Biochimica et Biophysica Acta, 423 (1976) 479–498

BBA 47054

EFFECT OF THE TRANSMEMBRANE ELECTRIC FIELD ON THE PHOTOCHEMICAL AND QUENCHING PROPERTIES OF PHOTOSYSTEM II IN VIVO

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(Received July 24th, 1975)

SUMMARY

The intermediate phase of fluorescence relaxation (1ms–1s) (Joliot, P., Joliot, A., Bouges, B, and Barbieri, G. (1971) Photochem. Photobiol. 14, 287–305), following a single saturating flash, is shown to be controlled by a slow phase of the reoxidation of Q^- by a secondary acceptor and, in vivo, by the transmembrane electric field.

The kinetics of reoxidation of Q^- are slowed by lowering the pH. This slowing effect is interpreted in terms of the reversible formation at low pH of QH which is not oxidizable by the secondary acceptor.

The electric field transforms Photosystem II centers into a non-quenching photochemically inactive state that cannot be attributed to an accumulation of Q^-. Centers are unequally sensitive to the field. A critical field strength can be defined for each center above which that center is blocked and below which the center is photochemically active. The transformation from the active to inactive state occurs over a narrow range of field strength.

Sensitive centers are blocked by the field in less than 1 ms and become active again in less than 10 ms as the field strength falls. Two hypotheses are proposed for the mechanism of blockage of centers by the field: (1) a field induced conformational change in the centers, (2) the formation or suppression of a dipole critical to the function of a center.

The activity of the ATP synthetase, determining the rate of relaxation of the field, was controlled by a light-dark treatment or by a chemical method using *p*-benzoquinone.

INTRODUCTION

Duysens and Sweers [1] have demonstrated that the quantum yield of Photosystem II fluorescence is dependent upon the redox state of the primary electron acceptor, Q.

More recently several authors have shown that additional factors modulate the fluorescence yield.

Homann [2] and Murata [3] have shown that Mg^{2+} controls the amplitude of the variable fluorescence yield. Similar control was observed by Bonaventura and Myers [4] following long preillumination with red or far-red light. These phenomena are probably linked to small structural changes of the thylakoid membrane.

A diminution of the fluorescence yield, induced by continuous illumination, has been interpreted as being linked to the formation of a proton gradient [5]. The number of oxidizing equivalents accumulated on the donor side of Photosystem II also modulates the fluorescence yield [6, 7]. Finally, Butler et al. [8] have shown that the chlorophyll cation radical of the Photosystem II reaction center is a fluorescence quencher.

The large number of phenomena that control the fluorescence yield becomes particularly apparent on studying the fluorescence relaxation following a saturating flash. At short times, a multiphasic rise in the fluorescence yield is observed [9]. The slower component (20–200 μs) has been interpreted by Zankel [10] and Joliot [11] as being due to an electron transfer on the donor side of Photosystem II.

Following the fluorescence maximum a fast decreasing phase ($t_{\frac{1}{2}} = 0.6$ ms) has been observed by Forbush and Kok [12] which corresponds to the reoxidation time of Q^-. A second decreasing phase (1 ms to 1 s) observed by Joliot et al. [6] is particularly prominent in whole algal cells. A final slow phase was observed by these same authors lasting 60 s and which is linked to the concentration of states S_2 and S_3, as introduced in the model of Kok et al. [13].

These relaxations play a role in the form of the fluorescence induction curve in continuous light following a period of dark adaptation. This induction curve is biphasic [14]. Joliot [15] and de Kouchkovsky and Joliot [17] have shown, using *Chlorella* and chloroplasts respectively, that the first phase is associated with the activation of Photosystem II, now interpreted as the appearence of states S_2 and S_3. The amplitude of this first phase is highly variable in living cells, yet is always larger than for chloroplasts. In vivo, this amplitude shows a biphasic dependence upon the illumination intensity, arising from the formation of states S_2 and S_3 plus an additional factor. As pointed out in ref. 6, this additional factor is also responsible for the intermediate phase of fluorescence relaxation (1 ms—1s) mentioned above.

Several interpretations can be given to this intermediate phase. Among these are: (1) a charge recombination of S_2 with Q^- for a fraction of the centers unable to oxidize Q^- by a secondary acceptor, as proposed in ref. 6; (2) a slow phase of reoxidation of Q^- by a secondary acceptor; (3) a dependence of the fluorescence yield on the transmembrane electric field relaxing in this time range.

We will show that interpretations (2) and (3) adequately explain the intermediate phase. The third arises from our observation that the intermediate relaxation phase is highly dependent on the duration of a dark period following illumination. A similar variability was observed in measurements of the rate of electric field relaxation in the same material, and led us to consider the possibility that the two were related.

In some early experiments, Witt [18] showed that a correlation existed between the increase in absorption at 515 nm and the fluorescence induction measured in continuous light. At this time, Witt suggested that a single substance was responsible for both phenomena. De Kouchkovsky [19] reinvestigated this problem and found only a partial correlation between these measurements. No interpretation was given

for these results except that the two were possibly indirectly related. More recently, the 515 nm absorbance increase has been shown by Junge and Witt [20] to be a natural probe which linearly measures the electric field across the thylakoid membrane. This interpretation, which is generally accepted, led us to reinvestigate the question of the relation of the 515 nm change (measured at 520 nm) to the fluorescence yield.

The 520 nm relaxation following a saturating flash, in dark adapted algae is characterized by four major phases: (a) a fast absorption increase occurring in less than 1 μs [21]. In our laboratory using *Chlorella*, we find 60 to 80 % of this phase is linked to Photosystem I activity and 20 to 40 % to System II activity [23]; (b) a slower increasing phase occurring in the time range 1 to 40 ms [22, 23]. Phase b depends only upon System I activity [23] (c) a fast absorption decrease is observed between 40 and 200 ms. This phase appears to be related to the phosphorylation activity [23, 24]; (d) a very slow decreasing phase ($t_{\frac{1}{2}} \approx$ 1.5–5 s) probably corresponds to the decay of the field by leakage through the membrane [23, 24].

The level of the breaking point between phases c and d has been interpreted as the minimum electrostatic potential necessary for the production of ATP [23, 24]. A preillumination of 1000 lux reduces the triggering level to zero without an appreciable change in the time constants *c* and *d*. Phase c can, however, be accelerated by higher intensity illumination. For algae left in the dark for an hour following preillumination, this triggering level rises to a point which sometimes exceeds the amplitude of the maximum field generated by a single saturating flash. Following a much longer dark period, this level has been observed to again decrease [23]. While we do not understand the mechanism which determines the triggering level, we have succeeded in defining the experimental conditions which permit varying its amplitude (see Materials and Methods). We stress that in the experiments that follow, the variation of the triggering level is used only as a tool for varying the field relaxation rate, and demonstrating its relation to the fluorescence relaxation.

MATERIALS AND METHODS

Measurements of the carotenoid band shift were made using the flash-detector differential spectrophotometer described by Joliot and Delosme [23].

