Biochimica et Biophysica Acta, 325 (1973) 213–229

BBA 46619

KINETICS OF LUMINESCENCE IN THE 10^{-6}–10^{-4}-s RANGE IN *CHLORELLA*

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(Received May 2nd, 1973)

SUMMARY

The decay of luminescence in the 6–600-μs range following a microsecond flash has been studied in *Chlorella*. The decay is highly polyphasic; three kinetic components are outlined, in confirmation of the results of K. L. Zankel (1971, *Biochim. Biophys. Acta* 245, 373–385).

Extrapolation of the decay to zero dark time suggests that a unique metastable species C_+^-, resulting from photochemical charge separation in the System II reaction center, is the substrate of the recombination reaction which gives rise to luminescence.

The fast (5–10 μs) and medium (50–70 μs) phases of the decay denote different stabilization steps, preceding relaxation of the centers by electron and proton transduction to the photosynthetic chain.

NH_2OH specifically inhibits the fast phase and enhances the medium phase. This effect is explained by assuming that the fast phase results from electron transfer from the water splitting system Z to the oxidized primary donor Y.

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), in the presence of NH_2OH elicits another fast phase. It is believed that DCMU affords a parasitic stabilization of C_+^- by forming a complex with Q^-.

INTRODUCTION

This study was primarily undertaken in the aim of investigating on a possible delay between light excitation and the onset of luminescence (or delayed light) emission in the System II part of the photosynthetic apparatus. Following the recombination hypothesis[1,2], quantum conversion and luminescence are expressed as follows:

$$h\nu \rightarrow \varepsilon_F \quad (1a)$$

$$C + \varepsilon_F \rightarrow C_+^- \quad (1b)$$

$$C_+^- \rightarrow C + \varepsilon_L \quad (2a)$$

$$\varepsilon_L \rightarrow h\nu' \quad (2b)$$

Quantum conversion results from creation of "fluorescence" excitons (ε_F)

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

when light is absorbed by the "collector" chlorophyll molecules of the photosynthetic unit (Eqn 1a), followed by trapping of ε_F by the photoactive reaction center (C) and conversion of the latter into its non-photoactive form (C_+^-) (Eqn 1b). The symbol C_+^- implies that, once they are separated by the photochemical act, the + and − charges exist for a while as a metastable, dipole-like pair. C_+^- may be taken as equivalent to the conventional symbol $Z-^+YChl\ Q^-$ as used in Eqn 5 below. Inversely, luminescence results from recombination of charges within the photoelectric dipole C_+^-, with conversion of its energy into a "luminescence" exciton (ε_L) (Eqn 2a), followed by the radiative decay of ε_L in the collector of the photosynthetic unit (Eqn 2b). C_+^- is thus the primary substrate of luminescence. Study of the kinetics of luminescence at very short times (t_L) following a pulse of actinic light is likely to yield evidence on the formation and disappearance of C_+^-. The above description of the primary events in the System II apparatus admittedly is schematic and incomplete; its purpose is to stress the opposite, almost symmetrical character of quantum conversion and luminescence.

Formation of C_+^- is certainly not a one-step process (as expressed by Eqn 1b), since it includes at least the electronic excitation of the reaction center chlorophyll and transfer of charge to one partner (primary acceptor or primary donor). Fluorescence lifetime measurements in the photoactive 0 state[3] indicate that the minimum formation time of C_+^- is of the order of 0.4 ns. Study of these minimum steps by luminescence kinetics is out of question in this t_L range due to the presence of very great fluorescence signal. More generally, formation of C_+^- might occur through several steps:

$$C+\varepsilon_F \rightarrow C_1 \rightarrow C_2 \rightarrow \cdots \rightarrow C_+^- \tag{3}$$

The latter scheme would seem to be contrary to the essence of the recombination hypothesis which rather requires that C_+^- be the earliest metastable state following quantum conversion, if it is to recombine without having to cross an activation barrier of excessive height. For the sake of completeness, however, this scheme must be considered. It predicts that, provided that the duration t_I of the actinic flash is shorter than the total lifetime of the process from C to C_+^- in Eqn 3, the luminescence signal L must rise to a transient maximum before decaying. This question was implicitly raised when Arnold and Davidson[4] conjectured that extrapolation of L to very short t_L could reach a sizable fraction of the prompt fluorescence signal F. When the present study was started, little data had been obtained under experimental conditions likely to permit observation of the transient maximum of L. Zankel[5] had studied luminescence excited with electronic flashes, but he could only monitor the signal beyond $t_L \approx 50\ \mu s$. While the present study was being completed, Haug *et al.*[6] published decay curves starting at $t_L \approx 100$ ns, they demonstrated in a clearcut way that the fastest kinetic component of luminescence seen under their experimental conditions was altogether distinct in amplitude and lifetime from "prompt" fluorescence. However, since these authors used the phosphoroscope method with rather long actinic pulses ($t_I \approx 1$ ms), their results were unlikely to exhibit the hypothetical delay in the formation of C_+^-.

The results to be presented here show that no delay exceeding a few microseconds is detectable when using microsecond flashes. Meanwhile, under such flashing light conditions the decay of L, monitored from 6 μs to a few 100 μs, displays

several fast phases (essentially confirming Zankel's results); they are modified in a significant manner in the presence of NH_2OH and (or) 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and under other experimental conditions. It is shown that this kinetic behavior is best discussed in connexion with two classes of events (stabilization and relaxation) which are part of the overall process of transduction of electrons and protons through the photosynthetic chain.

