Biochimica et Biophysica Acta, 590 (1980) 385—399

BBA 47838

A STUDY OF DARK LUMINESCENCE IN *CHLORELLA*

BACKGROUND LUMINESCENCE, 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA-TRIGGERED LUMINESCENCE AND HYDROGEN PEROXIDE CHEMILUMINESCENCE

JEAN LAVOREL

Laboratoire de Photosynthèse du C.N.R.S., 91190 Gif-sur-Yvette (France)

(Received October 22nd, 1979)

Key words: Luminescence; Chemiluminescence; Photosystem II; Delayed fluorescence; (Chlorella)

Summary

Dark luminescence, defined as the ability of completely relaxed (dark-adapted) photosynthetic systems to emit light, has been studied in *Chlorella*. Three main effects have been demonstrated. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea elicits a weak emission L_D of very long lifetime (several minutes); it is believed to result from a negative shift of redox potential of the secondary System II electron acceptor B producing in some centers a state Q^- (reduced primary acceptor), as postulated by Velthuys and Amesz ((1974) Biochim. Biophys. Acta 333, 85—94), which can recombine with an oxidizing equivalent in a state S_2 present in very small amount. As in photoinduced luminescence, this recombination excites chlorophyll which then emits light. A much stronger emission L_H is observed after injection of H_2O_2. Both signals are modified or suppressed by treatments specific of the oxygen emission system, such as: thermal denaturation at 50°C, NH_2OH, etc. In addition, a weak, permanent background luminescence L_0 has been observed; like L_D and L_H, it is a System II property and requires the integrity of the oxygen-evolving system. It is believed to reflect a very slow back flow of electrons from an endogeneous reductant pool to oxygen through part of the photosynthetic chain. Using flash preillumination, it is demonstrated that H_2O_2 is able to oxidize S_0 into

---

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; L_D, luminescence observed after addition of DCMU; L_H, luminescence observed after addition of H_2O_2; L_0, background luminescence; PQ, plastoquinone.

S_2, the latter giving rise to L_H; H_2O_2 does not act on S_1 (or much less). The reactive site of H_2O_2 seems to be the same as the binding site of NH_2OH. Evidence is given that the strong L_H signal in particular reveals a stable, low pH of the intrathylakoid phase in *Chlorella*.

Introduction

Luminescence of photosynthetic systems, also termed delayed fluorescence or delayed light emission, is the radiative decay of chlorophyll singlet states formed at the expense of electron-hole pairs recombining at the site of photochemical centers. In higher plants, luminescence is essentially of System II origin. It is ordinarily observed following light activation of the sample (e.g. with flashes): the responsible electron-hole pairs have been produced by the photochemical functioning of the System II centers and are in great majority stabilized as redox states of the primary electron and acceptor, as well as redox states of other neighboring electron carriers in the photosynthetic chain in partial equilibrium with the foregoing. In fact, luminescence is only allowed because of a slight imperfection of this stabilization (see Ref. 1 for a recent review).

This theory does not say that light activation is obligatory. One should be able to make fully relaxed (dark-adapted) photosynthetic systems luminesce either by triggering the recombination of charges once photochemically separated and quasi-permanently trapped or by feeding charges to the primary donor and acceptor, even if indirectly, at the expense of exogeneous redox substances. The first possibility (triggered luminescence) is suggested by the existence of charge-storing abilities operating on both sides of System II: the state S_1 of the oxygen-evolving system [2] and the state B^- (singly reduced) of the secondary System II acceptor [3,4] apparently are very stable in the dark. One could conceivably trigger their recombination by the addition of DCMU:

$$S_1QB^- \xrightarrow{\text{DCMU}} S_1Q^-B \rightarrow S_0QB + h\nu_L \qquad (1)$$

In Eqn. 1, $h\nu_L$ is a photon of luminescence, Q is the primary System II electron acceptor; the first reaction is the consequence of a negative shift of the redox potential of B brought about by its association with DCMU, an hypothesis proposed by Velthuys and Amesz [4]. The second possibility (chemiluminescence) is also suggested by the well-known ability of several classes of redox compounds (Hill reagents, artificial electron donors) to interact at different places with the photosynthetic chain in competition with or as substitutes of physiological carriers.

The expected triggered luminescence and chemiluminescence according to the two types of actions above explained were indeed observed for the first time in 1975 with fully relaxed *Chlorella* or isolated chloroplasts [5]. * As

* It must be mentioned that a chemiluminescence was already demonstrated in bacterial chromatophores by Fleischman and Mayne [6].

mentioned, DCMU was used for demonstrating the triggered luminescence; a relatively strong chemiluminescence was also observed in response to hydrogen peroxide, acting presumably as hole donor to the oxygen-evolving system. These effects were strikingly independent on the time which the sample had spent in darkness after its full relaxation. The very low level of the DCMU-triggered luminescence suggested that probably S_2 instead of S_1 was involved in Eqn. 1. The present report considerably expands the previous findings in *Chlorella* and attempts a comprehensive interpretation of the phenomena. It is intended to show that 'dark' luminescence, a coinage emphasizing the fully relaxed situation of the photosynthetic apparatus when it is observed *, can be an interesting new tool for the study of System II and of the elusive associated oxygen-evolving system.

Material and Methods

Algae (*Chlorella pyrenoidosa*) were grown and harvested as described previously [8]. For purpose of comparison, a limited number of experiments were done using two other species and several of their mutants isolated in this laboratory: *Aphanocapsa* 6714 and *Chlamydomonas reinhardtii* kindly supplied by F. Espardellier (see Ref. 9) and J. Garnier (see Ref. 10), respectively. Algae were used in their respective growth media.

