Measuring P700 Absorbance Changes around 830 nm with a New Type of Pulse Modulation System

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A new measuring system for monitoring absorbance changes around 830 nm is described, which was developed by modification of a commercially available pulse modulation fluorometer. All modifications concern the emitter-detector unit of the fluorometer, such that only this unit needs to be exchanged when changing from fluorescence to absorbance measurements and *vice versa*. The new system is shown to be well-suited for measuring redox changes of P700, the reaction center of photosystem I, in intact leaves and isolated chloroplasts. The observed kinetic changes at 830 nm in response to single turnover or multiple turnover saturating flashes are practically identical to those previously measured around 700 nm. The signal/noise ratio is sufficiently high to give well-resolved kinetics without signal averaging. When P700 is oxidized by far-red background light, valuable information on the state of the intersystem electron transport chain is given by the re-reduction kinetics induced by single or multiple turnover saturating flashes. Such measurements are facilitated by the use of poly-furcated fiberoptics. With intact leaves, almost identical responses are found when measuring through the leaf (transmission mode) or from the leaf surface (remission mode). Modulated chlorophyll fluorescence can be measured in parallel; application of saturation pulses for fluorescence quenching analysis produces transient P700 oxidation without oversaturating the measuring system. The information on the P700 redox state complements that obtained from fluorescence measurements, yielding a new practical tool in plant physiological research.

Introduction

Since its discovery in 1957 by Kok [1], P700 has been characterized in detail by absorbance measurements around 700 nm ([2–10]; for reviews see Ref. [11, 12]). P700 acts as the reaction center of PS I which, in contrast to PS II, can be driven by far-red light in the 700–730 nm range. P700 absorbance changes reflect the dynamic interaction of the two photosystems, mediated by the intersystem electron carriers PQ, Cyt *b/f* and plastocyanin.

Measurements of P700 redox changes at the main absorbance band around 700 nm are complicated by the overlapping changes of chlorophyll fluorescence, particularly with samples strongly scattering the measuring beam. For this reason, so far almost no practical application of P700 measurements has been made to study photosynthesis in intact leaves. There is, however, also the possibility of measuring P700 redox changes *via* the broad band increase in absorbance caused by the $P700^+$ cation-radical at 810–830 nm [13–16]. In this wave-length region chlorophyll fluorescence is not excited and fluorescence emission is small. Recently, there was a report by Weis *et al.* [17] on 820 nm absorbance measurements, to determine the extent of P700 oxidation in intact leaves during steady state illumination.

We have developed a new pulse modulation technique, originally for measuring changes in chlorophyll fluorescence yield with high sensitivity and selectivity [18, 19]. In principle, this modulation technique can also be used for measuring changes in transmitted or reflected light. Here, we describe a measuring system for monitoring $P700^+$ around 830 nm, which was derived from our original modulation fluorometer by small modifications. A first account on the system properties and performance is given. It will be demonstrated that the new system allows the recording of P700 redox changes in intact leaves with great precision and high time resolution. The information from such P700 measurements is complementary to that of chlorophyll fluorescence, which primarily reflects properties of PS II.

Abbreviations: PS, photosystem; P, reaction center chlorophyll; PQ, plastoquinone; Q_A and Q_B, primary and secondary acceptor of photosystem II.

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The Measuring System

The system for measuring absorbance changes around 830 nm was derived by modification of a pulse modulation fluorometer, the properties of which already were described in detail [18, 19]. Here, only the essential features of the pulse modulation measuring principle are recalled: Very short (1 μs) but intense pulses of measuring light are obtained from light emitting diodes (LED) and applied repetitively at 100 kHz. The light signal is detected by a fast PIN-photodiode, which displays a linear response over a very large range of light intensities. The photodiode pulse signal is amplified in two steps: The preamplifier close to the detector is AC-coupled, *i.e.* overlapping changes of slower, non-modulated signals are eliminated. A so-called "selective window amplifier" specifically amplifies the pulse response, ignoring all changes between the pulses. Logical elements assure that flash triggering is always in the midst between two consecutive measuring pulses. Special gating circuitry protects the amplifiers from oversaturation during a flash. These properties provide for an exceptionally large dynamic range, tolerating vast changes of overlapping, non-modulated background signals. Hence, the system is not disturbed even by severalfold natural sunlight.

For an application of the pulse modulation system to measure P700 by absorbance changes around 830 nm, some modifications of the original fluorometer were required. All modifications concern the emitter-detector unit:

1) The 650 nm LED for fluorescence excitation is substituted against a 830 nm LED (Type HE 8811, Hitachi).

2) The short-pass filter in front of the LED is exchanged against a far-red cut-off filter (RG 780, Schott). The purpose of this filter is to eliminate the short wave-length tail emission of the LED, which would interfere with the simultaneous measurement of chlorophyll fluorescence.

3) The RG 9 filter in front of the photodiode is exchanged against a RG 780 filter. With this filter, the photodetector is effectively protected against all visible and photosynthetically active light.

4) The pre-amplifier gain is lowered by about a factor of 50, such that with most samples at high measuring light intensity the final output signal is about 1.5 V which can be readily compensated by the automatic zeroing circuitry.

A schematic diagram of the new pulse modulation system for measuring 830 nm absorbance changes is presented in Fig. 1.

As with the chlorophyll fluorometer, also for the P700 measurements the optical connections between

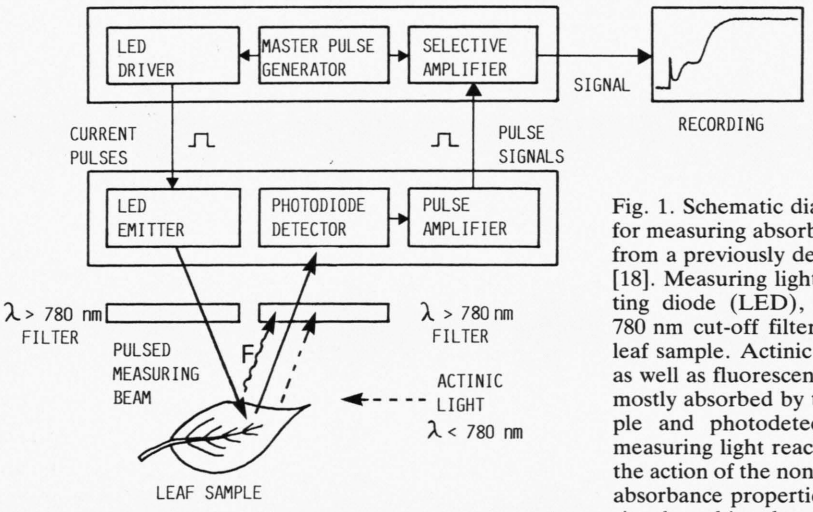

Fig. 1. Schematic diagram of the pulse modulation system for measuring absorbance changes around 830 nm, derived from a previously described pulse modulation fluorometer [18]. Measuring light pulses are generated by a light-emitting diode (LED), peaking around 830 nm. They pass 780 nm cut-off filters before and after interacting with a leaf sample. Actinic light, at wave-lengths below 780 nm, as well as fluorescence (*F*) excited by the actinic light, are mostly absorbed by the 780 nm cut-off filter between sample and photodetector. The modulated signal of the measuring light reaching the photodetector is modified by the action of the non-modulated actinic light on the 830 nm absorbance properties of the sample. Any non-modulated signal reaching the photodetector is rejected by the combination of an AC-coupled pulse amplifier and a selective amplifier. In practice, the various optical components are connected *via* flexible, multibranched fiber optics.