MATERIALS AND METHODS

Algae (*Chlorella pyrenoidosa*, Chick, Emerson strain) were cultivated and harvested as described previously[7]. They were used at an equivalent chlorophyll concentration of 25–50 $\mu g \cdot ml^{-1}$ at room temperature (20 °C), either in their culture medium or in 0.05 M phosphate buffer (pH 6.5). In some cases, H_2O in the buffer was replaced by ^2H_2O. For the high-NH_2OH-pretreated samples, the algae were incubated in the presence of 10^{-2} M NH_2OH for 0.5 h in darkness, then whashed 4 times by centrifugation and suspension in fresh culture medium.

Luminescence was monitored as previously described[8], except for the following details. The light source was a pulsed xenon laser (TRW Instruments, Model 83 X R) delivering flashes of 0.3-μs duration and power of 250 W at half-height. 75% of the light energy was concentrated in three green lines (5260, 5353, 5395 Å). The flashes were not saturating as indicated by the light curves for L (see Fig. 3) or fluorescence intensity F. The flow system included a flat, parallel-walled (0.4-mm thickness) quartz cell, ensuring homogeneous illumination of the algal sample. The luminescence signal, in the form of amplified photoelectron pulses, was stored into 200 points, with 3-μs resolution, using a multichannel analyzer (Intertechnique). 10000 decays had to be averaged in order to achieve a satisfactory compression of the statistical noise. As the photomultiplier was not turned off during the flash an artifact resulting from the intense fluorescence of the sample was recorded during approx. 4 μs; for this reason, the data has been considered reliable only for $t_L > 6$ μs; further correction was applied to the signal to take into account the dark count (corresponding to dark current) and the after pulses (resulting from the strong illumination of the photocathode by the flash). The integrated count of the correction from 6 to 600 μs was of the order of 5% of the L signal proper.

Two protocols were defined, depending on the number of flashes each algal sample received during operation of the pulsed laser at 10 Hz:

(1) 100 flashes (in symbol, 100 *). The algal sample was standing in the illumination cell for 10 s; then, it was renewed by a single stroke (2.5 ml) of the pump (Prominent, Type 0510);

(2) 1 flash (in symbol, 1 *). The algal suspension was continuously flown by gravity with the pump maintaining a constant head at such a rate that each sample in transit through the illumination cell could only see a single flash and that the linear displacement of the circular illuminated sample was not more than 1% of its diameter during the whole time span of measurement (approx. 450 μs).

RESULTS

(1) *Dark-adapted or light-adapted states*

In the dark-adapted state (1 *), a highly polyphasic decay is observed in the

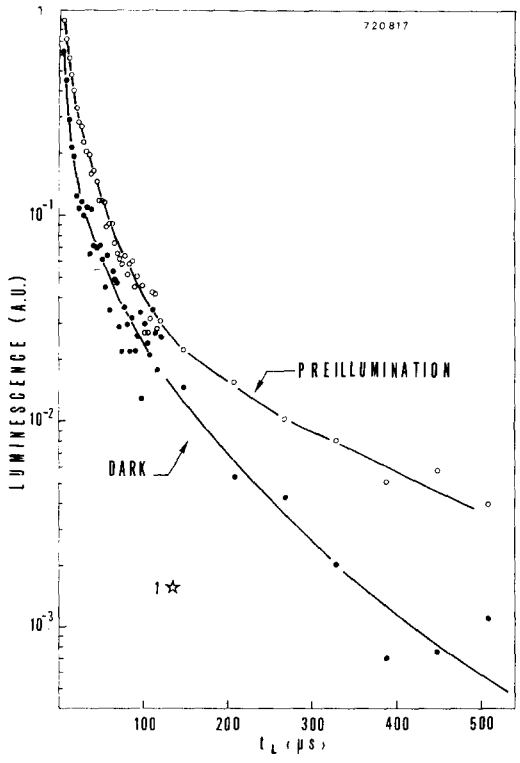

Fig. 1. Logarithm of luminescence intensity as a function of time (t_L) after the flash in *Chlorella*. Each sample received one flash (1 *) and was dark adapted or preilluminated as indicated (see text). Luminescence is expressed with the same arbitrary unit for both assays. Each recording is the average of 10000 decays and starts at $t_L = 6\,\mu s$.

10^{-6}–10^{-4}-s range (Fig. 1). Roughly speaking, three kinetic components may be outlined as indicated in Table I (line 1), where the results of a semi-quantitative analysis of decays in terms of exponential (first-order) components are given as lifetimes τ and amplitudes α. The large amplitude of the initial 6-μs component is noteworthy. The number of components is in agreement with Zankel's observation with chloroplasts[5], and the τ values are very similar. However, they are at variance with the results of Haug *et al.*[6]: τ of their fastest component is $\approx 35\,\mu s$ (estimated by myself). A notable difference in the experiment must be recalled: the latter authors used rather long light pulses. Preillumination with low intensity white light — followed by 2.5-s darkness during transfer of the sample into the illumination cell — induces an increase of α (by a factor of about 2) (Fig. 1) and of τ, in particular for the slowest phase (Table I, line 2). Under steady-state flashing light (100 *), the respective values of the kinetic parameters (α and τ) of the components are not much different from what is observed after preillumination (Table I, line 3). The effect of light adaptation is in agreement with observations made in sequence[9,5] or induction[10,11] experiments where L at the onset of illumination is invariably lower than in the steady-state light condition. The present results extend this property to the microsecond t_L range.

TABLE I

KINETIC CHARACTERISTICS OF LUMINESCENCE DECAYS

Amplitudes (α) and lifetimes (τ) have been calculated by standard graphical procedure from semi-log plots of decays such as shown on Figs 1–5. α values are expressed in fraction of initial amplitude ($\alpha_0 = 1.0$); τ values are expressed in μs.

