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THE EFFECT OF DITHIONITE ON FLUORESCENCE AND LUMINESCENCE OF CHLOROPLASTS

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SUMMARY

The kinetics of dithionite-induced changes in fluorescence and luminescence of isolated chloroplasts were studied as function of dithionite concentration and number of preilluminating short saturating flashes.

1. The kinetics of the dithionite-induced fluorescence increase were found to be of an apparently biphasic nature. A relatively small, rapid, increase (accounting for about 15% of the maximum increase that could be obtained with light) was followed by a "slow" increase. This second, "slow", phase, in contrast to the first phase, was strongly dependent on the number of preilluminating flashes. It was slowest after two flashes and most rapid after three.

2. The first phase of the dithionite-induced fluorescence increase was approximately correlated with reduction of the largest part of the total pool of primary and secondary acceptors of Photosystem II.

3. The kinetics of luminescence induced by dithionite were correlated with the rapid phase of fluorescence increase. The highest emission intensity of luminescence was observed after two flashes, but apart from a proportionality factor the kinetics were almost the same after different numbers of flashes. The results indicate that the luminescence is produced by a reaction between an oxidized product, which is the same after different numbers of preilluminating flashes, and reduced quencher, formed mainly during the "rapid" phase of the dithionite-induced fluorescence increase.

4. The results are discussed in relation to existing hypotheses, involving the existence of two primary acceptors of Photosystem II. It is argued that our results do not support these hypotheses and that the effects of dithionite may be explained in terms of a single primary acceptor (and quencher), reduced by dithionite *via* the secondary acceptor pool.

INTRODUCTION

It is generally assumed that at least one of the factors that determine the yield of chlorophyll fluorescence in algae and higher plants and in cell-free preparations of these organisms is the oxidation–reduction level of the primary electron acceptor Q, of Photosystem II. This yield is high when Q is reduced; in the oxidized state Q

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

quenches the fluorescence[1]. Reduction of Q can be brought about not only by light but also by the reductant sodium dithionite, as is indicated by the increase in fluorescence observed upon addition of this substance to spinach chloroplasts[1,2]. Addition of dithionite may also cause an increase in the emission of delayed fluorescence (luminescence); in this case, however, a preillumination is needed[3]. As will be discussed below, both phenomena appear to be related.

This paper presents a study of the kinetics of fluorescence and luminescence of spinach chloroplasts upon addition of dithionite, as a function of various parameters, such as number of preilluminating flashes and concentration of reductant. The results are in agreement with the concept that the increase of fluorescence and that of luminescence induced by dithionite have a common cause, *viz.* the reduction of the primary acceptor and that luminescence is produced upon reoxidation of the primary acceptor by an oxidized product of Photosystem II.

As will be discussed, the fluorescence kinetics may be explained by the assumption that the primary acceptor is reduced by dithionite *via* a secondary acceptor pool, which is in equilibrium with the primary one with an equilibrium constant of about 40. Our results do not support a model involving two primary acceptors as was proposed earlier by Joliot and Joliot[4].

MATERIAL AND METHODS

Chloroplasts were prepared from spinach obtained at a local shop, by a procedure based on the prescriptions of Walker[5]. 50 g of washed leaves were homogenised in 160 ml of a half-frozen solution of pH 6.5, containing 0.4 M sucrose, 0.01 M sodium pyrophosphate and 0.002 M $MgCl_2$, for 15 s in a blendor. The homogenate was filtered through four layers of fine mesh nylon net and centrifuged up to $6000 \times g$ for 90 s. To osmotically disrupt the chloroplasts the pellet was resuspended in 40 ml of ice-cold water. After addition of 40 ml 0.8 M sucrose the preparation was centrifuged for 2 min at $10000 \times g$. The pellet was resuspended in a small volume of 0.4 M sucrose and stored on ice in the dark. The chlorophyll concentration (4–8 mM) was determined according to Whatley and Arnon[6].

About 1 h before the experiment the chloroplasts were diluted to a concentration of $2.5 \cdot 10^{-5}$ or $5 \cdot 10^{-5}$ M chlorophyll by suspension in a solution of pH 7.8 containing 0.2 M sucrose, 0.06 M KCl, 0.04 M NaCl and 0.025 M sodium morpholinopropane sulfonate buffer. Solutions of other reagents that were added in the course of the experiments were prepared in the same medium as that of the chloroplast suspension. Hydroxylamine hydrochloride was neutralized with KOH.

