Biochimica et Biophysica Acta 890 (1987) 15–22
Elsevier

BBA 42078

Long-term delayed luminescence in *Scenedesmus obliquus* I. Spectral and kinetic properties

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(Received 11 September 1986)

Key words: Fluorescence; Long-term delayed luminescence; Chlorophyll spectrum; Spectrophotometry; (*S. obliquus*)

Long-term delayed luminescence varying from 0.3 s to several minutes has been investigated in wild-type cells of *Scenedesmus obliquus*. Complex decay kinetics as well as the comparison of excitation and emission spectra of long-term delayed luminescence with those of prompt fluorescence suggest the involvement of both Photosystem I and II in long-term delayed luminescence. A long-term intermediate showing maximal luminescence approx. 10 s after a 1 s excitation far-red light pulse is particularly attributed to the pigment system of Photosystem I. A mathematical model fitting the long-term delayed luminescence decay kinetics is presented.

Introduction

Delayed luminescence has been discovered in 1951 by Strehler and Arnold [1]. Detection of typical fluence rates smaller than 10^8 quanta/ml per s requires custom-made set-ups. As a valuable guide Lavorel [2] introduced the so-called 'τ-scale', which separates delayed luminescence in terms of three distinct regions of decay kinetics with half-lives ranging from 10^{-5} s up to several minutes. The domain larger than $t_{1/2} = 5$ s comprises very little data in the literature, even though valuable information may be extracted from this type of experiments [3,4].

The present continues an earlier investigation [5] of Long-term Delayed Luminescence (sometimes referred to as 'long-term delayed fluorescence'). Emission spectra and exciation spectra of short-term delayed luminescence often resemble those of prompt fluorescence [6,3]. Consequently, short-term delayed luminescence has been assumed to involve the same molecular species as prompt fluorescence: the lowest-excited singlet state of Chl *a* of PS II [7,8]. This has been claimed particularly for *Scenedesmus* by Haug et al. (Ref. 9; for further references, see Ref. 5). Various possible mechanisms for generating delayed luminescence have been suggested (for reviews, see Refs 10, 2 and 4). The most accepted explanation at present favors a thermally induced back-reaction of the transmembraneously accumulated charges as built up by the photosynthetic electron-transport chain. This, in turn, is taken to lead to a radiative charge recombination, giving rise either to delayed luminescence of "thermoluminescence" depending on the experimental conditions.

Nevertheless, there have been claims that some types of delayed luminescence originate from PS I (for reviews, see Refs. 4 and 11–13), even if the data are not as clear-cut as those supporting the

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; Chl, chlorophyll.

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involvement of PS II. A previous investigation [5] focussed on long-term delayed luminescence of tobacco leaves, and comparison of spectra of prompt fluorescence and long-term delayed luminescence also suggested processes different from a plain reversal of charge separation by PS II, postulating more complex molecular mechanisms.

Screening, selfabsorption and scattering as indispensable obstacles inherent in optical spectroscopy of opaque materials such as mature leaves of higher plants will truncate both spectra and kinetics in an uncontrolled manner. Therefore, in the present work we adopted the green alga *Scenedesmus obliquus*: suspensions of algae can be handled more conveniently and – due to strict standardization – allow a better control of various optical and physiological parameters such as extinction, temperature, uptake of exogeneous inhibitors or pH. In addition, there are several specific mutants available with well-defined defects which allow further analysis of the origin of long-term delayed luminescence.

Materials and Methods

Cultures of the unicellulr algae *Scenedesmus obliquus* strain D_3 were grown at a temperature of 30°C in liquid inorganic medium [14], aerated with 3% CO_2 in air. The cells were synchronized by a light-dark regime of 14 h white light of 15 $W \cdot m^{-2}$ and 10 h darkness. At the beginning of each light period the cultures were diluted automatically with a photoelectrically controlled dilution device [15] to a preset density. If not stated otherwise, cells were harvested for the experiments at the 16th hour of the life cycle.