Fig. No	*Experimental conditions*[a]		*Fast phase*		*Medium phase*		*Slow phase*		*ΣL (6–600 μs)*	*Line*
			α	τ	α	τ	α	τ		
1	1*, N.A.	Dark	0.83	6	0.16	45	0.02	130	1.0[b]	1
		Preillumination	0.83	12	0.14	50	0.03	360	1.67	2
—	100*, N.A.	Dark	0.68	13	0.27	60	0.04	220		3
2	1*, dark	$NH_2OH = 0$	—	6	—	60	—	—	—	4
		10^{-6} M	0.9	7	0.09	60	0.01	440	—	5
		10^{-5} M	0.71	7	0.24	80	0.05	285	—	6
		10^{-4} M	0.43	8	0.46	75	0.11	280	—	7
		10^{-3} M	0.27	6	0.6	70	0.13	130	—	8
3	1*, 10^{-2} M NH_2OH pretreated	$I = 100$	—	—	0.9	60	0.1	165	—	9
		50	0.2	4	0.68	69	0.12	150	—	10
		30	0.3	4	0.61	72	0.09	180	—	11
		10	0.34	5	0.58	75	0.08	210	—	12
—	10^{-3} M NH_2OH	1*	—	—	0.85	75	0.15	150	—	13
		100*	—	—	0.92	52	0.08	140	—	14
4	1*, 10^{-2} M NH_2OH pretreated	Control	0.28	6	0.61	75	0.11	190	1.0[b]	15
		$5 \cdot 10^{-5}$ M DCMU	0.68	8	0.22	110	0.11	190	0.76	16
4	100*, 10^{-3} M NH_2OH	Control	0.12	6	0.85	54	0.03	210	—	17
		10^{-5} M DCMU	0.51	4	0.46	63	0.03	200	—	18
—	1*, N.A.	Control	0.84	6	0.15	36	0.01	135	1.0[b]	19
		10^{-4} M DCMU	0.87	5	0.10	36	0.02	110	0.29	20
5	1*	H_2O[c], dark	0.88	20	0.11	125	0.09	> 600	1.0[b]	21
		^2H_2O[c], dark	0.98	12	0.018	85	0.002	480	1.62	22
		^2H_2O, preillumination	0.98	18	0.021	75	0.002	360	1.84	23
—	100*	Control	—	—	0.96	54	0.04	200	1.0[b]	24
	10^{-3} M NH_2OH	Ethanol (5.7%)	—	—	0.97	45	0.03	200	0.76	25

[a] 1* = one flash per sample, 100* = hundred flashes per sample. N.A. = no addition. I = relative intensity of the flash.

[b] 1.0 = ΣL normalized to one.

[c] H_2O or ^2H_2O as solvent in the buffer.

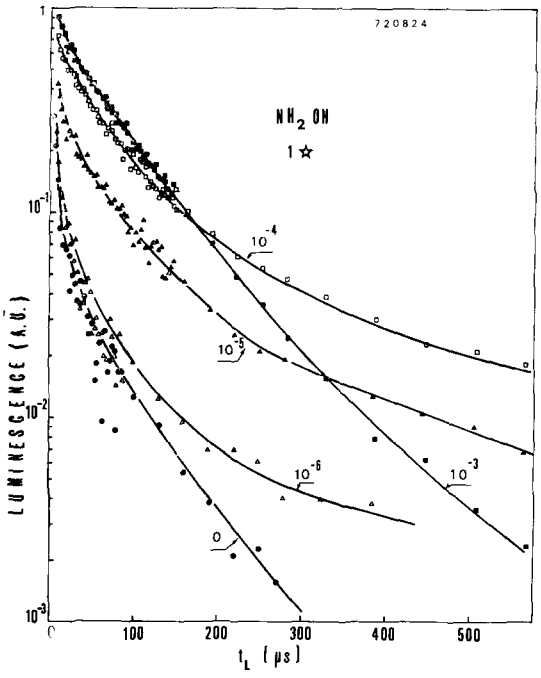

Fig. 2. Effect of NH_2OH on the luminescence decay in *Chlorella*. Molar concentrations of NH_2OH as indicated. Each sample received one flash (1 *). Other conditions as in Fig. 1.

(2) Effect of NH_2OH

NH_2OH is known to inhibit the slow phase and enhance the fast phase of the L decay[12]. Furthermore, this poison, when acting at a low concentration, delays, by two steps, the maximum of L[13] and that of O_2 emission[14] in sequence experiments; at a higher concentration, it inhibits altogether O_2 emission and it is photooxidized by System II in a simple one quantum process[15]. It is believed that, at low concentration, NH_2OH forms a complex on the donor side of System II and competes with Z for electron donation to the center[14] and that, in addition at a high concentration, it destroys or disconnects Z[16]. The effect of several concentrations of NH_2OH on the L decay at 1 * in the 10^{-6}–10^{-4}-s range is depicted in Fig. 2; three points are to be noted:

(a) all decay curves extrapolate fairly well to the same intensity at $t_L=0$;

(b) up to 10^{-4} M NH_2OH, the typical three-components pattern of the decay is essentially preserved, although with modifications: τ of the third (slow) phase is much increased and remarkably a of the first (fast) phase progressively decreases, while that of the second (medium) phase becomes more and more prominent (Table I, lines 4–7);

(c) at 10^{-3} M NH_2OH a qualitative change occurs: the medium phase is largely dominant — giving a nice first-order decay over almost two decades — the first phase has (almost) vanished and the third one is much reduced in amplitude (Table I, line 8).