The measurements were performed using the apparatus of Kraan *et al.*[7]. Where indicated, the chloroplast suspension was illuminated in a preillumination vessel. The suspension was subsequently mixed either once or twice with equal volumes containing the desired reagents, and immediately after the final mixing transferred to a measuring vessel to measure luminescence or fluorescence. The measuring vessel was 2 mm thick and contained 3.2 ml of suspension. The time lapse between the start of the final mixing and the complete filling of the measuring vessel was 0.75 s. The concentration of chlorophyll in the measuring cuvette was always $12.5 \cdot 10^{-6}$ M. Preillumination of the chloroplasts was applied in the form of a series of short flashes from a xenon flash tube, transmitted by a Balzers infrared

mirror and Corning CS 4-76 glass filter, at intervals of 1 s. The intensity of the flashes could be reduced by a factor of two without affecting the luminescence intensities observed, indicating that the flashes were saturating for the phenomena studied. The duration of the flashes was 4 μs at one-fifth of the peak. Luminescence, transmitted by Schott RG 645 and RG 630 filters, was measured by an EMI-9558 photomultiplier, situated close to the measuring cuvette.

Fluorescence yield was measured with exciting light of a band around 472 nm by means of another EMI-9558 photomultiplier. It was filtered by a Schott AL 688 interference and two RG 645 glass filters. When needed, the fluorescence was corrected for the luminescence signal seen by the same photomultiplier by subtracting the signal observed without excitation light. The maximal fluorescence yield, expressed as F_m/F_0, was routinely determined by illuminating dark adapted chloroplasts with a strong continuous blue exciting light (2.0 mW/cm^2), obtained by passing the beam from a tungsten halogen lamp through Balzers Calflex C, Corning 4-96 and Schott BG 18 filters. The value of F_m/F_0 obtained in this way was identical to the value obtained with the weak exciting light. (In this last case F_m was measured after a strong illumination in the presence of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 10 mM hydroxylamine[8].) The strong blue light was also used to determine fluorescence induction curves for Figs 3 and 4.

The signals were recorded on a Clevite-Brush 100-Hz recorder. The experiments were done at about 16 °C.

The solutions of $Na_2S_2O_4$ were prepared under N_2 and stored, under N_2, for at most 1 h. Freshly prepared dithionite solution and dithionite stored for 1 h gave identical fluorescence and luminescence effects. The apparatus was closed from the air and preflushed with N_2. No attempts were made to remove O_2 from the chloroplast suspension.

RESULTS

The kinetics of the fluorescence rise observed upon addition of 10 mM dithionite to dark-adapted chloroplasts are shown in Fig. 1A (broken line). After a small initial delay, the fluorescence yield showed a rapid increase for about 10 s, followed by a slower one. The rapid phase covered only an increase in fluorescence yield (ΔF) by about 60% of the initial level of fluorescence (F_0), whereas the maximum yield obtained by illumination (F_m) was 5.1 times the F_0 level. A rapid and a slow phase were also observed by Ikegami and Katoh[2].

The fluorescence kinetics were dependent upon the so-called S state[9] of the chloroplasts. The solid lines in Fig. 1A show the fluorescence kinetics with dithionite after preillumination with different numbers of short, saturating flashes of light. F_0 was somewhat different for different flash numbers, but the rate of the rapid increase was little affected by the number of flashes given. This was not true for the slow secondary phase: especially after 1 or 2 flashes, the rate was slowed down considerably, whereas after 3 or 4 flashes the rates were similar to that of the dark-adapted control. It may be noted that the variation in F_0 is similar to that reported by Joliot and Joliot[4] but the kinetics shown in Fig. 1A do not appear to agree with the model proposed by these authors, as will be discussed below.

