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Devices and Methods for Room-Temperature Fluorescence Analysis

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Phil. Trans. R. Soc. Lond. B 1989 **323**, 241-251
doi: 10.1098/rstb.1989.0007

Phil. Trans. R. Soc. Lond. B **323**, 241–251 (1989)
Printed in Great Britain

Devices and methods for room-temperature fluorescence analysis

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An overview of the various types of photochemical and non-photochemical fluorescence quenching *in vivo* is given. Devices and methods are outlined that allow specific information to be obtained from complex fluorescence responses. The importance of correlated measurements of other, independent photosynthesis signals is emphasized. It is shown that a recently introduced pulse-amplitude modulation fluorometer (PAM fluorometer) can also be used with modified emitter–detector units to measure absorbance changes. Examples are given for absorbance changes of the Hill reagent methyl purple, induced by single turnover flashes, and for P_{700} absorbance changes measured simultaneously with fluorescence. Correlated P_{700} and fluorescence measurements give deeper insights into the control of electron transfer from PQH_2 to cytochrome (cyt) *b*/*f* and into the intersystem acceptor-pool size of sun and shade leaves. Possible explanations for differences in pool sizes determined by P_{700} and fluorescence measurements are discussed. By using P_{700} reduction as an indicator, it is shown that in saturating light the plastoquinone (PQ) pool is already reduced within 50 ms, whereas the last phase of the fluorescence rise (I_2–P) takes about 300 ms and is paralleled by the re-reduction of P_{700}. It is concluded that I_2–P reflects removal of photochemical quenching at PSI and that 50 ms saturation pulses are appropriate to eliminate the relevant photochemical PSII quenching.

Introduction

Chlorophyll fluorescence is a complex indicator of the photosynthetic apparatus. It is controlled by a considerable number of different quenching processes, which may be photochemical or non-photochemical in nature. As shown in figure 1, various photochemical and non-photochemical quenching mechanisms may be distinguished. Although most photochemical quenching is due to PSII, some quenching (about 10%) by PSI photochemistry must be taken into consideration. PSII can operate in a linear or in a cyclic way. Only linear flow leads to water splitting and CO_2 reduction. Besides CO_2, other electron acceptors, in particular O_2, may be involved. Non-photochemical quenching can be dissipative or non-dissipative. If, as in the case of a state I → II shift, or with spillover, there is increased energy transfer from the highly fluorescent PSII to low fluorescent PSI, the corresponding quenching is non-dissipative. Dissipative non-photochemical quenching may occur in the antenna or at the reaction centres. In the antenna, zeaxanthin has been proposed to act as a quencher (Demmig *et al.* 1987). At the reaction centres, the ion radicals P^+_{680}, P^+_{700} and $Pheo^-$ as well as carotenoids are known to quench excitation energy (Butler 1972). Static, dissipative quenching by oxidized plastoquinone has also been suggested (Vernotte *et al.* 1979).

From the existence of such a wide variety of different quenching mechanisms, two conclusions are apparent. On the one hand, fluorescence is a very complex indicator and without proper precautions mistakes in the interpretation of data are easily made. On the other

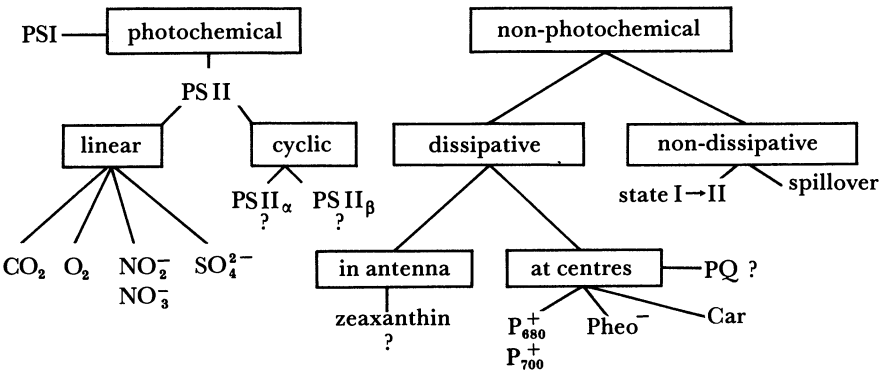

FIGURE 1. Various forms of photochemical and non-photochemical quenching that may contribute to overall quenching of chlorophyll fluorescence *in vivo*.

hand, however, fluorescence is a very powerful tool, as it carries information about almost all aspect of photosynthesis. To obtain specific information, appropriate measuring devices and methods are essential.