The device for measuring long-term delayed luminescence has been described in detail previously [5] and was slightly modified for measuring algae suspensions: Petri dishes of 100 mm diameter and 18 mm height were filled with 10 ml suspension (corresponding to $A = 0.25$) and placed on top of a vertical, red-light sensitive end-on photomultiplier tube (EMI 9658 B, S20 photocathode, 1 inch diameter) with 'shutter 1' in between for protection of the photomultiplier tube during irradiation with exciting light. Prior to illumination, cell suspensions wer adjusted to a chlorophyll concentration of 5 $\mu g \cdot ml^{-1}$ by dilution with the culture medium. Measurements were usually performed at 20 ± 1°C under dim green safelight (cf. action spectrum of long-term delayed luminescence in Fig. 6, bottom). Using a mechanically operated 'shutter 2' (Prontor Press) in series with a lens system, the suspension was uniformly illuminated from above for 1 s with monochromatic light of a given fluence rate. Light intensities were measured with a photometer/radiometer system (Ealing, Model 450). Excitation and Emission wavelengths were defined by interference filters (DIL, half-band widths of 8–10 nm, Schott, Mainz, F.R.G)

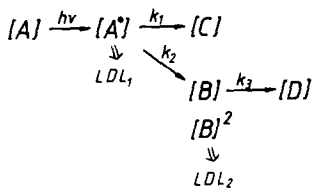
With a time lapse of approx. 0.3 s after terminating the exciting light the kinetics of long-term delayed luminescence were monitored typically for 1 min. The photomultiplier signal was amplified by a low-noise amplifier (Brookdeal, model 450) which was operated in the ratemeter mode, and fed either to a NIC 1174 special purpose microcomputer (and stored on magnetic tape for subsequent evaluation), or directly to a Hewlett-Packard strip chart recorder (7101 BM).

Absorption measurements and 4th-derivative analysis were performed with a self-made, computerized single-beam spectrophotometer described in detail previously [16]. Measurements of corrected excitation and emission fluorescence spectra were done with a Shimadzu correcting fluorimeter, model RF-502. The action spectrum for long-term delayed luminescence was measured according to Shropshire, Jr. [17].

Results and Discussion

In a previous study of long-term delayed luminescence in tobacco leaves [5] occasionally deviations from simple first-order kinetics of long-term delayed luminescence were observed, i.e., an initial, relatively fast decay, succeeded by a (smaller) optimum in long-term delayed luminescence, i.e., the build up of some kind of luminescing intermediate. Occurrence and conditions for its occurrence have been far from being clear. The same effect is observed in *Scenedesmus* as well, and was studied more thorougly in the present paper.

The dependency of the decay kinetics of long-term delayed luminescence of *Scenedesmus* as a function of fluence rate of the 1 s exciting light



$$LDL_{tot} = LDL_1 + LDL_2$$

$$\frac{d[A^*]}{dt} = -(k_1 + k_2)[A^*]$$

and

$$\frac{d[B]}{dt} = k_2[A^*] - k_3[B]$$

with the solutions $k_1 \gg k_2$

$$[A^*] = [A^*]_0 \, e^{-k_1 t} \approx LDL_1$$

$$[B] = [A^*]_0 \frac{k_2}{k_3 - k_1}(e^{-k_1 t} - e^{-k_3 t}) \approx \sqrt{LDL_2}$$

$$LDL_{tot} = [A^*]_0 \left(e^{-k_1 t} + m[A^*]_0 \left\{ \frac{k_2}{k_3 - k_1}(e^{-k_1 t} - e^{-k_3 t}) \right\}^2 \right)$$

with m = relative luminescence quantum efficiency.

Scheme I. Minimum model which crudely explains the crude shape of the long-term delayed luminescence kinetics, including the luminescent intermediate (top). The only light reaction is the excitation of some component A to A* (for details, see text). The solution of the differential equation (bottom) is visualized in Fig. 4.

