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EXTERNAL ELECTRIC FIELD EFFECTS ON PROMPT AND DELAYED FLUORESCENCE IN CHLOROPLASTS

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Summary

An electric field pulse was applied to a suspension of osmotically swollen spinach chloroplasts after illumination with a saturating flash in the presence of DCMU. In addition to the stimulation of delayed fluorescence by the electric field, discovered by Arnold and Azzi (Arnold, W.A. and Azzi, R. (1971) Photochem. Photobiol. 14, 233—240) a sudden drop in fluorescence yield was observed. The kinetics of this fluorescence change were identical to those of the integrated delayed fluorescence emission induced by the pulse. The S-state dependence of the stimulated emission was very similar to that of the normal luminescence. We assume that the membrane potential generated by the pulse changes the activation energy for the back reaction in Photosystem II. On this basis, and making use of data we obtained earlier from electrochromic absorbance changes induced by the pulse, the kinetics of the field-induced prompt and delayed fluorescence changes, and also the amplitude of the fluorescence decrease, which was about 12% for a nearly saturating pulse, are explained. Our results indicate that in those reaction centers where a decrease of the activation energy occurs the effect of a pulse can be quite spectacular: the back reaction, which normally takes seconds, is completed in a few hundred microseconds when a sufficiently strong pulse is applied. Measurements of the polarization of the stimulated luminescence supported the interpretation given above.

Only 2.8% of the back reaction was found to proceed via transition of reexcited chlorophyll to the ground state, both during the field pulse and in the absence of the field.

Abbreviations: DCMU, 3(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

It was first shown by Arnold and Azzi that luminescence of osmotically swollen chloroplasts could be stimulated by applying an external electric field [1]. The effect was surprisingly large: a 60-fold enhancement by a relatively small external field of 600 $V \cdot cm^{-1}$ has been reported [2] and in the present study even larger values were observed. A detailed description of the kinetics and field dependence of the stimulation was given by Ellenson and Sauer [2] who also carried out experiments with two successive pulses of opposite or equal polarity. Because the structure of the system and the kinetics and amplitude of the membrane potentials generated by the external field were insufficiently known, no satisfactory explanation of their data could be given.

From a recent study of the electrochromic absorbance changes induced by an external electric field and a careful consideration of the geometry of the swollen chloroplasts, we were able to determine the kinetics and the value of the membrane potentials generated by the external field pulses [3,4]. On this basis we have studied the luminescence stimulation by an electric field.

Chloropyll *a* luminescence in vivo is usually attributed to a reversal of Photosystem II electron transport, resulting in a recombination of charges in the reaction center and reexcitation of chlorophyll [5,6]. In terms of this hypothesis a stimulation of luminescence may be explained by two mechanisms: (1) a stimulation of the rate of back reaction and (2) an increase of the probability that a recombination leads to the emission of a photon. From the measurement of a field effect on prompt fluorescence we show in the present study that the first mechanism indeed occurs and is the sole cause of the stimulated luminescence.

A preliminary account of part of this work was presented at the 5th Int. Congress on Photosynthesis.

Materials and Methods

Spinach was grown in the laboratory at about 22° C and a light intensity of about 3000 lux. Chloroplasts were isolated as described elsewhere [7] and resuspended in a 50 mM Tricine (*N*-tris(hydroxymethyl)methylglycine) buffer (pH 7.8) containing 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$. The chlorophyll concentration, determined according to the method of Whatley and Arnon [8] was adjusted to 1 mM and the chloroplasts were stored at 0° C. Just before the measurement, the chloroplasts were diluted 200-times in distilled water.

The experimental set-up used for the measurements of changes in prompt and delayed fluorescence induced by an external field was similar to that earlier used to measure electrochromic absorbance changes [4]. Fluorescence and delayed fluorescence were filtered by a Scott AL 680 interference and RG 630 colored glass filter. In order to protect the photomultiplier from scattered light and fluorescence excited by the actinic flash, the photomultiplier was gated during the flash. The flash-induced absorbance changes at 320 nm were measured with a single beam apparatus described in Ref. 9.

