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Long-term delayed luminescence in *Scenedesmus obliquus*. II. Influence of exogeneous factors

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Long-term delayed luminescence varying from 0.3 s up to several minutes has been studied in wild type and several pigment mutants of *Scenedesmus obliquus* during the life cycle and under the influence of various exogeneous parameters such as herbicides, different pH values, temperature, preillumination, and the diurnal rhythm of synchronized cells. All these parameters investigated exhibit a specific and distinct impact. Therefore, long-term delayed luminescence may serve as some kind of assay of the 'status of vitality' of green living cells, i.e., as a fast and simple screening procedure of potentially harmful environmental factors, and also for herbicides. In addition, only intact chloroplasts show long-term delayed luminescence suggesting a possible assay for the physiological activity during isolation of chloroplasts.

Introduction

The present work continues our studies of long-term delayed luminescence in *Scenedesmus obliquus*. In the previous paper [1] we investigated the spectral and kinetic properties. Complex decay kinetics as well as the comparison of excitation and emission spectra of long-term delayed luminescence with those of prompt fluorescence suggested the involvement of pigment systems associated both with PS I and PS II in long-term delayed luminescence. Particularly a long term intermediate showing maximal luminescence at 720 nm approx. 10 s after excitation with a 1 s far red light pulse was attributed to pigments of PS I. During those studies we noticed a strong influence of exogeneous parameters such as pH, preirradiation conditions, temperature and various chemicals on long-term delayed luminescence. In the current paper we follow these effects more thoroughly. Various chlorophyll mutants of *Scenedesmus* allow further characterization of long-term delayed luminescence.

Materials and Methods

Culture conditions and a synchronization method were described previously [1]. In addition, thylakoids were prepared by mildly disrupting the cells according to Senger and Mell [2].

In order to transfer the algae cells into State 1 or State 2 they were illuminated for 10 min either with 'PS-I light' (Schott-filter AL 712 nm) or 'PS-II light' (Schott-filter DIL 640 nm). In both cases the light intensity was adjusted to 8 $W \cdot m^{-2}$. Prior to illumination, cell suspensions were ad-

Abbreviations: PBQ, parabenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS I and PS II, Photosystem I and Photosystem II; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; 2-4-D, dichlorophenoxyacetic acid; LDL_1, fast component of long-term delayed luminescence; LDL_2, intermediate component of long-term delayed luminescence [1].

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justed to a chlorophyll concentration of 5 $\mu g \cdot ml^{-1}$ by dilution with the culture medium. For the inhibitor experiments DCMU, PBQ, 2-4-D, CCCP, diphenylcarbazide, chloramphenicol, phenoxythiourea, dithionite and streptomycin were added to give a final concentration of 10^{-5} M.

The following buffers (0.02 M, photochemically inert) were used to study long-term delayed luminescence as a function of exogenous pH; all sodium salts, plus 0.1 M NaCl: pH 0, HCl; pH 2 sulphate; pH 4, acetate; pH 6–8 phosphate; pH 10 borate.

Device and conditions for measuring long-term delayed luminescence have been described in detail previously [3] and were slightly modified for measuring algae suspensions [1]. Absorption measurements were performed with a selfmade, computerized single-beam spectrophotometer [5].

Results and Discussion

Cells of synchronous cultures of *Scenedesmus o.* reach maximal photosynthetic capacity at the 'eighth hour', i.e., when cells have been illuminated with white light for eight hours in their diurnal rhythm [6]. At this stage fluorescence yield is minimal [4]. Between the 8th and 16th hour fluorescence yield increases and photosynthetic activity decreases (at the 14th hour the light is switched off); thereafter fluorescence yield decreases, and photosynthetic activity slightly increases again. More recently Krupinska et al. [7] studied the so-called light-induced 'state transitions' of the photosystem(s) of *Scenedesmus o.* by fluorescence induction (Kautsky effect). According to these authors the ability to perform state transitions follows the same time-course during the life cycle as the fluorescence yield. At the 8th hour no state transition can be induced; at the 16th hour it is maximal.