Oxygen was detected by the polarographic method described by Joliot and Joliot [25]. Flash excitation was provided by stroboslaves (General Radio, type 1539A) employing xenon flash lamps (3 μs width at half-height). Simultaneous measurements of oxygen activation and fluorescence induction following a dark period were made using modulated light according to the method described by Bennoun and Li [26]. For the latter measurements exciting light was filtered through one BG 38 (Schott) and two 4-96 (Corning)blue filters. The photomultiplier (Radiotechnique XP 1002) was placed close to the polarograph and detected the emitted fluorescence though one 2-64 (Corning) and two Rubylith Ulano red blocking filters.

The relaxation of the fluorescence yield following one or more saturating flashes was measured by a method described by Joliot [11]. Stroboslave detecting flashes were filtered by a 2 cm Schott BG 38 filter. These have a negligible actinic effect and were used to sample the fluorescence yield at variable times (10 μs–2 min)

following the saturating flash. The photomultiplier was protected by Ulano Rubylith and Wratten no. 70 filters which let pass the fluorescence emission at > 665 nm. In order to study this relaxation over an extensive time range a series of detecting flashes were given such that the time period between successive detecting flashes was a geometrical progression in which the time interval was successively doubled.

Chloroplasts were prepared from market spinach according to the method of Avron [27] and stored at −70 °C in 0.05 M Tris · HCl buffer (pH 7.8), containing 0.01 M NaCl, 0.4 M sucrose and 5 % dimethylsulfoxide. When used for 0_2 measurements, the chloroplasts were diluted to a chlorophyll concentration of 300 μg/ml using various buffers (see figure legends) to obtain the desired pH, and containing 0.4 M sucrose and 0.1 M KCl. Chloroplasts were prepared in the same manner for simultaneous fluorescence relaxation measurements, at 100 μg chlorophyll per ml.

Chlorella pyrenoidosa were grown on Knop medium containing Arnon's trace elements A_6 and B_6, illuminated by white fluorescence light of ≈ 3000 lux.

For simultaneous measurements of oxygen activation and fluorescence induction cells were suspended in 0.1 M phosphate buffer pH 7.0, containing 0.1 M KCl at a chlorophyll concentration of 150 μg/ml. For measurements of the 520 nm, absorbance change and fluorescence relaxation, cells were suspended in the same buffer containing 7 % Ficoll at chlorophyll concentrations of 65 and 150 μg/ml, respectively.

As pointed out in the Introduction, the triggering level, separating phases *c* and *d* of the 520 nm relaxation, was highly variable. Some control over this level could be obtained by the following treatment. Algae were stored overnight in the dark at 5 °C and then preilluminated in very weak white light (50 lux) for 2 h at room temperature, at which point the triggering level was of intermediate amplitude. Upon 3 min preillumination in stronger white light (1000 lux), the triggering level could be brought to zero. After 5 min of dark adaptation, a time sufficient for complete deactivation of the S states, the triggering level remained close to zero. In a subsequent dark period, this level increased during about one hour until it attained a level exceeding the maximum field produced after a single saturating flash. This evolution in the dark could be completely reversed by a subsequent illumination. We stress that the weak light preillumination was absolutely required to obtain a maximum dark evolution toward the slowly relaxing field condition.

All the experiments reported here have been performed at 20 °.

RESULTS

Shown in Fig. 1 is the fluorescence yield detected at the indicated times following each of a series of actinic flashes. At 450 ms, the sequence obtained closely resembles the oscillations of the sum S_2+S_3 as a function of flash number [6, 7]. At 1 ms and 20 ms, the flash sequence resembles that at 450 ms with the exception that these are, to a first approximation, superimposed on a constant background. This comparison suggests that at times shorter than 450 ms, one or more additional phenomena stimulate the fluorescence yield. This stimulation is to a first approximation additive to that of the sum S_2+S_3.

While it is probable that the reoxidation of Q^- is partly responsible for the decreasing fluorescence yield between 1 and 20 ms, as will be discussed later, it is unlikely that the relaxation 20 ms to 450 ms can be explained in the same manner. Another relaxation process is probably occurring in this time range.

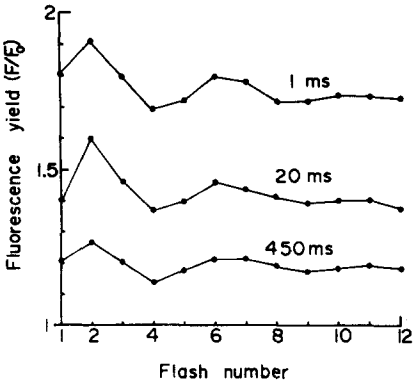

Fig. 1. Fluorescence yield measured 1, 20 and 450 ms following each of a series of saturating flashes given 450 ms apart. Algae were adapted to the rapidly relaxing field condition, consisting of 3 min preillumination followed by 5 min dark (see Materials and Methods).

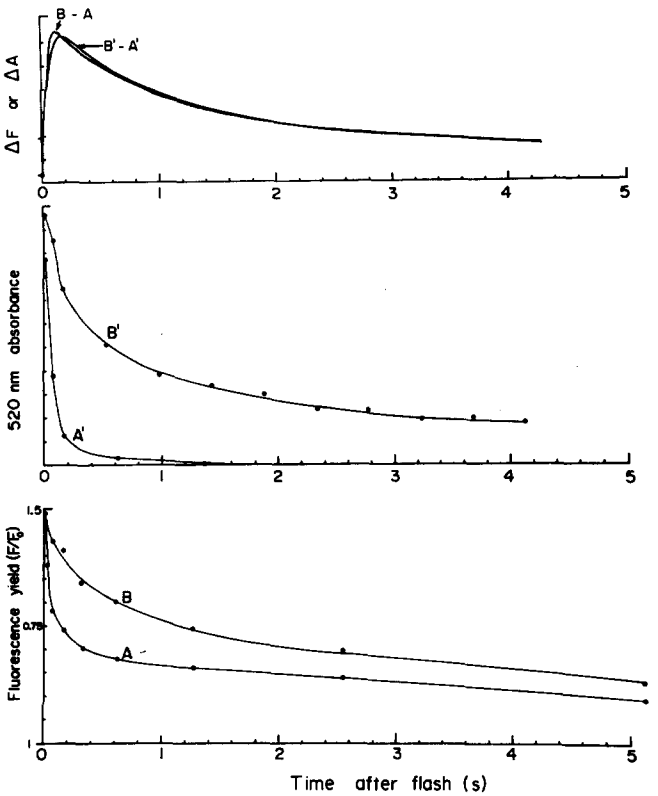

Fig. 2. Fluorescence and 520 nm relaxation following a single saturating flash. Curves A and A′, algae adapted to the rapidly relaxing field condition as in Fig. 1. Curves B and B′, the same algae after 25 m of dark adaptation. The first detecting flash was given 10 ms after the actinic for both measurements. The difference curves B–A and B′–A′ were normalized with respect to each other.

In Fig. 2, is shown a simultaneous study of the fluorescence and 520 nm relaxations following a single saturating flash. As in all the experiments in which simultaneous measurements were made using separate techniques, precautions were taken to assure that the algae were pretreated in the same manner.

For curves A and A′, algae were first preilluminated in very weak light as described in Materials and Methods, then preilluminated for 3 min in stronger light. The algae were placed in each apparatus in total darkness and a single saturating flash was given to a fraction of these after 5 minutes. For curves B and B′, the remaining algae were kept in the dark for 25 min at which time a saturating flash was given. This dark adaptation was of sufficient duration to allow a significant increase in amplitude of the 520 nm phase *d*.

