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Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer

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Abstract. A newly developed fluorescence measuring system is employed for the recording of chlorophyll fluorescence induction kinetics (Kautsky-effect) and for the continuous determination of the photochemical and non-photochemical components of fluorescence quenching. The measuring system, which is based on a pulse modulation principle, selectively monitors the fluorescence yield of a weak measuring beam and is not affected even by extremely high intensities of actinic light. By repetitive application of short light pulses of saturating intensity, the fluorescence yield at complete suppression of photochemical quenching is repetitively recorded, allowing the determination of continuous plots of photochemical quenching and non-photochemical quenching. Such plots are compared with the time courses of variable fluorescence at different intensities of actinic illumination. The differences between the observed kinetics are discussed. It is shown that the modulation fluorometer, in combination with the application of saturating light pulses, provides essential information beyond that obtained with conventional chlorophyll fluorometers.

Introduction

Chlorophyll fluorescence induction curves (Kautsky effect) reveal valuable information on the state of the photosynthetic apparatus in plants (for recent reviews see refs. [1–3]). However, the interpretation of induction curves is complicated by the existence of several mechanisms of "fluorescence quenching". The major quenching components are Q-quenching (q_Q), due to photochemical energy conversion at PS II reaction centers with the primary acceptor Q_A being oxidized, and energy-quenching (q_E), due to an increased rate of radiationless deexcitation upon "energization" of the thylakoid membrane [1–5]. "Energization" is believed to be closely related to the build-up of a transthylakoidal pH-gradient.

Two methods have been described to determine the relative contribution of q_Q and q_E to total quenching: First, Bradbury and Baker [6] estimated q_Q from measurements of the superimposed fast-induction kinetics occurring in response to the application of a second, strong illumination at appropriate times of the underlying induction curve (light-doubling method). This is based on the rationale that at any time during induction, illumination by

strong light will cause a transitory complete removal of q_Q. This phenomenon is reflected in an increase in variable fluorescence. Second, Krause et al. [5] reported separation of the two quenching components in isolated chloroplasts by measurement of the biphasic fluorescence rise induced by rapid injection of DCMU. In this system the rapid component reflects reduction of Q_A, i.e. relaxation of q_Q. Recently, Quick and Horton [7] showed in protoplasts that there is acceptable correlation between q_Q-determinations by the "light-doubling method" and by the "DCMU-method". By measuring modulated fluorescence [see refs. 7, 8], these authors were able to determine q_Q in a more direct way than originally described. Using a similar technique, Dietz et al. [9] examined q_Q during steady state photosynthesis under various limiting conditions.

The present communication reports on measurements of chlorophyll fluorescence induction in intact leaves by a new pulse modulation technique. This technique allows for continuous determination of q_Q and q_E and, hence, of the redox state and the energy status of the chloroplasts in the leaves. It is shown that at high light intensity the overall slow induction kinetics are governed by q_E, while at low light intensity the influence of the q_Q-component is prevailing. At high light intensity, electron transport activity, as expressed in q_Q, lags behind membrane energization, as expressed in q_E. The release of energy quenching is shown to be related to the onset of CO_2-fixation during induction.

Materials and methods

Potted plants of *Phaseolus vulgaris* were grown in the greenhouse at 20°C and 40 W/m^2 white light (Radium, HRI-E 400 W/D) at day/night cycles of 13/11 h. Measurements were done at 20°C in air.