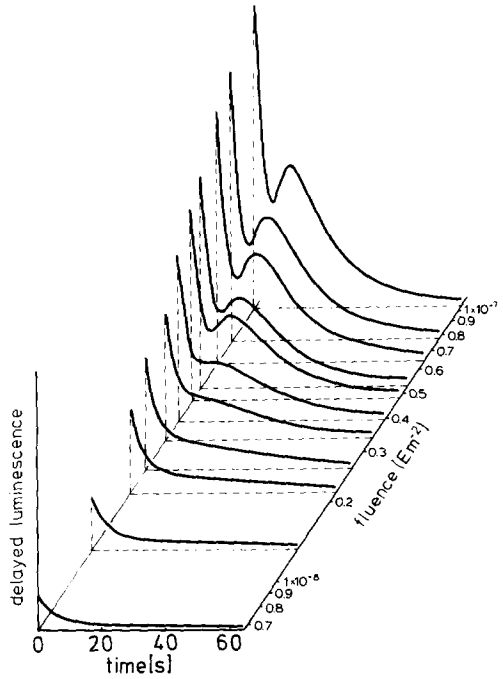


Fig. 1. Kinetics of long-term delayed luminescence as function of the fluence of a 1 s exciting light pulse of 721 nm. Light emission was monitored at 712 nm. At increasing fluence rates a luminescing intermediate builds up after approx. 10 s.

pulse is shown in Figs. 1 and 2. At defined ' medium' intensities, and with light emission monitored at 712 nm and excitation at 721 nm (Fig. 1) or 694 nm (Fig. 2), after about 10 s a long term luminescent intermediate is observed. When 694 nm exciting light is used, the intermediate appears to be less pronounced compared to 721 nm, and fades out at higher fluence rates (Fig. 2). Depending on the conditions used, the long-term delayed luminescence signal decays more or less rapidly to zero after a specific period of time.

In order to measure a crude 'action spectrum' for the generation of this intermediate, we adjusted the intensity of the exciting light at various wavelengths to generate the same initial height of the long-term delayed luminescence signal (corresponding to approx. 10^{-7} E · m^{-2}; Fig. 3. Clearly – at the light intensities chosen – the intermediate only developes when long-term delayed luminescence is excited between 700 and 730 nm, with a maximum at 720 nm; blue light turned out to be inactive at any light intensity. At higher intensities of red light the intermediate can still be seen using exciting light from a slightly wider wavelength range (extension, approx. ±10 nm).

In Scheme I we propose the minimum model, which crudely explains the shape of the kinetics, including the intermediate. Some species 'A' are excited to 'A*', which emit LDL_1. A*, in turn, decays to a non-luminescing species 'C' with a reaction constant k_1, and to a species 'B' with a reaction constant k_2. Subsequently, 'B' undergoes a bimolecular reaction, thereby generating LDL_2, and finally decays with a reaction constant k_3 towards another, nonluminescent product 'D'. This bimolecular reaction turned out to be an indispensible part of the model, and cannot be re-

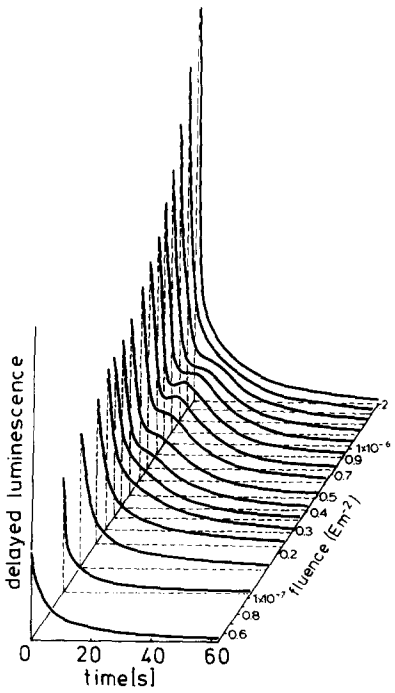


Fig. 2. Kinetics of long-term delayed luminescence as function of the fluence of a 1 s exciting light pulse of 694 nm. Light emission was monitored at 712 m. Approx. 10 s after the pulse medium fluence rates will generate some intermediate species. With higher fluence rates the intermediate fades away.