emitter-detector unit and the plant sample are *via* multibranched, flexible fiber optics. Different arrangements of the various fiber branches are used, depending on whether absorbance changes are measured from the surface (remission mode) or in the through-light (transmission mode). Fig. 2 shows schematic drawings of the two basic fiber optics arrangements. In Fig. 3 the light-induced induction kinetics are compared which are observed at 830 nm in the transmission and remission modes of measurement. The recordings were with the same sample, without any change in sample geometry. The two measuring modes give almost identical recordings, as well with respect to the amplitude as with respect to the kinetics of the absorbance changes. Hence, in first approximation the pathlength of the measuring beam within the leaf and the average actinic light intensity along the path of the measuring beam appear to be very similar for the two measuring modes. This somewhat surprising result may be explained by the fact that the 830 nm beam, which is hardly absorbed within the leaf, will undergo multiple scattering and reflections at the leaf-air interfaces, before it eventually escapes from the leaf *via* the upper or lower surface [20, 21].

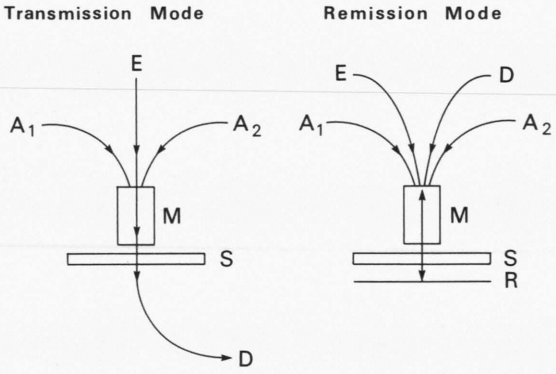

Fig. 2. Fiber optics arrangement and light pathways for measurements of $\Delta A(830)$ in the transmission and remission modes. Abbreviations: E, emitter (LED); D, detector; A_1 and A_2, actinic light sources; M, mixing pathway of fiber optics; S, sample; R, reflecting surface.

A special fiber optics arrangement and additional optical filtering are required when 830 nm absorbance changes are to be measured simultaneously with modulated chlorophyll fluorescence. In this case, it must be avoided that pulsed 830 nm light can penetrate to the fluorescence detector. For this purpose an additional short-pass filter ($\lambda < 760$ nm; Calflex X (special), Balzers) is inserted in front of the fluorescence detector. Fig. 4 shows the fiber optics arrangement for simultaneous absorbance and fluorescence measurements. Fluorescence is measured from the upper leaf surface, from which side also transmitted 830 nm light is collected and where short wavelength actinic light is applied. P700 is measured in the transmission mode, with the measuring beam entering at the lower leaf surface, from which side also far-red actinic light is applied.

Single turnover and multiple turnover flashes of saturating light play an important role for the analysis of P700 absorbance as well as of chlorophyll fluorescence [18, 19, 22]. We have used various kinds of light sources for applying saturating light. Saturating flashes with a half peakwidth of 14 µs were obtained from a discharge tube (XST 103, Walz). While these flashes cause single turnovers at PS II, double turnovers are likely for PS I. Multiple turnover flashes of saturating light were obtained from a prototype of a discharge flash lamp, equipped with special circuitry to produce almost square pulses of 5 ms to 50 ms duration (XMT 103, Walz). With

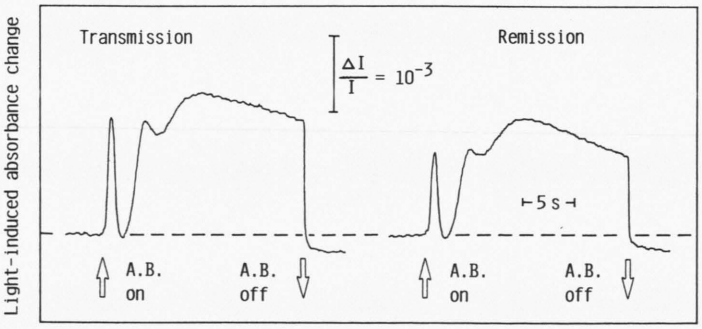

Fig. 3. Comparison of light-induced absorbance changes at 830 nm measured in the transmission and remission modes. A spinach leaf was illuminated repetitively for 30 s with 100 s dark-intervals, until a stable induction pattern was established in the transmission mode; then the fiber optics arrangement was changed for measurement in the remission mode under otherwise identical conditions. Actinic beam (A.B.), 150 W/m^2 blue light (Schott BG 18).

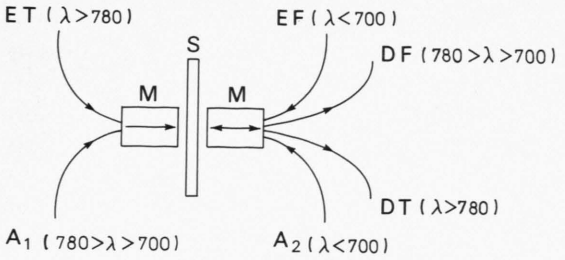

Fig. 4. Fiber optics arrangement and light pathways for simultaneous measurements of $\Delta A(830)$ and modulated chlorophyll fluorescence. Absorbance changes are measured in the transmission mode, with the measuring beam entering from the lower (abaxial) leaf surface. Fluorescence is measured from the upper (adaxial) leaf surface. Abbreviations: ET, emitter (830 nm LED) for transmission measurement, with 5 mm RG 780 (Schott) to remove wave-lengths below 780 nm. EF, emitter (650 nm LED) for fluorescence excitation, with a DT Cyan short-pass filter (Balzers). DT, detector of 830 nm transmission, protected by 5 mm RG 780. DF, detector of fluorescence, protected by 2 mm RG 9 (Schott) and a Calflex X (special) short-pass filter (Balzers). A_1, far-red actinic light, filtered through 3 mm RG 715 (Schott) and Calflex X special (Balzers). A_2, short-wave-length actinic light, filtered through DT Cyan (Balzers) or 2 mm BG 18 (Schott).

these pulses a defined amount of PS II turnovers can be elicited to probe the size of the intersystem carrier pool. In Fig. 5 the intensity profile of the multiple turnover flashes is depicted. For ignition of the discharge lamp an initial μs discharge at elevated voltage is required, which causes a brief overshoot preceding the actual square pulse. A stable intensity is reached with a half-time of 100 μs; the light-off decay proceeds with a half-time of 40 μs. For simultaneous measurements of 830 nm absorbance and modulated fluorescence using the "saturation pulse quenching analysis" [19], longer pulses of saturating light (800 ms) were obtained from a pulsed halogen lamp (FL 103, Walz).