The luminescence apparatus and protocol of experiments are similar to those described in Ref. 5, except for the following modifications that were introduced for an easier operation or for improving the signals. The Algal sample is in a light-tight, thermostatically-controlled glass resevoir (50 ml maximum) where it is kept in suspension by gentle magnetic stirring. The upper surface of the sample is seen by the photomultiplier through a flexible light guide of large section (SOVIS, 50 cm length, 1 cm diameter) inserted into a black plastic cover fitted to the reservoir. A mechanical shutter in front of the photomultiplier window enables one to check for the absolute level of the light emission. Reagents are expelled manually from hypodermic syringes; up to four syringes can be positioned by conical stops glued to their needles on the cover of the reservoir. This permits rapid successive injections and bubbling of the suspension with gas mixtures. The apparatus is easily operated in dim laboratory light, the latter contributing no more than 5% of the weakest background (L_0, see below). In the standard protocol, the temperature is set at 30°C, the volume of sample is 25 ml (approx. 75 $\mu g \cdot ml^{-1}$ chlorophyll) and final concentrations of DCMU or H_2O_2 are $2 \cdot 10^{-5}$ M or 0.2% (v/v), respectively. Because the algal sample had been exposed to the ambiant light prior to being introduced in the reservoir, it required about 10—15 min for relaxing to the dark-adapted condition; during this period the tail of the light-induced luminescence can be monitored at high sensitivity. In some cases, flash preillumination, with flash lamp (General Radio Stroboslave) positioned inside the reservoir, close to the suspension, was used a predetermined time before reagent addition. The above protocol (in particular with respect to temperature) was

* This is to be contrasted with the triggered luminescence reported by others (see e.g. Ref. 7) which requires prior activation by light and decays at about the same rate as the photoinduced luminescence.

devised in order to optimize the signals. The latter were identical in shape and similar in amplitude to those earlier reported [5] (in the range of 10^{-8}—10^{-9} A at photomultiplier voltage of 1300 V), the diference being explained by the change in geometry and replacement of the photomultiplier (EMI 9558).

Several comparison experiments were needed. Oxygen evolution in sequence of flashes was recorded as described in Ref. 8. Pretreatment with H_2O_2 was done by injecting the reagent in the electrolyte stream and watching for the extra amperometric signal (due to H_2O_2) to rise and subside before firing the flash lamp. Fluorescence at the zero level (dark adapted) and fluorescence induction were monitored in a set up similar to that described in Ref. 11.

DMBIB and 1-bromo-4-nitrothymol were kindly given by Dr. A. Trebst, and 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene by Dr. G. Renger.

Results and Discussion

General characteristics of dark luminescence signals

Fig. 1 shows recordings of the various dark luminescence signals discussed in this report.

An important result obtained with the new apparatus was to show that fully relaxed *Chlorella* cells emit a steady, low background of luminescence. It can be clearly shown by operating the photomultiplier shutter (see S and S′ in Fig. 1). This background signal (amplitude L_0 *) can be monitored for hours (Fig. 2); its half-time is approx. 2 h. Evidently, L_0 manifests the existence of a quasi-permanent, very low level reverse electron flow through the System II reaction center. The origin and site of coupling of the flux of positive charges on the donor side and of negative charge on the acceptor side remain speculative. However, there are reasonable assumptions. Concerning the donor side, one notes that L_0, as well as the other dark luminescence signals (except L_{H2}, see below) requires a functional state of the oxygen-evolving system (see Table Ib), which therefore is the likely coupling site. The flux of positive charges results presumably from a redox equilibrium between O_2 and the oxygen-evolving system, as postulated by Kok et al. [12], in particular maintaining a low concentration of S_2 state. We concluded earlier [5] that this situation could explain the small amplitude of the dark luminescence signals. In agreement with this view are the facts that L_0 is suppressed by removal of O_2 (see Table Ib) and by NH_2OH (see below for the discussion of this effect) as shown on Fig. 2. Another independent argument is our observation (unpublished) on the flash energy dependency of Y_2, the O_2 yield at the 2nd flash in sequence experiments, which cannot be explained by double hits alone but requires that a small amount of S_2, besides the majority: $S_0 + S_1$, is also present in the dark-adapted S states distribution. Concerning the acceptor side, it has been repeatedly assumed (e.g. Ref. 13) that the photosynthetic chain was coupled by another redox equilibrium to an endogeneous reducing system RH, probably at the side of the plastoquinone pool (PQ). This is also borne out by the inhibiting effect of L_0 of DBMIB (oxidized, see Fig. 4e) and benzoquinone

* The symbol L_0 and other similar symbols to be defined later shall be used rather freely to designate also the phenomenon itself.

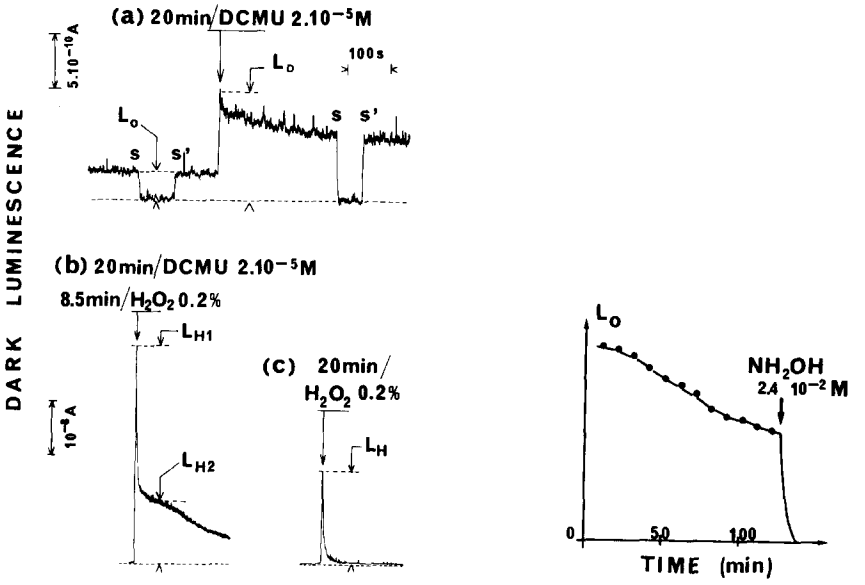

Fig. 1. Recording of dark luminescence signals of *Chlorella*. (a) Background luminescence (L_0) and DCMU-triggered luminescence (L_D); photomultiplier shutter on (S) and off (S') to demonstrate L_0; the cell suspension was incubated for 20 min in darkness prior to DCMU addition (arrow and indicated concentration). (b) H_2O_2 chemiluminescence (L_{H1}, L_{H2}) following DCMU triggering; as in (a), then 8.5 min after DCMU addition, H_2O_2 was injected (arrow and indicated concentration). (c) H_2O_2 chemiluminescence without prior DCMU triggering; cf. (b); note absence of the second wave (L_{H2}). Note the different vertical scale for recording (b) and (c) compared to (a) (20-fold reduction of amplifier gain). The experiments were made with the same batch of algae. Chlorophyll: 150 $\mu g \cdot ml^{-1}$. Temperature: 30°C.