In recent years, some important progress has been made in fluorescence analysis (Quick & Horton 1984; Schreiber *et al.* 1986; Weis & Berry 1987), initiated by the original work of Bradbury & Baker (1981) on light-doubling. Such progress has not only led to a better understanding of the mechanisms that govern fluorescence, but also to deeper insights into the functioning of photosynthesis, in particular the delicate regulatory mechanisms by which the overall system is optimized and protected against damage by environmental stress. In this paper we shall give an outline of the kind of integrative experimental approach that leads to specific information from fluorescence measurements. Emphasis will be on recent progress, in particular on the development of an emitter–detector probe for measuring P_{700} absorbance changes in parallel with chlorophyll fluorescence.

EXPERIMENTAL APPROACH

To obtain specific information from chlorophyll fluorescence, special measuring techniques and accessory devices are required. It is important that the system is sufficiently flexible to enable other photosynthesis signals to be measured in parallel. When the interpretation of a certain fluorescence parameter has been established and its amplitude calibrated by comparative measurements of appropriate, independent signals, fluorescence also provides quantitative, reliable information when measured alone. Figure 2 shows a scheme depicting the kind of approach taken in our laboratory at Würzburg. We have developed a special pulsed-amplitude modulation fluorometer (Schreiber 1986; Schreiber *et al.* 1986) based on pulse light-emitting diodes (PAM fluorometer) which has proved quite useful for the analysis of fluorescence compared with other procedures, such as infrared gas analysis, O_2 electrodes and photoacoustics. The main virtues of this fluorometer are its large dynamic range and its rapid response. Fluorescence yield is detected by a weak measuring beam, which by itself does not induce a fluorescence increase, and at the same time the system tolerates the highest actinic light intensities applied either continuously or in pulses. Practical application of the fluorometer is facilitated by a number of purpose-adapted accessory devices, in particular single-turnover and multiple-turnover flash lamps, a saturation pulse lamp and suitable data-acquisition systems. With the help of these devices specific information is obtained on certain

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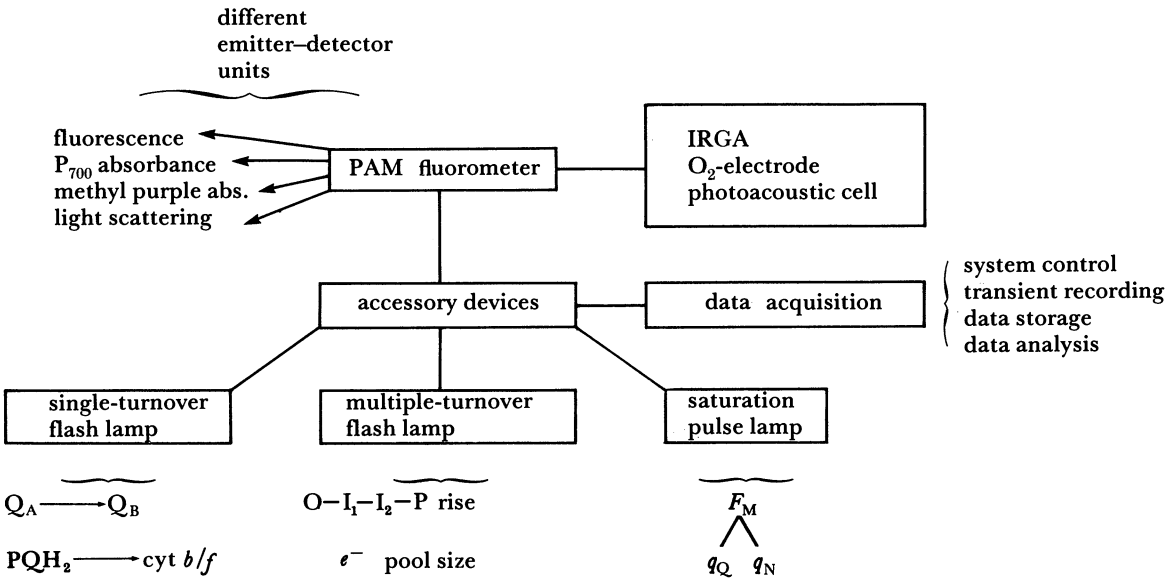

FIGURE 2. Integrative approach of analysing photosynthetic parameters with the PAM fluorometer in combination with a number of accessory devices and independent measuring devices. See text for explanation.

electron-transfer steps, on electron-carrier pool size, PSII heterogeneity and on various types of fluorescence quenching (q_Q and q_N).