System Performance

In Fig. 6 the performance of the measuring system is summarized for a variety of conditions, with P700 being either oxidized or reduced by continuous light, single turnover (ST) or multiple turnover (MT) flashes of light. The flash responses indicated in the upper part of the figure by the numbers 1, 2, 4, 5 and 6 are kinetically resolved at different time scales in the lower part of the figure. In the dark-adapted state, P700 is reduced. When illuminated, P700 becomes more or less oxidized, depending on the intensity, wave-length and duration of illumination. In traces 1 and 2 the responses to a multiple turnover and a single turnover flash of strong white light are compared. In this application, advantage is taken of the high time resolution (100 kHz) of the system and of the special gating circuitry, protecting the amplifier system from oversaturation during the actual flash. The gating time of about 100 μs, and some residual electronic disturbance by the flash discharge, limit the time at which a first reliable signal can be measured to about 180 μs after the flash. For multiple turnovers a special flash discharge lamp was used (see text accompanying Fig. 5). It is apparent from the traces 1 and 2 of Fig. 6 (lower part) that neither with a saturating single turnover nor with a multiple turnover flash it is possible to observe full oxidation of P700, as can be produced by far-red continuous light (trace 3). This is to be expected from the known rate constants for $P700^+$ re-reduction by plastocyanin [23, 24]. Most of $P700^+$ is re-reduced with half-times of 20 and 140 μs, *i.e.* in a time range which is not resolved by the instrument.

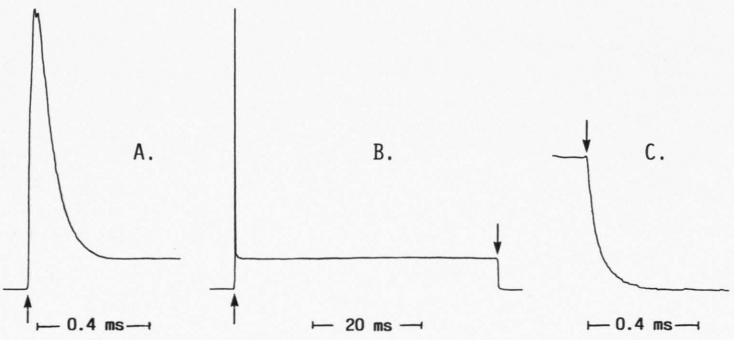

Fig. 5. Intensity profile of a multiple turnover flash. A. and C. Rapid time scales, to display on-off characteristics. B. Slow time scale, to display the overall square profile. Trace C (off-kinetics) are presented at fourfold sensitivity as compared to traces A. and B. The stabilized intensity (after about 2 ms) corresponds to 1500 W/m^2. A 50 ms flash was applied with a prototype of a new discharge flash lamp (XMT 103, Walz).