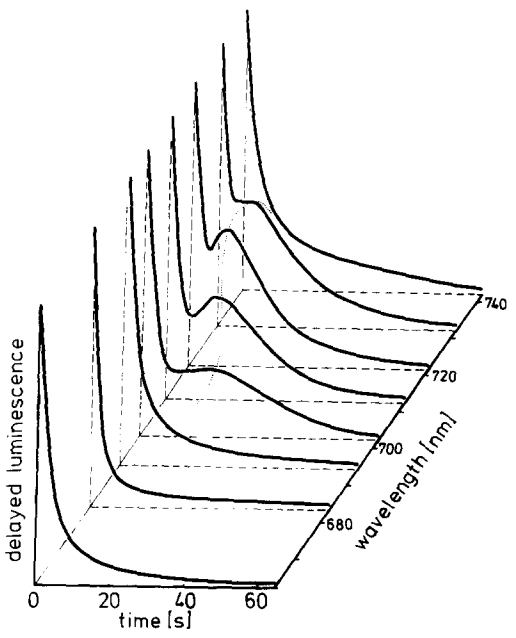


Fig. 3. Kinetics of long-term delayed luminescence as function of wavelength of the exciting light pulse of 1 s producing the same initial quantum emission (approx. 10^{-7} E·m^{-2}). The dashed line indicates the crude action spectrum for the production of fluorescing intermediate with a maximum around 720 nm.

placed by a monomolecular one. According to their small quantum efficiencies, long-term delayed luminescence reactions (empty arrows in Scheme I) do not significantly consume the substrates A* and B which simplifies the proposed scheme. Long-term delayed luminescence either originates in A* and B themselves, or is generated by energy transfer from these species towards pigment molecules with the proper spectral emission characteristics exhibited by Figs. 7 and 8.

The solution of the corresponding differential equation is given in Scheme I and visualized in Fig. 4. The parameter 'm' defines the contribution of LDL_2 to the total signal LDL_{tot} and can be defined as the 'relative luminescence efficiency'. The course of the actual kinetics is essentially fitted by this model, as the comparison with the measurement shows (dotted line, taken from Fig. 1). In addition, the model explains the decrease of the intermediate long-term delayed luminescence species with decreasing fluence rates of the ex-

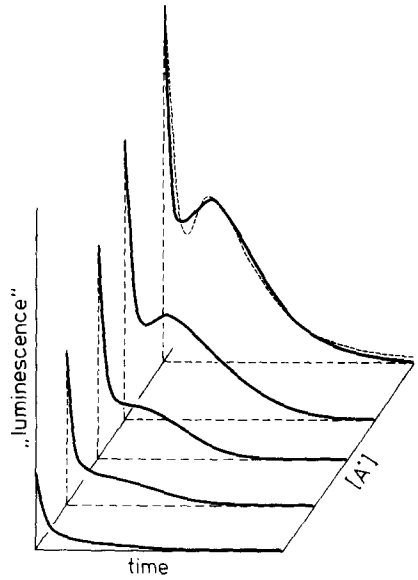


Fig. 4. Visualization of Scheme I: the minimum model which crudely explains the shape of the kinetics of long-term delayed luminescence. It also explains the fact that with lower 'intensities' of the exciting light, i.e., with decreasing A^*_0, the intermediate fades out.

citing light; the fluence of the exciting light is mimicked by varying the concentration of 'A_0^*' (all other parameters are kept constant). The curve was fitted with the help of a microcomputer (Commodore, model 8032). For this calculation no attempt was made to develope an automatic parameter-search program, which probably would result in a more satisfying fit of the kinetics.

The semilogarithmic plot (not shown) of the long-term delayed luminescence kinetics of *Scenedesmus*, excited with 421 nm light where no intermediate is build up, is complex and does not allow any further interpretation. It can neither be fitted by a first- or second-order kinetic, nor by a composition of two individual first-order kinetics, indicative of the involvement of several pigments or pigment systems in long-term delayed luminescence. This implication was further clarified by determining a true action spectrum for long-term delayed luminescence.