The potential of the PAM fluorometer has been further increased by the recent introduction of additional emitter–detector units that permit absorbance measurements under conditions identical to those of the fluorescence measurements. For this purpose, the light-emitting diode (LED) and the filters in front of emitter and detector are modified (see table 1). For example, to measure the absorbance change of the Hill reagent methyl purple (Hill *et al.* 1976), a yellow LED is used (peak emission at 580 nm) and in front of the detector a short-pass filter is placed that transmits the measuring beam, but not actinic light with wavelengths longer than 640 nm. As shown in figure 3 the bleaching of methyl purple caused by single electron transfers from PSII via PSI can be readily detected in this way. In the control, each single turnover flash produces a constant step of electron transfer. With the addition of the ADRY reagent 2-(3-chloro-4-trifluoromethyl)anilino–3,5-dinitothiophene (ANT-2p) the deactivation of the S-states in the H_2O-splitting enzyme system is accelerated (Renger 1973), leading to a loss of PSII quantum yield for linear electron transport.

TABLE 1. COMPONENTS OF EMITTER–DETECTOR UNIT FOR RECORDING FLUORESCENCE AND VARIOUS TYPES OF ABSORBANCE CHANGES

type of recording	type of LED	emitter filter	detector filter
fluorescence	Stanley H-3000 (650 nm)	Balzers DT Cyan $\lambda < 700$ nm	Schott RG 9 (1 mm) (Balzers Calflex C)[a] (Balzers Calflex X)[a]
P_{700}	Hitachi HLP50 RGB 800–850 nm	Schott RG 870 (3 mm)	Schott RG 780 (3 mm)
methyl purple	Stanley HAY 5566 (580 nm)	not required	Balzers DT Cyan $\lambda < 640$ nm
scattering	Stanley HBG 5566 (555 nm)	not required	Balzers DT Cyan $\lambda < 640$ nm

[a] These additional filters are required when modulated fluorescence and P_{700} are measured simultaneously.

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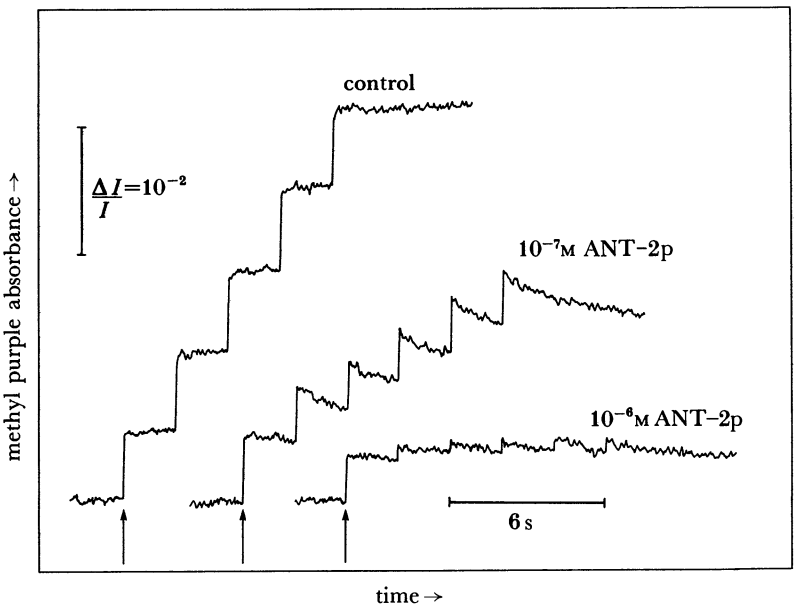

FIGURE 3. Reduction of methyl purple by isolated spinach chloroplasts (bleaching of about 580 nm monitored with a PAM fluorometer modified with respect to the emitter–detector unit). The effect of the ADRY reagent ANT-2p is demonstrated. See table 1 for modifications in the emitter–detector unit. Intact spinach chloroplasts were osmotically shocked and resuspended isotonically to a final chlorophyll concentration of 10 μg ml^{-1}.

COMPARATIVE MEASUREMENTS OF CHLOROPHYLL FLUORESCENCE AND P_{700} ABSORBANCE

The possibility of correlated or even simultaneous measurements of P_{700} and chlorophyll fluorescence opens new ways for the analysis of fluorescence and of photosynthesis in general. The information from P_{700} is complementary to that of fluorescence, as the latter responds primarily to properties of PSII, whereas P_{700} is at the core of PSI. As was first demonstrated by Harbinson & Woodward (1987) and also by Weis *et al.* (1987), P_{700} measurements can be readily carried out with intact leaves via the absorbance changes of the P_{700}^{+} cation radical around 820 nm. Recently we have reported on the properties and performance of a P_{700} emitter–detector unit operated in conjunction with the PAM fluorometer (Schreiber *et al.* 1988). We have shown that almost identical responses are obtained when P_{700} is measured by back-scattering from the leaf surface or by transmission through the leaf. The kinetic changes induced by single-turnover or multiple-turnover flashes are very similar to those reported by Haehnel on the basis of measurements at 703 nm (for a review, see Haehnel (1984)).