Fig. 2. Background luminescence (L_0) of *Chlorella* as a function of time in darkness. The cell suspension had been exposed to the ambiant laboratory light for about 1 h prior to being introduced in the luminescence apparatus, at zero time. The signal was not recorded until the light-induced luminescence had decayed, i.e. before approx. 10 min. NH_2OH was injected (indicated final concentration) at the time marked by an arrow. Chlorophyll: 150 $\mu g \cdot ml^{-1}$. Temperature: 30°C.

to be discussed later. The following scheme summarizes the working hypothesis for L_0:

$$O_2 \leftarrow \text{(oxygen-evolving system)} \ldots P\text{-}680 \Leftarrow Q \leftarrow B \leftarrow PQ \leftarrow RH \qquad (2)$$

Accordingly, a possible explanation of the very slow decay of L_0 could be a progressive depletion of the RH pool.

Upon addition of DCMU, the dark luminescence rises immediately to L_D (Figs. 1a, 4a and c), at least twice the L_0 level, but sometimes more. Thereafter, it decays very slowly ($t_{1/2}$ 5—10 min) to a level below L_0. A small, more rapid phase is often observed at the beginning.

Thus some variability is observed in the L_D pattern depending on uncontrolled parameters in the physiological states of the algae and indicating at least that the phenomenon is more complex than predicted by the working hypothesis ($QB^- \xrightarrow{DCMU} Q^-B$). It must be noticed that at any rate the decay is incomparably slower than that of Q^- by recombination after light activation in the presence of DCMU ($t_{1/2} \approx 1$ s, see e.g. Ref. 14). This raises a problem:

TABLE I

CHARACTERISTICS OF DARK LUMINESCENCE OF *Chlorella*

See detailed protocol in Material and Methods, L_O, background luminescence; L_D: DCMU-triggered luminescence: L_{H1} and L_{H_2}, H_2O_2 chemiluminescence in the presence of DCMU (first peak and 2nd wave, respectively); L_H, H_2O_2 chemiluminescence. Inhibition: +, complete; (+), partial, 0, no effect or stimulation (in the latter cases, amplitude relative to the control between parenthesis). ANT2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene.

	L_O	L_D	L_{H1}	L_{H2}	L_H
(a) Relative amplitude *	1	4.4	176	26	62
(b) Donor side					
Heat (50°C, 10 min)	+	+	+	(+) (0.56)	+
NH_2OH					
10 mM	+	+	(+)	(+) (0.24)	
50 μM		+	(+) (0.42)	0 (1.27)	(+) (0.56)
Anaerobiosis	+	+			(+) (0.56)
10 μM CCCP	+	+	(+) (0.16)	0 (1.2)	
1 μM ANT2p	+	+	(+) (0.21)	(+) (0.39)	
(c) Acceptor side					
15 μM DBMIB	+	(+) **			
0.2 mM benzoquinone	(+) ***				

* Representative experiment.
** See Fig. 4e.
*** Momentary inhibition, see text.

according to the working hypothesis and referring to Eqn. 2, Q is isolated by DCMU from the endogeneous pool RH, centers in state Q^-B (which were in state QB^- before DCMU addition) should react in a few second to exhaust any excess of S_2; consequently, L_D after a transient peak of several seconds should exhibit a plateau, limited by the slow generation of S_2, to die out with the exhaustion of Q^-B. This and other problems shall be discussed below.

The idea that the dark luminescence was inherently weak because of a very limited supply of holes on the donor side [5] led to experiments of the type: DCMU/t/H_2O_2, where H_2O_2 is added at time t after DCMU. It was expected that H_2O_2, known as a Photosystem II artificial donor [14], could also have an oxidizing action on the donor side due to its ambivalent redox character. Indeed the signal thus produced (Fig. 1b) is much stronger than L_D (see the relative amplitudes in Table Ia). The shape of the H_2O_2 signal is complex: the first peak, L_{H1}, appearing immediately upon the addition of H_2O_2, is followed by a second wave, L_{H2}. Here again, some variability is experienced. The dip between L_{H1} and L_{H2} is not always marked, in which case L_{H2} is still present as a plateau following the decay of L_{H1} or merely as a slower second phase of the overall decay (compare Fig. 6a and b). All dark luminescence signals are increasing function of temperature (this is the reason for the choice of 30°C in the protocol). The shape of L_{H2} is especially sensitive to temperature: at room temperature, it shows up as a plateau of relatively smaller amplitude, but of very long duration [5].

Omission of DCMU before H_2O_2 addition results in a signal, L_H, similar in shape to L_{H1}, but smaller by a factor of 2 or 3 (Fig. 1c and Table Ia); the time integral of the signal is comparable to, although smaller than the corre-

sponding quantity in the presence of DCMU. This is somewhat surprising: keeping in mind that DCMU is supposed to induce the reaction $QB^- \rightarrow Q^-B$, the ratio L_H/L_{H1} should be much smaller than observed. The fact that the amplitudes are of comparable magnitude may be understood if in both cases the limiting step is the oxidation of the oxygen-evolving system by H_2O_2. Noteworthy is the absence (or much reduced size) of a second component, analogous to L_{H2}, when H_2O_2 is used alone.

