KINETIC ANALYSIS OF THE FAST PHASE OF THE DELAYED FLUORESCENCE EXCITED BY NANOSECOND LASER PULSES IN CHLORELLA*

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A self-beating photon-counting apparatus for measuring the decay of delayed fluorescence in the microsecond time range has been constructed. The exciting light source is a nitrogen laser (wavelength, pulse duration, output energy and repetition time of pulses are 337.1nm, 2.5 ns, 1 mJ and from 10 ms, respectively), or a dye laser pumped by it. The functioning of the detector, consisting of a photomultiplier and 512-channel analyzer (with 20 μs minimum channel width), the pumping unit (for providing fresh samples) and the laser is synchronized. The decay of the delayed luminescence of Chlorella analyzed in terms of exponential components consists of four components with 3, 36, 160 and 600 μs decay times. The rate constants of the back-reactions of the stabilization process of the PS II reaction center complex on the donor side are 1.5×10^5, 1.0×10^4 and 2.0×10^3 s^{-1}. The depths of the three states of stabilization from the energy of the first excited singlet state of chlorophyll are 503, 530 and 554 meV.

1. Introduction

Delayed fluorescence (luminescence) from green plants was discovered by Strehler and Arnold [1]. Since its discovery, this delayed light has become a very effective tool in studying the primary events of photochemical energy conversion in the photosynthetic apparatus. It gives information about the pigment system and the charge separation processes, and is considerably influenced by characteristic membrane parameters, such as pH gradients and the electric field across the membrane [2, 3].

Delayed luminescence at room temperature is attributed to the multi-step back-reaction (recombination) of the photooxidized and photoreduced products of the light reaction [2–5] in the photosystem II (PS II). The recombination is always in competition with the various stabilization steps, at the end of which the light energy absorbed by the antenna pigments generates charge separation (electric dipole) in the reaction center (C_+^-)

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and is finally stored in photochemical form. The stabilization of the electric dipole needs several hundred microseconds (PS II turnover time). During this time, the decay of delayed luminescence is determined only by the stabilization steps and does not influence the kinetics of C_+^-. After this time the decay is determined by the deactivation process of C_+^-. Lavorel [2] calls the two types of luminescence "leakage type" and "deactivation type". The latter type of luminescence has been investigated extensively [2, 3]. Detection of "deactivation-type" delayed light does not require special techniques. On the other hand, few data are available so far about "leakage-type" luminescence.

The aim of our study was to construct an apparatus for measuring delayed luminescence in the microsecond (or longer) time-range and to attempt the analysis of decay curves, in other words the kinetic study of the steps of stabilization of the reaction center.

2. Experimental

For measuring the fast phase of delayed luminescence the exciting flash should be short (compared to the lifetimes of the stabilization reactions) and intensive (because of the very weak luminescence), and the apparatus should have a short response time.

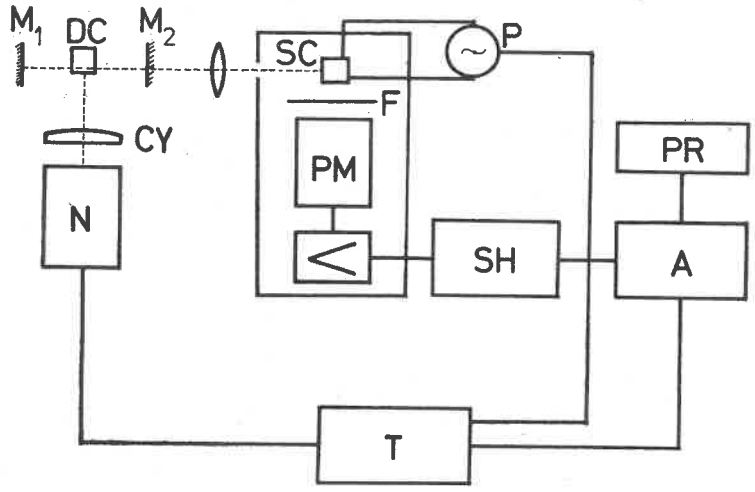

Fig. 1. Block diagram of the apparatus. N nitrogen laser, CY cylindrical lens, DC dye laser cuvette, M_1 and M_2 mirrors, SC sample cuvette, P pumping unit, F filter combination, PM photomultiplier, < amplifier, SH shaping unit, A multichannel analyzer, PR printer and T triggering unit