The reduction kinetics of P_{700} are controlled by the rate of electron transfer from PSII via reduced plastoquinone (PQH_2), the cytochrome (cyt) *b*/*f* complex and plastocyanin. It is generally assumed that the rate-limiting step between the two photosystems, and possibly of overall photosynthesis, is located between PQH_2 and cyt *b*/*f* and is controlled by the pH of the thylakoid internal space, into which the protons are supposed to be released (Witt 1979). Another important parameter determining this rate is the reduction state of the PQ pool. As shown in figure 4, when the chain is oxidized the half-time of P_{700}^{+} reduction is about 15 ms,

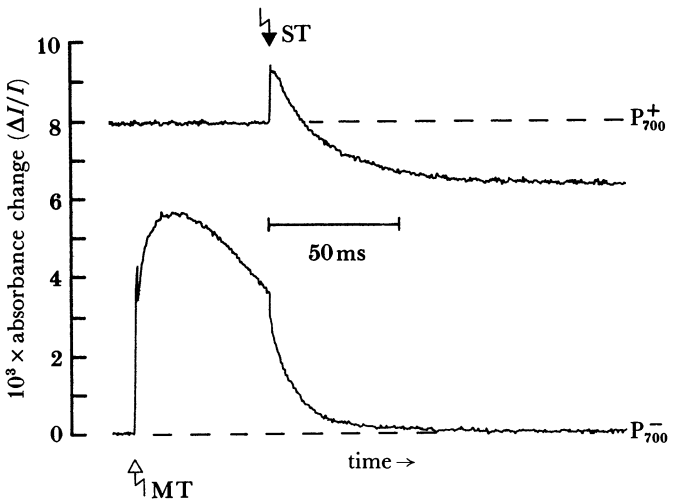

FIGURE 4. Kinetics of flash-induced P_{700} absorbance changes in a spinach leaf measured around 830 nm. (*a*) Single-turnover flash (XST 103, Walz) applied under conditions of P_{700} and the PQ pool being oxidized; presence of 3 W m^{-2} far-red background light (RG 715). (*b*) Multiple-turnover flash (XMT 103, Walz) of 50 ms duration in the course of which P_{700} is partly oxidized while the PQ pool is fully reduced (see figure 10). P_{700} re-reduction kinetics reflect the rate-limiting step of electron transfer from PQH_2 to cyt *b*/*f*.

whereas it is speeded up to about 5 ms when the PQ pool has become fully reduced by application of a multiple-turnover flash of 50 ms duration (see also figure 10 below).

By using repetitive multiple-turnover flashes we have determined the rate of P_{700} reduction simultaneously with chlorophyll fluorescence quenching during the recording of typical induction curves. Figure 5 shows the original traces of 830 nm absorbance (top) and fluorescence (bottom). In figure 6 the half-time of P_{700} reduction is compared with the change in non-photochemical fluorescence quenching. As expected, the internal acidification of the thylakoids, reflected by the initial increase in non-photochemical quenching, is paralleled by a significant increase in the half-time of P_{700} reduction (from 4 ms to almost 30 ms). However, whereas non-photochemical quenching relaxes by only about 20%, there is complete restoration of the 4 ms P_{700} reduction half-time. It should be noted that, with the 50 ms saturating light pulses, full reduction of the PQ pool can always be achieved, implying that the changing parameter in this experiment is the internal pH and not the PQ redox state.

This example shows how additional information from P_{700} measurements may lead to a more profound interpretation of the fluorescence results or allow deeper insight into the mechanisms of photosynthesis: either by some unknown change after about 2 min of illumination the H^+-release from PQH_2 has become insensitive to the internal pH, or the internal pH has indeed returned to its dark value, in which case one should conclude that in the given example only about 20% of non-photochemical quenching is 'energy dependent'. This finding may also have important consequences with respect to the controversial issue of localized against delocalized coupling of protolytic reactions with the thylakoidal ATPase (see, for example, Williams 1982).