Results and Discussion

Origin of the field-induced luminescence; S-state dependence

It was first shown by Zankel [10] that if adapted chloroplasts are subjected to a series of short saturating flashes, the intensity of the luminescence emitted at 90 or 500 μs after the flash exhibits a periodicity of four, related to the S-state mechanism at the donor side of Photosystem II. The luminescence induced by an external electrical field in a suspension of osmotically swollen chloroplasts (blebs) measured 0.5 ms after the flash showed a similar flash dependence as the normal luminescence (Fig. 1). The same result was recently also reported by Babcock et al. [11]. These observations confirm the idea that the luminescence induced by an electric field is due to a stimulation of the normal luminescence, originating from Photosystem II. Also, since the membrane potential is appreciable only at places where the bleb wall consists of a single membrane [3], the experiment indicates that the donor side of Photosystem II in the bleb wall is still functioning.

The question whether the field-induced luminescence originates from system II_α or system II_β (see Ref. 12) was examined by varying the intensity of the actinic flash in DCMU-treated blebs. If the intensity was lowered to such a level

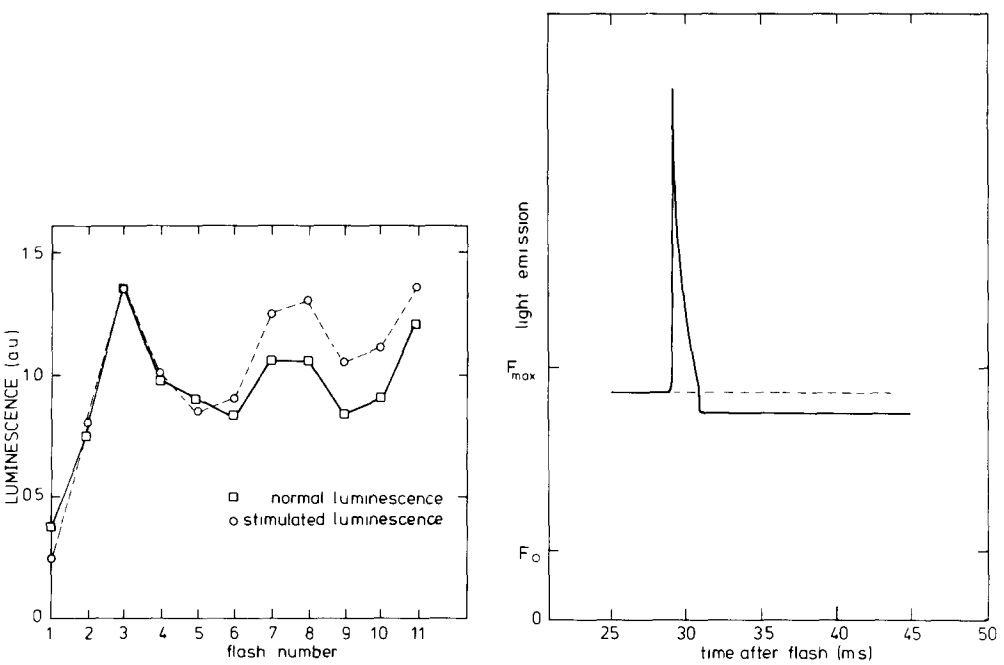

Fig. 1. Normal (□) and field-induced (○, F_{ex} = 360 V · cm^{-1}) luminescence in blebs, measured 0.5 ms after an actinic flash as a function of the flash number. The intensities are normalized at the third flash.

Fig. 2. Prompt and delayed fluorescence emission from a suspension of blebs after a saturating flash given at t = 0 in the presence of 5 μM DCMU. Fluorescence was excited by measuring beam (480 nm) from 25 to 45 ms after the flash. Solid line: 28 ms after the flash an electric field pulse of 3 ms, 1300 V · cm^{-1} was applied. The transient light emission observed during the pulse is due to enhanced luminescence. Dashed line: control measurement, no electrical field pulse was given.

that the flash-induced increase in fluorescence yield was about 35% of it maximum, the intensity of the field-induced luminescence was reduced to 60% of its maximum. Since the saturating light intensity for system II_β is about 3 times higher than that of system II_α, this suggests that the field-induced luminescence originates mainly from more abundant system II_α.