Fluorescence was measured with a prototype of a newly developed pulse amplitude modulation fluorometer (Model PAM 101 Chlorophyll Fluorometer; H. Walz, Effeltrich, Germany). Fluorescence was excited by 1 μsec light pulses from a light emitting diode applied at a frequency of 1.6 KHz. The integrated intensity of the modulated measuring beam was 10 mW/m^2, which is sufficiently low not to cause appreciable induction transients. Simultaneous illumination of the samples by two different actinic light sources and by the modulated beam, as well as detection of the fluorescence signal was accomplished via a polyfurcated fiberoptic system. Actinic illumination, derived from halogen lamps (Xenophot XLX 64634, Osram) was controlled by electromagnetic shutters (Compur electronic-m) (half-opening time 1 msec at 4 mm cross-section), and by suitable triggering circuits. Actinic white light of saturating intensity (2000 W/m^2) passing through a shortpass filter (Balzers DT Cyan) was applied repetitively, in short pulses (300 msec), to monitor the saturation level of variable fluorescence, $(F_V)_S$, during the courses of induction curves. Actinic illumination of variable intensity, consisting of

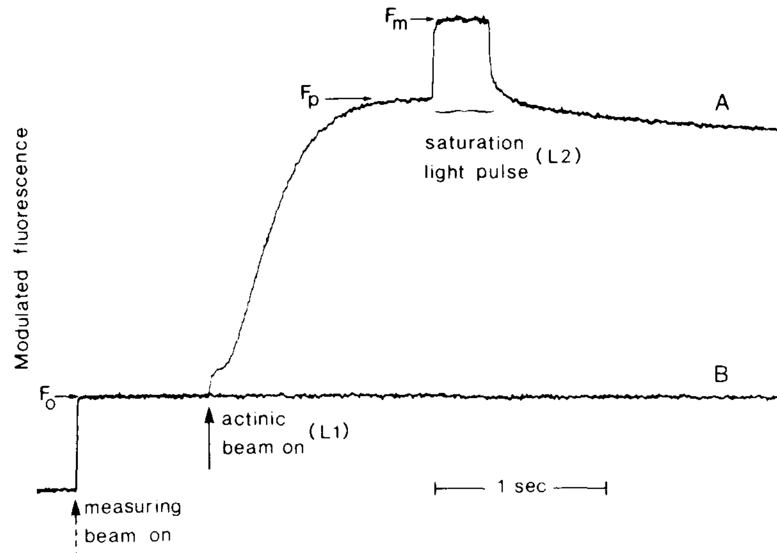

Figure 1. Comparison of modulated fluorescence responses of a *P. vulgaris* leaf (A) and a chlorophyll extract (B) upon application of actinic light of moderate and saturating intensities. The chlorophyll concentration of the leaf extract (ethanolic extract of bean leaf) was adjusted to give a fluorescence signal identical to the F_0 level of the leaf. Actinic light intensity (L1), 20 W/m²; intensity of saturation light pulse (L2), 2000 W/m².

heat-filtered white light (Balzers Calflex C), served to cause fluorescence induction. The photodiode detector of the fluorometer was protected by a far-red cut-off filter (Schott RG 9). The dynamic range of the modulated measuring systems (PAM 101, Walz) was large enough to allow reliable detection of the modulated signal against a large background of continuous signals from actinic illumination (stray light and fluorescence). This is demonstrated in Figure 1, where the modulated fluorescence response of a bean leaf (curve A) is compared to that of a chlorophyll extract (curve B). With the bean leaf, upon admission of continuous light (L_1) ($20\,W/m^2$) there is the expected rise of fluorescence yield from F_0 to a peak level. F_p, and a further rise to the maximal level, F_m, when the saturating beam L_2 ($2000\,W/m^2$) is switched on. Under identical conditions there are no changes in the fluorescence yield of the chlorophyll extract. When L_2 is admitted, the signal amplitude remains constant, despite a ratio of $1{:}10^6$ between modulated and continuous signals (as determined with a photodiode protected by a RG 9 filter). The given measuring system is free of the switching-on and switching-off artifacts found with conventional lock-in amplifier systems, thus allowing the resolution of rapid induction phenomena. The kinetic traces were recorded with a digital storage oscilloscope (Nicolet Explorer III) or with a chart recorder.