A comparison of curves A and B and A′ and B′ shows that corresponding to a slowed relaxation of the 520 nm absorption is a similar effect on the fluorescence relaxation. A more quantitative analysis can be obtained by comparing the difference curves B–A and B′–A′. In these curves, the influence of State S_2 on the fluorescence yield is eliminated as the concentration of this state, produced by a single saturating flash, is the same for both conditions (slowly and rapidly relaxing field).

The parallelism between these curves is evident, which means that the electric field is one of the phenomena controlling the fluorescence yield.

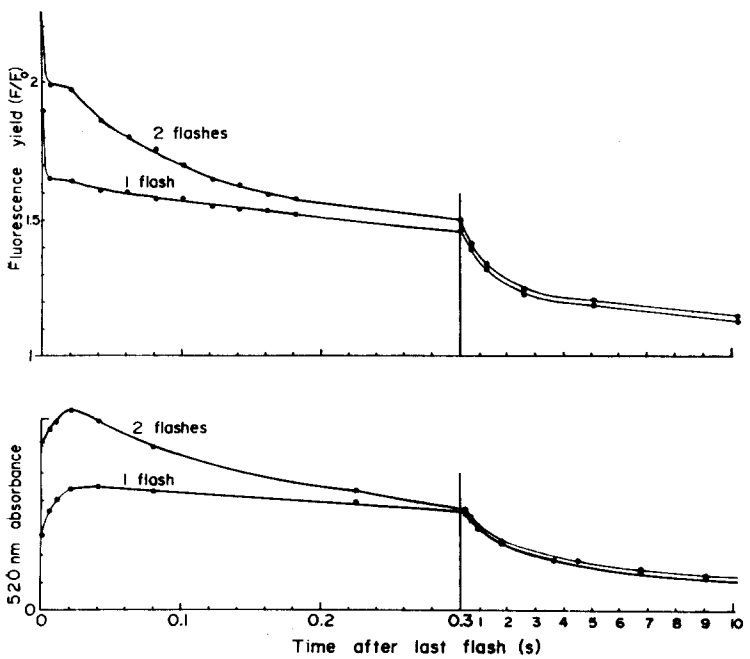

Fig. 3. Fluorescence and 520 nm relaxation following one or two saturating flashes spaced 225 ms apart. Algae were adapted to the slowly relaxing field condition by preilluminating in weak light followed by dark adaptation for 1 h. The first detecting flash is given 1 ms after the last actinic flash for both measurements.

In Fig. 3, the algae have been treated as in the previous experiment to develop a slowly relaxing field, except that the dark incubation period was lengthened to 1 h. At this time, no fast phase *c* was visible following a single saturating flash. On giving a second flash, 225 ms after the first, the field developed by the second flash is added to that remaining from the first. As previously described [23] the field generated by the first flash does not exceed the triggering level and only the slowly relaxing phase *d* is observed. The field attained following the second flash exceeds the triggering level and both phases *c* and *d* are observed. Less than one second following the second flash, the 520 nm relaxation is practically superimposable on that following the first flash alone. Similar behavior is observed for the fluorescence relaxation following one or two flashes. A slight difference remains in the long time range for the one and two flash fluorescence yields and is attributed to a slight difference in the S_2+S_3 concentrations. Nevertheless, in the time range 0–40 ms, it is apparent that the 520 nm amplitude increases while the fluorescence yield decreases. The phase *b* of the field relaxation is nonetheless visible as a small plateau in the fluorescence relaxation curves. The difference in the short time range between the 520 nm absorbance and fluorescence relaxations implies that an additional factor is controlling the fluorescence yield in this time range. To minimize this factor we have subtracted the single flash from the double flash curve for both 520 nm and fluorescence relaxations.

These two sets of curves were normalized to give the same amplitude for the difference curves, 2 flashes minus 1 flash. As observed in Fig. 4 (upper) these difference curves are similar.

A rough estimation of the kinetics of the short-time (0–40 ms) component can be obtained by taking the difference between the fluorescence yield and 520 nm absorption relaxations following one or two saturating flashes. These difference curves (Fig. 4, lower) show multiphasic relaxation with an overall half-time of 5–10 ms. They relax to a level elevated by the increased fluorescence yield of states S_2+S_3, not detected at 520 nm. This experiment shows that this component relaxes

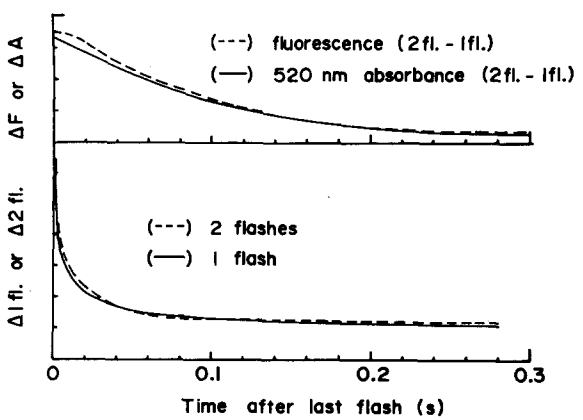

Fig. 4. Upper: Difference between 2 flashes and 1 flash as a function of time for the fluorescence and 520 nm relaxations of Fig. 3. The curves of the latter figure are normalized to give approximately the same amplitude for the difference curves. Lower: Difference between fluorescence and 520 nm absorption as a function of time for the 2 flash and 1 flash curves of Fig. 3.

identically after 1 or 2 flashes. We will demonstrate later that similar kinetics are observed in spinach chloroplasts which show no field-dependent fluorescence. Together these observations support the idea that this component probably corresponds to the tail end of the Q^- reoxidation kinetics.

The same experiment as shown in Fig. 3 was performed for algae adapted to show a fast relaxing field i.e. no phase d (data not shown). For these algae the fluorescence and 520 absorbance relaxations show a negligible difference between the one and two-flash curves. This result is due to the negligible field remaining 225 ms after the first flash. Thus no integration of the field occurs following excitation by the second flash.

An additional experiment was performed in which the evolution of the field from the fast-relaxing to the slowly relaxing condition, in the dark, was followed by detection of the 520 nm and fluorescence relaxations.

The slow phase evolves with the same kinetics as detected both by the 520 nm absorption and the fluorescence yield. The increasing phase d is half-maximal after 20 min and maximal after 45 to 60 min. While this time course is sometimes variable from one algal culture to another, a parallelism is always observed between the two methods for the evolution of phase d.

As pointed out in the Materials and Methods section, an illumination could transform algae from the slowly relaxing field condition to that of the fast-relaxing field. Fig. 5 shows the 520 nm absorbance change and fluorescence yield detected at 20 and 450 ms after each of a series of saturating flashes given 3.6 s apart. This 3.6 sec interval between actinic flashes was chosen to allow appreciable deactivation and mixing of the S states. In this way, the oscillation of the fluorescence yield was minimized which allows a more direct comparison of the fluorescence yield and 520 nm absorbance changes. The parallelism between these two kinds of measurements is obvious and shows a decreasing amplitude at 450 ms which corresponds to a progressive acceleration of the field relaxation. At 20 ms the principal difference observed between the second and fourth flashes is attributed to the decreasing amplitude of phase b [23].