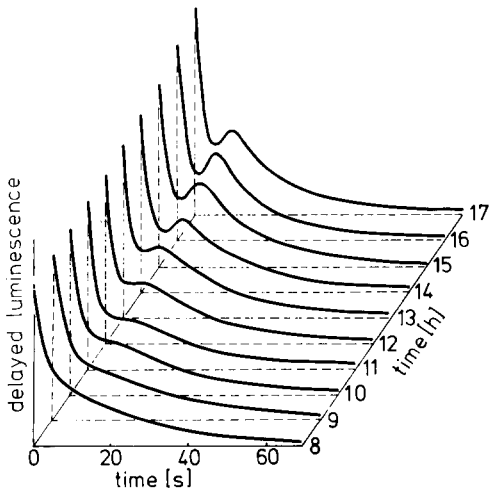

Fig. 1. Kinetics of long-term delayed luminescence as a function of the developmental stage studied in a synchronous culture of *Scenedesmus o.*

The diurnal rhythm is also reflected by the intermediate long-term delayed luminescence species, as shown in Fig. 1: between the 8th and the 16th hour it increases significantly, and subsequently it decreases again. In addition, the long-term delayed luminescence kinetics are susceptible to the wavelength of the preirradiating light as exemplified by Fig. 2D (16th hour of the life cycle): the long-term delayed luminescence intermediate is strongly enhanced by irradiation with 'light 1' (712 nm of 10^{-7} $E \cdot m^{-2} \cdot s^{-1}$), and this effect is reversible by 'light 2' (640 nm of $5 \cdot 10^{-7}$ $E \cdot m^{-2} \cdot s^{-1}$). This observation appears to be consistent with the dependence of prompt fluorescence on the wavelength of preirradiation [7]: State 1 is more fluorescent than State 2. In contrast to the situation in *Chlorella* [8], the initial height of long-term delayed luminescence kinetics is approximately the same in both of these 'states'. State transitions measured by fluorescence induction are typically measured after preirradiation periods of 10 min (cf. Ref. 7). However, light-induced conversion from State 1 to State 2 and vice versa in *Porphyra* occurs with half-times of 5 and 10 s, respectively [9], and in *Chlorella* [10] in comparable times. Similarly, the effect observed with long-term delayed luminescence is already saturated after an irradiation of only 1 min, utilizing comparable light intensities. Nevertheless, it remains questionable whether the effect of preirradiation on long-term delayed luminescence reflects state transitions as defined by fluorescence induction, or merely is a coincidence. The cycle of state transitions as shown in Fig. 2D can be repeated many times with one individual sample. In consistency with fluorescence induction, at the eighth hour also with respect to long-term delayed luminescence no 'state transition' is possible.

Preliminary measurements on young leaves from a beech tree showed a different pattern: a strong initial long-term delayed luminescence-signal after a 1 min actinic irradiation with 640 nm light, but an approx. 3 times smaller signal after a subsequent 712 nm irradiation. Saturating irradiations utilizing interference filters of intermediate wavelengths indicate an equilibrium of the red and far red light effect at 685 nm (curve not shown). It should be noted that the photoequilibrium of phytochrome is also close to this wavelength.

Further analysis of long-term delayed luminescence, particularly with respect to the intermediate species, was performed by treatment with various inhibitors of photosynthesis (applied immediately before the 1 s light induction), or by other treatments. First of all, only cells grown autotrophically show this intermediate, regardless of being synchronized or in batch culture. It has never been observed with heterotrophically grown wildtype cells, which are supposed to develop the complete photosynthetic apparatus in darkness. If light-grown cells, showing the intermediate, are incubated for some time in darkness, the intermediate completely disappears (Fig. 2A).

A wide variety of herbicides investigated all influence extent and time-course of long-term delayed luminescence in well reproducible and distinctive ways. However, chemically inert salts such as NaCl or KCl do not influence long-term delayed luminescence (i.e., in comparably small concentrations of 10^{-5} M). Concentrations of as much as 0.3 M of alkali salts are capable themselves of inducing long-term delayed luminescence in preilluminated spinach chloroplasts (cf. Ref. 11).