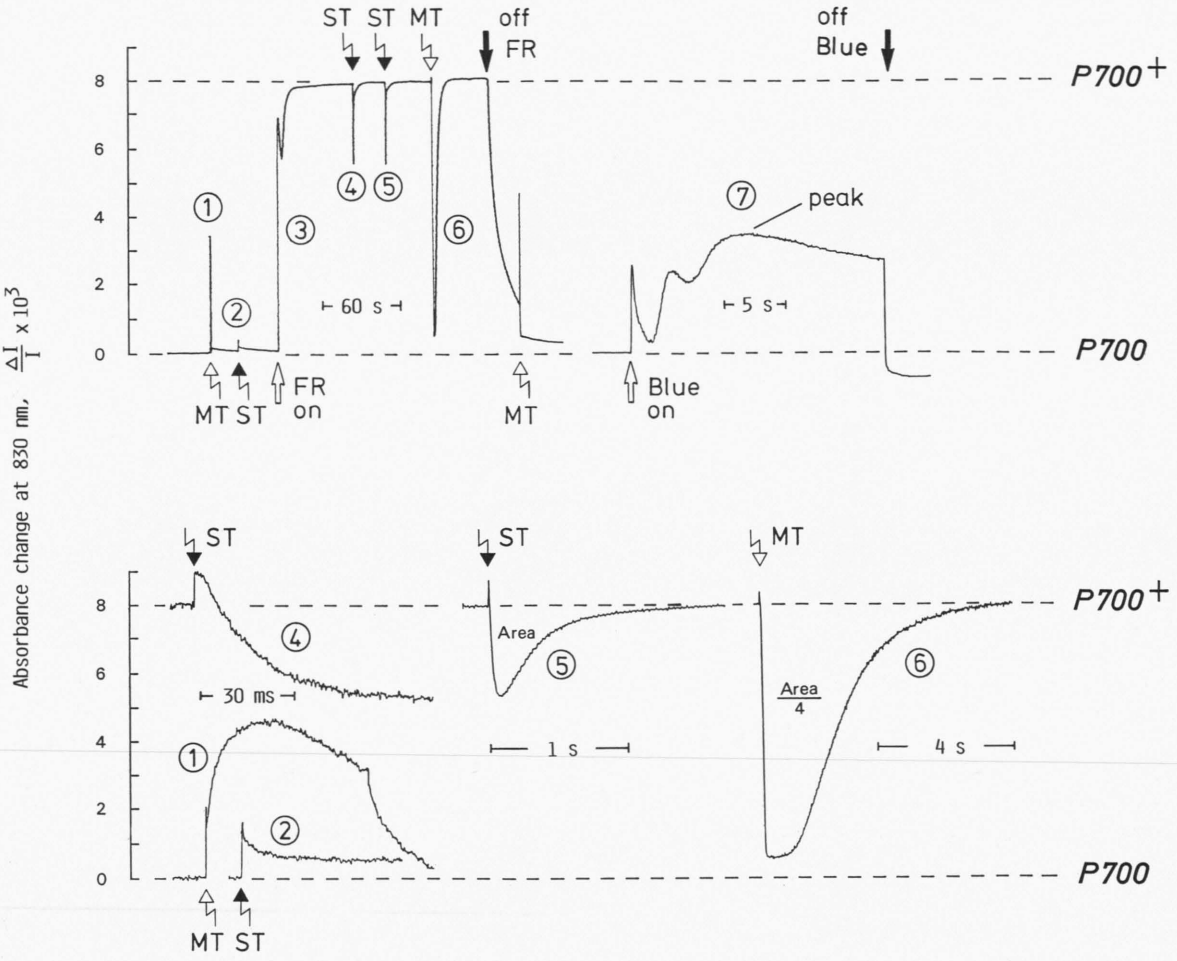

Fig. 6. Comprehensive display of various types of light-induced 830 nm absorbance changes of an intact spinach leaf measured with the new modulation system. The kinetic responses to single turnover flashes (ST), multiple turnover flashes (MT), continuous far-red light (FR) and continuous blue light are depicted. The flash responses (1, 2 and 4–6) are also displayed at an expanded time scale. Single recordings (no signal averaging) are presented. The duration of the multiple turnover flash was 50 ms (for further explanations, see text).

The biphasic oxidation kinetics in the 50 ms pulse (trace 1) can be interpreted as displaying in the rapid phase a population of P700 which is not tightly complexed with plastocyanin, while the slower phase reflects exhaustion of the PS I donor pool, consisting of plastocyanin and the Cyt *b/f* complex. The PS I donor pool becomes, however, re-filled by electrons originating from PS II, which are transferred by plastoquinol. This delayed arrival of electrons from PS II causes the partial re-reduction of P700 showing after approx. 20 ms. Upon termination of the 50 ms pulse, the relaxation kinetics reflect the rate limiting electron transfer from plastoquinol to Cyt *b/f* under conditions of a fully reduced PQ-pool, with a characteristic half-time of about 5 ms [4]. Under conditions of a fully oxidized PQ-pool, the corresponding half-time is considerably longer, as shown by the example of trace 4.

With far-red light almost exclusively PS I is excited. In trace 3 the oxidation of P700 by 13 W/m^2 far-red light is shown. The kinetics display a rapid phase followed by a dip and a slower phase. The final oxidation level after about 1 min with 13 W/m^2 illumination constitutes a saturation level already reached at far-red illumination with considerably lower intensity (about 5 W/m^2) (not shown). Inter-

estingly, it is still possible to reach a somewhat higher extent of oxidation by additional application of strong light flashes (see traces 4, 5, 6). Also flashes of far-red light produced this additional increase (not shown). Provided, a substantial excitation of PS II by the far-red light can be excluded, the fraction of P700 which can not be oxidized by continuous far-red light, could be an indication of cyclic electron flow around PS I.

When P700 is mostly oxidized in continuous far-red light, a single turnover flash produces the kinetic responses shown in traces 4 and 5 at different time scales. As described in detail by Haehnel [9], there is an initial lag period of about 3 ms which represents the time required for electron transfer from PS II to the Cyt *b/f* complex (trace 4). The following decay phase reflects the rate limiting step of plastoquinol oxidation at the Cyt *b/f* complex, with a half-time of about 20 ms, when the PQ-pool is almost completely oxidized [4, 9]. At a slower time scale also the far-red light driven re-oxidation of the P700 reduced by the flash can be observed (trace 5). As demonstrated by Haehnel and Trebst [22], the relative area covered by the reduction transient can be of considerable diagnostic value. This area will decrease with any decrease in PS II activity.

When a multiple turnover flash is applied under conditions of P700 and the intersystem electron transport chain being almost fully oxidized, there is rapid, almost complete re-reduction of P700, which becomes reoxidized by the continuous far-red background light after a certain lag-phase (trace 6). In the given example a 50 ms pulse of 1500 W/m^2 was applied. Considering the four times slower time scale for trace 6 in comparison with trace 5, the total area under the "long flash" induced reduction transient amounts to 17 times the area observed with a single turnover flash. Hence, a minimal intersystem carrier pool capacity of 17 electron equivalents can be derived. The ratio between long-flash and single-turnover flash areas varies considerably with the plant species and is particularly dependent on the light environment during growth. Sun plants display much larger ratios than shade plants (data not shown).

The P700 induction kinetics observed upon onset of blue actinic light (trace 7) are rather complex, displaying an oscillatory pattern. The kinetics depend on the state of preillumination. After longer dark adaptation P700 oxidation following the initial spike is rather slow (not shown). In the given example, a kinetic pattern is depicted which stabilizes with periodic light-dark cycles of 20 s light and 100 s dark. These were the conditions for recording of the difference spectra described below. It is apparent, that upon light-off there is some undershoot below the level of fully reduced P700, suggesting that with blue light some other component causes a slowly reversible absorbance decrease which overlaps the rapidly reversible absorbance increase by P700$^+$.

Difference Spectra

In Fig. 7 the light-dark difference spectrum in the 720 to 950 nm wave-length range of a spinach leaf is

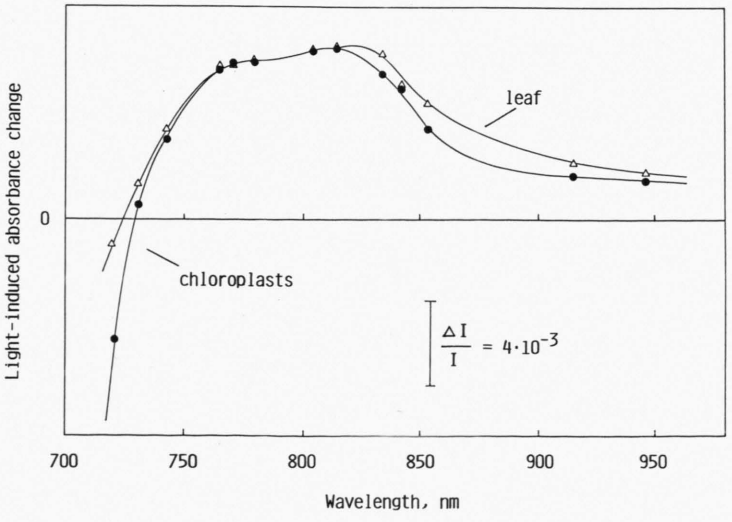

Fig. 7. Comparison of light-dark difference spectra of a spinach leaf and isolated spinach chloroplast. Illumination by 10 W/m^2 far-red light (RG 715, Schott). The envelopes of intact chloroplast were ruptured by hypotonic treatment. Presence of 5 mM ferricyanide in the chloroplast suspension buffer.

compared with that of isolated chloroplasts (class D) in presence of 5 mM ferricyanide. 10 W/m^2 far-red light was applied to induce maximal absorbance changes. Different wave-lengths of the measuring beam were obtained by interference filters (3–5 nm half-bandwidth) and by use of light-emitting diodes with appropriate emission spectra. The far-red induced absorbance change observed with the intact leaf is very similar to that measured with chloroplasts, with isosbestic points between 720 to 730 nm, and a broad maximum around 800 nm, which is composed of a pronounced shoulder at 770 to 780 nm and a peak at 815 to 825 nm.