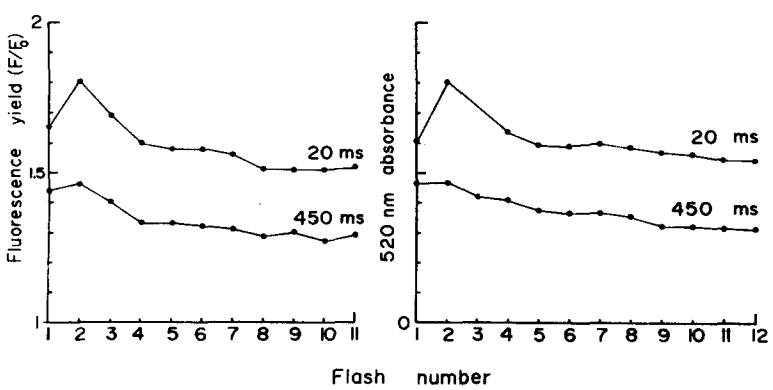

Fig. 5. Fluorescence yield and 520 nm absorption detected 20 and 450 ms following each of a series of saturating flashes spaced 3.6 s apart. Algae were adapted to the slowly relaxing field condition over a dark period of 1 h.

If flashes are given at a higher frequency than that for Fig. 5, then for the same number of flashes a lesser acceleration of the field relaxation was observed. This observation suggests that a slow thermal reaction is rate-limiting for the transformation from the slowly to the rapidly relaxing field.

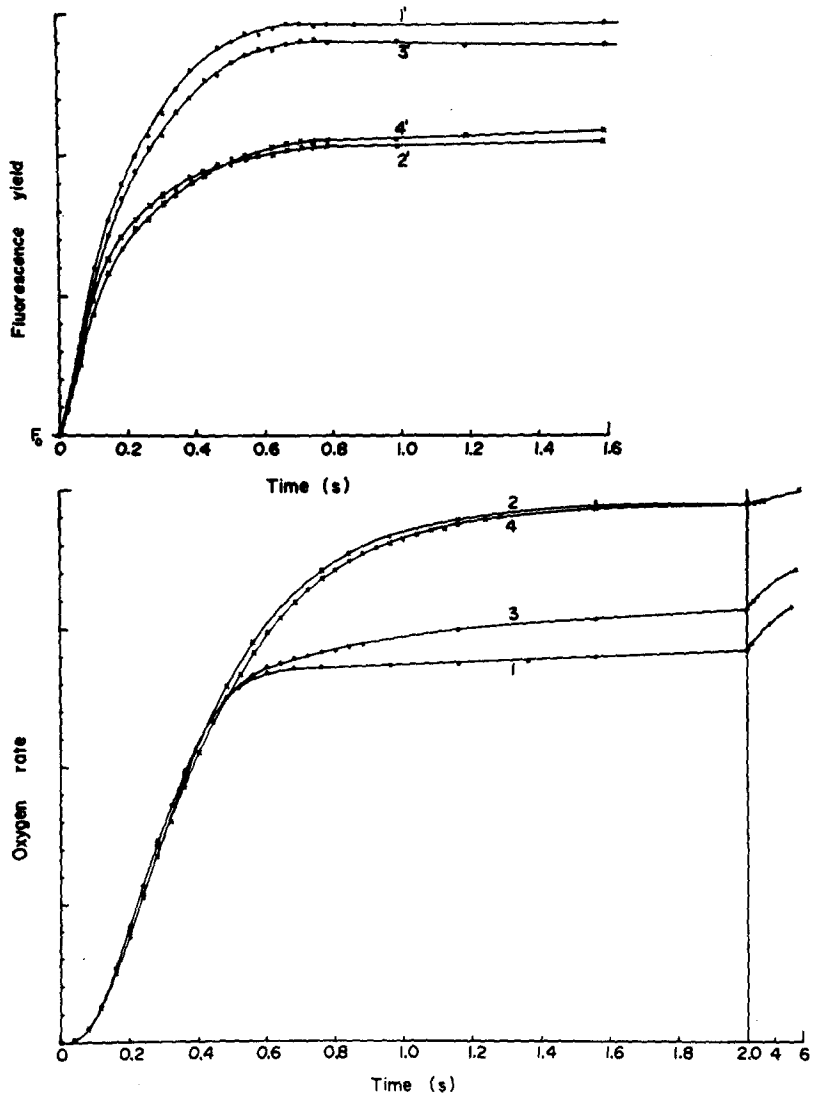

Fig. 6. Oxygen rate and fluorescence yield in weak modulated light following a dark adaptation of variable length. In all of the experiments presented in this figure, a single sample of algae was subjected to the light-dark cycle described chronologically below. Curves 1 and 1', algae were preilluminated in weak light (50 lux) (Materials and Methods) and adapted to the slowly relaxing field condition during 45 min in the dark. Curves 2 and 2', same algae preilluminated for 3 min followed by 5 min dark. Curves 3 and 3', same algae again dark adapted for 45 min. Curves 4 and 4', same algae again preilluminated for 3 min followed by 5 min dark. The preilluminating light for curves 2,2' and 4,4' was the measuring light.

Having established that the field modulates the fluorescence yield, we investigated whether a control by the field exists over the photochemistry, particularly the production of oxygen.

To this end, simultaneous measurements were made, using the same apparatus, of the oxygen activation and fluorescence induction following a dark period. The same algae were adapted alternately to fast and slowly relaxing fields, by conditioning the algae with a short or long dark adaptation period (see legend, Fig. 6). The amplitude of the field in steady state illumination is determined by the rate of photochemical formation of the field offset by the relaxation rate. Thus for the same light intensity, the field strength is higher for algae in the slowly relaxing field condition than for those relaxing rapidly.

As shown in Fig. 6, algae in the slowly relaxing field condition show an elevated amplitude for the first phase of fluorescence and a diminished steady state oxygen (25 % inhibited) rate as compared to algae in the rapidly relaxing field condition.

Thus, corresponding to the increase in the fluorescence yield, for the algae in the slowly relaxing field condition, is an inhibition of the steady state oxygen rate. A similar complementarity was observed by Delosme et al. [16] for centers blocked in the non-quenching state, Q^-. An important point is that the four oxygen activation curves are practically superimposed during the first 400 ms of illumination. These results indicate that for either 5 or 45 min dark incubation, centers show the same photochemical activity at the onset of illumination. In fact, there appears to be even a 5 % higher initial rate for the slowly relaxing algae than for those that relax rapidly. During the course of the subsequent illumination the inhibition develops with the accumulation of the field in the slowly relaxing algae.

A comparison of curves 1 and 3 and 1′ and 3′ indicates that during the second cycle of dark adaptation the inhibition attained for the steady state oxygen yield is not quite as great as that obtained during the first cycle. This difficulty in obtaining a complete reversibility was also observed in measurements of the 520 nm absorbance relaxation.

We have pointed out on several occasions that a transformation from the slowly relaxing to the rapidly relaxing field condition is progressively induced by continuous or flash illumination (Fig. 5). This acceleration progressively decreases the steady state level of the field and is probably responsible for the slow increase in the oxygen rate for times greater than 1 s, for slowly relaxing algae (curves 1 and 3, Fig. 6).