Therefore, neither the phosphoroscope method [5] nor the flash lamp techniques [6, 7] were used, but an apparatus with a pulsed nitrogen laser (Fig. 1). The flash duration, the output energy, the emission wavelength and the highest flash repetition rate are 2.5 ns, 1 mJ, 337.1 nm and 100 Hz, respectively. The synchronization possibilities are satisfactory as the jitter is less than 1 μs. The algal suspension was excited either directly by the UV laser beam or by a dye laser (POPOP) pumped with the N_2 laser. The

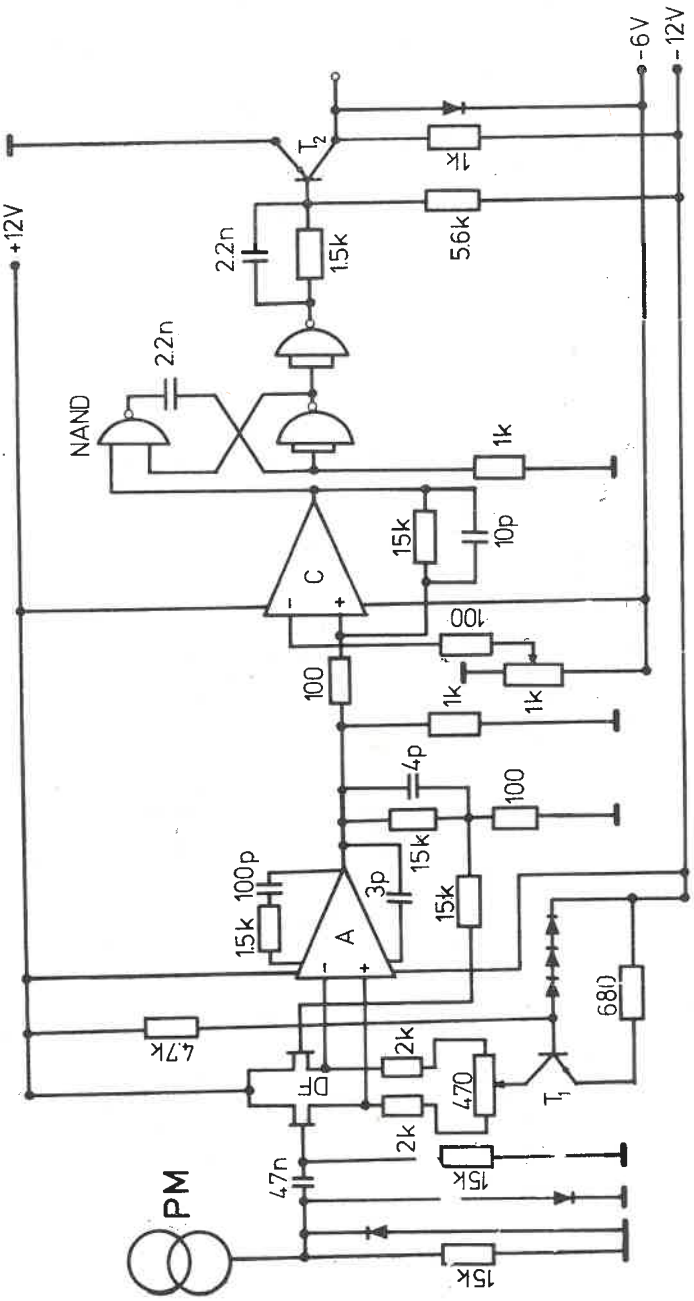

Fig. 2. Electric circuits of the amplifier and the shaping unit. PM photomultiplier, DF dual FET (TIS 26), A amplifier (F 709 PC), C comparator (F 710 PC), NAND gates (K 1 LB 553) and T_1 and T_2 transistors (BF 224 and OC 44 K, respectively)

chlorophylls can be excited effectively by using POPOP dye generating in the Soret band of chlorophyll absorption (420 nm).