Following standard procedure, we first measured a series of fluence-response curves (Fig. 5, covering more than five orders of fluence. They do not run in a strictly parallel manner, i.e., there are either several photoreceptors and/or screening pigments involved. The action spectra deduced for three different responses specified by the intersection of the fluence response curves with the horizontal lines 'a', 'b' or 'c' (Fig. 5) are given in Fig. 6, bottom; no essential differences are established. With the irradiation system utilized, no saturation of the signal was achieved at any wavelength. Due to these limitations at higher responses the spectra are incomplete (Fig. 6, bottom, b and c). For comparison, the absorption spectrum of *Scenedesmus* cells, including the 4th derivative in order to increase wavelength resolution, is depicted in Fig. 6, top, and the corresponding excitation spectrum of prompt fluorescence in Fig. 6, middle. Prompt fluorescence is essentially emitted by Photosystem II, in full agreement with the general assumption. Peaks in the blue and red part of this spectrum can readily identified by the 4th derivative of the absorption spectrum (Fig. 6, top), at least involving two Chl a species with Soret-bands at 444 and 479 nm.

The action spectrum of long-term delayed luminescence (Fig. 6, bottom) consists of a peak in the Soret-region between 400 and 430 nm (partly coincident with the peak at 413 nm in the fourth derivative of the absorption spectrum), a massive contribution by some ultraviolet-absorbing com-

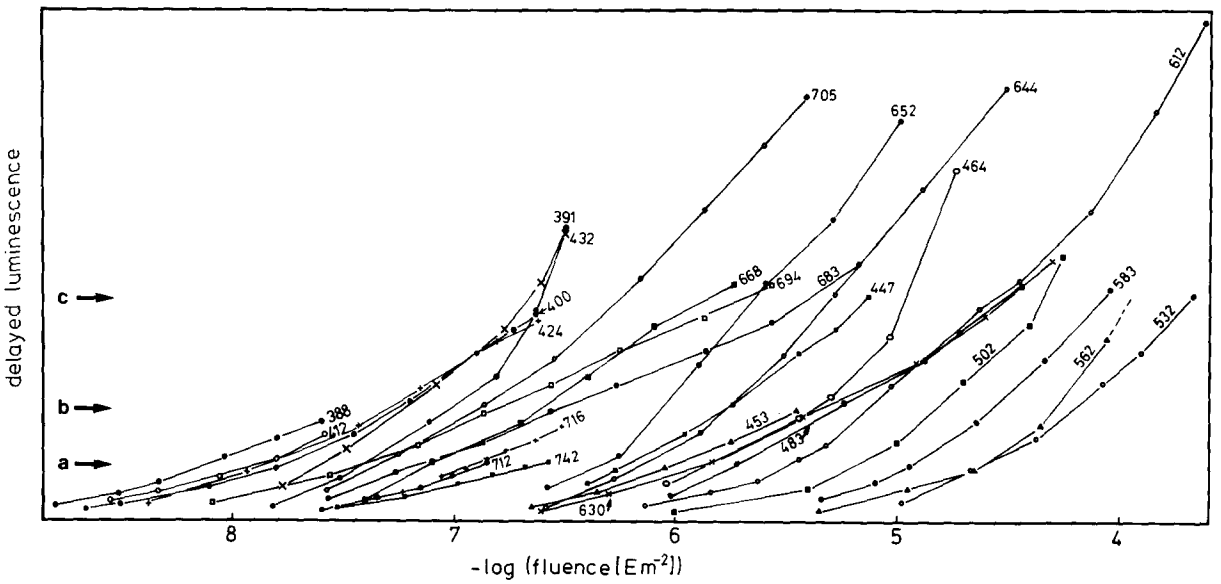


Fig. 5. Fluence-response curves of the initial quantum output of long-term delayed luminescence. More than five order of fluence are covered with the set-up used. Based on three different responses marked 'a', 'b' and 'c' true action spectra for the induction of long-term delayed luminescence are calculated and shown in Fig. 6, bottom