For high NH_2OH-pretreated algae (Fig. 3), the decay at 1 * is much the same

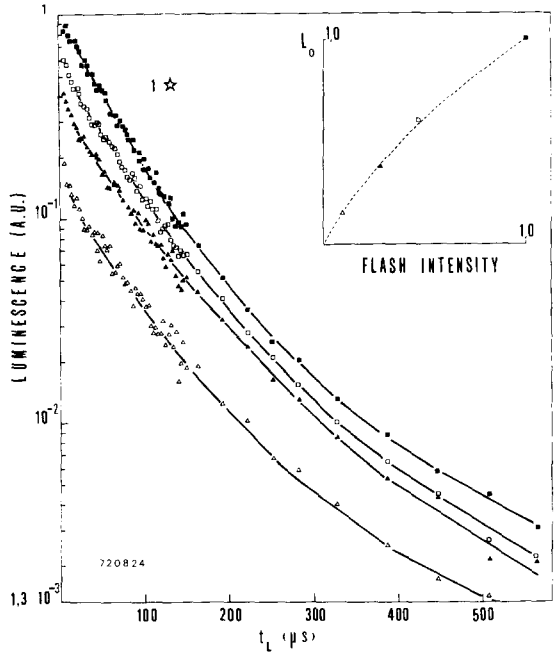

Fig. 3. Decay of luminescence in *Chlorella* pretreated with 10^{-2} M NH_2OH (see Materials and Methods). Each sample received one flash (1 *). Luminescence is recorded at several flash intensitities, in arbitrary units: ■, 100; □, 50; ▲, 30; and △, 10. Insert, light curve of luminescence intensity (L_0) extrapolated at $t_L = 0$. Other conditions as in Fig. 1.

as in the presence of the inhibitor (point c, above): the medium component is also dominant with a τ slightly shorter. In this experiment, the laser intensity was also varied by neutral filters; it is seen that the pattern of decay is almost unaffected (Table I, lines 9–12). The light curve (for this initial luminescence intensity, L_0) is of hyperbolic type, without any sign of an inflexion point (Fig. 3, insert). Clearly, the full intensity of the laser beam was far from saturating; this point was also controlled in a separate experiment by looking at the light intensity dependence of the F signal, during the flash.

Comparison of the protocols 1 * and 100 * shows that in the presence of 10^{-3} M NH_2OH, the decay preserves its simple, quasi-monophasic character, the only difference being that τ is shorter for the light-adapted state (100 *) than for the dark-adapted state (1 *) (Table I, lines 13 and 14).

It is interesting to note that the characteristic quasi-monophasic pattern of decay induced by high NH_2OH concentration does not depend, once produced, on the actual presence of the inhibitor. This fact suggests that the medium phase of the L decay does not depend on NH_2OH oxidation and that, consequently, the latter is not a good donor.

(3) Simultaneous effect of hydroxylamine and DCMU

The effect of DCMU in the 1–100-ms t_L range is complex; depending on the decay phase and condition of observation, this substance may inhibit or stimulate

luminescence (for a review, see ref. 17). In the short t_L range Zankel[5] observed that only the medium decay (in this case, $\tau \approx 35\ \mu s$) was unaffected by DCMU. The combination "NH_2OH+DCMU" was used in an interesting fashion by Bennoun[18] to show that, due to NH_2OH photooxidation, the center was left in a state ZChl Q^-, resulting in complete inhibition of luminescence and blocking of fluorescence at the high P level (in the 0.1–10-s t_L range). A different picture was seen by myself at shorter t_L using a higher concentration of NH_2OH than did Bennoun: addition of DCMU occasioned only a partial inhibition of luminescence (Lavorel, J., unpublished). This observation was confirmed and extended in the present study (Fig. 4). When DCMU is added to the high NH_2OH-treated or -pretreated sample, a fast component reappears, followed by the same sort of medium decay, albeit somewhat slower (Table I, lines 15 and 16). Three points are to be noted:

(a) the fast phase in the presence of DCMU extrapolates to the same initial level L_0 as in the control (NH_2OH-treated or pretreated);

(b) the decays are very similar, whether the sample received 1 * (Fig. 4, left) or 100 * (Fig. 4, right), in particular as regards the order of magnitude of the amplitude (Table I, lines 15–18). This is in contrast with the above-mentioned experiment[18] where, at $t_L > 20$ ms, complete inhibition of luminescence was found after several flashes;

(c) by comparison a much smaller effect of DCMU was observed in an experiment without NH_2OH (Table I, lines 19, 20); this evidently points to a different significance of the fast initial phase in the control and the medium phase in the presence of NH_2OH.

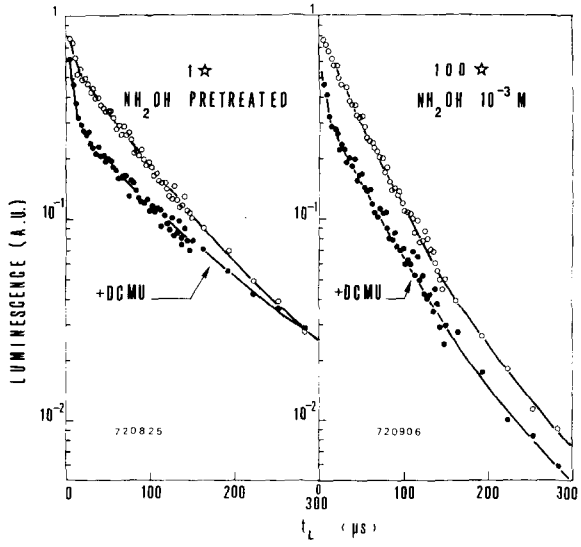

Fig. 4. Effect of DCMU on luminescence decay in *Chlorella* pretreated with 10^{-2} M NH_2OH (left) or in the presence of 10^{-3} M NH_2OH (right). Each sample received one flash (1 *, left) or hundred flashes (100 *, right). Note that L_0, the extrapolated medium phase at $t_L = 0$, is lower for DCMU than for the control. Other conditions as in Fig. 1.