Changes in prompt fluorescence induced by an external electric field

Dark-adapted samples of blebs were subjected to a saturating xenon flash in the presence of 5 μM DCMU. In these conditions reoxidation of Q^- is solely due to recombination with an oxidized component at the donor side of system II [13]. The fluorescence level, excited by a weak measuring beam, was monitored from 25 to 45 ms after the flash. Due to the relatively slow rate of reoxidation of Q^- the fluorescence level was almost constant during the time of the measurement (Fig. 2, dashed line). When during the measurement an electrical field pulse was applied, a strong transient signal was detected during the pulse, and the level reached after the field was turned off was lower than that of the control measurement without a field pulse (Fig. 2). The transient signal measured during the field pulse was also observed in the absence of the measuring light (Fig. 3), but not when the flash was omitted. Therefore, the transient is ascribed to field-induced luminescence. The peak intensity of the stimulated luminescence was about 500 times more intense than the normal luminescence intensity at the same time. The fluorescence decrease can be attributed to an enhancement of the rate of back reaction in Photosystem II by the pulse. This results in a decrease of the concentration of the reduced acceptor Q and consequently, in a decrease of the fluorescence level.

The maximum decrease in fluorescence induced by a pulse of 3 ms duration and a field strength of 1300 $V \cdot cm^{-1}$ was about 12% of the total variable fluorescence. A rough estimate of the maximum change to be expected can be obtained as follows. It was earlier concluded [3] that only about one third of the reaction centers are contained in the single membrane bleb wall. Furthermore, only in one half of each bleb wall the direction of the field is such that a stimulation of the rate of back reaction occurs; in the other half of the bleb wall the field is of opposite sign, and thus is expected to stabilize the charge separation. Thus the maximal effect of the external field expected from this model is a complete recombination in about 17% of the reaction centers. This corresponds to a decrease of the variable fluorescence by about 17% since all reaction centers in a given area of the bleb undergo a charge recombination, so that one may assume a linear relation between the decrease in fluorescence and the concentration of Q^- for the system as a whole. The observed 12% is in good agreement with this value and suggests that the effect of the applied field is not far from saturation.

Kinetic relation between the stimulated luminescence and fluorescence decrease induced by a field

As mentioned in the Introduction, the stimulation of luminescence may be due to an enhancement of the back reaction or to an increase of the probability that recombination leads to the emission of a photon. In this section we will show that the enhancement of the back reaction described above is the sole cause of the stimulated luminescence.

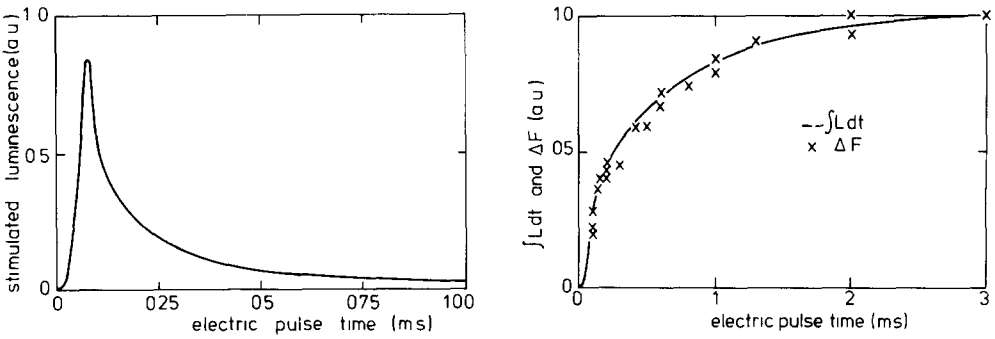

Fig. 3. Kinetics of the field-induced (1300 V · cm^{-1}) luminescence measured in DCMU-treated blebs. The field pulse was applied 3 ms after the actinic flash.