As is known, luminescence is very much dependent upon events occurring on the donor and acceptor sides of Photosystem II and respond sensitively to all experimental actions modifying or altering this segment of the photosynthetic chain. The same can be said for dark luminescence. Table Ib and c summarizes the results of treatments specific of the donor and acceptor sides of System II on dark luminescence. These treatments will be examined in detail in the following parts of this report. It is significant that all the above-defined signals (L_0, L_D, L_{H1}, L_H) respond in a similar way to these treatments. Noteworthy is the different behavior of L_{H2} which is often much less or not at all affected; the reason for this difference has not been elucidated.

Role of the acceptor side

The mere existence of L_D may be considered as a positive test of the hypothesis of Velthuys and Amesz [4]. Other observations are in favor of this point of view. (1) The dependency of L_D amplitude on DCMU concentration is characteristic of this inhibitor (Fig. 3, left); the concentration giving half the effect corresponds to a $P_{i(50)}$ of 6.4—6.5, in reasonable agreement with that for the photosynthetic electron transport [16]. Note that the rate of decay of L_D is depending on the DCMU concentration exactly as the initial amplitude. (2) Other Photosystem II inhibitors presumably acting at the same site as DCMU also produce a signal similar to L_D, as was recently shown in our laboratory by Van Assche [17]. The case of *o*-phenanthroline is depicted in

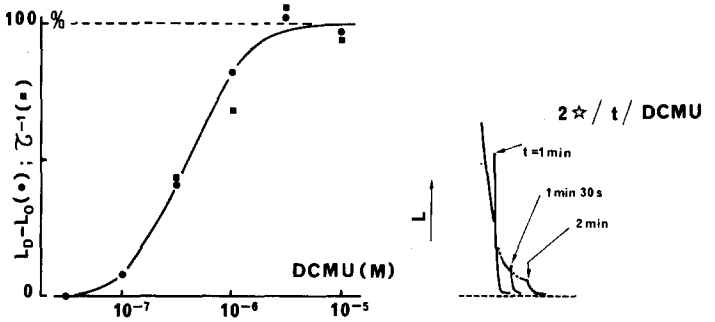

Fig. 3. DCMU-triggered luminescence (L_D) of *Chlorella*. (Left) Relative amplitude of $L_D - L_0$ and relative value of the reciprocal of decay lifetime of L_D (τ^{-1}) as a function of the log of DCMU concentration. Chlorophyll: 105 $\mu g \cdot ml^{-1}$. Temperature: 30°C. (Right) Effect of DCMU added during the decay of the flash-induced luminescence of *Chlorella*. The samples, after having relaxed in darkness in the luminescence apparatus for 3 min, were given two saturating flashes; the reagent was added at a variable time (t) after the flash pretreatment. The pictures are composite tracings relative to several experiments. Final concentration: DCMU: $2.5 \cdot 10^{-6}$ M; chlorophyll: 15 $\mu g \cdot ml^{-1}$. Temperature: 30°C.

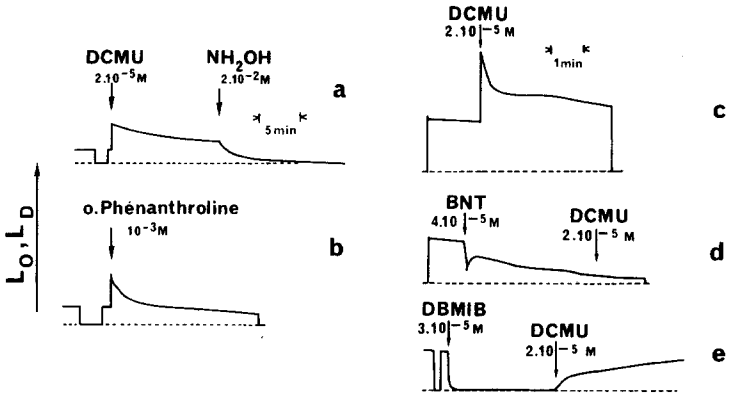

Fig. 4. Triggered luminescence and related phenomena in *Chlorella*. (a and b) Comparison of triggered luminescence (L_D) induced by DCMU (a) or *o*-phenanthroline (b); the reagents are added at time marked by arrows at the indicated final concentration; at several places, the shutter in front of the photomultiplier was turned on and off in order to record its dark current (horizontal dashed line). (c—e) are experiments with a different batch of algae than (a) and (b). (c) Compare with (a) (prominent fast initial phase). (d) Successive additions of 1-bromo-4-nitrothymol (BNT) and DCMU. (e) Successive additions of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) and DCMU. Chlorophyll: 150 $\mu g \cdot ml^{-1}$. Temperature: 30°C.

Fig. 4b. An interesting case of competition is found with 1-bromo-4-nitrothymol, which is believed to act at the same place as DCMU but with a different mechanism [17]; correspondingly, it merely produces a negative effect on L_0 and DCMU, added on top of it, has no longer any effect (Fig. 4d). (3) The site for DCMU binding probably involves a protein (see Ref. 19) which can be altered by mutation. We have observed that mutant C of *Aphanocapsa* which is resistant ot DCMU [9] correspondingly exhibits no L_D * whereas the wild type behaves just like *Chlorella* (not shown).