The luminescence quanta are detected by a photomultiplier (EMI 9558 A) protected from scattered light by a filter combination. To get a satisfactory signal-to-noise ratio and response to high frequencies, the photomultiplier is used as a photon-counter. The electric pulses are amplified, uniformized (shaping unit) and, finally, counted by a multichannel analyzer. The smallest channel-width is 20 μs and the maximum number of channels is 512. The counting rate of the analyzer is about 1 MHz. The functions of the laser, the analyzer and the pumping unit (providing fresh sample after each measurement) are

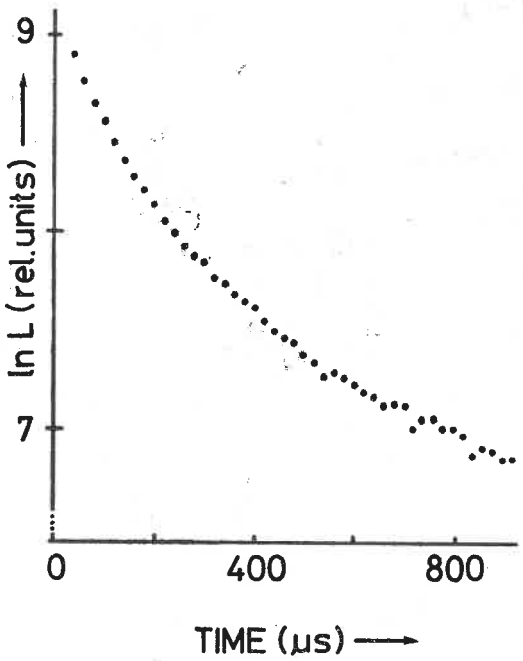

Fig. 3. Logarithm of luminescence intensity as a function of time in Chlorella

synchronized by an electric digital pulse generator (trigger). After completion of the repetitive measurements, the contents of the channels can be printed.

The electrical circuits of the amplifier and the shaping unit can be seen in more detail in Fig. 2. The signal is fed through a low-frequency cut-off filter to the input of a source-follower difference amplifier. The amplified signal enters the noninverting input of the integrated circuit comparator, giving very narrow (100 ns) pulses with standardized amplitudes (TTL levels) and extremely short (1–2 ns) rise and fall times. By varying the reference voltage on the inverting input, the greatest signal-to-noise ratio can be achieved. The pulses leaving the comparator are narrow and have small amplitudes to be fed directly into the analyzer. This is why we need a monostable multivibrator constructed from NAND gates, which makes the pulses broader (2 μs), and a simple transistor amplifier between the

comparator and the analyzer. The occultation time (dead time) due to oversaturation of the photomultiplier and the amplifier is about 40 μs. The green algae (Chlorella pyrenoidosa, Chick, Emerson strain) were cultivated and synchronized in our laboratory under standard conditions (see e. g. [9]). The measurements were performed at 20°C. Each point of the experimental decay curve of delayed luminescence is the average of 6,000 measurements with an accuracy of a few percent.

The decay of the rapid delayed luminescence is polyphasic, as can be seen in Fig. 3, where the logarithm of the relative intensity of luminescence (L) is plotted against time (t). This shows the existence of several luminescent components.

3. *Decomposition of the decay curves, discussion*

A decomposition of the decay curves into first-order components of the form

$$L = \sum_i \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \tag{1}$$

is generally accepted. Here the sum of the amplitudes α_i is normalized to unity, and τ_i is the lifetime of the i-th component. Zankel [6] observed three components, which lifetimes of 10, 35 and 200 μs. Lavorel [8, 9] could distinguish a fast ($\tau_1 = 10$ μs), a medium

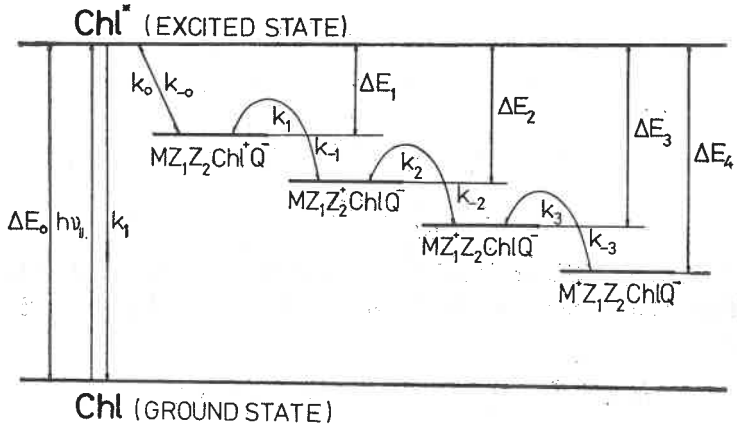

Fig. 4. Energy level diagram of microsecond time scale reactions in the PS II reaction center

($\tau_2 = 50$–70 μs) and a slow ($\tau_3 = 200$ μs) phase. The correlation of these lifetimes to the half-times of the stabilization reactions seems by no means definite.