Fig. 4. Kinetics of the electrical field-induced changes in fluorescence (crosses; measured as in Fig. 2 with pulses of different duration) and time-integrated luminescence (solid line; measured as in Fig. 3).

First of all, the complex kinetics of the luminescence intensity during the pulse (Fig. 3) were found to be identical to the kinetics of the recombination rate. This is shown in Fig. 4, where the kinetics of the field-induced fluorescence changes are compared to those of the time-dependent stimulated luminescence. Thus we may conclude from Fig. 4 that the proportionality constant between the luminescence intensity and the recombination rate did not change during the field pulse. The value of this constant was determined by dividing the integrated luminescence emitted during the field pulse by the fraction of Q^- reoxidized during the pulse. The corresponding value in the absence of the field was measured under identical conditions and at the same time after the flash. In this case, the recombination rate was determined from the absorbance changes of Q^- at 320 nm [14], to avoid complications by the non-linear relation between Q^- and fluorescence yield under these circumstances. The proportionality constant between luminescence intensity and recombination rate was found to be identical within experimental error (20%) in the presence and absence of the field. Thus we conclude that the field accelerates the recombination of charges without modifying the probability that recombination results in luminescence emission. In those reaction centers where a stimulation occurs, the back reaction is completed in a few hundred microseconds (Fig. 4), whereas it normally takes several seconds. This means that the total stimulation of the rate of the back reaction by the pulse is by more than a factor of 10^4.

Determination of the luminescence yield

The absolute value of the probability that recombination results in luminescence can be determined by comparing the luminescence and fluorescence intensity, measured with the same detection system. Then the quotient of the luminescence and fluorescence signals can be expressed by:

$$\frac{L(t)}{F(I)} = \frac{\eta_L k_{L\,exc}(t)}{\eta_F k_{F\,exc}(I)} = \frac{\eta_L \eta_{exc} k_{rec}(t)}{\eta_F \frac{1}{\eta_{Q^-}} k_{Q^-}(I)} \qquad (1)$$

where $L(t)$ is the luminescence intensity observed at time t after a flash, $F(I)$ is the fluorescence intensity observed at an illumination intensity I, η_L and η_F are the fluorescence yields of the chlorophylls excited by charge recombination and by light absorption, respectively, and $k_{Lexc}(t)$ and $k_{Fexc}(I)$ denote the rates of chlorophyll excitation by the processes as observed under the respective conditions of the two measurements. $k_{Lexc}(t)$ is an unknown fraction η_{exc} of the measured decay rate $k_{rec}(t)$ of Q^-. Actually most excitation will be retrapped and the relevant value of η_L is presumably close to η_{F0}, the fluorescence yield observed when all centers are in the state Q. However, it is more convenient to disregard retrapping, so that η_L becomes η_{Fmax}, the fluorescence yield observed when all center are in the state Q^- [6]. The corresponding lower value of η_{exc} then obtained directly indicates the fraction of the observed Q^- oxidation rate, $k_{rec}(t)$, which proceeds via transition of reexcited chlorophyll to the ground state. $k_{Fexc}(I)$ may be determined from the inital rate of Q reduction $k_Q\text{-}(I)$ upon illumination (with the same intensity I) of a dark adapted sample in the presence of DCMU, taking the quantum yield η_{Q^-} of this reaction into account. The value of $k_Q\text{-}(I)$ was determined from the initial kinetics of the area above the fluorescence induction curve (see Ref. 15). The value of η_{Q^-} was assumed to be about 0.9.

