Photosynthesis Research 11:173–182 (1987)

Regular paper

A solid-state, portable instrument for measurement of chlorophyll luminescence induction in plants

ULRICH SCHREIBER and ULRICH SCHLIWA

Lehrstuhl Botanik I, Universität Würzburg, Mittlerer Dallenbergweg 64, D-87 Würzburg F.R.G.

Key words: chlorophyll luminescence, luminometer, phosphoroscope, photosynthesis, delayed fluorescence

Abstract. A newly developed compact instrument is described for the measurement of chlorophyll luminescence induction in plants. The instrument operates with a pulsed light emitting diode (LED) as light source and a photodiode as luminescence detector. A special emitter-detector geometry provides for high irradiance of the sample and efficient collection of luminescence by the detector. With insertion of appropriate filters the same probe is also suited for measuring prompt chlorophyll fluorescence. The instrument shows considerable flexibility with respect to pulse frequency, relative lengths of light/dark intervals and luminescence sampling periods. Due to a selective amplifier system only that part of luminescence is processed which is induced by the individual excitation pulses. By this approach, the problem of "slow phase accumulation", encountered with conventional phosphoroscopes, is eliminated. Some examples are given for system operation, demonstrating satisfactory performance in measurements with intact leaves and isolated chloroplasts.

Introduction

Chlorophyll fluorescence methods have been widely applied in plant physiological work [for recent reviews see refs. 7, 13]. Important advantages are the non-intrusive character of fluorescence probes, rapidity of data acquisition, small sample requirement and the detailed information carried by the fluorescence signals. Besides the so-called "prompt fluorescence" which is observed upon chlorophyll excitation, green plants do also emit "delayed fluorescence" in the dark following illumination [15].

Delayed fluorescence, more shortly called "luminescence", originates from charge recombination at PS II reaction centers, requiring activation energy. The height of the activation energy barrier may be lowered either by a membrane potential or by a transmembrane proton gradient [for reviews, see refs. 1, 4, 8–10]. Hence, chlorophyll luminescence carries important information on PS II charge separation rate, as well as on the membrane gradients driving photophosphorylation.

In the past, practical application of chlorophyll luminescence in plant physiological work has been restricted by the fact that the luminescence signal is very weak (about 1/1000 of the fluorescence signal) and that it rapidly decays in darkness. Most work so far has been carried out with

sophisticated instrumentation, involving phosphoroscopes, photomultipliers and special amplifier systems.

Here we wish to report on a newly developed, compact luminescence apparatus for field and laboratory work, which employs a light emitting diode (LED) as pulsed light source and a photodiode as detector. Due to a special emitter-detector geometry and a highly selective amplifier system, the sensitivity of the new device matches that of much larger and more costly conventional instruments.

Description of the new instrument

The new instrument consists of two parts: First, a luminescence probe, containing the light emitting diode, the photodiode and the signal preamplifier. Second, a power and control unit, containing rechargeable batteries, the main electronic circuitry for LED light control and for selective signal amplification. Figure 1 shows the general system organization in a block diagram. The LED driver is controlled by the same master oscillator which also determines, with the appropriate delays, the periods for integration and sampling of the luminescence signal. The processed signal can be directly recorded (oscilloscope, chart recorder) or further analysed by microcomputer systems.