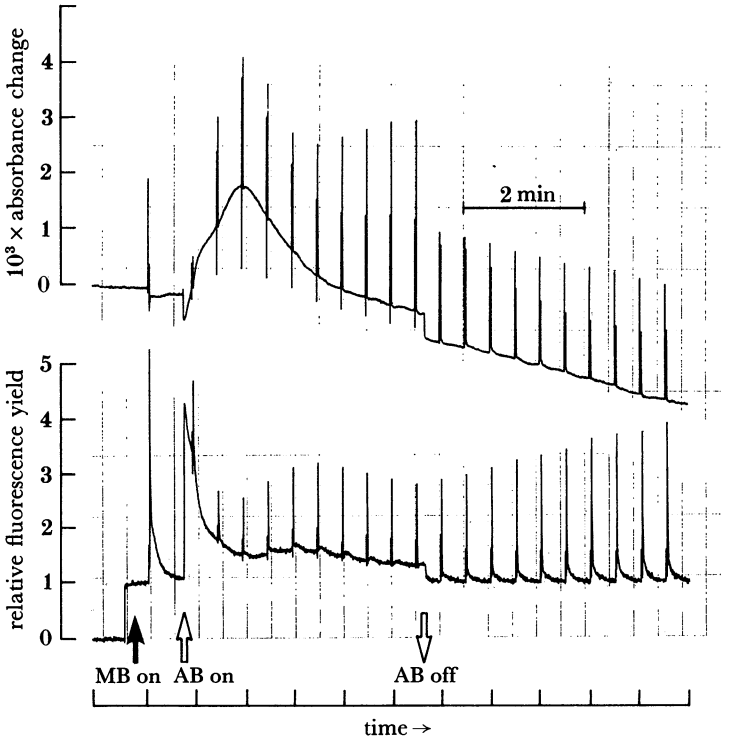

FIGURE 5. Simultaneous recordings of light-induced changes in P_{700} absorbance and chlorophyll fluorescence in a spinach leaf. Every 30 s a saturating light pulse (50 ms duration, 2500 W m^{-2} white light, XST 103, Walz) was applied, leading to transient P_{700} oxidation and elimination of photochemical quenching. The actinic beam (50 W m^{-2}; Schott BG 18) was interrupted for 200 ms, starting 10 ms before each saturation pulse, with the aid of a photographic shutter (Compur electronic-m). MB, measuring beam; AB, actinic beam. For each saturation pulse, the P_{700} relaxation kinetics were recorded separately with a digital storage oscilloscope (Nicolet Explorer III) and the half-time of re-reduction determined (see figure 6).

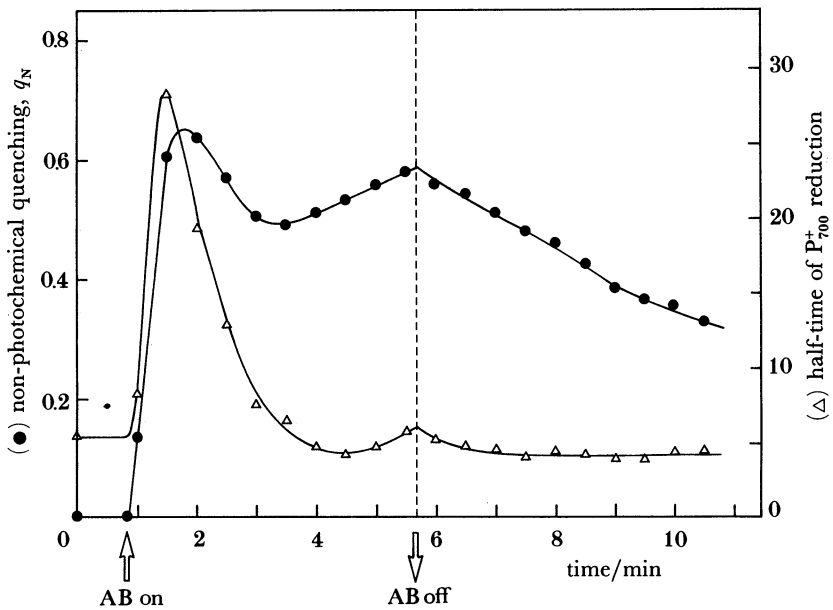

FIGURE 6. Comparison of non-photochemical quenching, q_N, and the half-time of P^+_{700} re-reduction during induction kinetics in a spinach leaf. The data were obtained on the basis of the experiment described in figure 5 (see legend of figure 5).

Comparative measurements of acceptor pool sizes by fluorescence and P_{700}

Provided that a fluorescence induction curve leads to maximal fluorescence yield, the complementary area between the F_M line and the fluorescence curve is supposed to be a measure of the acceptor pool size (Murata *et al.* 1966). It is well known that sun leaves display larger electron-carrier pools than shade leaves (Björkman 1973; Schreiber *et al.* 1977). In figure 7 the fluorescence-rise kinetics of *Asarum* (shade plant) and *Helianthus* (sun plant) are compared. According to the complementary areas above the induction curves, one should conclude that the electron carrier pool of *Helianthus* exceeds that of *Asarum* by a factor of about 1.6.