Therefore, if for $F(I)$ the maximum fluorescence level F_{max} is taken we have:

$$\eta_{exc} = \frac{L(t)\ k_Q\text{-}(I)}{0.9F_{max}(I)\ k_{rec}(t)} \tag{2}$$

Similarly, by integrating Eqn. 2 during the pulse time we can write for η_{exc} during the field pulse:

$$\eta_{exc} = \frac{\int L(t)\ \mathrm{d}t\ k_Q\text{-}(I)}{0.9F_{max}(I)\ \Delta Q^-} \tag{3}$$

where ΔQ^- is the fraction of reaction centers which have recombined during the pulse. The value of η_{exc} thus obtained from our measurements, both in the presence and absence of an electrical field, is 0.028 ± 0.005 at about 30 ms after the flash. This value may not be time-dependent, otherwise one could simply integrate all luminescence emitted after a saturating flash and assume a nearly complete turnover of Q^- (i.e. $\Delta Q^- = 1$), so that the Q^- decay rate need not be determined (cf. Ref. 10). However, this determination, either from the 320 nm absorbance change or from the fluorescence induction area, not only provides more information but also avoids the notorius difficulty of the socalled head- and tail cut-off effects [5] in the determination of the integrated luminescence.

The value of the excitation yield of the back reaction suggests that 97% of the charge recombinations do not involve a complete reversal of the photosynthetic process and constitute a leakage of electron from Q^- to an oxidized donor. On the other hand the same percentage is obtained in the presence of an electrical field which enhances recombination at least 10^4 times. This suggests that the leakage reaction may occur only from a very primary stage, possibly $P\text{-}680^+$pheophytin$^-$ [16], preceding the charge separation across the membrane, which is completed already in the state $P\text{-}680^+Q^-$ [17].

A possible route for this leakage reaction was recently suggested [16] in terms of the charge pair mechanism [18]. By reversal of the secondary electron transfer reactions the primary charge pair is produced three times more often in a 'virtual triplet state' than in a single state. The triplet state can recombine only to the triplet of P-680, which reaction constitutes the leakage reaction, whereas the singlet state may recombine to singlet excited P-680, which reaction can lead to delayed fluorescence emission. Apparently the rate constants involved are such that the recombination via the triplet state is the predominant process.

Kinetics of the changes in prompt and delayed fluorescence induced by a field pulse; model calculations

We have shown that the observed changes in delayed fluorescence induced by a field pulse are due to an enhancement of the rate of back reaction. A more detailed description, however, has to explain the origin of this enhancement and its complex kinetics (Fig. 3). In principle, the lag phase observed when the field is applied can be ascribed to the fact that the potential across the membrane is not built up instantaneously but requires a certain RC time [3]. This, together with an exponential dependence [19,20] on the membrane potential of the rate of the back reaction, can account for the initial kinetics. The decrease of the luminescence signal during the pulse may be explained by a depletion of luminescence precursors in those areas of the bleb wall where the membrane potential is high. We have carried out model simulations of the field-induced kinetics of prompt and delayed fluorescence taking into account the following points.

1. The luminescence intensity is proportional to the rate of recombination k_{Q^-} of the system II reaction centers. This rate can be expressed as

$$\frac{dQ^-}{dt} = -k_{Q^-} \cdot Q^- \tag{4}$$

In the absence of an electric field the rate ($k^0_{Q^-}$) was determined to be 0.4 s^{-1} by measuring the kinetics of the absorbance changes at 320 nm at the same time after the flash as the field was applied.

2. Since the reaction P-680* Q $\rightleftharpoons$ P-680$^+$Q$^-$ spans the total membrane dielectric [17], we may expect that the energy difference (expressed in eV) between the energy level of a luminescence precursors state and that of P-680* is changed by an amount V, where V equals the local membrane potential expressed in volts. Therefore, the equilibrium constant between these states is changed by a factor exp (V/kT) (where k is Boltzmann's constant, see also Refs. 19 and 20) and consequently the value of k_{Q^-} in the presence of a membrane potential is approximately given by

$$k_{Q^-} = k^0_{Q^-} \exp(V/kT) \tag{5}$$

3. As was shown earlier [3], upon the onset of an external field F_{ex} a local membrane potential is induced which is given by

$$V = \tfrac{3}{2} r F_{ex} \cos\phi \, (1 - e^{t/\tau}) \tag{6}$$

where r is the radius of the bleb and ϕ is the angle subtended by the direction

of the applied field and the normal at the bleb surface. The external field is applied at $t = 0$; the membrane potential is built up exponentially with an RC time τ.