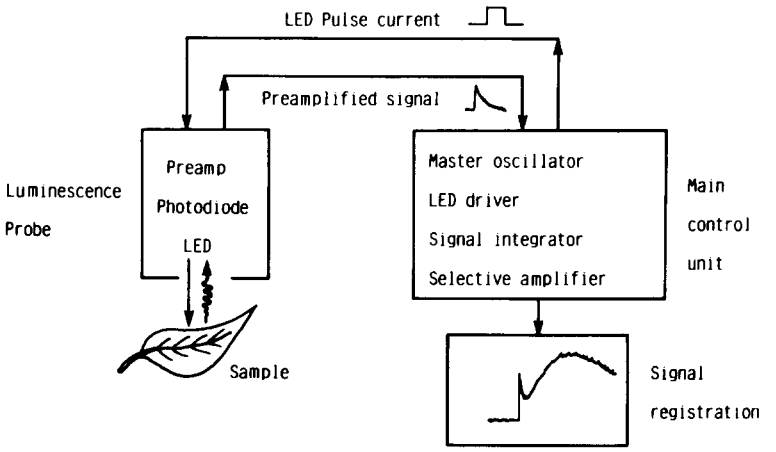

Figure 1. Block diagram of the solid-state chlorophyll luminescence measuring system. The instrument consists of a luminescence probe, containing a light emitting diode (LED) for pulsed excitation, a photodiode as detector and a preamplifier. The probe, which is enclosed in a shielded metal housing, is connected via flexible leads to the main control unit. The leads carry the pulse current for the LED and the preamplified signal. In the main control unit, which is battery powered (± 6V), a master oscillator with variable frequency provides synchronised triggering signals for the circuitry controlling LED driver, signal integrator and selective amplifier. The analog output signal is in the range of 0–2V.

The luminescence probe

Most information on the photosynthetic performance of a leaf by luminescence is obtained from so-called induction curves, i.e. when the leaf is illuminated following a period of darkness. While "luminescence induction" shows some similarity to the well known "Kautsky effect" of prompt fluorescence [see refs. 2, 5, 6, 11], the two types of induction phenomena are not identical. To observe pronounced luminescence induction curves, high light intensity is required. This aspect is essential for the design of the luminometer probe, particularly when low power solid state devices (LED and photodiode) are to be used.

In Figure 2 the luminometer probe is depicted in a schematical drawing.

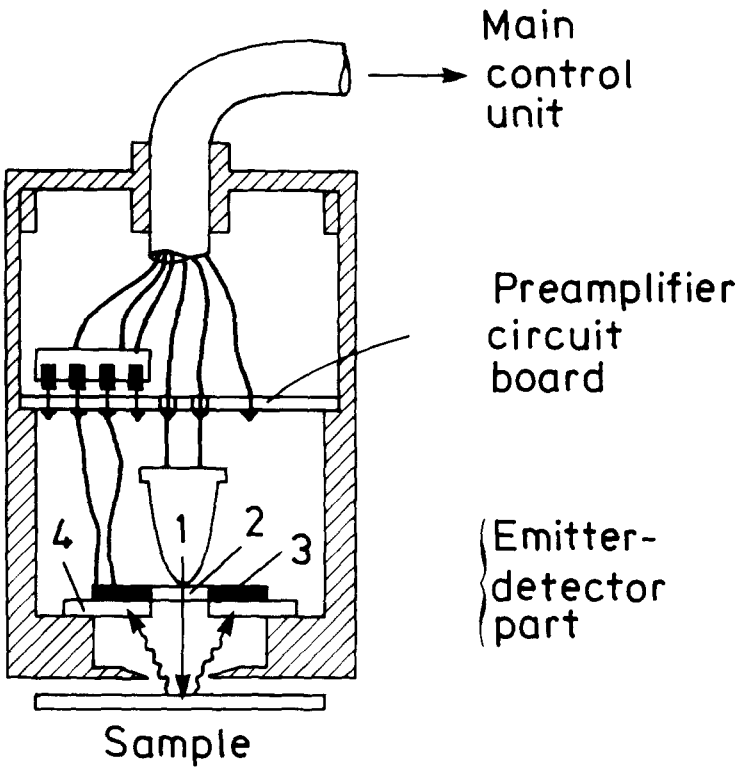

Figure 2. Schematic drawing of luminescence probe. The light-emitting diode (1) irradiates the sample via a central hole of the photodiode silicon chip (3), which receives a large solid angle of luminescence radiation from the sample. Between LED (1) and sample, a short-pass optical filter (λ < 680 nm) (2) may be inserted, as well as a long-pass filter (λ > 700 nm) (4) between sample and photodiode (3) These filters, which prevent reflected excitation light to reach the detector, are essential if the same probe is to be used for also measuring prompt fluorescence. The electronics for signal preamplification are located on a printed circuit board in close arrangement to the emitter-detector part of the probe.