(4) Effect of various treatments

The recombination hypothesis has gained much strength from the finding of Barbieri *et al.*[9] that luminescence was partly under the control of the S states of System II — a result essentially confirmed by Zankel[5] in the 10–100-μs t_L range. For this reason and also since it is known that the pH in the vicinity of the center affects luminescence[19], substitution of ^2H_2O to H_2O, once a fashionable tool in the kinetic studies of photosynthesis, should deserve a renewal of interest. The effect of ^2H_2O on L at 1 * is shown in Fig. 5. In this experiment, the supporting medium is a phosphate buffer (see Materials and Methods). Comparing Figs 1 and 5, one sees that luminescence is dependent on the ionic composition of the medium — a fact already observed by Kraan[20] at longer t_L: the L decay is much faster in the culture medium as compared to the phosphate buffer (Table I, compare lines 1 and 21). In ^2H_2O, the pattern of kinetic components is not much different from that in H_2O, the amplitude is larger in the whole t_L range and preillumination has a lesser effect: ΣL (6–600 μs), *i.e.* the sum of light or the time integral of the L signal from 6 to 600 μs, is increased by 67% in ordinary water and by only 14% in heavy water (see also Table I, lines 22 and 23). This is in contrast with observations made with about 10-ms actinic pulses (to be published) where the effect of ^2H_2O is more pronounced.

Recently, evidence has been presented to consider luminescence at $t_L \approx 1$ ms

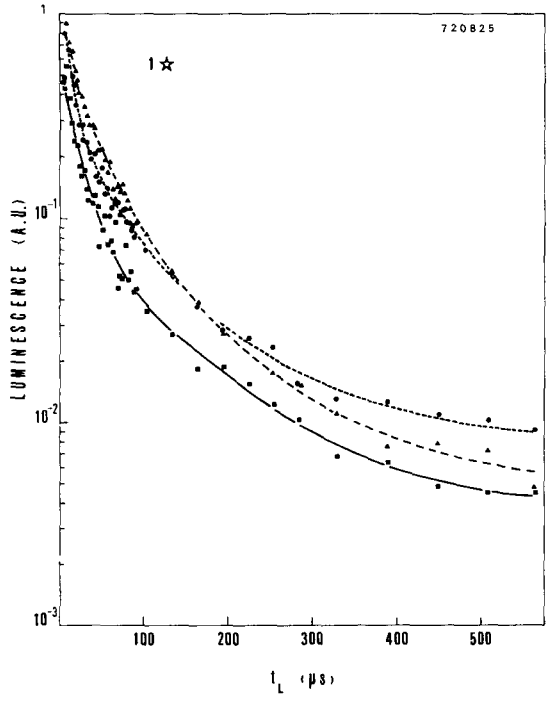

Fig. 5. Decay of luminescence in *Chlorella* suspended in phosphate buffer with H_2O (■) or ^2H_2O (●, ▲). Algae are dark adapted (■, ●) or preilluminated (▲) (see text). Each sample received one flash (1 *). Other conditions as in Fig. 1.

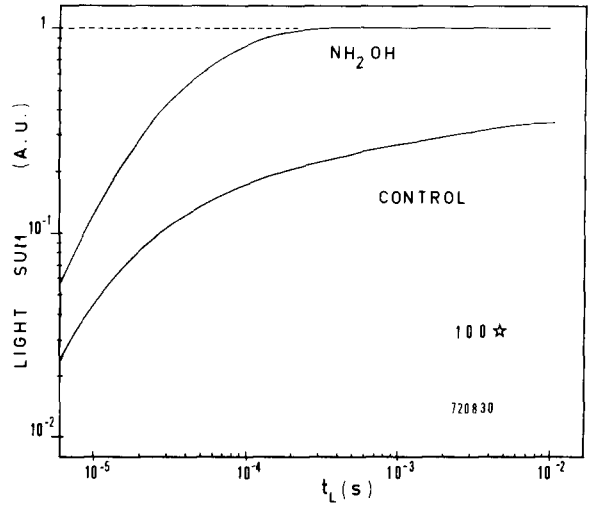

Fig. 6. Sum of light, *i.e.* luminescence intensity integrated from 6 μs to t_L in *Chlorella*. Log–log plot. Each sample received hundred flashes (100 *). Lower curve, dark-adapted control; upper curve, 10^{-3} M NH_2OH. Other conditions as in Fig. 1.

as a good index of the electric membrane potential[11,21]. In this connection, the effect of ethanol has been tested. This substance, at low concentration, abolishes the field indicating absorbance change at 515 nm[22]; it presumably is able to penetrate the cell wall of *Chlorella*. In the presence of 5.7% ethanol, the L decay of NH_2OH-treated algae is a little faster and the amplitudes lower than in the control (Table I, lines 24 and 25).

(5) Sum of light

ΣL (t_{L1}, t_{L2}) has been little considered in the literature, yet it is of much significance. Assuming a time-invariant exciton yield for the recombination reaction (Eqn 2a), one sees that ΣL is proportional to the amount of C_+^- which has disappeared by the luminescence route. This measurement is severely underestimated and its meaning uncertain if this sum is truncated, as is often the case for obvious technical reasons. This difficulty stems from the very strong polyphasicity of the L decay in the whole t_L scale. The effect of truncation at t_{L1} (head-cut-off) is especially important from this point of view.