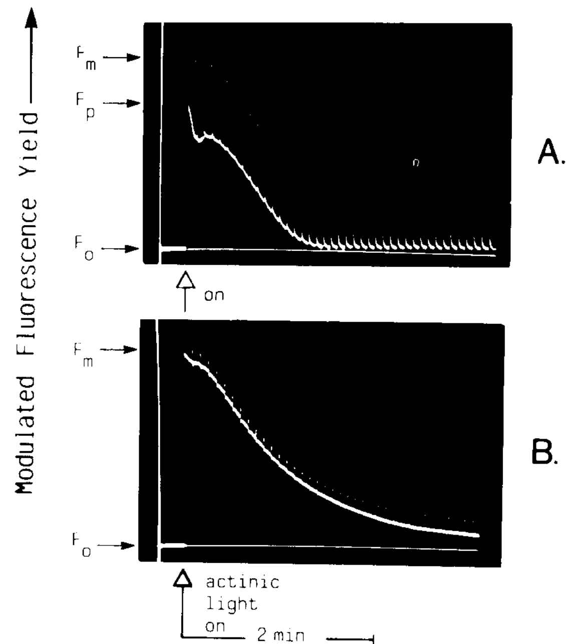

Figure 2. Original oscilloscope traces of induction curves of *P. vulgaris* with repetitive application of saturation light pulses. Panel A, actinic light intensity, 15 W/m². Panel B, actinic light intensity, 100 W/m². Saturation pulses of 2000 W/m² intensity were applied every 5 sec. At the given oscilloscope sweep rate, the fluorescence levels reached during saturation pulses (F_s) are represented by the dotted lines. All photochemical quenching is suppressed by the saturation pulses. The observed changes in F_s are believed to reflect primarily changes in energy-dependent fluorescence quenching, q_E. After dark-adaptation, $q_E = 0$ and $F_s = F_m$.

Results

Figure 2 shows oscilloscope recordings of fluorescence induction curves of a bean leaf measured with the pulse modulation system at a high (100 W/m²) and at a low (15 W/m²) intensity of white light (L_1), superimposed by repetitive flashes of saturating white light (L_2). These recordings contain two types of induction kinetics: First, the fluorescence levels reached during the L_2-flashes reflect F_s, i.e. the saturated fluorescence yield at 100% Q_A^-. Hence, the kinetics of the curve connecting the F_s levels with consequent L_2-pulses (F_s- or $(F_v)_s$-curve)* correspond to the development of energy-quenching, q_E. In this report all quenching different from q_Q is defined as q_E, although we are aware of non-photochemical quenching mechanisms different from energy-dependent fluorescence quenching [see reviews in refs. 1, 2]. Recent data of Quick and Horton [7] suggest that within the range of light intensities used in the present study, these alternate types of quenching play a minor

*Please note that for quantitative analysis of fluorescence changes the characteristic levels of *variable* fluorescence yield, F_v, $(F_v)_s$ and $(F_v)_m$, are decisive (see Fig. 3).