The sample is illuminated by the LED through a central hole of a silicon photodiode chip. In this way a particularly close arrangement of LED and detector with respect to the sample is given, and, consequently, there is optimal utilization of the LED light and efficient collection of luminescence by the photodiode. This emitter-detector geometry gives better results than the previously described "piggy-back" arrangement [14], where a phototransistor was put on the back of an LED. A short-pass filter ($\lambda < 680$ nm) may be inserted between the LED and the sample, and the photodiode may be covered by a long-pass filter ($\lambda > 700$ nm), also with a central hole. In this way, prompt fluorescence can be measured by the same probe, with only minor loss in luminescence signal. Using a Stanley USBR 5501 light emitting diode (emission peak at 650 nm) and a Balzers DT Cyan short-pass filter ($\lambda < 680$ nm) the probe gives an average light intensity of up to 70 W/M^2, which is close to saturating for many plants. Our system employs a Vactec VTS 3072 N silicon photodiode and Schott RG 9 long-pass filter, into both of which 4 mm central holes were drilled with the aid of brass tubing and carborundum powder.

The measuring principle and electronic circuitry

Chlorophyll luminescence displays the same emission spectrum as fluorescence, from which it may be separated upon darkening, as fluorescence life time is about 1 nsec, while luminescence decays with several phases from the μsec range to several minutes. The different decay phases reflect different aspects of primary PS II reactions. It is the range of about 200 μsec to 5 msec (so-called "msec luminescence") which has been most extensively studied by the conventional phosphoroscope method. With this method, the sample is repetitively illuminated by light pulses and the detector is only open to the sample during the intermittent dark periods. An inherent property of the phosphoroscope method is accumulation of luminescence originating from preceding illumination cycles. Hence, as has been pointed out by Mar et al. [12], the measured signal is heterogeneous with respect to various decay components.

In the newly developed solid-state luminometer, the use of a light emitting diode as pulse light source provides considerable flexibility with respect of the choice of pulse frequency, light interval, dark interval and of the actual period during which luminescence is sampled. Furthermore, a selective amplifier system was designed, in order to minimize the contribution of slower decaying components. This was achieved by introducing a "reference measuring period" at the end of each measuring cycle. With each consecutive cycle only the signal exceding this reference signal is integrated. By this means the system is also rendered relatively insensitive to external light reaching the detector. In Figure 3 the measuring principle of the solid-state luminometer is further illustrated. An oscillator-driven

clock repetitively counts from 1 to 10, providing the appropriate triggering signals for the electronic components (LED driver, SI, SR, S1, S2) which control the various functions of the system. In the given example, the light interval lasts for 2 clock units; 1 unit is given between light-off and beginning of signal integration, which lasts for 5 units; this is followed by a 2 unit reference sampling period; the signal levels reached at the end of the integration and reference periods are sampled by sample-hold circuits