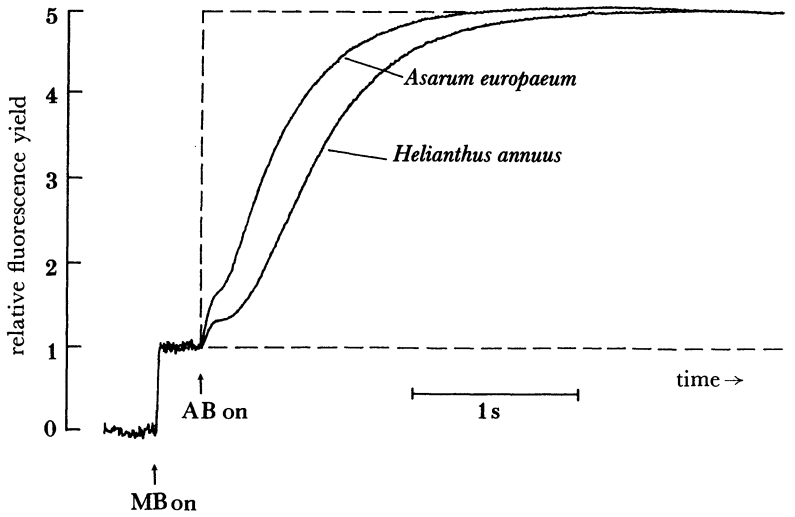

Figure 7. Comparison of light-induced fluorescence rises in *Asarum europaeum* and *Helianthus annuus*. The areas above the fluorescence curves, bounded by the broken lines, are a measure of the acceptor pools reduced by actinic illumination. MB, measuring beam; AB, actinic beam.

For comparison, figure 8 shows the pool size determined by P_{700} absorbance measurements. Far-red background light is applied to oxidize P_{700} and to empty the intersystem chain. Then either a saturating single-turnover flash or a saturating multiple-turnover flash is given. With single-turnover flashes the 'reduction areas' are approximately equal for *Helianthus* and *Asarum*. With multiple-turnover flashes, however, the reduction area for *Helianthus* is more than twofold that of *Asarum*. Making the assumption that at the given far-red background intensity there is no limitation at the PSI acceptor side and ignoring possible cycling of electrons from PSI back into the PQ pool, these data would suggest intersystem pool sizes of about 23 electrons in *Helianthus* and of about 13 electrons in *Asarum*, yielding a ratio of 1.8. However, the above-mentioned assumptions concerning PSI acceptor side and cyclic flow are not necessarily correct. There is some difference between the pool-size determination by fluorescence and P_{700}, which deserves further investigation.

In figure 9 the kinetics of pool reduction upon illumination of a spinach leaf disc are shown, as determined from the fluorescence rise kinetics and from P_{700} reduction. To avoid back-cycling of electrons from PSI into the PQ pool, the sample was infiltrated with antimycin A (Moss & Bendall 1984). It is interesting to note that the pool, as determined from P_{700}

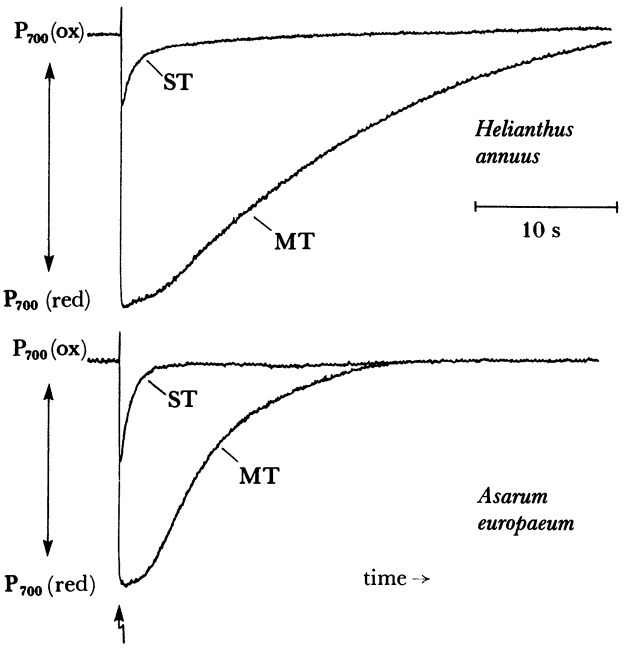

FIGURE 8. Intersystem pool size determination in *Helianthus annuus* and *Asarum europaeum* by means of P_{700} absorbance measurements. Far-red background light (3 W m^{-2}, RG 715, Schott) is applied to pre-oxidize P_{700} before triggering of single-turnover (ST) or multiple-turnover (MT) flashes of saturating light and to re-oxidize P_{700} following flash illumination. See text for explanation.