The difference spectra of Fig. 7 are in agreement with previously published spectra for P700$^+$ [25, 26]. With the use of far-red light and the presence of ferricyanide in the chloroplast experiment, conditions were given favoring predominant changes of P700. On the other hand, with strong blue light both photosystems are excited and the complex induction kinetics (see *e.g.* trace 7 in Fig. 6) reflect the interaction of the two photosystems, controlled by the changing properties of intersystem electron transport and the transthylakoidal gradients. Under such conditions, an overlapping of absorbance changes not caused by P700$^+$ would not be surprising. In Fig. 8 recordings of blue-light induced absorbance changes are displayed for a number of selected wave-lengths and in Fig. 9 the resulting difference spectra are shown. Upon onset of strong blue illumination there is a rapid absorbance increase followed by a decline, consisting of two components which are most easily distinguished at low wave-lengths. The decline leads to a 1. dip, from where absorbance increases again, leading *via* a 2. dip to a peak, which is followed by a slow decay. Upon light-off, absorbance rapidly drops even somewhat below the original dark level. It is

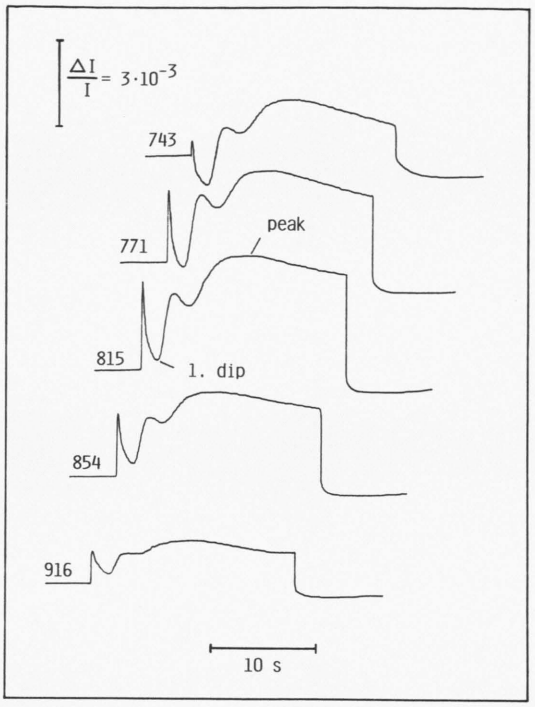

Fig. 8. Kinetics of blue-light induced absorbance changes at different wave-lengths in the far-red region. A spinach leaf was illuminated repetitively for 20 s with 100 s dark-intervals, until a stable induction pattern was established and the displayed transients were recorded. Blue-light (BG 18, Schott) at an intensity of 150 W/m^2.

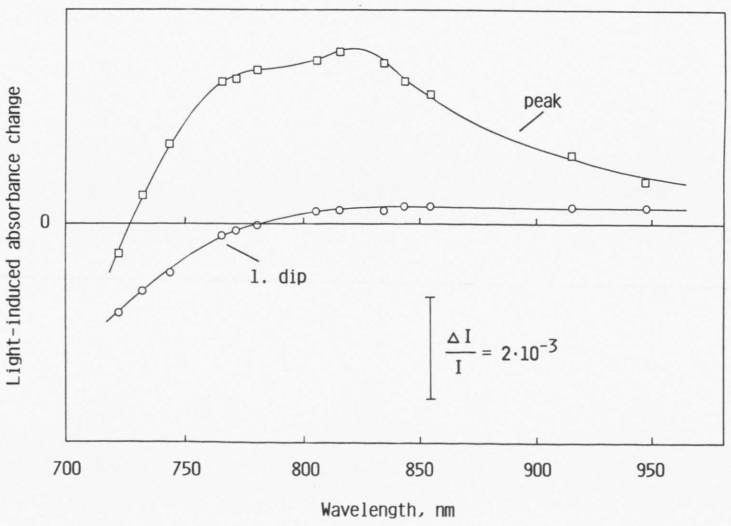

Fig. 9. Difference spectra of blue-light induced absorbance changes in a spinach leaf. Data were obtained from original recordings as displayed in Fig. 8. The changes in absorbance from the dark level to the levels of the first dip and of the peak are plotted.

already apparent from the traces in Fig. 8 that there are at least two types of absorbance changes contributing to the complex induction kinetics. Particularly at low wave-lengths, the rapid absorbance increase, likely to be caused by $P700^+$ formation, is overlapped by an only slightly slower absorbance decrease of so-far unknown origin. This absorbance decrease causes a signal drop below the dark level. The close similarity of the transients following the 1. dip at all wave-lengths suggests that the process inducing the rapid absorbance decrease does not display significant variations at longer illumination times. The maximal positive absorbance change in blue light (peak level) shows a difference spectrum (see Fig. 9) almost identical to that found with far-red illumination (see Fig. 7). Hence, even with strong blue illumination, the major absorbance changes above 800 nm appear to be caused by $P700^+$. Fig. 9 also shows the difference spectrum for the transient change from the dark level to the level of the 1. dip. The amplitude of this negative transient increases gradually with wave-lengths decreasing below 800 nm. Above 800 nm it is almost constant up to 950 nm. Preliminary experiments with isolated chloroplasts revealed that this transient can be eliminated by valinomycin (data not shown). Hence, formation of a membrane potential may be involved.