4. The value of τ is determined by the specific membrane capacity C_s and the specific resistance of the internal and external media, ρ_s according to

$$\tau = \tfrac{3}{2}\rho_s C_s r \tag{7}$$

The value of C_s was taken to be 1.7 $\mu F \cdot cm^{-2}$ [4] and ρ_s was determined experimentally.

5. By dielectric breakdown the value of V is limited between ±1 V [3].

6. The blebs have a certain size distribution. The typical size distribution shown in Ref. 3 was used. The relative amount of reaction centers per surface area was taken to be equal for all blebs.

Based upon these assumptions computer simulations of the field-induced luminescence and fluorescence changes were carried out. In view of the clearly oversimplified model and the fact that no adjustable parameters remain, the results were satisfactory: the general features of the observed luminescence curves such as the intial delay, the subsequent rise to a maximum level and the slower decrease are reproduced (Fig. 5). The delay time and the time after which the maximum level was reached were the same as the experimentally observed ones within a factor of two. The subsequent decrease in the experimental curves was systematically slower than in the simulations. This can be expected, however, since the charge recombination involves secondary reactions at the donor side of system II which may not be accelerated by the pulse.

In agreement with the observations, the kinetics of the field induced luminescence became slower at decreasing values of the applied field strength, and the maximum level obtained decreased (Fig. 5). The latter effect is more clearly

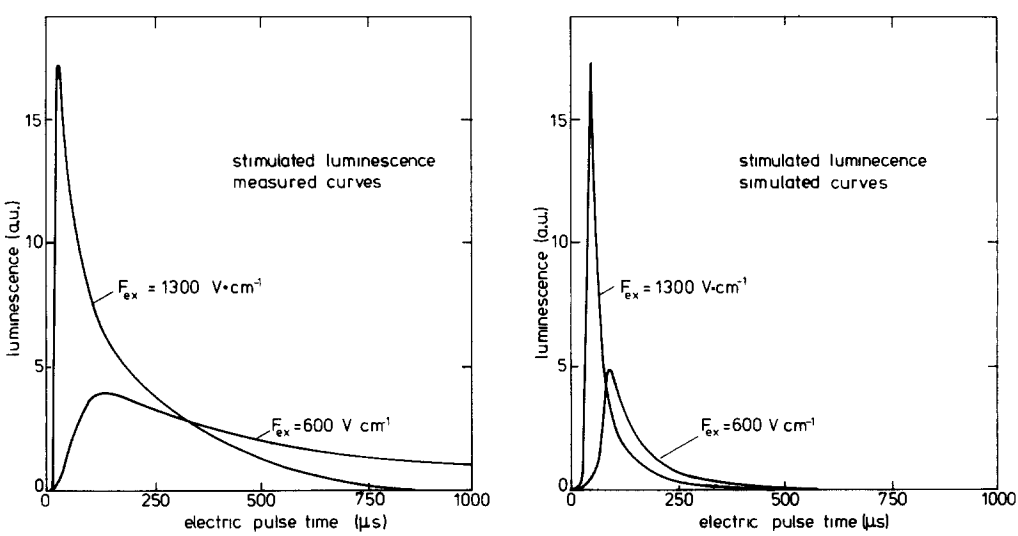

Fig. 5. Measured (left) and simulated (right) kinetics of the field-induced luminescence stimulation at the indicated field strength. The time between the flash and the onset of the field pulse was 0.5 ms. The simulation was obtained by the assumptions outlined in the text.