The function ΣL (6 μs, t_L) = f (t_L) has been plotted in Fig. 6 for a high NH_2OH-treated sample and a control sample. In the former case, the decay is well completed at $t_L \approx 1$ ms, whereas, in the latter case, ΣL is still increasing by 40% between 1 and 10 ms. In a separate experiment, this residual increase was found to drop to about 5% between 10 and 100 ms. Thus assuming that the normal decay is practically over at $t_L \approx 100$ ms and correcting the ΣL values by extrapolation to $t_{L1} = 0$, one finds that the ratio of $\Sigma L(0, \infty)$ in the presence of NH_2OH to that in the control is of the order of 3.5. The importance of the head-cut-off effect is illustrated by the following remark: if the analysis was limited to $t_L \approx 1$ ms, the conclusion would have been that the luminescence was totally inhibited by NH_2OH! Another useful conclusion from

Fig. 6 is that approx. 50% of the luminescence is already emitted at $t_L \approx 50$–100 μs.

DISCUSSION

Some of the concepts that will be used in the following section have been presented or proposed, and discussed more thoroughly in a report which is being published elsewhere[17].

(1) Unicity of the luminescence substrate C_+^-

As indicated above at several places, there is reasonable evidence that the same initial level of luminescence intensity L_0 is common to many experimental conditions. One might thus conjecture that, in every circumstance, a unique species or metastable state C_+^-, resulting from the System II photochemical charge separation, is the substrate of the luminescence or recombination reaction (Eqn 2a). Incidentally, the present data do not support the view (Eqn 3) that C_+^- is not the immediate product of the photochemical act; at least in the microsecond range, no delay in the appearance of luminescence was found. The postulate of the unicity of C_+^- must be qualified in a least two senses:

(a) C_+^- has probably four subspecies C_+^- (Sn) ($n=1$, 4) depending on the S state of the reaction center, *i.e.* the number of + charges accumulated on Z (ref. 23). The oscillations of the L sequence[9,5] suggest that the luminescence ability of the four subspecies increase from S_1 to S_4:

$$S_1 < S_2 < S_3 < S_4 \tag{4}$$

The inequalities in Eqn 4 are the consequence of the increase of electrostatic energy of the dipole C_+^- from S_1 to S_4 (due to accumulation of + charges on Z) and of the resulting decrease of activation energy for recombination[17].

(b) While it is postulated that a unique species C_+^- is found at the same concentration at $t_L=0$, under identical actinic light conditions, it does not follow that the corresponding L_0 must be invariable with respect to other experimental factors. L being the rate of the recombination reaction expressed as radiative decay, it can be modified by several factors besides the concentration of C_+^-. Important factors are the activation energy for recombination, which is dependent on the electro-osmotic state of the thylakoid membrane[11], and the fluorescence yield Φ (see below, L relation).

(2) Stabilization, relaxation and System II turnover

The significance of the fast phase of the L decay is best discussed in terms of a general kinetic scheme which is depicted on Fig. 7. Two classes of kinetic events must be distinguished.

Firstly, stabilization. It evidently counteracts the tendency towards recombination and loss of photochemical efficiency. C_+^- is the first member of this class since it is already strongly stabilized as compared to the excited state of the reaction center chlorophyll. Further stabilization results from depletion of C_+^- by several (reversible) steps to give various species (in arbitrary number and kinetic relationship in Fig. 7). Protonation equilibria on the Q and Z sides of the reaction center, as

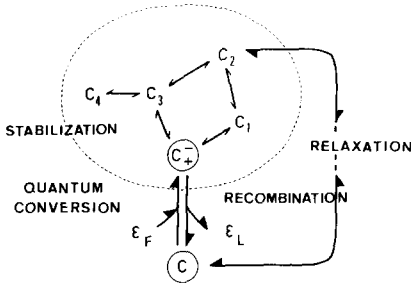

Fig. 7. General kinetic scheme of stabilization and relaxation (see text). The C's are various forms of the reaction center: photoactive form (C), luminescence substrate (C_+^-), stabilized forms (C_1, C_2, C_3, C_4).

introduced by Kraan[20], are essential steps of stabilization. Other natural or "parasitic" steps will be considered below.

Secondly, relaxation. Its effect is to restore the photoactive state C of the reaction center; its meaning is electron and hole transduction through the carriers of the photosynthetic chain. The complex bilateral character of relaxation (hole to the water end, electron to the System I end) is greatly simplified in Fig. 7. The relaxation time constant is the turnover time of System II. It cannot be defined in a simple way, since relaxation has a periodicity of four for the + charges, whereas it is presumably a one-step process for the − charges. One may assume that the limiting process is the reoxidation of Q^- with a lifetime of the order of 0.1–1.0 ms (ref. 24). This critical value thus separates, in the time domain, the stabilization area from the relaxation area.

As a consequence of this analysis, the fast phases of the *L* decay in the 100-μs t_L range are likely to reflect several stabilization steps, since their lifetimes are distinctly shorter than the System II turnover time. It is readily understood why, in the data of Haug *et al.*[6], the fast phase did not show up conspicuously. The reason is similar to that already given in the introductory part: if the duration t_I of the actinic pulse is much larger than the mean lifetime of stabilization, a quasi-equilibrium or steady state is already established in the stabilization area at $t_L = 0$, when the observation begins. Note that all steps, whether of stabilization or relaxation, are considered to be essentially reversible (except possibly for the $S_4 \rightarrow S_0$ reaction which yields oxygen) in order for C_+^- to exist, even in very small concentration, at all t_L values when a luminescence signal can be detected. Lastly, it may be noticed that, in keeping with the definition of relaxation as the restoration of C, recombination must also be viewed as a special type of relaxation.