Only the variable part of the fluorescence curves has been plotted which means that any difference in the F_0 level has been neglected in this figure. However, a consistent elevation of the F_0 level was observed for algae adapted to the rapidly relaxing field condition. This difference in the F_0 level was variable and was of the order of 3–10 %.

There are several ways to explain the decrease of the photochemical rate with the increased field: (1) a decrease of the quantum yield of the photoreaction; (2) a decrease in the efficiency of energy transfer between the antenna and trap of photosystem II; (3) a decrease in the number of active Photosystem II centers.

An experiment which permitted an unambiguous choice of the third hypothesis consisted of giving a sequence of saturating flashes (160 ms apart) to the same algae adapted to the rapid or slowly relaxing field condition. (Fig. 7). Algae in the slowly relaxing field condition are inhibited 34 % for the 25th flash. By extrapolating the

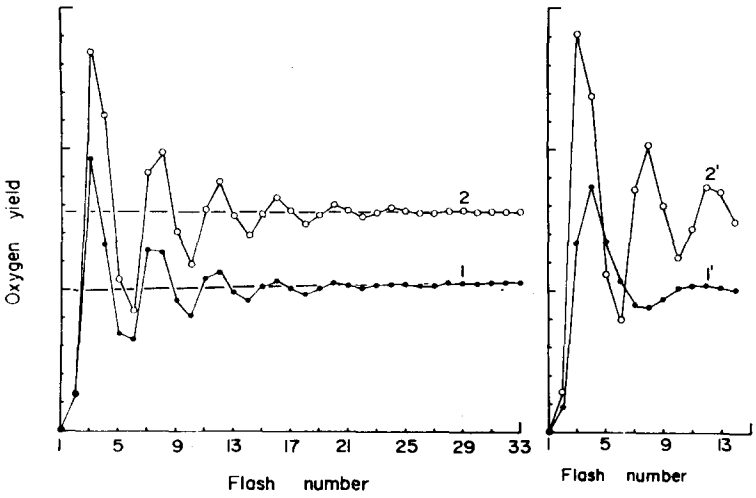

Fig. 7. Left: Oxygen yield for each of a series of saturating flashes spaced 160 ms apart. The algae used in this experiment are the same as those of Fig. 6. Curves 1 and 2, algae were subjected to the same light-dark treatment as those of Curves 1 and 2 of Fig. 6. The average oxygen yields are indicated by dashed lines. Right: Theoretical oxygen yield sequences computed from the model of Kok et al. Curve 2′: miss parameter, $\alpha = 0.17$; double hit parameter, $\beta = 0.075$. Initial condition: $S_0 = 0.35$, $S_1 = 0.65$. Curve 1′: miss parameter = 0.17 for the 1st flash, 0.37 for the 2nd flash, 0.47 for the 3rd and remaining flashes. This variable miss parameter has been chosen to simulate the increasing inhibition of Photosystem II centers by the field. Same double hit parameter and initial S_0, S_1 condition as in Curve 2′.

steady-state to the first several flashes of the sequence an inhibition of 37 % is obtained. Despite this considerable steady-state inhibition, the second flash yield is practically identical for the two sequences; this observation is in accord with the superposition of the rates at the beginning of the O_2 activation. In the saturating flashes that we used, every center received at least several photons (each center received on the average 15 photons per flash). Thus any variation in the transfer between antenna and trap or quantum yield variation in the center would not modify the number of centers successfully undergoing a photoreaction.

We conclude that the development of the field blocks photosystem II centers in a non-quenching, non-photoactive form. Qualitatively there is little difference in the damping of the oscillations despite the appreciable inhibition.

Quinone treatment

We have found that another way to induce a slowly relaxing field consists of treating algal cells with low concentrations of *p*-benzoquinone (10^{-4}–$3 \cdot 10^{-4}$ M) for a short period (1–15 m). The quinone is then removed either by reduction with ascorbate or by removal through rapid dialysis or centrifugation.

The effect of such treatment on the 520 nm relaxation following a single saturating flash is shown in Fig. 8. Quinone treated algae show a half relaxation of 1.6 s as opposed to 80 ms for untreated cells. Phase b is clearly apparent attaining a maximum at 40 ms. The quinone has either irreversibly blocked fast phase c or has raised the triggering level above which this phase appears. This disappearance of

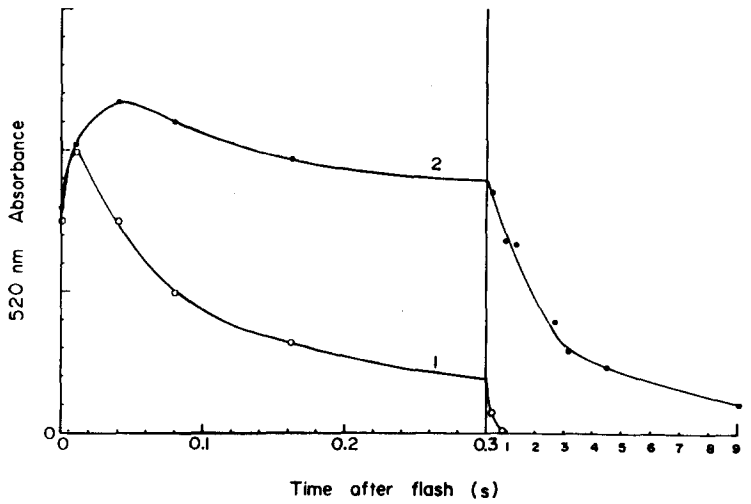

Fig. 8. 520 nm relaxation for algae pretreated with *p*-benzoquinone. Curve 1, control algae in the rapidly relaxing field condition; Curve 2, same algae (68 μg chlorophyll/ml) treated with 10^{-4} M quinone for 3 min at which point the quinone was reduced with $2 \cdot 10^{-4}$ M ascorbate.

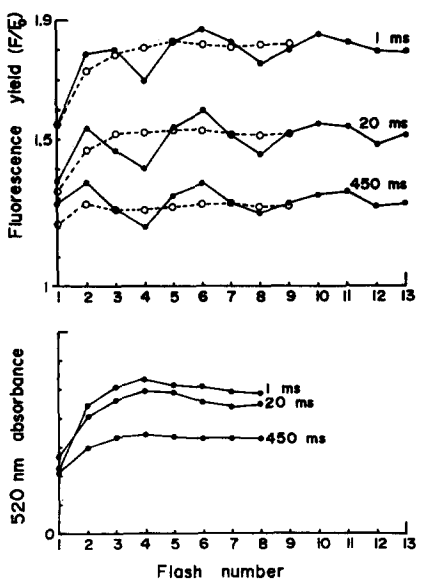

Fig. 9. Fluorescence yield and 520 nm absorption of quinone-treated algae detected at 1, 20 and 450 ms after each of a series of saturating flashes spaced 450 ms apart. Algae (120 μg chlorophyll/ml) were treated with $3 \cdot 10^{-4}$ M quinone for 3 min at which point the quinone was reduced with $4 \cdot 10^{-4}$ M ascorbate. Upper and lower ●–●, algae dark adapted for 10 min; upper (○---○), algae were preilluminated by 13 flashes spaced 450 ms followed by 20 s dark. This preillumination has no significant effect on the 520 nm flash sequence.

phase c appears to be a direct effect on the coupling factor rather than on the electron transport chain.