Several aspects of L_D and similar phenomena, however, are not explained by the working hypothesis and, so far, are not understood. (1) We mentioned in the foregoing that the L_D decay was much too slow compared to the rate of recombination. Comparing DCMU and *o*-phenanthroline (Fig. 4a and b) presents another problem: while the initial amplitude is about the same in both cases, the decay is much faster in the case of *o*-phenanthroline compared to that of DCMU. At any rate and whatever the mechanism of decay, one would expect at least that the time integral of luminescence, ΣL_D, to be identical in both conditions, because the total light emission should be simply related to the amount of Q^- present after the trigger and this presumably is identical in both cases (saturating inhibitor concentrations). (2) Another instance where the invariance of ΣL breaks down is observed when, unlike in the standard protocol, DCMU is added while a normal, photoinduced luminescence is still being evolved (Fig. 3, right). Only at the shortest time is a large stimulation seen (already in Ref. 20); the most important effect is an acceleration of the

* Oxygen evolution in mutant C of *Aphanocapsa* is not completely insensitive to DCMU (half-effect concentration: $3.5 \cdot 10^{-5}$ M). It is interesting that DCMU even at $7 \cdot 10^{-5}$ M did not elicit any L_D response.

decay. Clearly ΣL_D at all times, after DCMU addition, is much smaller than the residual ΣL, if, at the same t value, DCMU had not been added. The accelerating effect is probably identical to a faster DCMU-induced deactivation of S_2, the dominant species of the oxygen-evolving system in *Chlorella* in this time range, already documented in O_2 emission studies [21]. One might speculate that the lack of invariance of ΣL is related to an additional effect of DCMU on the yield of luminescence, i.e. the yield of exciton production. (3) The most serious problem is that Q^- when monitored by fluorescence does not seem to decay after DCMU triggering. The latter induces a substantial increase ($\Delta\Phi$) of the minimum fluorescence yield prevailing in the dark-adapted state. This well-known effect in fact stands as one of the experimental basis of the Velthuys and Amesz hypothesis. But we find that the resulting level ($\Phi_0 + \Delta\Phi$) is absolutely stable in the time range of the L_D decay (not shown). Similarly, the Q pool, measured by the complementary area, above the fluorescence induction curve, does not change during this time (Etienne, A.L., unpublished results).

Dark luminescence was assumed to require the presence of negative charges on the acceptor side quasi-stabilized in the form of QB^-, and perhaps also in the form of a partly reduced plastoquinone pool in slow equilibrium with B. In *Chlorella*, it is not easy to modify chemically the redox balance of carriers on the acceptor side in a controlled way. Yet, the effect of such oxidant as DBMIB is interesting. Addition of the latter immediately kills L_0 (Fig. 4e). DCMU added on top of it slowly restores some dark luminescence. The inhibiting effect means either that QB^- is, even if slowly, in redox equilibrium with the PQ pool and is in fact indirectly oxidized by DBMIB via this pool, or that DBMIB can directly oxidize QB^- as suggested in Ref. 22. The subsequent rise of dark luminescence after addition of DCMU can be understood assuming not only that QB is now isolated from PQ but also that part of the DBMIB which has been reduced while oxidizing the PQ pool now slowly feeds electrons to QB, which again implies a direct redox interaction between QB and DBMIB. Benzoquinone has the same immediate action on L_0 as does DBMIB; but secondary, complex phenomena also occur: after a while, the dark luminescence rises and reaches a level several times L_0, thereafter slowly declining to zero (not shown).

Role of the donor side

The functional integrity of the oxygen-evolving system is a prerequisite for dark luminescence. This is simply shown by the moderate thermal treatment (10 min at 50°C) which specifically destroys the oxygen-evolving system: it also completely suppresses L_0, L_D and L_{H1} (Table Ib). This observation is important because it disposes of the objection that the H_2O_2 signal might result from a trivial peroxidation of the bulk chlorophyll [23].

NH_2OH at high concentration provides another simple means to act on the oxygen-evolving system of *Chlorella* in a destructive way [24], meanwhile functioning as an artificial donor [25]; at low concentration, it is assumed to bind to S_0 and S_1 (or alternatively to reduce S_1 into S_0) [26] very slowly and by so doing it temporarily inhibits the oxygen-evolving system. Its role as an electron donor (or hole scavenger) may only be evidenced indirectly in whole

cells, e.g. by fluorescence [14] or luminescence [27] or O_2 emission kinetics [28]. 10 mM NH_2OH suppresses L_0 and L_D, L_{H1} is partly inhibited (Table Ib). Its immediate effect is also seen in Figs. 2 and 4a; the relatively fast inhibition can be explained by the reduction (see Ref. 28) of the low steady-state amount of S_2 by NH_2OH. Low concentrations of NH_2OH (50 μM) have also proved to be active on L_D and to a lesser extent on L_{H1} (Table Ib) and L_H (not shown). The implications of the effect of NH_2OH at low concentration on L_{H1} and L_H is that either H_2O_2 and NH_2OH compete for the same binding site on the oxygen-evolving system, which, when occupied by NH_2OH, hinders the oxidation of the oxygen-evolving system by H_2O_2, or alternatively, if NH_2OH is believed to simply reduce S_1 to S_0, that H_2O_2 is less effective for oxidizing S_0 than S_1. We shall see below that the second term of the alternative is probably wrong.

Anaerobiosis (bubbling with N_2) results in much the same effect as NH_2OH (Table Ib). This can be understood assuming that, in *Chlorella*, anaerobiosis activates a reducing path which prevents the small concentration of S_2 necessary for dark luminescence to build up. An independent experiment on fluorescence induction in *Chlorella* in the presence of DCMU demonstrates this point (Fig. 5). It is seen that in air the variable fluorescence rise is very limited because recombination competes strongly with the very slow forward photochemical reaction (note the extremely low exciting light intensity used in this experiment). In N_2 on the contrary, the full fluorescence rise is obtained, and the subsequent decay of fluorescence yield in darkness is prevented. The effect of anaerobiosis in *Chlorella* is thus exactly equivalent to that of NH_2OH in Bennoun's classical experiment [14]. Note that this reducing path is specific of the donor side: no spontaneous rise of fluorescence which would indicate a reduction of Q has ever been observed in our strain of *Chlorella*. A final argument in favor of the hypothesis that dark luminescence reflects a small, steady concentration of S_2 is the inhibitory effect of CCCP and 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (Table Ib). These compounds (ADRY reagents (ADRY, acceleration of the deactivation reactions