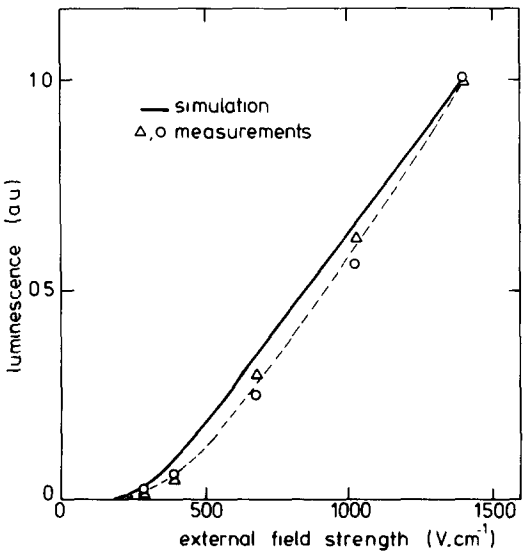

Fig. 6. Measured and simulated field dependence of the maximum amplitude of the stimulated luminescence in the presence of DCMU. The time between the actinic flash and the onset of the field was 53 ms (△) and 2.2 ms (○). The amplitudes were normalized at a field strength of 1400 V · cm^{-1}.

shown in Fig. 6 where the measured and simulated field dependence of the stimulated luminescence are plotted. The agreement between the simulated and measured curves is good; the discrepancy may e.g. be due to an overestimation of the external field strength or to a deviation from the assumed size distribution. From Eqn. 7 it follows that the kinetics of the field-induced luminescence can be influenced also by changing the specific resistance ρ_s of the medium. This was indeed observed, both in the simulations and in the experiments, although the actually observed effect was somewhat smaller than that predicted by the simulation (data not shown). The simulation of the field-induced fluorescence changes showed that under the conditions used, a field of 1300 V · cm^{-1} was sufficient to generate a complete back reaction in 45% of the reaction centers in the bleb walls during a 3-ms pulse. Referring to the reasoning given above this corresponds to a fluorescence change of about 15% which is close to the observed value of 12%. It should be noted that when the time between the flash and the onset of the pulse was increased from 0.5 to 50 ms, the observed intensity of the field-induced luminescence decreased by a factor of about three, although the concentration of Q^- remained virtually the same (Fig. 2). The kinetics of the stimulated luminescence and the value of k_Q- did not change. The cause of this effect was not investigated.

From simulations performed under different conditions, the following picture of the process emerged. We consider a single bleb as a globe with the North pole facing the positive electrode. Then the field will only enhance the luminescence intensity in the Northern hemisphere. For field strengths normally applied (400—1300 V · cm^{-1}), at each time during the pulse the stimulated light is emitted from a relatively small area on the Northern hemisphere with about the same latitude: the reaction centers at higher latitude have already

recombined, whereas the local membrane potential for the reaction centers at lower latitude is (still) too low to induce a significant stimulation. The area from which the stimulated light is emitted moves as a luminescent ring from the North pole towards the equator. As the field strength is increased, the ring moves faster and is thinner and brighter (Fig. 5), and approaches the equator more closely before it disappears.

Polarization of the field-induced luminescence

The above described movement of the source of the stimulated luminescence emission could be tested by measurement of luminescence polarization. Since the transition moments of the fluorescing chlorophyll molecules are preferentially oriented relatively flat in the membrane [21], the observed luminescence should reveal a net polarization: initially, when the light is emitted from the area near the North pole, the luminescence should be polarized perpendicular to the direction of the externally applied field. As the luminescence moves towards the equator, the degree of the polarization (defined as $L_\perp/L_{/\!/}$) should diminish. As discussed in the previous section, the higher the externally applied field strength, the smaller the area from which the light is emitted and the closer it reaches the equator. This means that the initial polarization should increase, and the final polarization should decrease with increasing field strength.