Binary Oscillations in $P700^+$ Re-Reduction

In the past, in order to resolve the time course of flash-induced P700 changes by measurements around 700 nm, it was necessary to apply repetitive techniques and signal averaging for improvement of the signal/noise ratio. As a consequence, the observed kinetic changes reflected the properties of the sample in a more or less pre-illuminated state. There are, however, aspects of photosynthetic electron transport which are revealed only when a dark-adapted sample is illuminated and when the response to single periods of illumination can be registered. For example, dark reoxidation of the secondary PS II acceptor Q_B is slow and the two-electron gate function of Q_B can be demonstrated only when, starting from the dark state, a sample is illuminated by saturating single turnover flashes [27, 28]:

$$Q_A\ Q_B \xrightarrow{1.\ \text{flash}} Q_A\ Q_B^- \xrightarrow{2.\ \text{flash}} Q_A\ Q_B^{2-} \xrightarrow[+\ 2H^+]{+\ PQ}$$

$$Q_A\ Q_B + PQH_2.$$

Electrons are released in pairs into the PQ-pool at even flash numbers, while at uneven flash numbers a single electron is stored in form of the semiquinone anion. The resulting binary oscillation damps out after about 10–15 flashes. Hence, the operation of this two-electron gating mechanism is obscured when repetitive flash illumination is applied.

For measuring P700 absorbance changes around 830 nm a very strong measuring beam can be applied without any actinic effect on the sample. This feature, together with the exceptional sensitivity and selectivity of the new modulation system, provides for a sufficiently high signal/noise ratio to analyze P700 flash responses with single recordings, *i.e.* without averaging. In Fig. 10 flash-relaxation kinetics are presented, corresponding to trace 2 of Fig. 6, for a sequence of saturating flashes. A spinach leaf was preilluminated at 20 °C for 1 min with 2 W/m² far-red light and then rapidly cooled to 5 °C, before flashes were applied every 3 s. In the intact leaf such pretreatment was required for establishing a sufficiently oxidized initial state of Q_B and of the PQ-pool. It is apparent that with the 1. flash there is a component of $P700^+$ which is not re-reduced within 400 ms following the flash. The same is true, although to a lesser extent, for the 3. and 5. flash, while with the 2. and other even numbered flashes

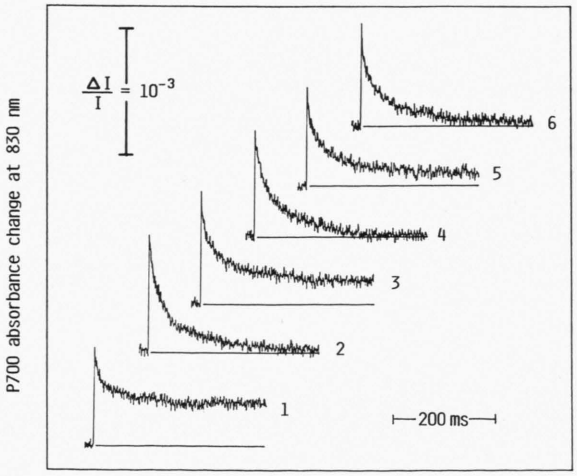

Fig. 10. Relaxation kinetics following illumination by single turnover saturating flashes applied every 3 s. A spinach leaf was preilluminated at 20 °C for 1 min with 2 W/m² far-red light (RG 715) and then rapidly cooled to 5 °C before the flash series was started. Note, the different extent of P700 reduction following even and uneven numbered flashes.

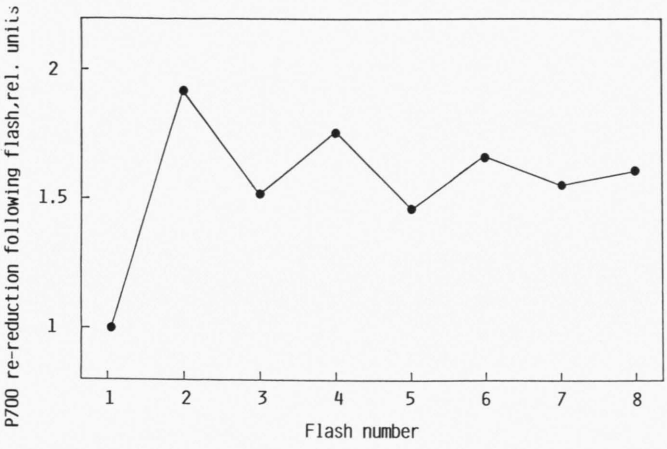

Fig. 11. Absorbance change caused by P700 re-reduction within 400 ms following a series of saturating single turnover flashes. Data obtained from the original traces displayed in Fig. 10. See legend of Fig. 10 for further details.

there is practically full re-reduction within 400 ms. In Fig. 11 the extent of the absorbance decrease observed between 2 ms and 400 ms following a flash is plotted *versus* flash number, displaying binary oscillations damping out with increasing flash number. It should be pointed out that the flashes induce single turnovers at PS II, while at PS I the flash duration (half peak width 14 μs) should allow a substantial amount of double turnovers at those centers where P700 is tightly coupled with plastocyanin (20 μs relaxation component) [24]. Actually, these double turnovers at PS I may help to observe the Q_B-gating mechanism *via* P700 absorbance changes (W. Haehnel, personal communication). A limitation of $P700^+$ re-reduction by the state of Q_B can be expected only if the immediate PS I donor pool contains less electrons than are transported by P700 during the flash.

With isolated chloroplasts, oxidation of the PS I donor pool can be assured by addition of ferricyanide. As shown in Fig. 12 and 13 under these conditions the Q_B gating mechanism is most clearly expressed in the re-reduction kinetics of P700.

In the past, the properties of electron flow *via* Q_B have been studied primarily by chlorophyll fluorescence measurements [28–30]. Actually, from fluorescence measurements the conclusion was drawn that a substantial part of PS II is not feeding electrons into the PQ-pool *via* Q_B [31, 32]. The new measuring system may allow to solve some of the open questions related to this controversial aspect of PS II heterogeneity by correlated measurements of P700 absorbance changes and chlorophyll fluorescence.

Simultaneous Recordings of $P700^+$ and Fluorescence

As outlined in the section on "The Measuring System", by appropriate arrangement of the fiber optics

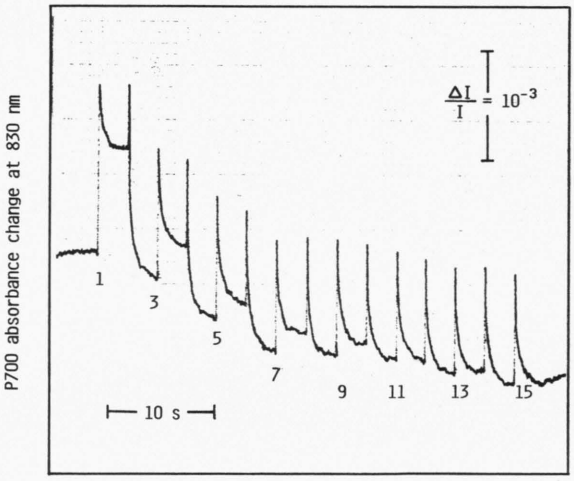

Fig. 12. Absorbance changes at 830 nm in isolated spinach chloroplasts induced by a series of single turnover saturating flashes. Presence of 5 mM ferricyanide. Temperature, 15 °C. Envelope-free chloroplasts were obtained by hypotonic treatment of intact chloroplasts. Chlorophyll concentration, 100 μg/ml. The measurement was carried out with a modified suspension cuvette (KS 101, Walz), with the fiber optics end-piece directly covering the sample, thus preventing the reflection from the usual plexiglas cone window.