In Fig. 9 (lower) is shown the 520 nm absorption for dark-adapted quinone-treated algae at 1, 20 and 450 ms following each of a series of saturating flashes. Because of the slow relaxation of the field, there is a partial summation of the 520 nm absorption for the first four flashes.

In Fig. 9 (upper) is shown the fluorescence yield detected at 1, 20 and 450 ms, following each of a series of saturating flashes. The oscillatory pattern indicative of states S_2 and S_3 is apparent at 450 ms. At 1 and 20 ms is added an increasing component to this oscillatory pattern.

To distinguish this component more clearly, algae were preilluminated to

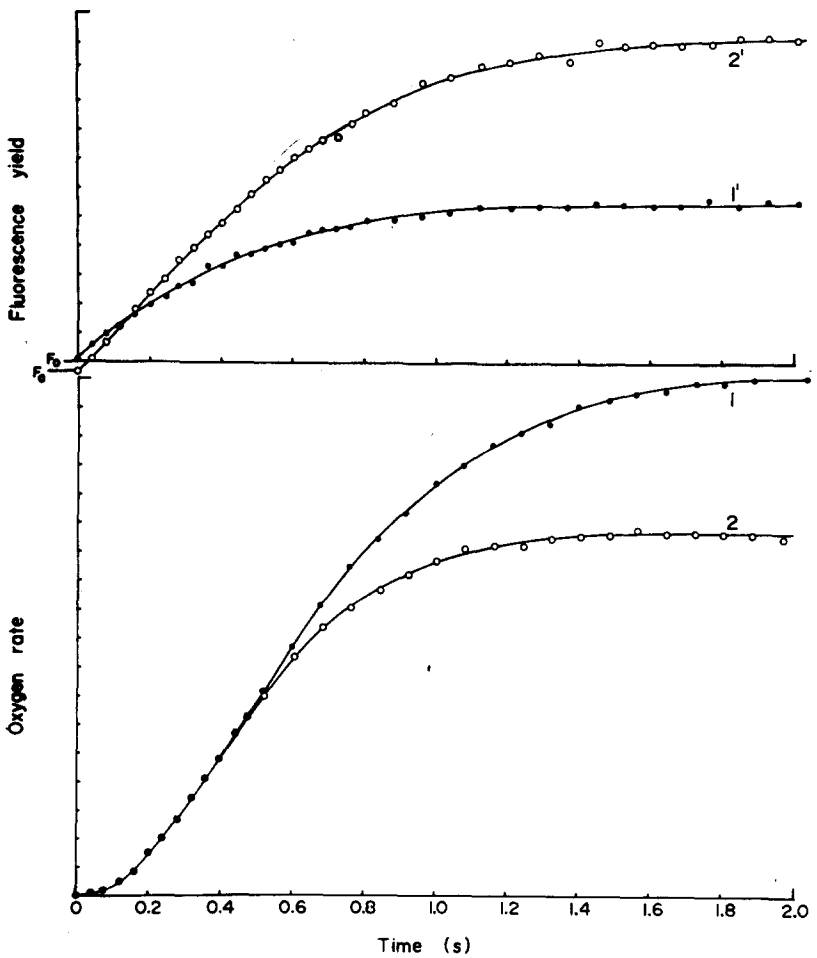

Fig. 10. Oxygen rate and fluorescence yield in weak modulated light following dark adaptation with and without quinone pretreatment. Curves 1 and 1′, untreated algae in the rapidly relaxing field condition. Curves 2 and 2′, algae were treated for 15 min with 10^{-4} M quinone while sedimented on the platinum electrode of the oxygen polarograph. The quinone was washed out with fresh quinone-free buffer. Algae were dark adapted for 10 min before the measurements.

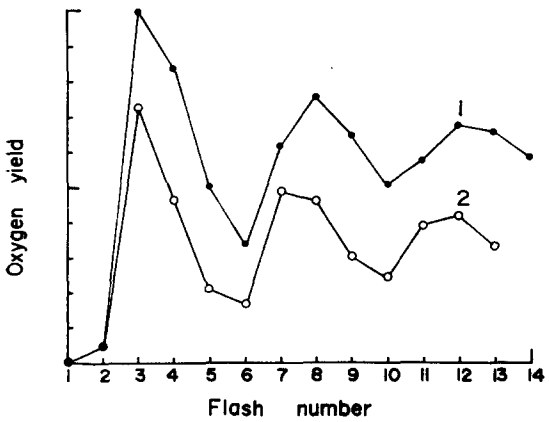

Fig. 11. Oxygen yield for each of a series of saturating flashes spaced 320 ms apart, with and without quinone treatment. Curve 1, untreated algae in the rapidly relaxing field condition; Curve 2, algae (160 μg chlorophyll/ml) were treated with $3 \cdot 10^{-4}$ M quinone for 1 min at which point the quinone was reduced with $4 \cdot 10^{-4}$ ascorbate. Algae were dark adapted for 10 min before the measurement.

minimize the amplitude of the oscillations. This preillumination consisted of an activation by 13 saturating flashes, 450 ms apart followed by 20 s darkness. During the dark period, there is a complete relaxation of the 520 nm absorption change and only a partial deactivation of states S_2 and S_3. Following such preillumination the fluorescence and 520 nm absorption sequences evolve in a parallel manner. As pointed out earlier, there are additional factors which determine the amplitude of the fluorescence curves. For all curves in this figure, S_2+S_3 contribute to the fluorescence yield, while at 1 and 20 ms is added the fluorescence contribution due to the tail-end of the Q^- reoxidation kinetics. These two phenomena are invariant during the flash sequence, following preillumination, as evidenced by the parallelism of the fluorescence and 520 nm curves.

Oxygen rate and fluorescence induction at the onset of weak illumination were measured for algae in the fast-relaxing field condition and following pretreatment with 10^{-4} M quinone. (Fig. 10). Analogous to the behavior of algae in the slow-relaxing field condition, a large inhibition (30 %) and a higher fluorescence yield are observed at the end of the activation period following quinone pretreatment. Also, as observed earlier, the oxygen rates are practically superimposable at the beginning of the activation period. This experiment shows that quinone treatment does not by itself inhibit photosystem II centers. We pointed out earlier that the F_0 for algae in the slowly relaxing field condition is lower than for those which relax rapidly. A similar relation is observed for quinone-pretreated algae (Fig. 10) relative to untreated algae.

Oxygen yields during a flash sequence were studied for untreated and quinone-pretreated algae (Fig. 11). These latter show an average inhibition of 48 %. In other experiments, a large decrease in the damping of the oscillating pattern was often observed for quinone pretreated algae.