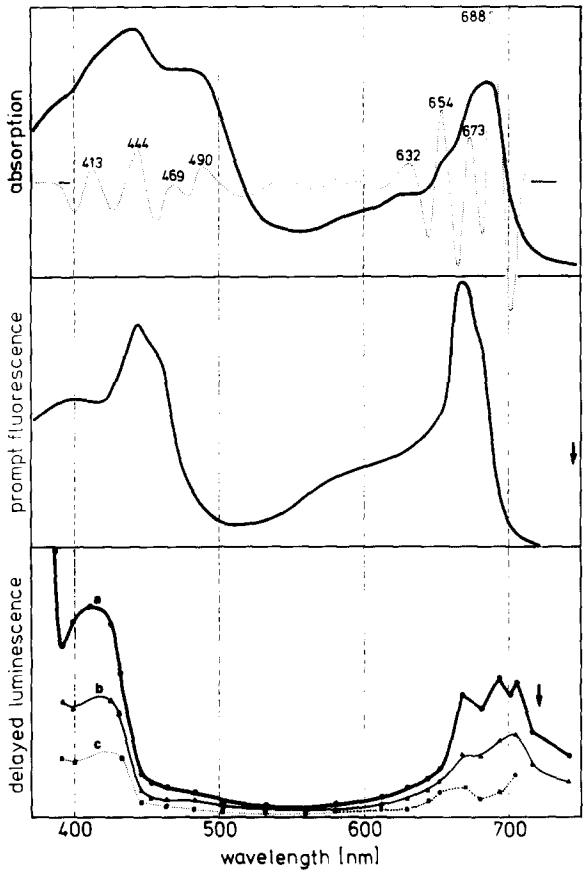


Fig. 6. Top: absorption spectra of wild-type *Scenedesmus o.* Total extinction is approx. $A = 1$. The fourth derivative (dotted line) was taken with $dx = 3.0$, 3.1, 3.2, and 3.3 nm (Ref. 21); wavelengths indicated are based upon calibration with argon lines. Middle: corrected excitation spectrum of prompt fluorescence. The measurement has been performed under steady-state conditions. Excitation wavelength: slit width, 5 nm; emission wavelength, 750 nm; slit width, 15 nm. Bottom: action spectra derived from the fluence response curves in Fig. 5 as based upon three different responses ('a', 'b', 'c').

ponent(s), and a relatively broad peak in the far-red region. This, again, reflects the involvement of several pigments, particularly the 'long-wavelength antenna' of PS I (fluorescence emission at 725 nm; Ref. 20). To summarize, excitation spectra of prompt fluorescence and long-term delayed luminescence are significantly different, suggesting quite distinctive molecular species and mechanisms involved in these two processes. Based upon identical optical properties of the (macro) environment (range corresponding to the optical wavelength of approx. 700 nm) of the pigments or pigment-systems involved in these processes, trivial optical reasons such as scattering, screening or selfabsorption cannot account for the observed differences. This, again, suggests a different molecular origin of prompt fluorescence and long-term delayed luminescence: long-term delayed luminescence does not plainly represent the reversal of the initial steps of light-induced charge separation in Photosystem II. Kinetics and spectral extension of long-term delayed luminescence into the far-red region suggest the (additional) involvement of components generally attributed to PS I.

Experiments performed by other workers emphasize the diversity of pigments giving rise to prompt and delayed light emission: pronounced variations of fluorescence may occur without corresponding changes of delayed fluorescence [23]. Prompt fluorescence is quenched by carotenoids [18], whereas delayed fluorescence is not affected [19]. Since excitation produced by charge recombination always starts out from the reaction center(s), the transfer of excitation energy to the reaction center chlorophyll may be significantly faster – and therefore the predominant reaction to occur – than to the antenna chlorophylls. Thus, Amesz and Gorkom [3] hypothesized in their review "that delayed luminescence may originate largely from chlorophyll molecules in the vicinity of the reaction center" (of PS I and PS II, Ref. 4), whereas prompt fluorescence is known to originate in the bulk chlorophylls mainly associated with PS II. This would explain the distinct reactivities in response to various exogenous parameters, and long-term delayed luminescence might offer itself as a sensitive and specific probe of reaction-center activity.

The next experiments focussed on the emission spectrum of long-term delayed luminescence. We monitored the emission spectrum based upon the initial height (i.e., the rate of quantum emission) of the long-term delayed luminescence kinetics. As before, luminescence was excited for 1 s with 412 nm light of 10^{-8} E · m^{-2} · s^{-1}, where no luminescing intermediate developes. In order to select a particular emission wavelength, long-term delayed luminescence was monitored with the proper interference filter sandwiched between petridish-

sample and photomultiplier cathode. Taking into account the transmittance of the interference filter used, we obtained the corrected emission spectrum depicted in Fig. 7B (dots). In some consistency with the excitation spectrum in the red, its extraordinary width also suggests the involvement of diverse luminescing species.