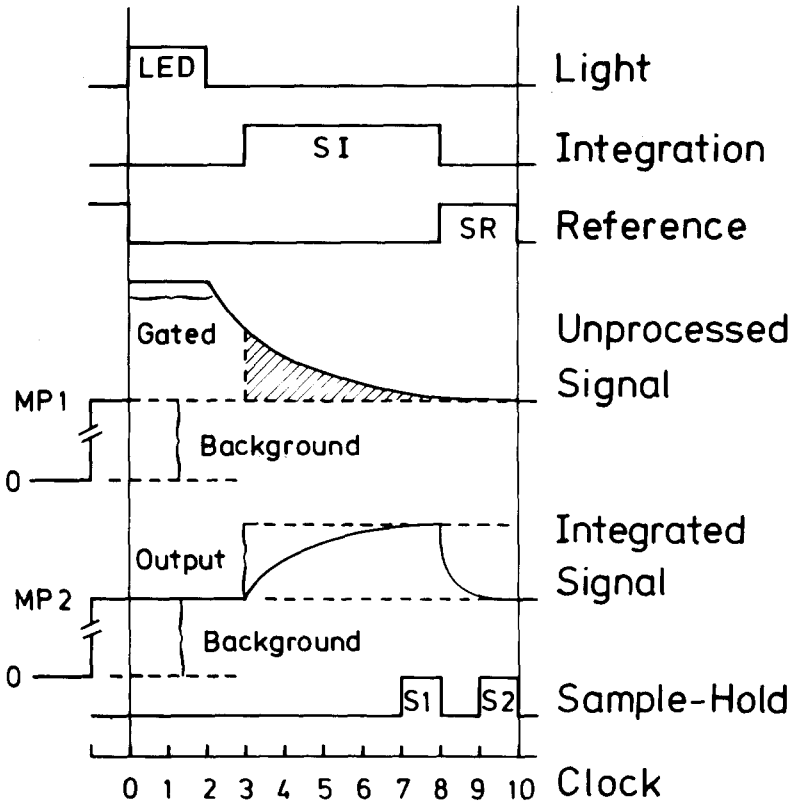

Figure 3. Measuring principle of solid-state luminometer. A clock, which is repetitively triggered by a master oscillator, divides one measuring cycle into 10 equal periods. By appropriate electronic circuitry (see Fig. 4) the various functions for processing the luminescence signal are strictly controlled via electronic switches. In the given example the periods are divided as follows: LED light pulse and gating of preamplifier, period 0–2; signal integration (S I), period 3–8; sampling of integrated signal (S 1), period 7–8; reference signal measurement and reset of integrator output (S R), period 8–10; sampling of reference signal (S 2), period 9–10. The diagram also shows schematically the time courses of the unprocessed signal, as observed before integration (measuring point MP 1 in Fig. 4) and of the integrated signal (MP 2 in Fig. 4). The signal change induced by the last LED pulse is distinguished from the non-specific background signal. The background signal is defined by the reference measurement (SR) at the end of each cycle. The output signal is given by the integrated signal, ignoring the background signal.

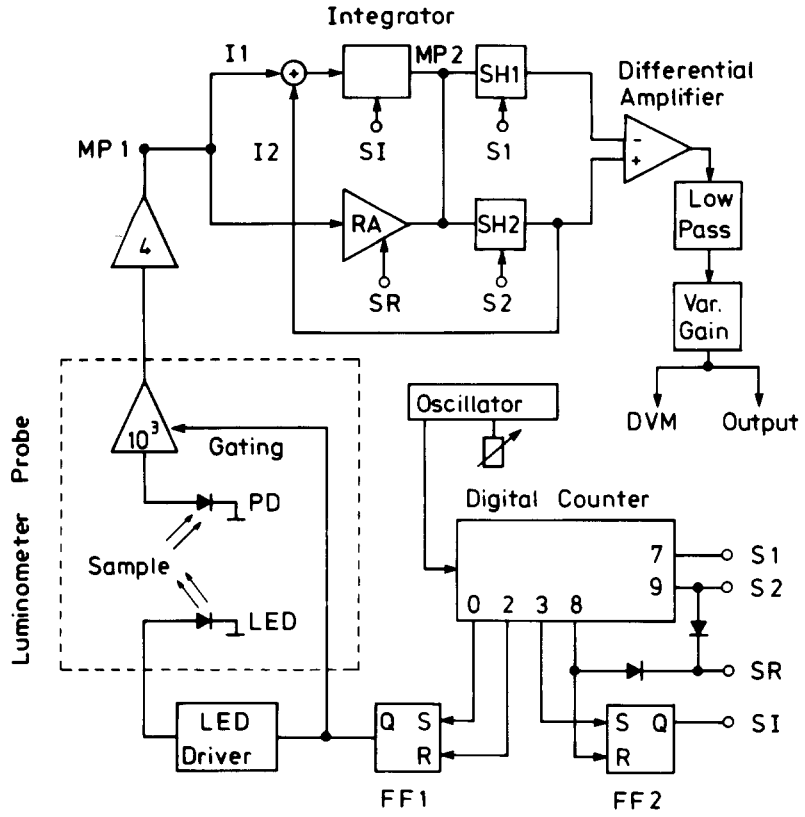

Figure 4. Basic electronic circuitry of the solid state luminometer. A master oscillator with variable frequency triggers a digital counter, which provides the switching signals for LED driver, preamplifier gating, signal integration (S I), reference measurement (S R) and signal sampling (S 1 and S 2). The inverted signal obtained at the output of the reference unity gain amplifier (R A) and stored in the sample-hold SH 2 is compensating (as current I 2) the signal arriving from the preamplifier (as current I 1) at a current summing point in front of the integrator. The difference of the signals stored in the sample-hold circuits SH 1 and SH 2 is measured. For further explanations, see text.

cuits during the clock periods 7–8 and 9–10, respectively. The figure also shows the general time courses of the signals before and after integration, distinguishing between the specific signal change induced by the last LED pulse and non-specific background signal.