Absence of field effect on chloroplasts

Upon illumination following dark adaptation, chloroplasts show a much

smaller first fluorescence rise phase than do *Chlorella*. In addition, the intermediate phase of fluorescence yield relaxation following a single saturating flash is much less developed than for *Chlorella*. For chloroplasts in which the 520 nm absorbance change relaxes with a half-time of 20 ms, no appreciable effect of 10^{-7} M Gramicidin plus 0.1 M KCl was observed on the fluorescence relaxation kinetics despite acceleration of the 520 nm relaxation. These observations lead to the conclusion that, in our hands, the fluorescence yield in chloroplasts, unlike *Chlorella*, is insensitive to the field. The chloroplasts we use have a broken outer membrane. We have not yet checked whether completely intact chloroplasts show a field-dependent fluorescence yield.

Effect of pH on fluorescence relaxation

We have remarked on several occasions, in this section, that another phenomenon, in addition to the field, controls the fluorescence yield between roughly 1 and 100 ms. To isolate this phenomenon we used chloroplasts that are insensitive to the

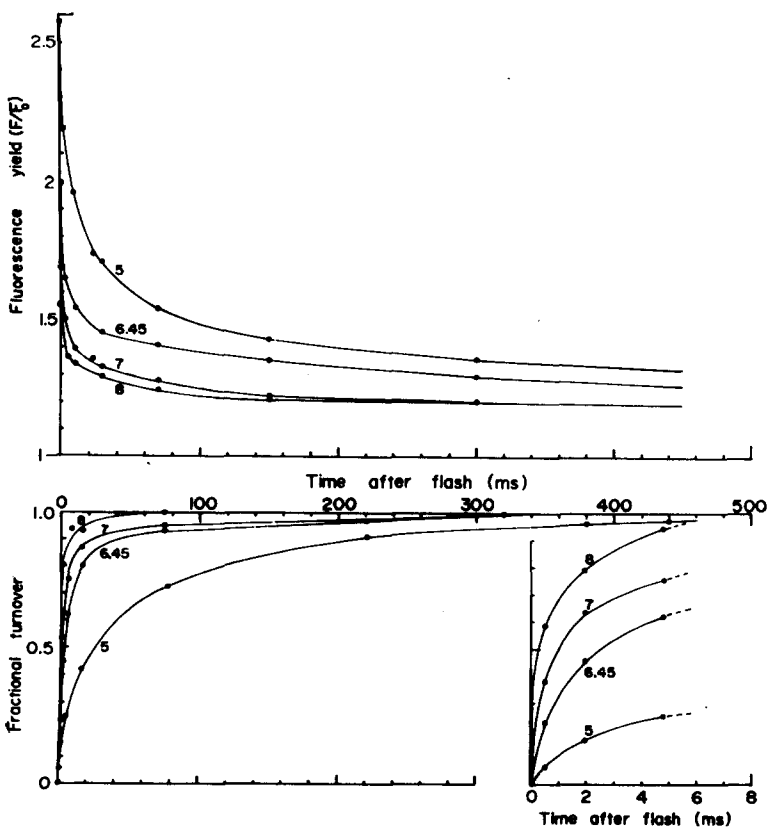

Fig. 12. Effect of pH on the fluorescence relaxation and oxygen detected turnover time ($S'_1 \rightarrow S_2$) following a single saturating flash. Chloroplasts were placed in the following buffers all of which contained 10^{-7} M Gramicidin D, 0.1 M KCl and 0.4 M sucrose: pH 5.0, 50 mM succinate; pH 6.45, 50 mM phosphate; pH 7.0, 50 mM phosphate; pH 8.0, 50 mM Tris.

field. To absolutely guarantee the absence of any field effect the chloroplasts were treated with 10^{-7} M Gramicidin and 0.1 M KCl, in which the 520 nm absorption lifetime is shorter than 2 ms. Simultaneous turnover measurements were performed on an oxygen polarograph and by the flash detected fluorescence method used throughout this paper. For the oxygen measurements, dark-adapted chloroplasts were given a saturating flash followed, a variable time later, by a pair of flashes, 320 ms apart. Detection of oxygen on the third flash as a function of the variable dark interval gives the $S_1' \rightarrow S_2$ turnover in Photosystem II. This turnover time was studied as a function of pH and a definite correlation is demonstrated in Fig. 12 between the oxygen-detected turnover time and the fluorescence yield relaxation following a single flash. Nevertheless, there appears at low pH a very slow component in the fluorescence relaxation which is not observed in the oxygen turnover experiments.

This parallelism demonstrates that the Q^- reoxidation kinetics are progressively slowed on going from pH 8.0 to 5.0. In addition these curves are roughly biphasic, consisting of a rapid phase of about 0.5 ms and a slow phase $\geqslant$ 30–40 ms; the proportion of each depends on the pH. A comparison of these fluorescence relaxation curves with that obtained for *Chlorella* (Fig. 4) after correcting for the field effect, indicates that a similarly decaying phenomenon occurs in the time range 1–100 ms. The kinetics obtained for *Chlorella* resemble most closely those obtained for chloroplasts at pH 7.0 suggesting that this is approximately the internal pH in whole cells.

DISCUSSION

Effect of pH on the reoxidation of Q^-

Bouges-Bocquet [28] and Velthuys and Amesz [29] have shown that an intermediary acceptor B exists between Q and the pool, A. Two reactions may be written for the reoxidation of Q^- : $Q^- B \rightarrow QB^-$ and $Q^-B^- \rightarrow Q B^=$. The half-times for these reactions are $\approx$ 0.5 ms.

To explain the decrease in the luminescence emission upon lowering of the extrathylakoid pH, Kraan et al. [30] proposed that Q^- could be protonated at low pH and that only Q^- is active in the charge recombination responsible for luminescence emission. We propose the same hypothesis to interpret our results for the pH dependence of the Q^- reoxidation time, i.e. that Q^- is reoxidized by B whereas QH is not. The fact that, at pH 7.0, the reoxidation of Q^- is fast, suggests that at this pH little QH is formed. This hypothesis is not in contradiction, with the results of Fowler and Kok [31] who at pH 7.0 did not observe an uptake of H^+ upon reduction of Q in the presence of DCMU (3(3,4-dichlorophenyl)-1,1-dimethylurea).

Effect of the field on Photosystem II

Emrich et al. [36] have proposed that the field perturbs the absorbance spectra of all the pigments contained in the thylakoid membrane (electrochromic effect). We have found that the fluorescence yield is another probe of the transmembrane field. While we have not explored in detail, particularly at low field strength, the exact function by which the fluorescence yield varies with the field, our data imply that, as a first approximation, the function is a linear one, as was demonstrated for the electrochromic effect.

The function relating the fluorescence yield to the transmembrane electric

field appears to be practically independent of the physiological state of the algae and of the algal culture used. What does vary are the kinetics of relaxation of the electric field which induce corresponding charges in the fluorescence yield.

Wolff [37] and Witt [38] have reported a small displacement of the fluorescence emission spectrum due to the transmembrane electric field. The displacement is not the phenomenon we observe in that we detect practically all the emitted fluorescence using wide band-pass filters (see Materials and Methods). Furthermore, Joliot and Joliot [32] have shown that in the Q^- form, the field does not modify the fluorescence yield. This experiment is consistent with our conclusion from the saturating flash experiment (Figs. 7, 11) that the primary effect of the field is a blockage of the Photosystem II centers in a non-photochemical, non-quenching form.