We cannot necessarily expect a direct correlation of excitation and emission spectra of long-term delayed luminescence typical for prompt fluorescence (however, we cannot exclude it either). Excitation and emission spectra of long-term delayed luminescence might be due to different molecular species. In order to analyze the composition of the emission spectrum, we first investigated the emission spectrum of prompt fluorescence of *Scenedesmus* on the basis of individual Lorentz-shaped bands, adopting the known wavelengths of maximal fluorescence of Photosystem II and I (685 and 725 nm, Fig. 7A; the choice of free wavelength positions of all four individual components would introduce ambiguity; i.e., various different parameter sets would fit the measured emission spectrum of long-term delayed luminescence).

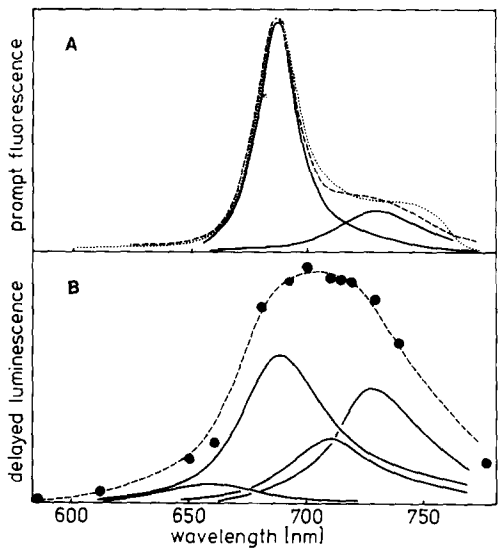


Fig. 7. (A) Dotted line: measured emission spectrum of prompt fluorescence. Dashed line: Calculated spectrum composed of two individual Lorentz-shaped bands at 685 and 775 nm (solid lines). (B) Dots: corrected emission spectrum of the initial components of delayed luminescence (LDL_1). This spectrum was fitted (dashed line) by superposition of varios Lorentz-shaped bands (solid lines).

Again, the calculations were performed with a CBM 8032 personal computer, utilizing a homemade parameter search program in this case. Two additional components with peaks occurring at 712 and 660 nm were introduced in order to explain the measured long-term delayed luminescence emission spectrum. After proper adjustment of all parameters defining width, height and wavelength of the individual components we obtained a reasonable good fit (Fig. 7B), comprising pigments of PS I and PS II.

As mentioned above, when measuring long-term delayed luminescence kinetics we noticed a significant change of the long-term delayed luminescence intermediate with the emission wavelength. Therefore, we measured the long-term delayed luminescence kinetics with respect to the intermediate long-term delayed luminescence component and obtained the spectrum depicted in Fig. 8. As a measure for the intermediate we utilized the ratio of sections a and b as indicated in Fig. 8. Based on known spectral properties, it appears to be chiefly related to the pigment system of PS I rather than PS II [20]. However, the molecular identity of the intermediate remains obscure, except that it undergoes a bimolecular reaction, as pointed out above. Under no circumstances we were able to observe the intermediate without the initial long-term delayed luminescence signal taking place. In consistency with Scheme I, a molecular species A* giving rise to emission of LDL_1, is the indispensable prerequisite for LDL_2 to occur. This interpretation is supported by experiments with the mutant C6E of *Scenedesmus*, which only contains active PS I: it does not show any long-term delayed luminescence signal. Only further experiments will allow a definite mutual assignment of spectra, pigments and mechanisms.

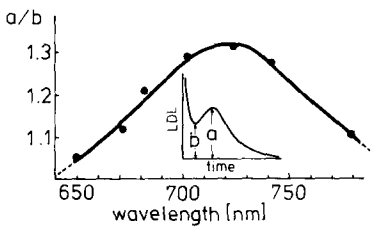


Fig. 8. Dependence of the intermediate (assayed by the a/b ratio as indicated) on the wavelength of emission. A maximal 'hump' is observed between 700 and 750 nm.