DCMU as specific blocker of electron flow between PS II and PS I suppresses the long-term delayed luminescence intermediate, but doubles the initial long-term delayed luminescence intensity (Fig. 2B). This appears to be consistent with the spectral analysis: the intermediate appears to originate in pigments primarily associated with PS I, in contrast to the initial long-term delayed luminescence, which is attributed to PS II. As in fluorescence induction, blocking the electron flow

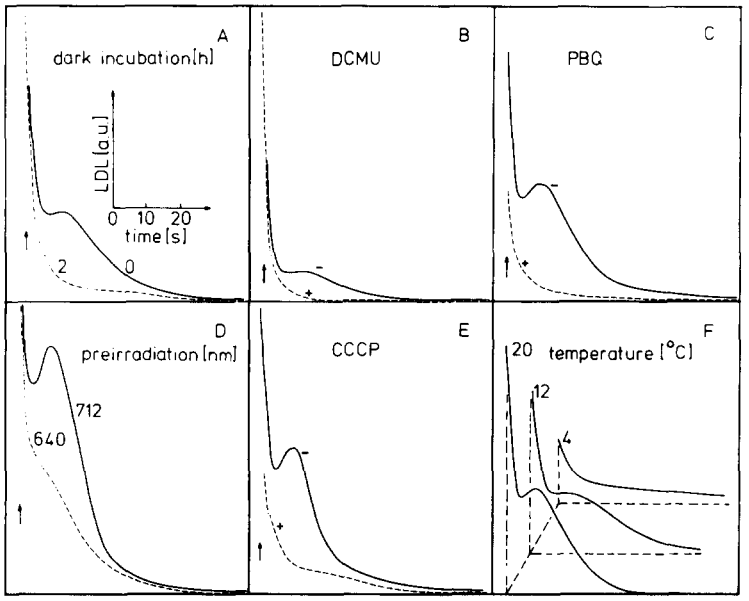

Fig. 2. Influence of various treatments and inhibitors on the kinetics of long-term delayed luminescence (LDL) of *Scenedesmus o.* (kinetics not normalized). (A) Cells grown autotrophically in a batch culture (labelled '0'), and after subsequent incubation of 2 h in darkness (labelled '2'). (B) Synchronized cells before (−) and after (+) treatment with DCMU. (C) before (−) and after (+) treatment with PBQ. (D) Long-term delayed luminescence kinetics after preirradiation with 712 nm light of 10^{-7} E·m^{-2}·s^{-1} or 640 nm light of $5 \cdot 10^{-7}$ E·m^{-2}·s^{-1} for 1 min. (E) Before (−) and after (+) treatment with CCCP. (F) Examples for the dependency of the kinetics of long-term delayed luminescence on temperature.

after PS II inhibits useful photochemistry, and gives rise to increased PS II luminescence. In contrast to DCMU, PBQ (Fig. 2C), CCCP (Fig. 2E) and dithionite (kinetics not shown) decrease the initial amplitude of long-term delayed luminescence, but suppress the intermediate likewise. Other herbicides tested such as chloramphenicol, phenoxythiourea, streptomycin, diphenylcarbazide or 2-4-D (kinetics not shown) exhibit a somewhat smaller but significant impact, typically decreasing both the initial and the intermediary luminescence.

PBQ is an electron acceptor competing with plastoquinone, which enhances the photochemical activity of PS II, and thereby reduced long-term delayed luminescence. CCCP is known to increase the proton permeability of biological membranes. The quite significant reduction of long-term delayed luminescence by CCCP can be taken to suggest the involvement of the chemiosmotic status of the thylakoid membrane in long-term delayed luminescence. Dithionite as a powerful, but unspecific reducing agent indiscriminately reduces all components accessible, thereby decreasing long-term delayed luminescence in general. This result is in line with the finding of Velthuys and Amesz [12] who observed an enhancement of delayed fluorescence of preilluminated chloroplasts by dithionite in the dark. They interprete their results by a specific reduction of the Q-acceptor pool of PS II, causing recombination luminescence. Subsequently the acceptors remain in the reduced state and no long-term delayed luminescence can be induced by illumination.

Interestingly, herbicides assumed not to influence the primary photosynthetic steps, nevertheless affect long-term delayed luminescence: e.g., 2-4-D as 'auxin-type herbicide', or even antibiotics such as streptomycin or chloramphenicol known as specific blockers of protein synthesis in chloroplasts (kinetics not shown).

Temperature also is capable of influencing intensity and kinetics of long-term delayed luminescence (Fig. 2F). With decreasing temperature, the initial intensity decreases and – consequently in terms of the model [1] – the intermediate progressively disappears. However, the quantum yield of long-term delayed luminescence, i.e., the (time-integrated) total number of photons emitted, stays approximately constant, i.e., the emission rate decreases, but the emission lasts longer (calculations not shown). From kinetics as shown in Fig. 2F, we determined the Arrhenius' plot for the initial intensity (plot not shown). Above 17°C the data deviate from a straight line, indicating more complex mechanisms. However, below this temperature they obey Arrhenius' equation yielding an activation energy of 33.4 $kJ \cdot M^{-1}$.