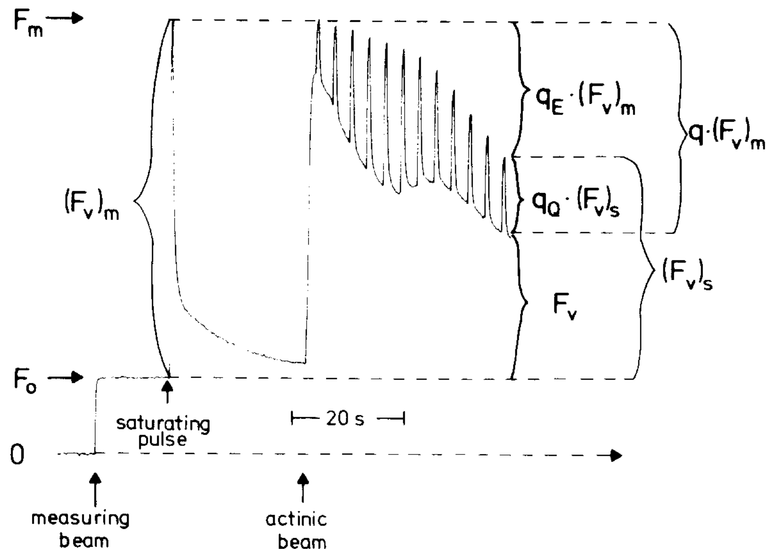

Figure 3. Definition of quenching coefficients and terminology of characteristic fluorescence parameters. F_o, fluorescence intensity of dark adapted sample with measuring beam if negligible actinic intensity. F_m, maximal fluorescence intensity obtained with dark adapted sample upon application of saturating light pulse. $(F_v)_m$, maximal variable fluorescence of dark adapted sample. F_v, variable fluorescence at any given time during induction. $(F_v)_s$, maximal variable fluorescence at any given time during induction. For further explanations, see text.

role. Second, the fluorescence levels displayed shortly before application of the L_2-pulses correspond to the normal fluorescence induction curve (F- or F_v-curve) which is determined by Q-quenching, q_Q, as well as by q_E. Even without calculating the precise contributions of q_E and q_Q, it is evident from these recordings that at the high light intensity the overall slow fluorescence kinetics follow closely q_E, while this is only partially true for the low light intensity. Approaching the steady state, the amplitude of the F_v-curve becomes about half that of the $(F_v)_s$-curve at the high light intensity and about 0.1 at the low light intensity. In a first approximation, assuming a linear relationship between fluorescence yield and the percentage of Q_A^-, this would mean that in the steady state 50% of PS II centers are closed (because of Q_A^-) at the high light intensity and 10% at the low light intensity. Hence this method provides rapid, semi-quantitative information on photosynthetic electron transport activity in leaves.

For a more detailed analysis continuous plots of the PS II reduction state (in first approximation Q_A^-) and of energy dependent fluorescence quenching may be derived from the fluorescence parameters given in the F_v- and $(F_v)_s$-curves. Figure 3 illustrates the derivation of expressions for Q_A-reduction and energy quenching from an original recording. The measuring beam is sufficiently weak not to induce a fluorescence increase beyond F_o. With a dark adapted sample, in which energy dependent quenching may be assumed to be

zero, a saturating light pulse induces a maximal increase of variable fluorescence $(F_v)_m$, corresponding to $q_Q = 0$ (100% Q_A^-). In the following dark period the fluorescence decay reflects reoxidation of Q_A^-. Upon application of continuous actinic light there is the typical inductive change of variable fluorescence, F_v, which at any given time is suppressed relative to the maximal variable fluorescence, $(F_v)_m$, by the given amount of fluorescence quenching:

$$F_v = (F_v)_m - q(F_v)_m \tag{1}$$

where q is the quenching coefficient which may vary between $q = 0$ (100% Q_A^-, $q_E = 0$) and $q = 1$ (full suppression of F_v). Following determination of saturated variable fluorescence, $(F_v)_s$, by saturating light pulses, it is possible to differentiate between the photochemical and non-photochemical parts of the total quenching:

$$F_v = (F_v)_s - q_Q(F_v)_s \tag{2}$$

$$(F_v)_s = (F_v)_m - q_E(F_v)_m \tag{3}$$

$$F_v = (F_v)_m - q_E(F_v)_m - q_Q(F_v)_s \tag{4}$$

with $0 \leqslant q_Q \leqslant 1, 0 \leqslant q_E \leqslant 1$.

By substituting $(F_v)_s$ in (4) by (3) one obtains after a transformation:

$$F_v = (1 - q_E)(1 - q_Q)(F_v)_m \tag{5}$$

In this form it can easily be seen, that the fluorescence yield is modulated by two independent factors.

In practice, expressions for $(1 - q_Q)$, q_Q and q_E are most helpful:

$$(1 - q_Q) = \frac{F_v}{(F_v)_s} \tag{6}$$

$$q_Q = \frac{(F_v)_s - F_v}{(F_v)_s} \tag{7}$$

$$(1 - q_E) = \frac{(F_v)_s}{(F_v)_m} \tag{8}$$

$$q_E = \frac{(F_v)_m - (F_v)_s}{(F_v)_m} \tag{9}$$

In first approximation, plots of $(1 - q_Q)$ reflect the relative extent of Q_A-reduction, and plots of $(1 - q_E)$ follow the lowering of fluorescence yield by proton accumulation within the thylakoids.