TABLE I

APPROXIMATE WAVELENGTHS OR WAVELENGTH RANGES OF EXCITATION AND EMISSION OF PROMPT FLUORESCENCE, AND OF THE FAST AND INTERMEDIATE COMPONENTS OF DELAYED LUMINESCENCE ($LDL_{1,2}$)

	Excitation	Emission
Prompt fluorescence	444, 665	685, 725
LDL_1	400–430, 650–740	665–750
LDL_2	690–740	675–770

The approximate wavelengths or wavelength ranges of both excitation and emission spectra of prompt fluorescence, and of the initial and intermediate components of long-term delayed luminescence are listed in Table I.

We are now investigating long-term delayed luminescence of *Scenedesmus* as a function of various parameters such as temperature, age during the diurnal life cycle, exogeneous pH, various herbicides, and deficiencies of essential trace elements and components. Preliminary results suggest that virtually all these parameters are capable of influencing shape and extent of long-term delayed luminescence kinetics. Particularly, so-called 'state transitions' of the photosystems appear to exhibit a strong impact. Utilizing different mutants of *Scenedesmus* with specific defects in their photosynthetic apparatus, we are studying the (light-dependent) development of the potency of exhibiting long-term delayed luminescence.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 305).

References

1 Strehler, B.L. and Arnold, W. (1951) J. Gen. Physiol. 34, 809–820
2 Lavorel, J. (1975) in Bioenergetics of Photosynthesis (Govindjee, R. ed.), pp. 225–314, Academic Press, New York, San Francisco, London
3 Amesz, J. and Gorkom, H.J. v. (1978) Ann. Rev. Plant Physiol. 29, 47–66
4 Malkin, S. (1977) in Primary Processes of Photosynthesis., (Barber, J., ed.), pp. 351–430, North Holland Biomedical Press
5 Schmidt, W. (1985) Photobiochem. Photobiophys. 9, 89–97
6 Inoue, Y. and Shibata K. (1982) in Photosynthesis, Vol. 1 (Govindjee, ed.), pp. 507–533, Academic Press, New York, San Francisco, London
7 Robinson, H.H. and Crofts, A.R. (1983) FEBS Letters 153, 221–226
8 Rutherford, A.W. and Inoue, Y. (1984) FEBS Letters 165, 163–170
9 Haug, A., Jaquet, D.D. and Bell, H.C. (1972) Biochim. Biophys. Acta 283, 92–99
10 Clayton, R. (1969) Biophys. J. 9, 60–76
11 Björn, L.O. (1971) Photochem. Photobiol. 13, 5–20
12 Goedheer, J.C. (1963) Biochim. Biophys. Acta 66, 61–71
13 Sane, P.V., Desai, T.S. and Takake, V.G. (1980) Z. Naturforsch. 35c, 289–292
14 Senger, H. and Bishop, N.I. (1972) Proc. 2nd Int. Cong. on Photosynthesis Res., Vol. 1, (Forti G., Avron M., Melandri A., eds.), pp. 678–687, W. Junk, The Hague
15 Senger, H., Pfau, J. and Werthmüller, K. (1972) in Methods of Cell Physiology, Vol. V., (San Pietro, A., ed.), pp. 301–323, Academic Press, New York and London
16 Schmidt, W. (1982) Anal. Biochem. 125, 162–167
17 Shrophire, Jr., W. (1972) in Phytochrome (Mitrakos, K., Shrophire, Jr., W. eds.), pp. 161–181, Academic Press, London
18 Duysens, L.N.M., Van der Schatte Olivier, T.E. and Den Haan G.A. (1972) Int. Congr. Photobiol., 5th, Bochum, abstract 277
19 Van Best, J.A. and Duysens, L.N.M. (1977) Biochim. Biophys. Acta 459: 187–206
20 Brinkmann, G. and Senger, H. (1981) in Photosynthesis, Vol. 3 (Akoyunoglou, G., ed.), pp. 337–346, Balaban Int. Science Services, Philadelphia
21 Butler, W.L. and Hopkins, D.W. (1970) Photochem. Photobiol. 12, 439–450
22 Velthuys, B.R. and J. Amesz (1973) Biochim. Biophys. Acta 225, 126–137
23 Den Haan, G.A. (1977) Ph.D. thesis, University of Leiden