Several mutants of *Scenedesmus o.* are available for further analysis of long-term delayed luminescence. The C2-A′ mutant can be heterotrophically grown in darkness without significant chlorophyl formation, but requires light for the development of the photosynthetic apparatus. However, the 1% of chlorophyll present compared to the total chlorophyll of the light-grown mutant, should be sufficient for measuring long-term delayed luminescence. Nevertheless, no long-term delayed luminescence is detectable at all, indicating that the pigment present is not yet in its 'active form'. However, a few seconds of irradiation with light of the proper wavelength and moderate intensity are sufficient to generate the potency of long-term delayed luminescence (Fig. 3). Under the conditions used (421 nm, $2 \cdot 10^{-7}$ $E \cdot m^{-2} \cdot s^{-1}$) the effect is saturated after 400 s of irradiation; no intermediate of long-term delayed luminescence (LDL_2) is observed. Within 5 min after turning off the lights the potency of exhibiting long-term delayed luminescence asymptotically declines to approx. 40% of the saturation level and then remains stable.

Using an illumination intensity of $2 \cdot 10^{-7}$ $E \cdot m^{-2} \cdot s^{-1}$ for 2 min at each wavelength, we determined a crude action spectrum for the light-induced potency of long-term delayed luminescence in the C2-A′-mutant (Fig. 4, top). Peak positions reflect the involvement of various photoreceptors. Clearly, these are not identical with the 'bulk pigments' reflected in the absorption spectrum, but probably include protochlorophyllide(s) (dashed line in FIg. 4, top) and pigments of PS II.

At least it appears to be safe to attribute the long-term delayed luminescence signal to pigments of both PS I and PS II of the developing or the fully developed photosynthetic apparatus itself. In contrast to prompt fluorescence, antenna chlorophylls appear to be less involved in long-

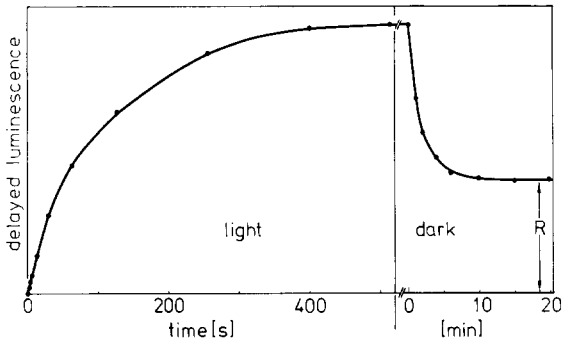

Fig. 3. Induction of the 'potency' for long-term delayed luminescence (initial rate of quantum emission) in the mutant C2-A' of *Scenedesmus o.* by blue light (421 nm, $2 \cdot 10^{-7}$ E·m^{-2}·s^{-1}). Under these conditions, after approx. 400 s of irradiation the system is saturated. However, within a few minutes in darkness, at room temperature, this potency will decline to a stable level R of approx. 40% of saturation.

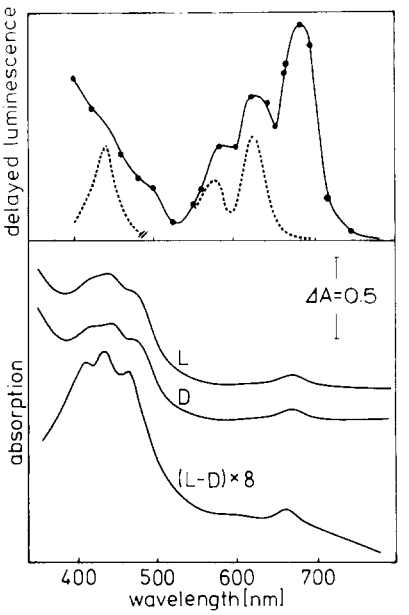

Fig. 4. Top: Crude action spectrum for the synthesis of some component(s) capable of emitting long-term delayed luminescence in the mutant C2-A'. The initial quantum output of long-term delayed luminescence (excitation 721 nm, emission at 712 nm), observed after 2 min of irradiation with actinic light of $2 \cdot 10^{-7}$ E·m^{-2}·s^{-1}, is given as a function of wavelength. The dotted line indicates the absorption spectrum of protochlorophyllide [15], (note various scales). Bottom: Light-induced absorption spectrum: D, before and L after 2 min of blue light irradiation (421 nm, $2 \cdot 10^{-7}$ E·m^{-2}·s^{-1}).