(3) The fast phase ($\tau \approx 5$–10 μs). Effect of NH_2OH

Experimental evidence points to a specific action of NH_2OH on the Z side of System II (refs 14–16). We have also seen (Fig. 2) that this poison gradually suppressed the fast phase of the *L* decay when acting at concentrations of 10^{-6}–10^{-4} M and that, at higher concentrations, the fast phase was irreversibly destroyed. An explanation can be found along the following line:

Hypothesis 1. The fast phase reflects the transfer of a + charge from a primary

donor Y (*cf.* ref. 18), which could be the center chlorophyll itself, to the Z water-splitting device:

$$Z\text{–}{}^{+}YChl\,Q^{-} \rightleftharpoons {}^{+}Z\text{–}YChl\,Q^{-} \tag{5}$$

Hypothesis 2. At low concentration, NH_2OH forms a complex on the donor side of System II (ref. 14). In so doing, NH_2OH prevents the transfer of + charges from Y to Z. NH_2OH somehow occupies a site in between Y and Z:

$$Z\text{–}YChl\,Q + NH_2OH \rightleftharpoons Z\,(NH_2OH)\,YChl\,Q \tag{6}$$

In this position, NH_2OH is not photooxidized fast enough to compete significantly with recombination in the 100-μs t_L range.

Hypothesis 3. Above 10^{-3} M, NH_2OH splits the Z moiety off the reaction center[16], which is left in the form YChl Q. As in Hypothesis 2, NH_2OH is only moderately efficient as a donor.

Hypothesis 2 is essential in order to explain why the fast phase is diminished in the presence of NH_2OH. If NH_2OH was merely taking the place of H_2O on Z, the step Eqn 5 would still occur, leaving the fast phase intact. The extent of association of NH_2OH with the center according to the equilibrium Eqn 6 can be appreciated from the vertical position of the decay curve at $t_L = 50$–$100\,\mu$s (medium decay) as a function of NH_2OH concentration (see Fig. 2). Assuming complete association at 10^{-3} M, a rough estimate of approx. $10^5\ M^{-1}$ for the association constant of NH_2OH with the reaction center is obtained by this procedure.

Note that the enhancement of L in the presence of NH_2OH at $t_L \approx 0.1$–1.0 ms, which was not well understood previously because of the head-cut-off effect, is readily explained in the present hypothesis when the decay can safely be extrapolated to zero t_L: as noted above, the same amount of C_+^- is produced by the flash, with or without NH_2OH, and the only effect of the inhibitor is to block the fast stabilization step on the donor side of the reaction center thereby allowing enhanced recombination. The comparison of ΣL in the control and in the presence of 10^{-3} M NH_2OH (Fig. 6) also demonstrates clearly that, in the latter condition, a larger fraction of C_+^- relaxes by recombination.

(4) The medium phase ($\tau \approx 50$–$70\ \mu s$)

In the presence of low concentration of NH_2OH, the behavior of the medium phase is complementary to that of the fast phase: it develops and gains prominence in the exact measure of the disappearance of the fast phase (Fig. 2). At first sight, it might be assumed that, according to Hypotheses 1 and 2, this behavior simply reflects the competition between NH_2OH and Z for electron transfer to ^{+}Y. This interpretation meets with two objections. Firstly, it gives no explanation for the medium phase in the absence of NH_2OH–but, it might be argued that, in the latter condition, the nature of that phase is different. Secondly, when, after high NH_2OH treatment, the medium phase is fully developed, it is clearly independent on the presence of NH_2OH (Figs 2 and 3). A tentative explanation could be to attribute it to the Q side of the System II reaction center, for instance as reflecting a protonation step (*cf.* ref. 20):

$$\cdots Q^- + H^+ \rightleftharpoons \cdots QH \tag{7}$$

[the change in pattern of L decay in different supporting media (Figs 1 and 5) would then result from a modification of the intracellular pH]. The conspicuous extension of the medium phase in the Z split system (compare 10^{-4} and 10^{-3} M NH_2OH in Fig. 2) would suggest that the equilibrium Eqn 7 is strongly displaced towards the protonation side as compared to its position in the intact system. Another possibility would be that the process is entirely controlled by recombination in this t_L range (in other words, luminescence would be of the "deactivation type" — see below); in this case, it should be attended by a (limited) $\Delta\phi$ decay of similar speed (*i.e.* of lifetime $\approx$1 ms). To my knowledge, this point has not been settled. Obviously, this explanation cannot hold for the medium phase of the intact system.

(5) The slow phase ($\tau > 100$ μs). Modulation by the fluorescence yield

As pointed out above, luminescence should be modulated by the fluorescence yield according to the L relation[2]:

$$L = (\Phi) \cdot J \tag{8}$$

where J is the net rate of production of ε_L and (Φ) the yield of radiative decay of ε_L, provided that (Φ) is identical with, or closely correlated to the macroscopic fluorescence yield[17]. Zankel[5] has interpreted the slow phase in this sense. Actually, this modulation should operate along the whole L decay; it will however become the dominant factor in a t_L range where the reoxidation of Q^-, which is assumed to control the fluorescence level, is the limiting kinetic factor. This should typically occur during the System II relaxation phase (see above). One notices that, according to Eqn 8, (Q^-) being a factor in both (Φ) and J, the corresponding lifetime should be, roughly speaking, half that of the decay of (Q^-) (ref. 17). This approximately agrees with the actual lifetime of the slow decay.