Corresponding measurements of luminescence are shown in Fig. 1B. The rapid

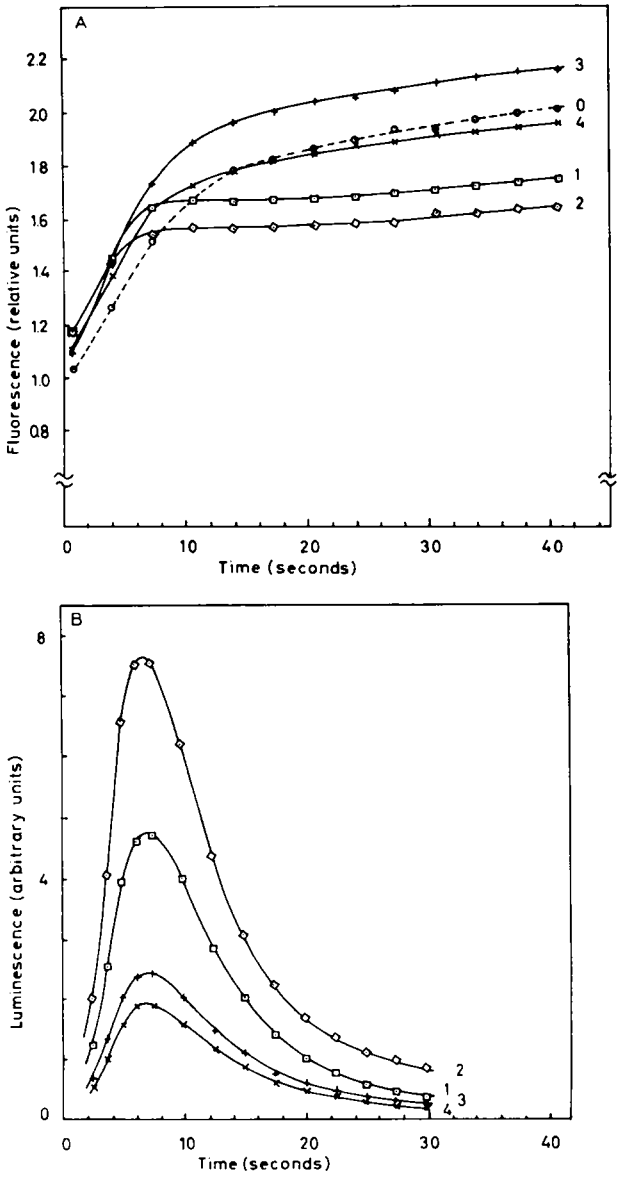

Fig. 1. (A) Fluorescence yield of dark-adapted and preilluminated chloroplasts as a function of time after the addition of dithionite to a final concentration of 10 mM. The chloroplasts had been stored in the dark at 0 °C for more than 1 h. Dithionite was added as an equal volume of a solution of 20 mM. Preillumination was given as a series of short saturating flashes 20 s before the addition of dithionite. Number of preilluminating flashes: □—□, 1 flash; ◇--◇, 2 flashes; +—+, 3 flashes; × —×, 4 flashes; ○---○, no preillumination given. Fluorescence was excited by light with a band around 472 nm, applied discontinuously, 0.5 s for each measuring point. The total amount of light given during the measurement of a curve was too low to affect the result significantly. The curves given in the figure are each the average of four experiments. The fluorescence plotted in relative units, taking 1 for the fluorescence yield of dark-adapted chloroplasts to which buffer instead of dithionite solution was added. In the same units the maximal fluorescence yield, measured with the same chloroplasts illuminated with strong blue actinic light, was equal to 5.1. Chlorophyll concentration $12.5 \cdot 10^{-6}$ M; pH 7.8; temp. 16 °C. For further details see Material and Methods. (B) Luminescence intensity of preilluminated chloroplasts as function of time after the addition of 10 mM dithionite. Same conditions and same chloroplast preparation as for (A). The curves given in the figure are the (calculated) average of four experiments. Symbols as for (A).

phase of the fluorescence increase was approximately paralelled by the kinetics of the luminescence increase induced by dithionite. The luminescence emission, however, reached a maximum after about 7 s and declined afterwards. These results suggest that the stimulation of luminescence by dithionite is caused by a reduction of quencher. No luminescence was observed without preillumination[3], in agreement with the concept that luminescence is produced upon back reaction of a reduced with an oxidized product of Photosystem II generated in the light. The decrease of luminescence after 7 s is apparently caused by exhaustion of the oxidized product, since there is no comparable decrease in the fluorescence yield. Comparison of the yields observed after various numbers of flashes shows that the highest luminescence emission was obtained after the second flash, the lowest after the fourth flash. This pattern is similar to the one obtained for other kinds of luminescence[10–12]. The corresponding fluorescence curves indicate that the dependence on flash number cannot be explained by differences in the amount of reduced quencher produced by dithionite. Apart from a proportionality factor the kinetics of luminescence were almost independent of flash number. This suggests that the same reactants are involved in the production of luminescence after different numbers of flashes.