term delayed luminescence: antenna mutants are not affected in their long-term delayed luminescence-signals. The mutant '-LHC', without a significant content of chlorophyll *b*, exhibits a long-term delayed luminescence not significantly different from that of wild-type cells. The mutant C-6 E, heterotrophically grown in darkness, contains only chlorophyll *a* and PS I, but does not show any long-term delayed luminescence (data not shown). This supports the assumption that the presence of PS II is a prerequisite for long-term delayed luminescence to occur, even if pigments of PS I appear to be involved (cf. Ref. 1).

An attempt to trace the long-term delayed luminescence signal of *Scenedesmus* back to cellular components basically failed. Thylakoid membranes were prepared by mild disruption according to Senger and Mell [2]. Nevertheless, the long-term delayed luminescence-signal – as observed in vivo – was essentially lost. This is probably due to the destruction of the thylakoids causing the breakdown of the transmembraneous pH gradient leading to the loss of long-term delayed luminescence. Only a minute, fast long-term delayed luminescence component is preserved, decaying in some tens of a second, i.e., being classified as delayed luminescence of medium lifetime [13] which is not well accessible with our set-up. Long-term delayed luminescence-kinetics cannot be restored when all components separated by breakage are simply mixed together. This supports a structural integrity as indispensible prerequisite for long-term delayed luminescence to occur. Isolated spinach chloroplasts exhibit long-term delayed luminescence like intact leaves indicating an undisturbed thylakoid structure and well-preserved organelles. Therefore, long-term delayed luminescence might serve as sensitive assay for a successful isolation of intact chloroplasts.

This result is in good agreement with the pH dependency of long-term delayed luminescence (Fig. 5). The kinetics were monitored immediately after pH adjustment. Consistent with the pH dependency of short-term delayed luminescence [14], and according to the model (Scheme 1 in Ref. 1), the long-term delayed luminescence signal of both the initial and the intermediate component deceases with increasing exogeneous proton concentration, and vice versa. This further supports

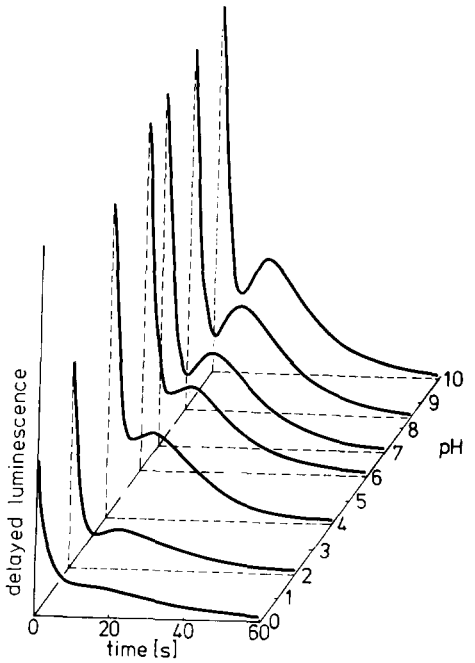

Fig. 5. Kinetics of long-term delayed luminescence as a function of pH of the exogeneous medium. Excitation wavelength, 721 nm (10^{-7} E·m^{-2}·s^{-1}); emission wavelength, 712 nm. The kinetics were measured immediately after adjustment of the pH.

the hypothesis that a proton gradient across the thylakoid membrane is a prerequisite for long-term delayed luminescence to take place. Lower external pH values result in a decreased transthylakoid proton gradient and electrical potential difference, which counteracts charge recombination, and hence leads to a diminished signal of long-term delayed luminescence.

Concluding, the present data demonstrate a strong dependency of kinetics and intensity of long-term delayed luminescence on various exogeneous and intrinsic factors. A distinct intermediate of long-term delayed luminescence ('LDL_2') is particularly responsive to various conditions, and exclusively occurs in light-adapted cells. It is suppressed by various herbicides, regardless of their action on the initial phase of long-term delayed luminescence (enhancement or suppression). The indispensible requirement for long-term delayed luminescence to take place is an intact chloroplast. In addition to this, the particular dependency of long-term delayed luminescence on the exogeneous pH or on preirradiation conditions is taken to reflect its intimate correlation with a well-functioning thylakoid membrane.

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