantly suppressed only at subfreezing temperatures (see Fig. 6). Therefore, the term "thermal phase" is somewhat misleading, as it suggests a particular thermal control, which actually is not apparent.

For an understanding of the quenching at I_1, it is important that the separation between photochemical and consequent rise phases becomes more pronounced at lower temperatures. Hence, it is unlikely that the existence of the I_1 level is due to a rapid, thermally controlled reoxidation of PS II acceptors. The dip phase, I_1-D, could well represent such a reoxidation reaction, as its slowing down at lower temperatures is accompanied by some rise in I_1. However, the finding of well separated I_1 and I_2 levels at low temperatures, when I_1-D is slowed down, rules out that the "dip process" is the main cause for the fluorescence quenching at I_1.

At the maximal intensity of 5200 W/m^2 approximately one quantum is absorbed per PS II reaction center every 50 μsec. As the reoxidation of Q_A at room temperature is characterized by a half time of 400–600 μsec, at maximal intensity Q_A reduction should parallel the shutter opening. Indeed, the I_1-level can be reached within 800 μsec. Another argument for full Q_A reduction at I_1 is the observation, that the fluorescence yield measured after a saturating single-turnover flash does not exceed the I_1-level (9).

On the other hand, there is the effect of DCMU and of other PS II inhibitors, which cause an increase of I_1 and eventually eliminate all thermal phases. On first sight, this effect seems to indicate a pathway of rapid acceptor reoxidation at I_1, which becomes blocked by the inhibitors. However, such interpretation is contradicted by the saturation characteristics of I_1 with light intensity and at low temperatures which were already discussed above. It is also impor-

tant no note, that the dip phase does not become slowed down at inhibitor concentrations which raise the I_1 level. Hence, as already concluded from the behaviour at low temperatures the dip process cannot be responsible for the lowering of I_1.

In the presence of DCMU, the I_2-P phase is eliminated, resulting in a lower peak level as compared to the control. The same change was found with addition of electron acceptors which are known to accept electrons from PS I. Considering the hypothesis of Vernotte *et al.* [15], it appears likely that oxidized plastoquinone can quench fluorescence "statically", *i.e.* independently from the "dynamic quenching" by photochemical energy conversion at PS II reaction centers. Hence, I_2-P could reflect the reduction of plastoquinone, which at saturating light intensity should be determined by the rate of PQH_2/PQ exchange at the Q_B-protein [17, 23] and by the rate of PQH_2-reoxidation *via* PS I.

The "thermal phase" described by Delosme [14] corresponds to the D-I_2 rise in our study. In some of Delosme's traces (*e.g.* Fig. 7b of ref. [14]) one may distinguish a slower phase, which should correspond to our I_2-P phase but this feature did not play any role in his discussion of the "thermal phase". In this context it is curious to note that in future work, when reference to Delosme's R-quenching was made, that part of the thermal phase was meant which corresponds most likely to I_2-P and not to D-I_2 (see *e.g.* ref. [15, 24, 25]). Delosme concluded that a quencher R becomes eliminated in the course of the "thermal phase", closely correlated with the reduction of the pool of secondary PS II acceptors. However, it is obvious that the D-I_2 rise cannot involve the removal of the same kind of "statical PQ-quenching" as suggested above for the I_2-P phase. It has to be emphazised, that effective PS I acceptors which should slow down PQ-reduction do not slow down D-I_2 (see Fig. 9).

Inhibition of PQ-reduction by DCMU not only blocks the I_1-I_2 transient but also removes the involved quenching mechanism. Hence, to maintain Delosme's hypothesis, one would have to assume that R is identical with the secondary acceptor Q_B [17, 26], which actually also was denoted as "R" by Velthuys and Amesz [16]. It could be argued that R-quenching requires PQ binding to the B-protein, which is prevented by competitive binding of DCMU. Then D-I_2 could reflect the reduction of Q_B, and the two subphases could arise from the two step reduction to Q_B^- and $Q_B^=$. An argument against this interpretation is the relative insensitivity of D-I_2 with respect to electron acceptors which should slow down $Q_B^=$ accumulation and, hence also at least the second subphase of D-I_2.

The present study of induction kinetics in saturating light was initiated by recent advancements in the use of the "saturation pulse method" in plant physiological work (see Introduction). The results presented above lead to conclusions which may be important for the practical applications of this method:

1) Even with extremely high light intensity, it is not possible to reach the maximal fluorescence yield within less than about 200 msec illumination.

2) The I_2-P phase, which most likely reflects removal of statical PQ-quenching, does not involve a change in photochemical quenching. Hence, it should not be considered, when photochemical quenching is evaluated. Applying saturation pulses of about 40 msec duration would yield a maximal fluorescence level corresponding to I_2 and, hence, the contribution of the I_2-P phase would be eliminated. Alternatively, as with a given species the contribution of this phase to the total variable fluorescence is relatively constant, a corresponding correction could be made.

3) In principal, it would be advantageous not only to measure the maximal level of fluorescence obtained in a saturation pulse but to resolve the rise kinetics. Additional information may be drawn from an evaluation of the different rise phases.

4) Although with the present state of information a detailed interpretation of the D-I_2 transient is not possible, the presented results favour the conclusion that this part of the fluorescence rise is unlikely to reflect elimination of "normal" photochemical quenching. Photochemical quenching, involving electron transfer to Q_A, should be completely suppressed at I_1. In the following contribution, results will be presented which argue for a type of dissipative quenching which is correlated with a decreased rate of electron donation from the watersplitting enzyme system.

Acknowledgements

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