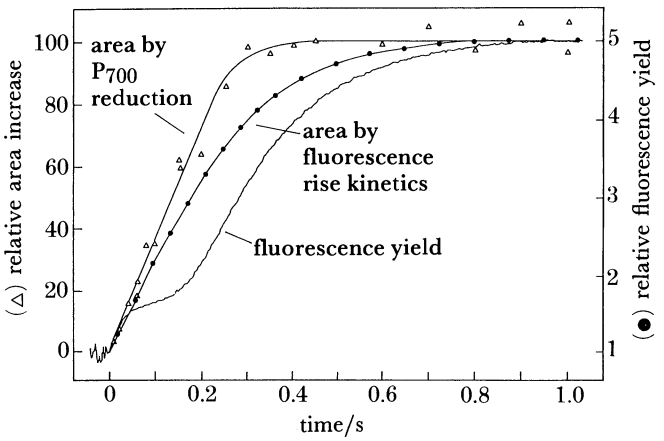

FIGURE 9. Kinetics of acceptor pool reduction upon actinic illumination, as determined via P_{700} or fluorescence (spinach leaf infiltrated with a solution of 3×10^{-6} M antimycin A). Pool reduction was determined via area measurements, as described for figure 7 and 8. Each point of P_{700} 'reduction area' corresponds to a separate measurement with a dark-adapted leaf disc sample illuminated for different times (10–1000 ms).

measurements, fills with distinctly more rapid kinetics than the one seen by fluorescence. This could be explained by an acceptor pool of PSI adding to the fluorescence area. Alternatively, one could speculate that PSII can reach an acceptor pool that does not donate to PSI. Cyclic electron flow around PSII would simulate such an acceptor pool (Schreiber & Rienits 1987; Schreiber & Neubauer 1987).

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The polyphasic fluorescence rise in saturating light

We have shown in previous work that with increasing light intensity the fluorescence rise kinetics approach a saturation pattern (Schreiber 1986; Neubauer & Schreiber 1987; Schreiber & Neubauer 1987). In saturating light (above 1000 W m^{-2}) a 'photochemical phase' O–I_1 is followed by 'thermal phases' I_1–I_2–P. The thermal phases cannot be speeded up by any further increase of light intensity; they were shown to be limited by the electron donation rate to PSII. A number of treatments that are known to affect the PSII donor side were found to suppress the I_1–I_2–P rise preferentially. The I_2–P rise can be selectively eliminated by Hill reagents and by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which led to the conclusion that it is likely to reflect the elimination of non-photochemical fluorescence quenching by PQ when this becomes reduced to PQH_2 by PSII.

By comparative measurements of P_{700} reduction it can be shown that our previous conclusions about I_2–P reflecting PQ reduction have to be modified (Neubauer & Schreiber 1987). In figure 10 the relative extent of intersystem pool reduction is plotted against the length of a saturating light pulse, as determined by the P_{700} reduction area (see figure 8). To avoid any limitation at the PSI acceptor side, the sample (spinach leaf disc) was infiltrated with methyl viologen. It is apparent that the pool fills up within about 50 ms, which corresponds to the time required for completion of the I_1–I_2 phase. In figure 11 simultaneous recordings of fluorescence and P_{700} changes induced upon onset of saturating light are shown. The I_2–P phase in fluorescence kinetically corresponds to P_{700} reduction.

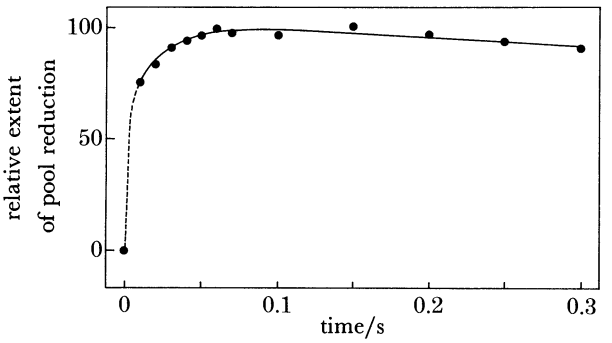

Figure 10. Relative extent of intersystem pool reduction induced by saturating light pulses of varying length, as measured via P_{700} (spinach leaf disc infiltrated with 2×10^{-4} M methyl viologen). Far-red background illumination was 3 W m^{-2} (RG 715, Schott). Saturating light pulses of varying length, 2500 W m^{-2} (XMT 103, Walz). Pool reduction was determined as described for figure 8.