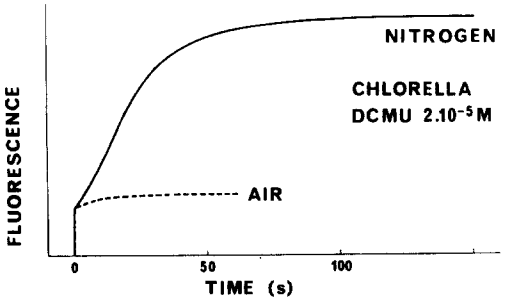

Fig. 5. Effect of anaerobiosis on fluorescence induction of *Chlorella* in the presence of $2 \cdot 10^{-5}$ M DCMU at extremely low exciting light intensity. Continuous curve (N_2): after bubbling the suspension with N_2 for more than 30 min; note the time scale: from the rate of fluorescence rise, it can be deduced that the exciting light intensity was equivalent to one photon hit per center every 30 s. Dashed curve (air): control in air with same intensity. Chlorophyll: 75 $\mu g \cdot ml^{-1}$; flat cell of 0.43 mm thickness. λ: 480 ± 10 nm; λ: 685 ± 15 nm. Temperature: 25°C.

of the water-splitting enzyme system Y), see Ref. 29) are known to accelerate the deactivation of the higher S states.

Concerning the mode of action of H_2O_2, we earlier speculated [5] on the possibility that this substance might act both as an oxidant on the donor side ($H_2O_2 + 2\,H^+ + 2\,e \rightarrow 2\,H_2O$) and as a reductant on the acceptor side ($H_2O_2 \rightarrow O_2 + 2\,H^+ + 2\,e$). Recently, Velthuys and Kok [30] gave evidence for a catalase-like cycle involving these two redox systems and the states S_0/S_2 (and also the states S_{-1}/S_1, S_{-1} being an hypothetical extra state of the oxygen-evolving system more reduced than S_0). In the foregoing, we noted that the experiment with NH_2OH (low concentration) suggested a specific binding of H_2O_2 to the oxygen-evolving system prior to its oxidation by this reagent. Another evidence in favor of the same idea is that the L_{H1} amplitude is saturated at fairly low concentration of H_2O_2, suggesting a limited amount of the target component and similarly, upon successive additions of 0.06% H_2O_2, only the first one elicits a L_{H1} peak (not shown). A more decisive experiment to understand the mechanism of action of H_2O_2 is depicted in Fig. 6. A flash pretreatment was given before adding DCMU and H_2O_2 (Fig. 6, top). The rationale behind this protol is similar to that in Forbush et al. [31]: the first part of the protocol forces a well-defined initial distribution of the centers in the states S_0 and S_1; on the ground that S_0 is practically only formed photochemically from S_3 and that deactivation is mostly a one-step process ($S_i \rightarrow S_{i-1}$), the second sequence of n flashes will result after subsequent deactivation (3 min) in a

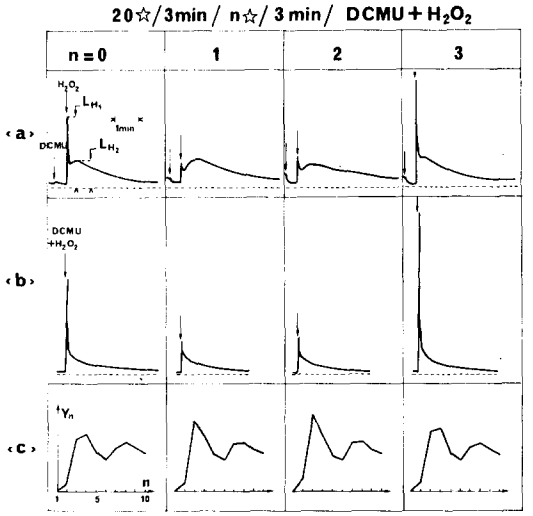

Fig. 6. Dark luminescence of *Chlorella* induced by the addition of DCMU and H_2O_2 (final concentration, respectively $2 \cdot 10^{-5}$ M and 0.95% in (a), 0.5% in (b)) following a flash pretreatment. Protocol indicated at the top, where n is the number of flashes given 3 min before reagents addition (see text). (a—c) are experiments made with different batches of algae. In (a) DCMU addition precedes H_2O_2 addition by 30 s (arrows); in (b) both reagents are added simultaneously. Note the difference in size and shape of second wave (L_{H2}) between the two assays. (c) As a control, O_2 emission (Y_n) in sequence of flashes with same protocole: flash sequence started 3 min after the n flash pretreatment without addition of DCMU and H_2O_2 (saturating electronic flashes; flashing period: 1 s). Chlorophyll: 15 $\mu g \cdot ml^{-1}$ (a) and (b). Temperature: 30°C.

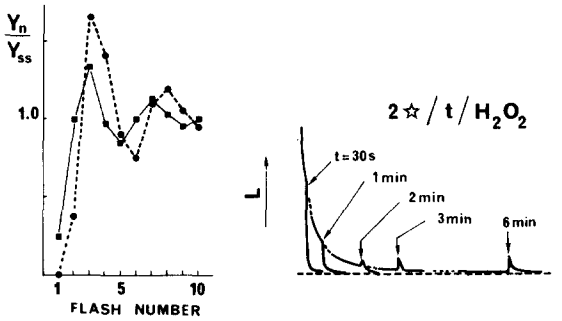

Fig. 7. (Left) Oxygen evolution of *Chlorella* in sequence of flashes following pretreatment with H_2O_2. 100 µl of H_2O_2 (20%, v/v) was injected in the electrolyte stream (flow rate 9 ml · min^{-1}); as fresh electrolyte was continuously admitted, the algal sample kept in the dark was submitted to H_2O_2 for about 8 min, as evidence by an extremely high amperometric current; 10 mm after the injection, the current had returned to a normal value and a first sequence was run (■———■); a second sequence was run 3 min later (●- - - - -●). Saturating flashes; flashing period: 1 s. Temperature: 18°C. (Right) Effect of H_2O_2 added during the decay of the flash-induced luminescence of *Chlorella*. Same protocol as Fig. 3 (right). Final concentration: H_2O_2: 0.5% (v/v). Chlorophyll: 15 µg · ml^{-1}. Temperature: 30°C.