Experimentally these effects are indeed observed as is illustrated in Fig. 7. The field-induced luminescence was partly polarized in the expected direction (perpendicular to the direction of the applied field) and during the pulse the degree of polarization decreased. A similar observation was recently reported by Farkas et al. [22]. As was expected, the initial degree of polarization increased with increasing field strength: maximum values of 2.0 and 1.5 were

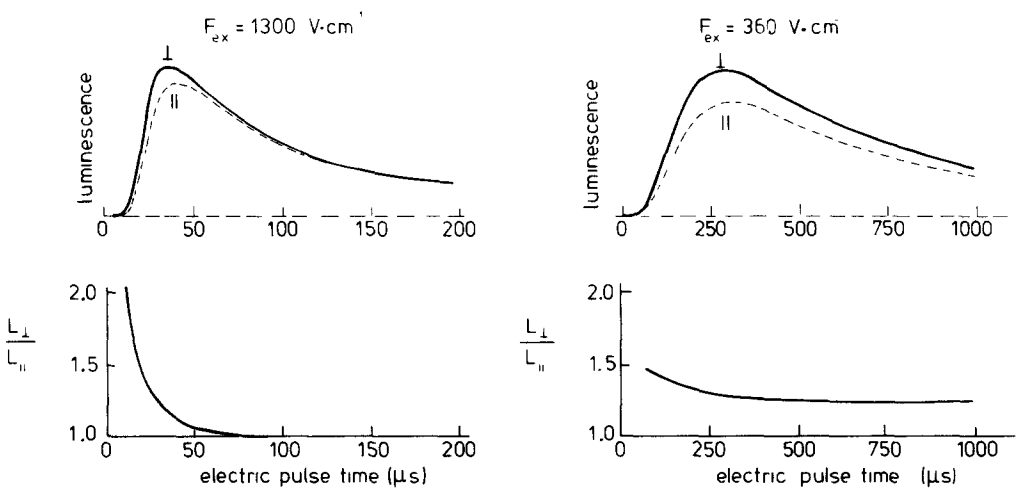

Fig. 7. Polarization of the electrical field-induced luminescence at the indicated field strengths. $L_{/\!/}$ and $L_\perp$ are the luminescence intensities measured through a polaroid filter light polarized parallel and perpendicular to the direction of the applied field, respectively. The time between the actinic flash and the onset of the field was 0.5 ms. Addition: 5 μM DCMU.

obtained with applied field strengths of 1300 and 360 $V \cdot cm^{-1}$, respectively (Fig. 7), whereas the degree of polarization measured when the luminescence intensity had dropped to 25% of its maximum was 1.00 and 1.20, respectively. In the largest blebs, the polarization should drop to values below 1, but the contribution of these blebs to the net effect decreases during the pulse. At the end of the pulse most luminescence comes from the more abundant smaller blebs, where the polar regions still contribute to the emission.

Luminescence induced by a second pulse

The luminescence induced by a second field pulse following the first one, with the same or opposite polarity was studied. The results obtained agreed with those reported by Ellenson and Sauer [2] and can be explained by the model discussed above. When a second field pulse was given following a pulse of the same polarity, the induced luminescence was much lower than that observed when the first pulse was omitted. When the polarity of the second pulse was reversed, the induced luminescence intensity was not affected by the first pulse. The explanation of the observations is that the first pulse diminishes the concentration of luminescence precursors in e.g. the upper bleb halves only, so that a second pulse of the same polarity can produce only little luminescence. If the polarity of the second pulse is reversed, however, no effect of the first pulse is to be expected since the concentration of luminescence precursors in the lower bleb halves is not affected by the first pulse.

We extended these experiments by applying the first field pulse during the time a flash was given, in order to investigate whether a membrane potential has an additional effect on the efficiency of the charge separation. Arnold and Azzi [23] postulated such an effect to explain part of their results but their experimental procedure did not allow an unambiguous conclusion. Our experiments revealed no significant effect on the efficiency of the primary charge separation: if the flash was given in the presence of a high field-induced membrane potential, just before the field was turned off, no effect of this field was observed on the luminescence intensity induced by a second field pulse applied a few ms later. It should be noted that the interpretation of the results of Arnold and Azzi are complicated by the fact that DCMU was not present in their experiment. During the first few hundred μs after the flash when the primary electron acceptor Q is still in the reduced state, a relatively large decrease of luminescence precursors can be induced by a field. After this time the electron is transferred to secondary acceptors and the decrease of luminescence precursors by a field can be expected to be smaller.

Acknowledgements

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