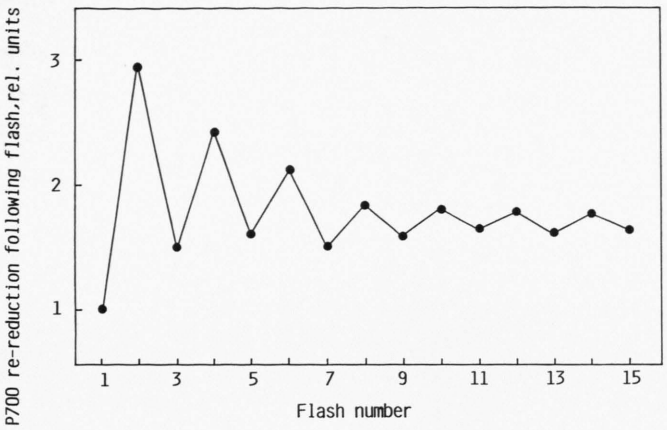

Fig. 13. Absorbance change caused by P700 re-reduction following a series of saturating single turnover flashes. Data obtained from the original traces of 830 nm absorbance changes displayed in Fig. 12. See legend of Fig. 12 for further details.

and choice of optical filters it is possible to measure absorbance changes around 830 nm and modulated chlorophyll fluorescence simultaneously.

This is demonstrated in Fig. 14 and 15, where simultaneous recordings of fluorescence and $\Delta A(830)$ of an intact spinach leaf are shown. In Fig. 14 A, B the induction kinetics upon illumination with continuous white light are compared for a dark-adapted leaf (A) and a preilluminate leaf (B). Both chlorophyll fluorescence and $\Delta A(830)$ reflect the interplay of the two light reactions, viewed from PS II and from PS I, respectively. Upon onset of light, there is a sudden rise of fluorescence, when the PS II acceptor Q_A becomes reduced, and at the same time, P700 becomes first oxidized and then rapidly reduced again, as electrons from PS II have passed the intersystem electron transport chain (t 1/2 about 20 ms). The reoxidation of P700, and consequently of the intersystem carrier pool including the PS II acceptor Q_A, is limited by the PS I acceptor side, which requires light activation [33–35]. This feature is reflected by the almost parallel oxidation of P700 and decline of fluorescence during the first 70 s in the dark-adapted sample and during the first 25 s in the preilluminated sample. It may be noted that the M_1 peak in fluorescence, which is most pronounced after

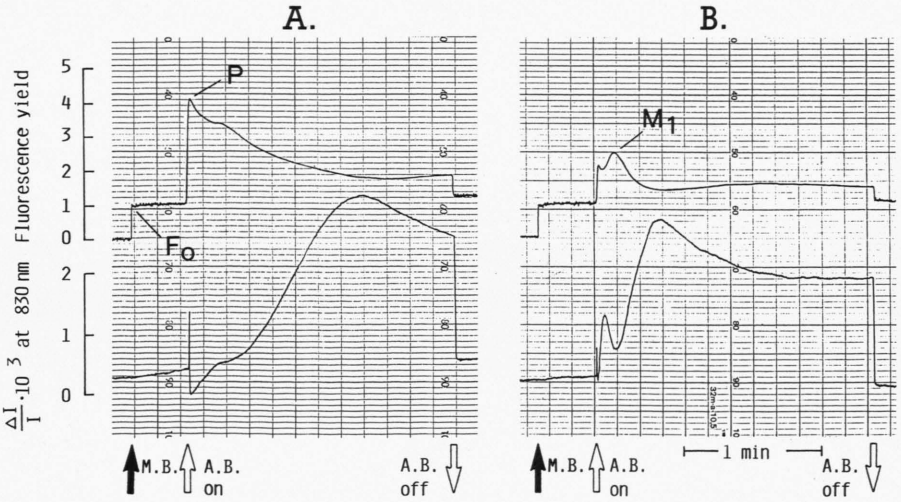

Fig. 14. Simultaneous recordings of modulated chlorophyll fluorescence and absorbance changes at 830 nm. Object: Intact spinach leaf. A. Leaf dark-adapted for 2 h. B. Leaf preilluminated for 2 min and than 10 min dark-adapted before recording. Abbreviations: M.B., measuring beam for excitation of chlorophyll fluorescence. A.B., actinic beam, 150 W/m^2 blue light (BG 18). F_0, dark-fluorescence level. P, peak fluorescence observed upon actinic illumination. M_1, the first of a series of secondary maxima observed in dark-light induction curve, particularly pronounced in preilluminated leaves.

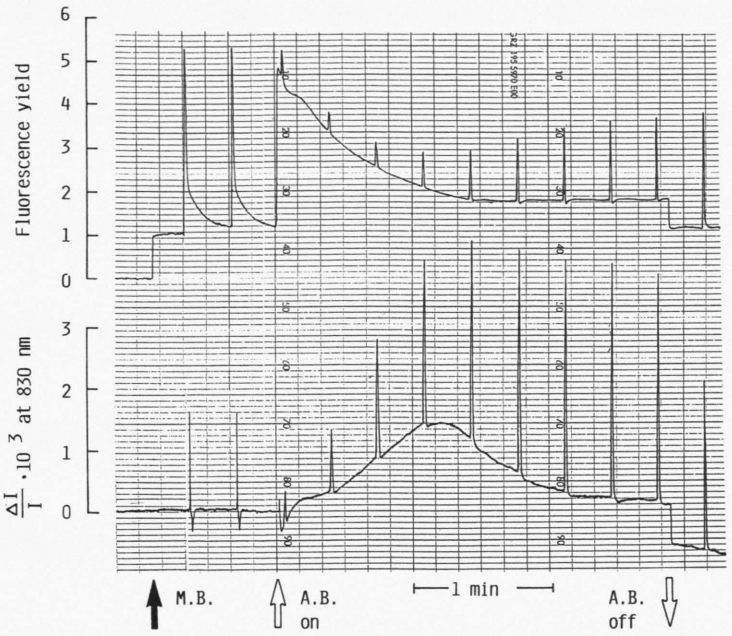

Fig. 15. Simultaneous recordings of modulated chlorophyll fluorescence and 830 nm absorbance changes with repetitive application of saturating light pulses to distinguish photochemical and non-photochemical quenching components. Object: Dark-adapted intact spinach leaf. Onset of the fluorescence measuring beam (M.B.) and on/off of the actinic beam (A.B.) are indicated by arrows. The spikes are the responses to 800 ms pulses of saturating white light (1000 W/m^2; DT Cyan and Calflex X (special), Balzers). Actinic light, 150 W/m^2 (BG 18, Schott).