The phenomenon we observe is independent of light induced ion movement (e.g. Mg^{2+}) as our effect can be observed immediately after a saturating flash, while significant ion movement, affecting the fluorescence yield requires extensive illumination. Furthermore, as pointed out by Bennoun [39] the addition of Mg^{2+} to Mg-free Chlamydomonas chloroplasts induces an increase in the fluorescence yield (F_0 and F_{max}) and of the number of active Photosystem II centers whereas we observe a decrease in the number of active centers with increasing fluorescence yield.

It is known [33], that when centers are partially blocked in the Q^- form, a larger inhibition is observed for the oxygen yield per flash than for the photochemical rate in weak light. We observe a similar situation for algae in the slowly relaxing field condition (37 % inhibition for flashes, 25 % inhibition for weak light). While we have adjusted the photochemical turnover rate to be of the same order of magnitude for modulated and for flashing light, a more valid comparison requires a precise measure of the field strength in both excitation conditions.

The photochemically blocked, non-quenching properties of the field-inactivated centers could correspond to blockage of an electron transfer from Q to B and thus an accumulation of Q^-. If such were the case then one would predict that centers in the Q^- form would undergo a charge recombination with a $t_{\frac{1}{2}}$ of 0.7 s [34], and more rapidly still in the presence of the field (0.5 s) [32]. In Fig. 2, the difference curve (B–A) for the fluorescence relaxation is parallel to that for the 520 nm absorption (B′–A′) over a period greater than 4 s. If the fluorescence yield were determined by the state of Q^-, then one would have expected, due to the back reaction, a more rapid relaxation of the fluorescence yield than for the 520 nm absorption.

We conclude that the field-inactivated form of the photosystem II centers does not correspond to an accumulation of Q^-. On the other hand, if the field were to act on the donor side by preventing an electron transfer to oxidized chlorophyll, one would expect either an accumulation of oxidized chlorophyll or the rapid formation of chlorophyll · Q by charge recombination. As both of these are quenching states, this model cannot explain the fluorescence stimulation that we observe. Thus it is likely the field inhibits neither the reoxidation of Q^- nor the reduction of chlorophyll$^+$, and must act directly on the ability of Photosystem II centers to undergo a photoreaction.

We propose two hypotheses to explain our results. A first hypothesis is that the field induces an intra or inter molecular displacement within the reaction center. One can define two states, active (Ac) and inactive (In), related by a field dependent equilibrium constant, $Ac \underset{}{\overset{K(f)}{\rightleftharpoons}} In$.

The fact that the fluorescence yield and 520 nm absorbance relax with the same kinetics (Figs. 2 and 3) means that the equilibration of the centers between their active and inactive forms occurs without a visible delay (< 10 ms). Furthermore, the blockage of the centers occurs within less than 1 ms, as determined by the same experiments. If we assume that all centers are equivalent with respect to their sensitivity to the field (same $K(f)$) then one should observe for an oxygen flash sequence an increase of the miss parameter as defined by the model of Kok et al. [13] equal to $In/Ac+In$.

In Fig. 7 is compared an experimental and theoretical oxygen flash sequence for algae in the rapidly and slowly relaxing field condition. Flashes were given every 160 ms, a time much longer than that required for the equilibration $Ac \rightleftarrows In$.

For fast relaxing algae a theoretical sequence is calculated with a miss parameter of 0.17, a double hit parameter of 0.075, and initial conditions $S_0 = 0.350$ and $S_1 = 0.650$. A satisfactory fit is obtained with the experimental sequence. For the slowly relaxing algae, a theoretical sequence was calculated with a miss parameter of 0.47 for the third and later flashes, which gives an experimentally observed inhibition of 37 % relative to the uninhibited sequence. This theoretical sequence is obviously very highly damped relative to the experimental result. A similar result was obtained for the oxygen flash sequence using untreated and quinone-pretreated algae. Despite a 50 % inhibition of the latter no increase in the damping is observed.

The fact that there is little difference in the damping of the experimental sequences (Figs. 7 and 11) means that contrary to the simplified hypothesis proposed above, the blockage of the centers observed here is not a phenomenon which affects all centers equally, e.g. centers can have widely differing $K(f)$. We conclude, contrary to the simplified hypothesis proposed above, that centers have widely differing sensitivity to the field (different $K(f)$) and that those centers blocked by a certain field strength remain blocked throughout the flash sequence as long as the field does not fall below this level. Furthermore, the range of field strength over which the transformation from the active to inactive state occurs is narrow as evidenced by the very limited effect on the misses in the inhibited flash sequence.

This concept must be reconciled with the fact that, for the centers considered as an ensemble, the fraction of centers blocked is a continuous function of the field. One is led to the conclusion that at every level of the field there are centers that undergo the active-inactive transition.

We have concluded that centers are not equally sensitive to the field. A study of the stimulation of the luminescence emission as a function of the field strength in *Chlorella* cells, led Joliot and Joliot [32] to the same conclusion. A limiting case is observed for our isolated chloroplasts in which centers are totally insensitive to the field. An unknown factor is thus responsible for the variable sensitivity of the centers to the field. This variable factor could be the number or spatial distribution of charges associated with the center, resulting in a variable force upon that center to undergo a conformational change.

In this first hypothesis, we have thus proposed a conformational change to explain the field-induced blockage. In a second hypothesis, not involving large molecular or atomic displacement, one might imagine an electronic perturbation similar to the electrochromic effect, yet which affects the center directly. Such field-reaction center interaction could involve the creation of a dipole or the suppression of

a preexisting dipole resulting in an inactivation of the photoreaction. It is not surprising that the primary photoreaction would be inhibited by the field as the primary charge separation is itself opposed by the transmembrane field. It is then obvious that a diminished probability of charge separation in the chlorophyll excited state would result in an increased excited state lifetime and fluorescence yield. Among the variable factors that could account for the difference in field sensitivity between centers could be:

(1) the orientation of the center with respect to the field;
(2) the distance between the photosystem II donors and acceptors;
(3) the dielectric constant of the environment surrounding the center.

As in the first hypothesis, to explain the very limited effect of the field on the miss parameter, one must admit that for a given center, the quantum yield goes from zero to practically 1 over a narrow range of field strength. The value of the field for which this transition occurs is highly variable among the centers.

We have already remarked that in the slowly relaxing field condition or for quinone pretreated algae, the F_0 level is lower than for rapidly relaxing untreated algae. For the cells in the latter condition the triggering level is quite low, which probably means that the ATP synthetase functions in a reversible manner. Thus if ATP has accumulated during the 3-min preillumination which transforms cells to the rapidly relaxing field condition, then one should observe an ATPase activity linked to proton movement from the outside to the inside of the thylakoid. A weak electrostatic transmembrane potential could be generated, at which point the inward movement of protons is balanced by the outward leakage of cations. This electrostatic potential would elevate the F_0 level and 520 nm absorbance. For algae in the slowly relaxing field condition or pretreated with quinone, the ATP synthetase functions irreversibly (high triggering level) and no proton movement and thus no field formation would occur in the dark. This interpretation is in accord with the observation of Carmeli et al. [35] of a light-induced activation of the ATPase which functions as a proton translocator in the presence of ATP.

ACKNOWLEDGEMENT

Bruce Diner gratefully acknowledges the support of the Helen Hay Whitney Foundation.

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