According to our present knowledge, the mechanism leading to the "leakage-type" luminescence can be summarized as follows (Fig. 4). Upon absorption of a quantum, $h\nu_{II}$, chlorophyll goes into an excited state ($\Delta E_0 = 1800$ meV) which may be de-excited by emitting fluorescence with rate constant $k_f = 1/15$ ns $= 6.67 \times 10^7$ s^{-1} [10], or by trans-

ferring energy to the reaction center to cause a charge separation, $M-Z_1Z_2\mathrm{Chl}^+Q^- - \mathrm{PQ}$. Here Z_1 and Z_2 are two secondary electron donors and Q is the primary electron acceptor. Charge pools (M, an unidentified species and plastoquinone, PQ) can be found on both sides of the reaction center complex. The charge separation is very fast ($k_0 = 2\times 10^9\ \mathrm{s}^{-1}$), but the back-reaction is rather slow ($k_{-0} = 1.3\times 10^3\ \mathrm{s}^{-1}$), corresponding to the small delayed light yields [11, 12]. The depth of this energy trap, ΔE_1, can be calculated from either the Boltzmann relationship or the maximum efficiency for light energy capture in photosynthesis ($\Delta E_1 = 500$ meV) [6, 11, 13]. The stabilization steps consisting in electron transfer on the donor side are separated from those of the acceptor side as they are independent events; they are shown in Fig. 4. The rate constants of the reactions leading to the dipole $M^+Z_1Z_2\mathrm{Chl}Q^-$ are $k_1 = 1/6$ μs $= 1.67\times 10^5\ \mathrm{s}^{-1}$, $k_2 = 1/35$ μs $= 2.85\times 10^4\ \mathrm{s}^{-1}$ and $k_3 = 1/200$ μs $= 5\times 10^3\ \mathrm{s}^{-1}$ [14]. The PS II turnover time is approximately 600 μs, equivalent to the time needed for reoxidation of Q^- by the plastoquinone pool: $D^+\,\mathrm{Chl}\,Q^- - \mathrm{PQ} \overset{k_4}{\rightarrow} D^+\,\mathrm{Chl}\,Q - \mathrm{PQ}^-$. ($D = MZ_1Z_2$ and $k_4 = 1/600$ μs $= 1.67\times 10^3\ \mathrm{s}^{-1}$).

Within the frame of the above picture the decay of the leakage type luminescence can be quantitatively explained if the probability of finding the reaction center in the $M-Z_1Z_2\mathrm{Chl}^+Q^- - \mathrm{PQ}$ state after a very short excitation is considered as a function of time. In the kinetic equations the back-reactions should be taken into account, together with the fact that the luminescence does not influence the time-dependence of the different states. Thus:

$$\dot{x}_1 = -k_1x_1 + k_{-1}x_2, \tag{2}$$

$$\dot{x}_2 = k_1x_1 - (k_{-1}+k_2)x_2 + k_{-2}x_3, \tag{3}$$

$$\dot{x}_3 = k_2x_2 - (k_{-2}+k_3)x_3 + k_{-3}x_4, \tag{4}$$

$$\dot{x}_4 = k_3x_3 - k_{-3}x_4. \tag{5}$$

Here x_i ($i = 1, 2, 3, 4$) is the probability of finding the + charge on the i-th place on the donor side (these states are shown in Fig. 4) and $\dot{x}_i$ denotes time derivation. The initial conditions are:

$$x_1|_{t=0} = 1 \quad \text{and} \quad x_2|_{t=0} = x_3|_{t=0} = x_4|_{t=0} = 0. \tag{6}$$

We are interested only in the time variation of x_1:

$$x_1 = \alpha_1 \exp(-\kappa_1 t) + \alpha_2 \exp(-\kappa_2 t) + \alpha_3 \exp(-\kappa_3 t) + \alpha_4, \tag{7}$$

where the amplitudes α_i and the rate constants κ_i are rather complicated functions of k_i and k_{-i}. κ_i results from the characteristic equation of the system (2)–(5) and α_i can be calculated from equation (6) as the solution of a linear system equations with four variables. These functions were treated by a computer technique to obtain a fit of experiment and theory (see later).