In Figure 4 plots of $(1 - q_Q)$ and $(1 - q_E)$ are compared with the corresponding F_v curves for three different light intensities. The $(1 - q_E)$ curves and F_v curves correspond to the $(F_v)_s$ and F_v curves displayed in Figure 2, respectively. The following points may be made:

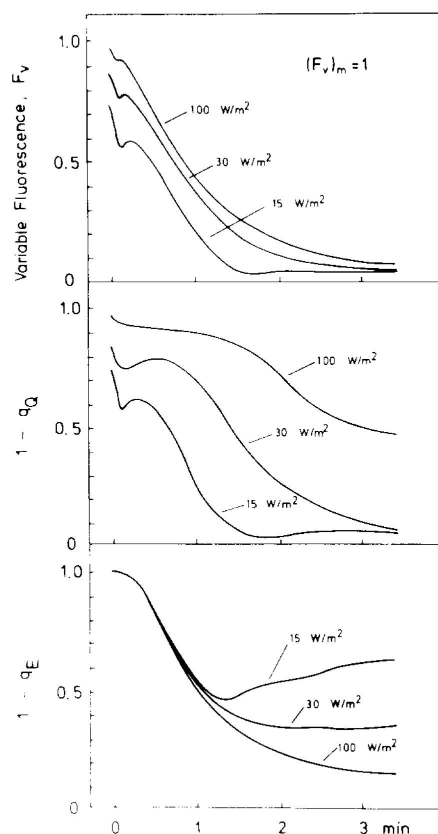

Figure 4. Plots of variable fluorescence, $(1 - q_Q)$ and $(1 - q_E)$ derived from fluorescence induction curves with repetitive saturation light pulses. Original induction curves recorded as in Figure 2 at three different light intensities.

(a) The $(1 - q_E)$ curves display a relatively extended lag-phase (about 15 sec) before any appreciable decay sets in. Interestingly, the duration of this lag-phase, as well as the rate of the ensuing decay, are almost identical for the three different light intensities;

(b) simultaneous with the lag-phase in the $(1 - q_E)$ curves, there is the $P - S_1 - M_1$ transient [for nomenclature see e.g. ref. 10] in F_v and a corresponding transient in $(1 - q_Q)$. This finding confirms previous results of Bradbury and Baker [6] who concluded that the $P - S_1 - M_1$ transient is mainly caused by changes in q_Q.

(c) As already noted in the discussion of Figure 2, the major decay kinetics of $M_1 - S_2$ may be caused to a variable extent by q_E or by q_Q, depending on the experimental conditions, as e.g. on light intensity. For example, after 80 sec at $100\,W/m^2$: $F_v = 0.32$, $(1 - q_Q) = 0.88$ and $(1 - q_E) = 0.37$. And after 80 sec at $15\,W/m^2$: $F_v = 0.07$, $(1 - q_Q) = 0.11$ and $(1 - q_E) = 0.47$. At

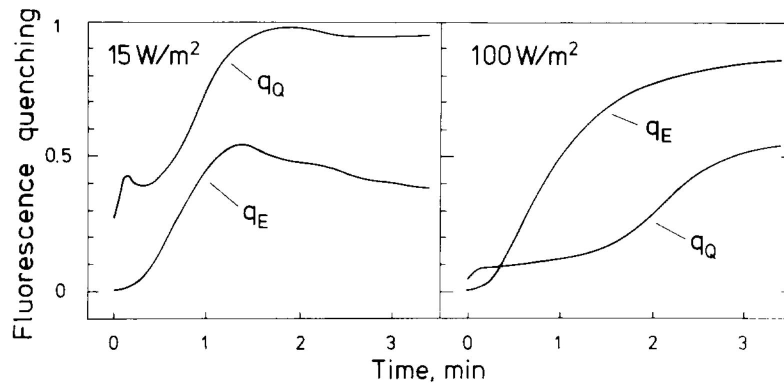

Figure 5. Comparison of the time courses of q_Q and q_E during induction at different light intensities. Original induction curves recorded as in Figure 2.

low light intensity, the overall fluorescence induction curve is quite closely related to q_Q-changes, while at high light intensity there is close correspondence to q_E-changes. Similar observations were made by Quick and Horton [7] who determined q_E and q_Q during the course of slow fluorescence oscillations in protoplasts.