A difficulty, however, subsists. The L relation predicts that the light curve should be sigmoidal at low intensity[7], which has been observed by several authors[8,10,20,25]. Instances of monotonous light curves have also been reported[10,26] and the one shown in the present paper (Fig. 3) is of this type. Furthermore, according to Mauzerall[27], Φ keeps increasing for nearly 100 μs after a 1-ns actinic flash and, with the above interpretation of the L relation, an increase in L — in fact, a delay (see Introduction) — should have shown up in my experiments. There is no obvious explanation for these discrepancies. It might be necessary to drop the assumption basic to the L relation that ε_L, once created by a recombining reaction center, is able to escape from the corresponding "open" unit and to reach neighboring, possibly "closed" (fluorescent) units; the L relation would then take on a simpler meaning, (Φ) being predominantly the fluorescence yield ϕ (0) of an open unit, with only minor contribution from the average local yield.

(6) The mode of action of DCMU

The effect of DCMU on the L decay in the Z split system (high NH_2OH treated or pretreated) possibly offers a clue on its mode of action (Fig. 4):

Hypothesis 4. DCMU forms a complex with C_+^- on its Q side:

$$\cdots Q^- + DCMU \rightleftharpoons \cdots Q^- - DCMU \quad (9)$$

The C_+-DCMU complex is still able to recombine, although at a lower rate, and to be stabilized by protonation.

In others words, this complex is another, "parasitic" luminescence species that is formed during the fast phase seen in Fig. 4 (alternatively, one could assume that equilibrium Eqn 9 occurs also with the oxidized form Q and that the complex undergoes a rearrangement when Q is reduced). Complexation affords some stabilization since the extrapolated L_0 is smaller for C_+^--DCMU than for C_+^- (see legend of Fig. 4); in Hypothesis 4, blocking of electron transfer to plastoquinone (PQ) is considered of steric nature rather than a consequence of this special stabilization. Note that *a fortiori* the observed reduction in amplitude of the medium phase would not be consistent with the assignation of this phase to the electron transfer from Q^- to the PQ pool, as cessation of this transfer in the presence of DCMU would then result in enhanced recombination and luminescence.

Several facts may be cited in support of Hypothesis 4. If DCMU combines only with Q^-, or if its affinity to Q^- is larger than to Q, addition of the poison should shift the redox equilibrium to the left side:

$$Q^- + H^+ + \tfrac{1}{2}PQ \rightleftharpoons Q + \tfrac{1}{2}PQH_2 \quad (10)$$

An instance of this effect may be seen in the experiment of Fig. 8 (experimental details in ref. 28): addition of DCMU to a chloroplast suspension during the dark reoxidation of Q^- following illumination results in a transient increase of $\Delta\Phi$ (a similar effect has been observed, indirectly on luminescence, by Clayton[29]); if DCMU was merely blocking the electron flow, one would only expect the decay of $\Delta\Phi$ to stop. Another instance is perhaps the well-known increase of the dark-adapted 0 level of fluorescence[30]. The same explanation holds, assuming that even in this state the PQ electron pool is not completely empty: the fact that preillumination with System I light enhances the oxygen burst supports the latter assumption[31].

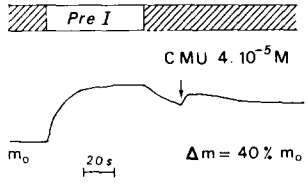

Fig. 8. Effect of DCMU added in the dark after preillumination in chloroplasts (spinach). Flow method (details in ref. 28). m_0 is the dark level of fluorescence. During preillumination in the main reservoir (pre I), the suspension is continuously sampled in the flow circuit, the fluorescence level increases and reaches a steady value (Δm). Note that DCMU was added in the reservoir about 30 s after the preillumination light was turned off.

Normally, in the t_L domain which is considered in this report, luminescence must be of the "leakage" type[17]: for the obvious reason of overall efficiency, recombination must be a quite negligible pathway for the decay of the electron-hole pool standing in the stabilization area (see Fig. 7) and the main decay must occur by relaxation. Such is not the case when the relaxation electron flow is blocked or, at

much larger t_L values, when the states S_2 and S_3 disappear by deactivation; the recombination might well be the only possible pathway for relaxation. In such cases luminescence is of the "deactivation" type and should obey the law:

$$L = -\frac{d(C_+^-)}{dt} \tag{11}$$

which has been verified, in particular in the DCMU system[17].

In this connection, experiments of Fig. 4 with 100 * needs further explanation, since the amplitude of the signals are not much different than in the 1 * experiment. Bennoun[18] has shown that, in the presence of DCMU and NH_2OH (low concentration), luminescence at $t_L > 20$ ms was completely inhibited after several flashes, which he explained by the accumulation of the reaction center in the inactive form ZChl Q^- resulting from NH_2OH oxidation. If this is applied to the present experiment, one would have expected a very small signal, since only a few out of the 100 flashes would have been operative. One might argue that the high NH_2OH case is different and that NH_2OH in the Z split system is only a poor electron donor (which is certainly true, as shown above). But, NH_2OH photooxidation in this condition is a well documented fact[15]. However, A. L. Etienne (personal communication) in this laboratory has recently shown that a large number (at least twenty) of saturating flashes were necessary in the case of high NH_2OH+DCMU system to exhaust the NH_2OH oxidizing capacity. The reason could be that after each flash, a fraction only of C_+^- is irreversibly blocked as C^- (or equivalent stabilized forms) due to NH_2OH oxidation and the remaining fraction presumably recombines. This suggests that the relaxation transfers of charges out of C_+^- are not truly independent phenomena and that, due perhaps to electrostatic interaction, the departure of the + charge is slowed down when the − charge is immobilized by complexation of Q^- by DCMU (subsequent protonation on that side should ease this constraint and permit transfer of the + charge to the artificial donor). The case in point (Fig. 4) is probably explained by this situation of competition between recombination and NH_2OH oxidation together with experimental conditions (low flashing frequency and non-saturating light) allowing a large turnover time for each center.

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