preillumination, corresponds to a distinct phase of transient P700 reduction. Previous work has shown that the early fluorescence transients leading to the M_1 peak are almost exclusively governed by the redox state of Q_A [19, 36]. At longer illumination times also non-photochemical mechanisms, in particular so-called energy-dependent quenching, affect fluorescence yield. Actually, the internal acidification of the thylakoids, which causes energy-dependent quenching, at the same time will favor P700 oxidation, as the rate limiting electron transfer step between PQH_2 and Cyt *b/f* becomes slowed down [37].

Photochemical and non-photochemical fluorescence quenching can be distinguished by application of saturating light pulses which transiently remove all photochemical quenching, as Q_A becomes completely reduced [19, 36, 38]. With the new modulated measuring system, it is possible to apply saturation pulses also with simultaneous recordings of fluorescence and P700. Fig. 15 shows such recordings with a dark-adapted spinach leaf, where saturation pulses were applied every 20 s. During the first 70 s of continuous illumination P700 oxidation goes in parallel with increases of both photochemical and non-photochemical quenching. Thereafter, P700 is re-reduced again, while relaxation of non-photochemical quenching indicates de-energization of the thylakoid membrane and increasing photochemical quenching reflects stimulated electron transport rates. Obviously, once the Calvin cycle enzymes and the thylakoidal ATP-ase are activated, the overall rate is not limited by intersystem electron flow. This was already concluded by Heber and co-workers on the basis of rate measurements in intact leaves [39, 40]. With prolonged illumination, the photosynthetic apparatus achieves a state of P700 being mostly reduced and the PS II acceptor side being mostly oxidized, *i.e.* a state in which both photosystems can operate at high quantum yields. Any accumulation of oxidized P700 would cause a drop of quantum yield, as $P700^+$ dissipates excitation energy into heat.

The P700 response to pulses of saturating light is more complex than that of chlorophyll fluorescence. With fluorescence, it may be assumed that all PS II centers become closed during a saturation pulse, as Q_A-reoxidation is slower than quanta absorption [18, 41]. On the other hand, it is not possible to oxidize all P700 by a pulse, as P700 becomes rapidly re-reduced by components of the intersystem carrier pool and by PS II (see Fig. 6). The amplitudes of the transient spikes of P700 oxidation, observed with application of saturation pulses (Fig. 15), give some qualitative information on the relative rates of electron flow from and to P700. They are favored by a

well activated PS I acceptor side, by a low intersystem electron transport rate (photosynthetic control) and by weak PS II activity.

Conclusions

As has been previously recognized by Weis *et al.* [17], absorbance changes around 830 nm give valuable information on the redox state of P700, the reaction center chlorophyll of PS I, in intact leaves, complementing the information from chlorophyll fluorescence which primarily relates to properties of PS II. In the past, measurements of P700 redox changes have been almost exclusively reserved to biophysically oriented research groups, with sophisticated spectroscopic equipment, well suited for the study of chloroplasts and sub-chloroplasts preparations. With the development of the new pulse modulation system, described in this report, the instrumental requisites for a practical use of P700 measurements in plant physiological work are given. It was shown that by small modifications of a commercially available instrument it is possible to obtain a measuring system with which absorbance changes around 830 nm can be monitored, the properties of which are practically identical to those around 700 nm, as *e.g.* characterized by the extensive work of Haehnel [8–10, 22–24]. Important advantages of the new system are:

1) As the 830 nm measuring light is almost not absorbed, a very high intensity can be used without any actinic effect. Hence, a high signal/noise ratio can be obtained without signal averaging.

2) There is no disturbance by overlapping changes of chlorophyll fluorescence, as the pulse modulated measuring beam does not excite fluorescence, and as the fluorescence signal originating from non-modulated actinic illumination is effectively eliminated by the selective amplifier system.

3) Good signals are obtained from intact leaves and there is no significant difference in light-induced responses when measurements are carried out in the transmission or remission mode.

4) As with the chlorophyll fluorometer, also the new system for P700 measurement is largely insensitive to non-modulated overlapping signal changes which may be caused by ambient day-light or by strong actinic light, even when applied in form of saturating flashes or pulses.

5) There is sufficient spectral separation between the excitation wave-length of chlorophyll fluorescence (650 nm) and the 830 nm measuring beam, such that modulated fluorescence and the modulated 830 nm signal can be monitored simultaneously. It is even possible to apply saturation pulses for fluorescence quenching analysis, without affecting the 830 nm absorbance measurement.

6) As the new instrument can be operated by batteries and as the measurements are not disturbed by ambient day-light, it is also suited for field measurements.

We are aware of the fact that not only P700 causes absorbance changes around 830 nm. An absorbance increase in this wave-length region may as well be due to formation of $P680^+$ [14, 15], of I^-, the pheophytin anion radical [42], and of the chlorophyll triplet excited state [43]. It may be assumed that under physiological conditions it is unlikely that $P680^+$, I^- and $^3Chl\ a^*$ accumulate. Under extreme conditions, however, possible contributions of these states to the overall 830 nm absorbance change should be taken into consideration.

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Note added in proof: After submission of this paper our attention was drawn on a very interesting recent report by Harbinson & Woodward (Plant, Cell and Environment **10,** 131–140 (1987)) which also describes the use of absorbance changes around 820 nm to monitor the redox state of P700. These authors, like Weis *et al.* (Ref. [17]) and ourselves, have developed a solid-state measuring system based on infra-red light emitting diodes. There is, however, a basic difference of our pulse modulation system, combined with a selective window amplifier, and the conventional lock-in amplifier system used by Harbinson & Woodward: With the conventional system, rapid transients in non-modulated background signals induce transient switching artifacts which interfere with the measurement of rapid absorbance changes. Hence, the report of Harbinson & Woodward deals with relatively slow transients, while in the present paper flash-induced rapid absorbance

changes play a major role. Our results confirm and extend the previous findings and conclusions of Harbinson & Woodward, in particular that absorbance changes around 820 nm mainly reflect the redox state of P700 in intact leaves as well as in isolated chloroplasts.

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