In Figure 5 the time courses of q_Q and q_E during induction are directly compared for two different light intensities. At 15 W/m^2 the development of q_Q and q_E is almot parallel, except for the rapid initial changes in q_Q. On the other hand, at 100 W/m^2 there is a distinct lag period before Q-quenching follows energy quenching. This behaviour relates to the balance between electron transport activity (open PS II reaction centers) and phosphorylation activity (membrane energization). Obviously, during induction at higher light intensity, electron transport activity lags behind membrane energization.

In Figure 6, induction curves of F_v and $(F_v)_s$ are shown in the presence and absence of external CO_2. While in the presence of CO_2, following an initial dip, the (F_v)-curve rises again to a high stationary level (panel A) $(F_v)_s$ remains low in the absence of CO_2 and is only induced to rise again, when CO_2 is added (panel B). From the result of this experiment it becomes apparent, that the time course of the $(F_v)_s$ curves carries information on the onset of the reductive pentose phosphate cycle which can not readily be obtained from ordinary fluorescence induction curves (F_v curves). Obviously, the rate and extent by which the initially developed energy quenching is released in the course of induction may be taken as a measure for the utilization of stored membrane energy in photophosphorylation and ATP-dependent electron transport.

Discussion and conclusions

This report demonstrates that the use of a newly developed pulse modulation

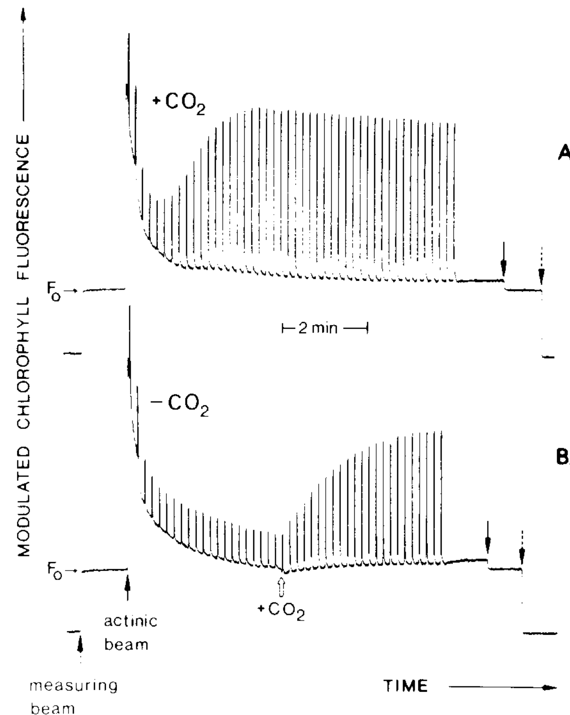

Figure 6. Induction curves of *P. vulgaris* with repetitive application of saturation pulses in dependence of the availability of CO_2. Panel A, the leaf was exposed to air with 0.03% CO_2. Panel B, CO_2 was removed from the air stream by soda lime; where indicated (+ CO_2) the stream of CO_2-free air was exchanged against a stream of air. Stream rate, $20\,l \cdot h^{-1}$. Actinic light intensity, 30 W/m^2.