distribution with S_0 minimum when $n = 1$ or 2 and S_0 maximum when $n = 0$ or 3, as may be verified by O_2 emission sequences (Fig. 6c). From the fact that L_{H1} is minimum for $n = 1$ or 2 and maximum for $n = 0$ or 3, it may be concluded that the main interaction of H_2O_2 with the oxygen-evolving system is

$$S_0 + H_2O_2 + 2\,H^+ \rightleftharpoons S_2 + 2\,H_2O \qquad (3)$$

Incidentally, the two experiments shown in Fig. 6 illustrates the variability of the L_{H2} component; it is also another instance of a clear distinction between L_{H1} and L_{H2}, the latter not being a function of n. The above interpretation agrees with that in Ref. 30 and extends it to the case of *Chlorella*. An independent confirmation is provided by an electrode experiment where an algal sample was submitted to a pulse of H_2O_2 (Fig. 7, left, see legend) before performing a flash sequence. It is seen that Y_2 is relatively larger and Y_4 smaller than in the control sequence. This control is simply a second flash sequence run 3 min after the first one, showing that everything has returned to the usual situation and that consequently the anomalous pattern of the first sequence was indeed due to a modification of the initial S states distribution by H_2O_2. It must be added that the result of the electrode experiment is not always as clear as in Fig. 7. A prominent Y_2 is not always seen albeit, even so, Y_4 is relatively small. We often observe a binary oscillation *, starting with Y_3 high, Y_4 low, etc, as reported in Ref. 26. In all cases however, a subsequent assay gives the normal pattern.

The reverse of Eqn. 3 as regards the S_0/S_2 couple, involving now the reducing redox system of H_2O_2 (see Ref. 30).

$$S_2 + H_2O_2 \rightleftharpoons S_0 + O_2 + 2\,H^+ \qquad (4)$$

* This was taken as evidence [26] for a reduction of S_4, the precursor state of O_2, into S_2 by H_2O_2. This is unlikely because, in our experiments, the binary oscillations may be seen even though H_2O_2 has been washed out. Furthermore, our hypothesis that the main effect is the oxidation of S_0 into S_2 (Eqn. 3) is sufficient: in Kok's model, given an initial distribution where S_0 and S_2 are predominant and in about equal amounts, it is readily seen that the oscillation must be binary.

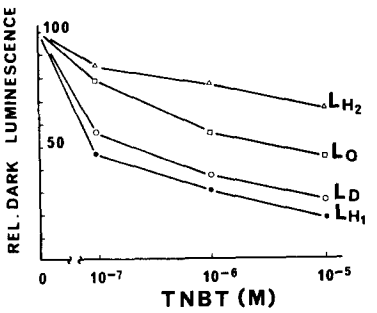

Fig. 8. Effect of tri-*N*-butylstannic chloride (TNBT) on dark luminescence in *Chlorella*: background luminescence (L_0), DCMU-triggered luminescence (L_D), H_2O_2 chemiluminescence (first peak L_{H1}, second wave L_{H2}). The algal sample was incubated in the dark with TNBT at the indicated concentration for 15 min prior to recording the dark luminescence signals. Chlorophyll: 75 $\mu g \cdot ml^{-1}$. Temperature: 30°C.

can also be demonstrated in an experiment (Fig. 7, right) where H_2O_2 is added during the decay of a two flashes-induced luminescence. At $t = 30$ s, when S_2 is relatively abundant, the above reaction results in an immediate inhibition of the photoactivated luminescence. L_H properly is seen later ($t > 3$ min) when the photoactivated luminescence has subsided and the ratio S_0/S_2 is very high. The reaction from right to left in the equilibrium Eqn. 4 might also be an attractive possibility for the slow steady production of S_2 that we postulate to explain L_0 and L_D.

In isolated chloroplasts, dark luminescence in general is low and L_H in particular is only seen when the pH of the suspending medium is below 6 (unpublished results). The likely explanation is that, as expressed in Eqn. 3, the oxidation of S_0 into S_2 by H_2O_2 requires an acid intrathylakoid phase. The implication then is that in dark-adapted *Chlorella* this phase is maintained at a pH substantially below neutrality. Joliot and Joliot [34] have independently reached the same conclusion; the mechanism they envisaged is a pH gradient quasi-stable in the dark driven by the membrane-bound ATPase hydrolysing ATP of mitochondrial origin. An important tool in their demonstration is tri-*n*-butylstannic chloride ($[CH_3(CH_2)_3]_3SnCl$), a potent ATPase inhibitor. In conformation of the above views, we have observed that L_{H1} is sensitively suppressed by tri-*n*-butylstannic chloride (Fig. 8) whereas L_{H2} is almost not affected. Of significance also is the substantial inhibition of L_0 and L_D by tri-*n*-butylstannic chloride, pointing also to a pH effect for the generation of S_2 by O_2 (Eqn. 4, right to left) as suggested in the foregoing. It is interesting that 10^{-5} M tri-*n*-butylstannic chloride has no effect on the O_2 emission of *Chlorella* in sequence of flashes (not shown).

Conclusions

Concerning the acceptor side, our studies on dark luminescence support the hypothesis of Velthuys and Amesz [4] in a first approximation, but actually raise several problems. Of special concern is the negative evidence derived from fluorescence: after DCMU triggering in the dark-adapted state, there is absolutely no decay of Q^- associated to the decay of L_D. This is to be con-

trasted with evidence by Wollman and Thorez [32] showing that in dark-adapted *Chlorella* approx. 50% of the centers are in the state QB^-, which, after DCMU triggering, can readily back react with S_2 and S_3 formed photochemically. We have no explanation for this discrepancy. Concerning the slow decay of L_D, the only explanation we can think of is that it could indirectly result from the presence of uncoupling impurities in the DCMU solution [3], in which case, not unlike the effect of tri-*n*-butyltin chloride (Fig. 8), the decay of L_D would be related to a slow collapse of the otherwise dark-stable proton gradient, consequently slowing down the rate of S_2 production (e.g. Eqn. 4 right to left) which we assume to control dark luminescence.