This is another example demonstrating that P_{700} measurements can help in the interpretation of fluorescence data. On the basis of the results of figures 10 and 11, it appears that the I_1–I_2 phase parallels PQ reduction whereas I_2–P reflects P_{700} reduction. There remains the question of what kind of quenching is involved: photochemical or non-photochemical? For reasons discussed in detail previously (Neubauer & Schreiber 1987; Schreiber & Neubauer 1987) it is unlikely that part of Q_A is still oxidized at I_1. Possibly, the quenching which is eliminated during I_1–I_2 corresponds to the R-quenching discovered by Delosme (1967) with Q_B being identical to R. Once Q_A is reduced, $Pheo^-$ may be able to transfer its electron directly to Q_B. This could correspond to Joliot's Q_2 (Joliot & Joliot 1977). In this sense I_1–I_2 would correspond to removal of photochemical quenching at PSII. The terminal phase I_2–P, which

parallels P_{700} reduction in the experiment of figure 11, may well reflect removal of photochemical quenching at PSI. Under strongly reducing conditions there is a light-induced increase of PSI fluorescence (Ikegami 1976). As P^{+}_{700} quenches fluorescence as well by non-photochemical means as P_{700} quenches it photochemically, I_2–P should normally reflect the exhaustion of the PSI acceptor pool. *In vivo*, a block at the PSI acceptor side and a corresponding increase in PSI fluorescence can be visualized only when extremely strong light is applied under conditions when the Ferredoxin–NADP–oxido-reductase is not yet activated.

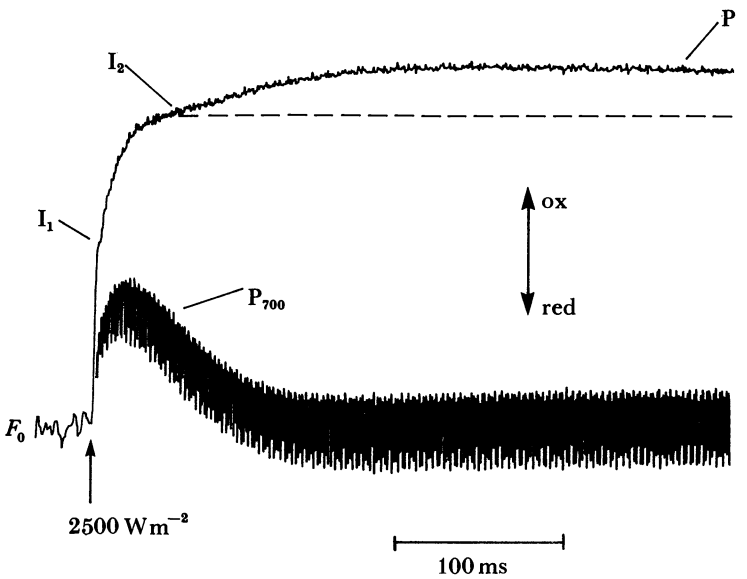

FIGURE 11. Simultaneous recordings of fluorescence and P_{700} absorbance changes in a spinach leaf during illumination by a pulse of saturating light. Previous work established that the primary acceptor of PSII, Q_A, is already fully reduced at I_1 (Schreiber & Neubauer 1988); the I_1–I_2 rise parallels PQ reduction (see figure 10). It is suggested that I_2–P reflects an increase of PSI fluorescence, as the quenching PP^{+}_{700} becomes reduced and photochemical quenching is suppressed upon exhaustion of PSI acceptors.

These findings may be important for the practical application of saturation pulses in the quenching analysis to distinguish photochemical and non-photochemical quenching. Experiments with methyl viologen (figure 10) show that even in presence of a very efficient PSI acceptor the PQ pool can be rapidly reduced by saturating light. In this case, only the I_2–P phase becomes suppressed. It would therefore be sufficient and even more appropriate to apply a 50 ms pulse of saturating light, to obtain with I_2 the relevant value of F_M for calculation of photochemical and non-photochemical quenching. The multiple-turnover flash lamp used in the present study (see experiments of figures 4–6, 8 and 10) and described elsewhere (Schreiber *et al.* 1988) produces such pulses and at the same time provides the possibility of sampling the 50 ms fluorescence level and holding it for 500 ms, so that it can be recorded on a slow recorder. Using such pulses is also advantageous with respect to the non-intrusiveness of the method, as the actinic effect of the saturation pulses is minimized.

We thank Ulrich Heber, Wolfgang Bilger and Jörg Kolbowski for stimulating discussions, Ulrich Schliwa and Annette Weber are thanked for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 176 and La 54/32.

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