In Figure 4 the basic electronic circuitry of the solid state luminometer is shown. A digital counter (1 out of 10) is driven by a master oscillator with variable frequency. It is this counter which divides a total interval, the length of which depends on the frequency, into 10 equal periods (see Figure 3). At the different outputs of the counter, trigger pulses are provided at multiples of 1/10 intervals, which in combination with appropriate logic gates initiate the following functions: Light-on (output 0),

light-off (output 2), start of signal integration (output 3 via switch S I), stop of signal integration (output 8 via reset of FF 2 flip-flop), sampling of integrated signal (output 7 via switch S 1) sampling of reference signal (output 9 via switch S 2), activation of reference amplifier linked to reset of integrator output to reference level (outputs 8 and 9 via switch SR). All switches are in CMOS. The same trigger pulses, which control the LED driver via the FF 1 flip-flop, are also employed as gating pulses for the preamplifier, which gives about 1000 fold amplification when not gated. Further amplification (another factor 4) occurs at the input of the main control unit. At the entrance of the signal integrator, the inverted reference signal from a preceding cycle (see Figure 3), which was stored in a sample-hold (SH 2), is added via a current summing point to the actual signal. Hence, integration only involves the luminescence signal exceding that at the end of the preceding measuring cycle. The CMOS switches S I and SR determine the periods of time (see Figure 3) during which either the integrator (inverting) or the reference amplifier (RA, inverting at unity gain) are active. At the end of the integration period, upon operation of switch S 1 the integrated signal is stored on a sample-hold (SH 1), which is connected to the inverting input of a differential amplifier. Into the non-inverting input of this amplifier the reference signal is fed, which is stored on a sample-hold (SH 2) upon operation of switch S2. The differential signal passes an RC filter with variable time constants. It is further amplified with variable gain before reaching the digital voltmeter display and instrument output.

Decisive for the performance of the new instrument is the introduction of a reference sampling period at the end of each measuring cycle. As this reference signal is electronically subtracted from the total signal observed after the next light pulse, only the specific luminescence excited by this pulse will be integrated and further processed. By this means any background signals from ambient room light (up to about 1000 times the pulse stimulated luminescence), as well as offset voltage from the preamplifier are effectively compensated. Most importantly, this compensation also eliminates the contribution from slowly decaying luminescence, which has been accumulating from preceding light pulses.

Instrument performance

The performance of the solid state luminometer is demonstrated by the original recordings depicted in Figures 5 and 6. Figures 5 shows luminescence induction curves of intact spinach leaves. The effects of light intensity, temperature and of NH_4Cl are shown. The observed induction transients display close similarity to those described in the literature for intact cells [see refs. 2, 5]. The pronounced initial spike, which typically occurs at high light intensity, may be considered expression of the rapid