fluorometer, in combination with the repetitive application of saturation pulses, can yield important new information which is not available from conventional fluorescence induction curves. Practical applications of the chlorophyll fluorescence induction method in plant physiological work have for a long time been restricted for several reasons: (a) The complexity of the fluorescence quenching mechanisms and, hence, the uncertainty on the interpretation of certain fluorescence transients or states, particularly in the slow parts of the induction curves; (b) the fact, that with prolonged illumination variable fluorescence becomes rather small and fluorescence changes hard to evaluate; (c) the possibility, that changes in one type of fluorescence quenching will overlap with different or even opposite changes in another type of quenching, such that the kinetic information may be distorted or even suppressed. Recent progress by work of Krause et al. [5], Bradbury and Baker [6, 11], Horton and co-workers [7, 8, 13] and our own laboratory [9] has led to techniques which may overcome these restrictions to a great extent. The newly developed pulse amplitude modulation system, introduced in the

present paper, allows to differentiate continuously between q_Q and q_E (see Figures 2–6). Due to the extreme selectivity of this measuring system for the pulse modulated signal, even very strong light-saturation pulses may be applied. The resulting information may be readily analysed on the basis of simple relationships (as depicted in Figure 3). While a deconvolution of a fluorescence induction into the q_Q and q_E components gives detailed information, also the original recorder traces already allow valuable insights.

In particular, on the basis of the presented results some conclusions can be drawn which may help to understand the mechanisms governing chlorophyll fluorescence induction:

(a) Energy-dependent quenching (q_E) was shown to develop after an appreciable lag-phase, extending to the M_1-peak [for nomenclature see e.g. ref. 10]. Hence, the transient fluorescence changes observed up to that point in the induction curve, appear to be caused mostly by changes in Q-quenching (q_Q). This result does not *per se* exclude the possibility of more rapid changes in non-photochemical quenching during the fast part of the induction curve ($O - I - D - P$ transients). However, although there is no problem in applying a saturation pulse during fast induction, in this way reliable information can not be obtained, as any increase in q_E will be only transient and removed again by the saturation pulse, which drives the system to the F_m-level. This aspect has to be considered when evaluating corresponding results of Bradbury and Baker [11].

(b) The identification of the $P - S_1 - M_1$ transients with preferential changes in q_Q, is in line with former findings of Bradbury and Baker [6, 11]. These transients are likely to be caused by transient reoxidation of the electron transport chain by a limited pool of acceptors (possibly NADP) which becomes available only following an activation step [possibly of ferredoxin-NADP reductase, see ref. 12]. Such interpretation agrees with former suggestions by Horton [8, 13].

(c) The kinetics of q_E (represented in the $(F_v)_s$-curves) display properties which make it unlikely that q_E directly reflects the transthylakoidal ΔpH. From experiments with isolated chloroplasts it is known that this gradient is established upon illumination without lag and at a rate which is considerably faster than that of q_E-development [see e.g. refs. 8, 13]. One may conclude that actual "membrane energization", which should reach a high level within a few seconds, will induce rather slow secondary membrane changes, which are correlated with an increase in q_E. The rate and extent of this slow, secondary change will be related to the size of the ΔpH [4, 14], while the rate with which the ΔpH is established may play no role, as long as it is distinctly faster than the secondary change. This interpretation accounts for the relative indifference of early $(F_v)_s$-curve kinetics to different light intensities.

(d) At low light intensities, the overall rates with which energy-dependent and photochemical quenching develop are rather similar and, hence, are also closely correlated with the corresponding decay of fluorescence from F_p to a

low steady-state level. The higher the light intensity, the more the development of q_Q is retarded with respect to q_E. Consequently, at higher light intensities the fluorescence response is governed by energy-dependent quenching.

It may be said that in combination with the saturation pulse method, originally proposed by Bradbury and Baker [6], the newly developed pulse modulation fluorometer has opened new ways of analysing chlorophyll fluorescence changes. As the system is not affected even by extreme levels of white light, it may be employed under conditions identical to those generally applied with gas exchange measurements. Hence, the fluorescence behaviour and CO_2-fixation can be immediately compared under the natural light conditions of the plant. Such experiments are currently being undertaken in our laboratory under a variety of physiological conditions.

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