The probability of finding Q in reduced form is $\exp(-k_4 t)$. Thus, the observed luminescence is

$$L = [\alpha_1 \exp(-\kappa_1 t) + \alpha_2 \exp(-\kappa_2 t) + \alpha_3 \exp(-\kappa_3 t) + \alpha_4] \exp(-k_4 t). \quad (8)$$

The decomposition given by this equation can be compared to Eq. (1). If $k_{-i} \ll k_j (i, j = 1, 2, 3)$, i. e. the back-reaction rates are negligible compared to those of the forward reactions, then $\kappa_i = k_i$ and $\alpha_4 = 0$. In this case, Eq. (8) yields the components discussed above. If, however, not every back-reaction rate is small, decomposition as in Eq. (1) will not give the true rate constants because $\kappa_i \neq k_i$.

The theoretical curves given by Eq. (8) were fitted to the measured decay. The k_i values were taken from the literature (discussed above) and the k_{-i} values were varied to get a good approximation. The curves were normalized to the value measured (or calculated) at 40 μs after the flash (Fig. 5). A satisfactory agreement of experiment and

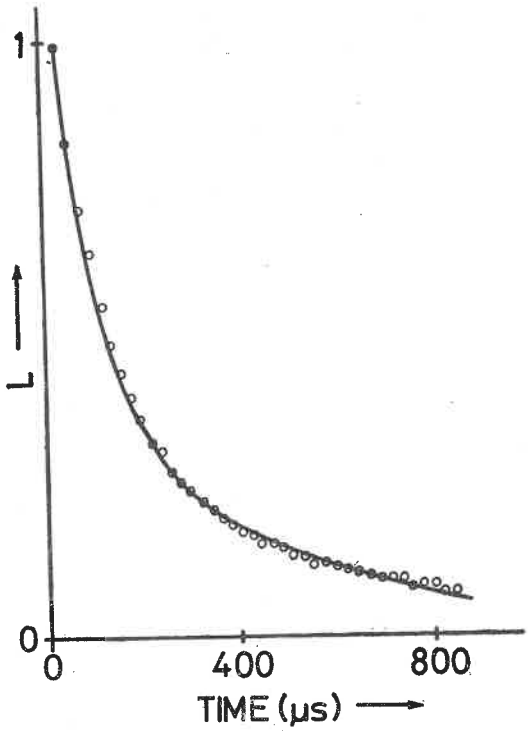

Fig. 5. Fit of the theoretical curve (Eq. 8) to the experimental points. ○ Measured values and —— theoretical curve

theory can be obtained with $k_{-1} = 1.5 \times 10^5\ s^{-1}$, $k_{-2} = 1 \times 10^4\ s^{-1}$ and $k_{-3} = 2 \times 10^3\ s^{-1}$; $\alpha_1 = 0.479$, $\alpha_2 = 0.270$, $\alpha_3 = 0.175$ and $\alpha_4 = 0.076$; $\kappa_1 = 3.33 \times 10^5\ s^{-1}$, $\kappa_2 = 2.6 \times 10^4 s^{-1}$ and $\kappa_3 = 4.5 \times 10^3\ s^{-1}$. According to these results, the mechanistic decomposition given by Eq. (1) may be supported. Our decay times (3, 36 and 160 μs) obtained by simulation

correspond fairly well with the approximate half-times of the forward reactions (6, 35 and 200 μs). The deviations can be attributed to the back-reactions.

From the k_{-i} values, reasonable approximations can be found for the depths of the different stabilization states. Using the Boltzmann relationship (with $kT = 26$ meV)

$$\frac{k_{-i}}{k_i} = \exp\left(-\frac{\Delta E_{i+1} - \Delta E_i}{kT}\right), \tag{9}$$

$\Delta E_2 = 503$ meV, $\Delta E_3 = 530$ meV and $\Delta E_4 = 554$ meV. The energy gaps fall in the order of magnitude of the thermal energy. Thus, the energy loss due to the stabilization processes is not considerable, as it reaches only a few percent of the absorbed energy.

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