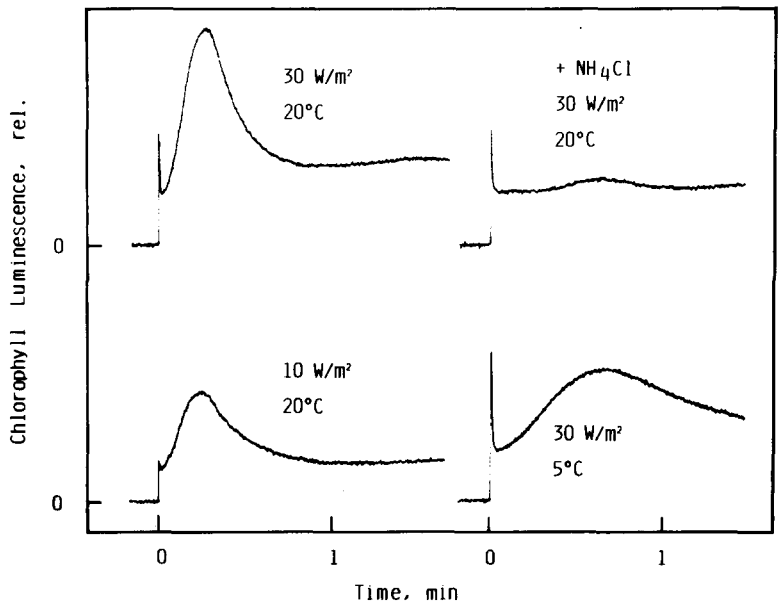

Figure 5. Luminescence induction curves of intact spinach leaves. Conditions are varied as indicated in the figure. NH_4Cl was added by applying a 200 mM solution for 5 min to the upper surface of the leaf, the cuticle of which was partially removed by gentle rubbing with carborundum powder. LED pulse frequency, 200 Hz; pulse length, 1 msec; signal integration from 0.5 msec to 2.5 msec following LED pulse.

build-up of a transmembrane potential [16] while the main peak reflects electron transport driven development of a proton gradient, as confirmed by the inhibitory effect of NH_4Cl. It has been shown that the peak amplitude is a good measure of electron transport activity: When leaves are damaged to varying extents by heat-treatment, peak amplitude and assimilation rate are linearly correlated [3].

In Figure 6 luminescence induction kinetics of spinach chloroplasts are depicted. The differences between intact and osmotically shocked chloroplasts, with and without acceptors, as well as the effect of the electron transport inhibitor DNP-INT are shown. In confirmation of previously published data [2, 5, 16] the results emphasize the different requirements for the rapidly induced and the more slowly induced components of luminescence. Only the latter component requires active electron transport exceding the acceptor capacity of the plastoquinone pool.

Concluding remarks

The presented examples of system performance demonstrate that the newly developed solid-state chlorophyll luminometer is suited for field

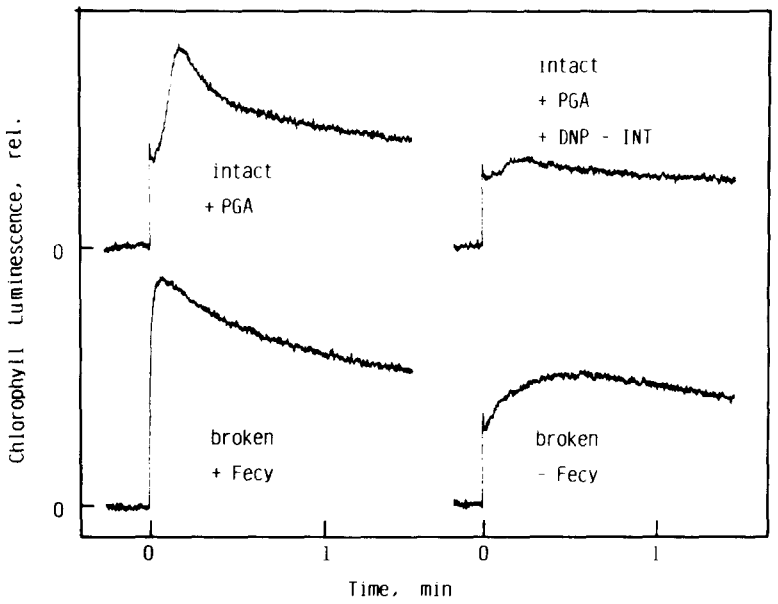

Figure 6. Luminescence induction curves of isolated spinach chloroplasts. Conditions are varied as indicated in the figure. Broken chloroplasts were obtained by osmotic rupture in 5 mM $MgCl_2$ and resuspension in isotonic buffer. The additions were at the following final concentrations: PGA, 2 mM; DNP–INT, 10^{-5} M; Fecy, 2 mM. Chlorophyll concentration, 100 μg/ml. The samples were contained in a vital with transparent bottom which was set on the luminometer probe.