Surprisingly the conclusions concerning the donor side seem more clearcut. The effect of NH_2OH (low concentration) on L_H and the flash pretreatment experiment (Fig. 6) taken together show that NH_2OH and H_2O_2 compete for the same binding site of the oxygen-evolving system. The alternative considered also in Ref. 26 (NH_2OH reduces S_1 to S_0) is obviously ruled out by our results. If then we speculate that this binding site might also be that for H_2O, the flash pretreatment experiment (together with the electrode experiment (Fig. 7)) suggest the following possibility. While occupying the H_2O site, H_2O_2 oxidizes S_0 into S_2; S_2 thus made chemically can be further oxidized into S_4 photochemically, giving rise to O_2 emission. If this picture is correct, which depends ultimately on the assumption that H_2O and H_2O_2 compete for the same binding site of the oxygen-evolving system then the S states do not have the meaning of the successive oxidation states of a $2\,H_2O$ complex (e.g., $S_0 \equiv H_2O$, H_2O, $S_1 \equiv H_2O$, OH, etc.). It is even conceivable that water binds only at the end of the S cycle and that its oxidation only takes place during the $S_4 \rightarrow S_0$ transition. An interesting consequence is that the protons released during the successive S state transitions might not come, directly, from water.

A final practical conclusion is derived from the fact that the H_2O_2 chemiluminescence is specifically dependent upon the integrity of the oxygen-evolving system. We are considering using to advantage this property to devise a screening test for mutants of algae. Preliminary tests with Photosystem II-deficient mutants of *Chlamydomonas* [10] suggest this possibility: three such mutants (Fl 50, Fl 52 and Fl 54) were assayed for L_{H1} and Fl 50 only showed a negative response, possibly indicating a deficiency of the oxygen-evolving system (also Campagne, J. and Etienne, A.L., unpublished results).

Acknowledgements

This work has been in part supported by a contract with the Communauté Economique Européenne (contract No. 013-76-ESF). The author is indebted to Dr. A. Trebst for the interpretation of the DBMIB effect and to A.L. Etienne and J. Lavergne for a critical reading of this manuscript.

References

1 Amesz, J. and Van Gorkom, H.J. (1978) Annu. Rev. Plant Physiol. 29, 47—66
2 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457—475
3 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250—256
4 Velthuys, B.R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85—94

5 Etienne, A.L. and Lavorel, J. (1975) FEBS Lett. 57, 276—279
6 Fleischman, D.E. and Mayne, B.C. (1973) in Current Topics in Bioenergetics, Vol. 5, pp. 77—105
7 Malkin, S. (1977) in Primary Processes in Photosynthesis (J. Barber, J., ed.), pp. 349—431, Elsevier/North-Holland, Amsterdam
8 Lavorel, J. and Lemasson, C. (1976) Biochim. Biophys. Acta 430, 501—516
9 Astier, C., Vernotte, C., Der-Vartanian, M. and Joset-Espardellier, F. (1980) Plant Cell Physiol., in the press
10 Garnier, J., Guyon, D. and Picaud, A. (1979) PLant Cell Physiol. 20, 1013—1027
11 Haveman, J. and Lavorel, J. (1975) Biochim. Biophys. Acta 408, 269—283
12 Kok, B., Radmer, R. and Fowler, C.F. (1975) Proc. 3rd Int. Congress on Photosynthesis, Rehovot, Israel (Avron, M., ed.), pp. 485—496, Elsevier, Amsterdam
13 De Kouchkovsky, Y. (1964) in Carnegie Institution of Washington Year Book, Vol. 63, pp. 447—453
14 Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357—363
15 Inoue, M. and Nishimura, M. (1971) Plant Cell Physiol. 12, 739—747
16 Zweig, G., Tamas, I. and Greenberg, E. (1963) Biochim. Biophys. Acta 66, 196—205
17 Van Assche, C.J. (1979) in Advances in Pesticide Science(Geissbühler, H., ed.), pp. 494—498, Pergamon Press
18 Trebst, A. and Draber, W. (1979) in Advances in Pesticide Science (Geissbühler, H., ed.), pp. 223—234, Pergamon Press
19 Renger, G. (1976) Biochim. Biophys. Acta 440, 287—300
20 Clayton, R.K. (1969) Biophys. J. 9, 60—76
21 Bouges-Bocquet, B., Bennoun, P. and Taboury, J. (1973) Biochim. Biophys. Acta 325, 247—254
22 De Kouchkovsky, Y. (1975) Proc. 3rd Int. Congress on Photosynthesis, Rehovot, Israel (Avron, M., ed.), pp. 1081—1093, Elsevier, Amsterdam
23 Goedheer, J.C. and Vegt, G.R. (1962) Nature 193, 875—876
24 Cheniae, G.M. and Martin, I.F. (1970) Biochim. Biophys. Acta 197, 219—239
25 Bennoun, P. and Joliot, A. (1969) Biochim. Biophys. Acta 189, 85—94
26 Bouges, B. (1971) Thèse de 3e Cycle, Paris, France
27 Etienne, A.L. (1974) Biochim. Biophys. Acta 333, 497—508
28 De Haan, G.A. (1976) Thèse, University of Leiden
29 Renger, G., Bouges-Bocquet, B. and Delosme, R. (1973) Biochim. Biophys. Acta 292, 796—807
30 Velthuys, B. and Kok, B. (1978) Biochim. Biophys. Acta 502, 211—221
31 Forbush, B., Kok, B. and McGloin, M. (1971) Photochem. Photobiol. 14, 307—321
32 Wollman, F.A. and Thorez, D. (1976) C.R. Acad. Sci. Paris 283, 1345—1348
33 Blein, J.P., Ducruet, J.M. and Gauvrit, C. (1979) Weed Res. 19, 117—121
34 Joliot, P. and Joliot, A. (1980) Plant Physiol., in the press