oriented investigations on intact leaves as well as for laboratory work on isolated chloroplasts. The solid state components of the system provide for great compactness, small size, low power consumption, portability, low cost and great flexibility with respect to the programming of dark/light and sampling periods. By virtue of a selective amplifier system, only that part of luminescence is processed which is induced by each separate light pulse. Hence, the new system avoids the problem of signal heterogeneity, generally encountered with conventional phosphoroscope systems. In agreement with a suggestion by Itoh and Murata [6] it appears likely that the thus observed luminescence will preferentially reflect those PS II reaction centers which are open before application of each light pulse. Only these centers will give charge separation and, hence, will show charge recombination and concomitant luminescence generation. In addition to this "precursor" requirement, luminescence yield also depends on the "modulating parameters" of transmembrane activation energy [9, 16]. Both requirements, oxidized PS II acceptors and an energized thylakoid membrane, are in line with the notion of luminescence as a indicator of photosynthetic activity. This conclusion may only apply to that part of luminescence which is observed during the main peak of the induction kinetics, following the initial, rapid spike.

Acknowledgments

We gratefully acknowledge support by the Deutsche Forschungsgemeinschaft and by Ulrich Heber. Wolfgang Bilger is thanked for stimulating discussions.

References

1. Amesz J and van Gorkom HJ (1978) Delayed fluorescence in photosynthesis. Ann Rev Plant Physiol 29:47–66
2. Bauer R and Wijnands MJG (1974) The inhibition of photosynthetic electron transport by DBMIB and its restoration by p-phenylenediamines; studied by means of prompt and delayed chlorophyll fluorescence of green algae. Z Naturforsch 29c:725–732
3. Bilger W and Schreiber U (1985) Chlorophyll-Lumineszenz als Indikator Hitze-induzierter Schädigung der Photosynthese in intakten Blättern. Mitteilungsband Botaniker-Tagung Wien 1984 (F. Ehrendorfer, Herausgeber), S. 154
4. Govindjee and Jursinic PA (1979) Photosynthesis and fast changes in light emission by green plants. Photochemical and Photobiological Reviews. Vol 4, pp. 125–205. New York: Plenum Press
5. Itoh S (1977) Temperature dependencies of the rate of electron flow and of the formation of the high energy state in spinach chloroplasts and leaves. Plant & Cell Physiol 18:801–806
6. Itoh S and Murata N (1973) Correlation between delayed light emission and fluorescence of chlorophyll a in system II particles derived from spinach chloroplasts. Photochem photobiol 18:209–218
7. Krause GH and Weis E (1984) Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. Photosynth Res 5:139–157
8. Lavorel J (1975) Luminescence. In Govindjee, ed Bioenergetics of Photosynthesis. pp. 223–317. New York: Academic Press
9. Lavorel J, Lavergne J and Etienne A-L (1982) A reflection on several problems of luminescence in photosynthetic systems. Photobiochem Photobiophys 3:287–314
10. Malkin S (1977) Delayed luminescence. In Barber J, ed Primary Processes of Photosynthesis. pp.349–431
11. Malkin S and Barber J (1978) Induction patterns of delayed luminescence from isolated chloroplasts. I. Response of delayed luminescence to changes in the prompt fluorescence yield. Biochim Biophys Acta 502:524–541
12. Mar T, Brebner J and Roy G (1975) Induction kinetics of delayed light emission in spinach chloroplasts. Biochem Biophys Acta 376:345–353
13. Renger G and Schreiber U (1986) Practical applications of fluorometric methods to algae and higher plant research. In Govindjee, Amesz J and Fork DC (eds) Light emission by plants and bacteria. Academic Press, New York, in press
14. Schreiber U, Groberman L and Vidaver W (1975) Portable, solid-state fluorometer for the measurement of chlorophyll fluorescence induction in plants. Rev Sci Instr 46:538–542
15. Strehler BL and Arnold W (1951) Light production by green plants. J Gen Physiol 34:809–820
16. Wraight CA and Crofts AR (1971) Delayed fluorescence and the high-energy state of chloroplasts. Eur J Biochem 19:386–397