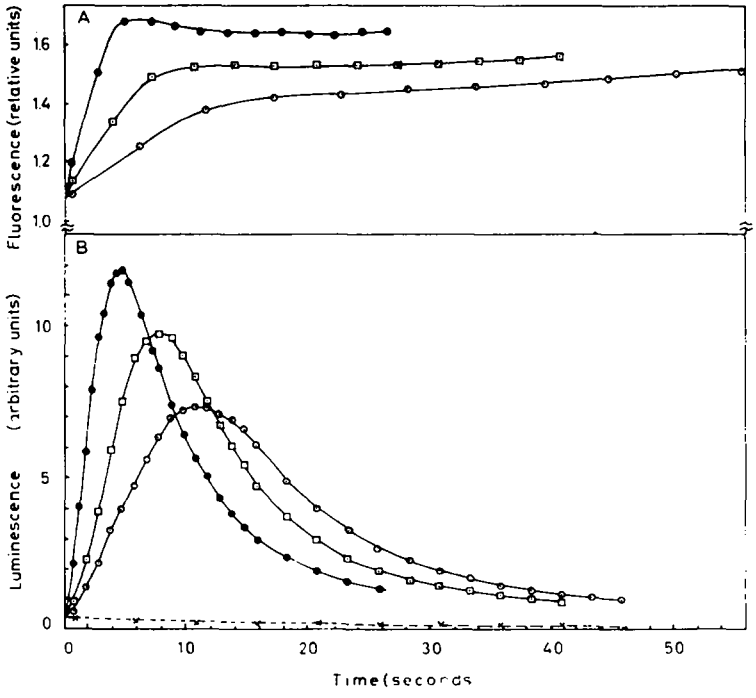

Fig. 2. (A) Fluorescence yield of preilluminated chloroplasts as a function of time after the addition of three different concentrations of dithionite. Same conditions as for Fig. 1A except for dithionite concentration. Dithionite concentration (final concentration): ●—●, 20 mM; □–□, 6 mM; ○—○, 2 mM. Preillumination: 2 flashes. Same units as for Fig. 1A; maximum fluorescence yield: 4.6. (B) Luminescence intensity of preilluminated chloroplasts as function of time after the addition of three different concentrations of dithionite. Same conditions and same chloroplast preparation as for (A). Broken line: no dithionite added (buffer added instead).

The effect of different concentrations of dithionite on luminescence and fluorescence is shown in Figs 2A and 2B. The broken line gives the emission intensity without addition of dithionite. At each concentration of dithionite there was a good correspondence between the time course of the fast phase of the fluorescence increase and of the increase in luminescence. The amplitude of the fast phase of the fluorescence increase was dependent on dithionite concentration; it was larger at higher concentrations. With 20 mM dithionite, the highest concentration used for Fig. 2, a temporary decrease in fluorescence was observed after the first rapid phase.

The biphasic kinetics of the fluorescence increase, as observed in Fig. 1A, might suggest that these kinetics reflect the reduction of more than one quencher, which differ in their reducibility by dithionite, and whose reactivity with dithionite is differently affected by the S state of the chloroplasts. However, as will be discussed below, it is not necessary to explain our results by such an assumption; Figs 3 and 4 indicate that it may be sufficient to assume that there is only one Q.

Fig. 3 shows the kinetics of the increase of fluorescence brought about by illumination with light of relatively high intensity, both in the absence of dithionite (Curve A), and after incubation with 10 mM dithionite (Curves B and C). As reported earlier[2], the rate of the fluorescence rise increased with the time of incubation with dithionite, and the corresponding area above the fluorescence induction curve was reduced. Because the photochemical rate of Photosystem II and emission of fluorescence are "complementary"[13] the area above the fluorescence induction curve may be taken as a measure for the degree of reduction of the total acceptor pool of Photosystem II (refs 14 and 15). In the experiment of Fig. 4, ΔF, measured with weak excitation light, and area, measured with strong excitation light, were determined at various times after the addition of two different concentrations of dithionite. Fig. 4A indicates that the reduction of the total acceptor pool and of the fluorescence quencher are correlated, and, particularly, that both are apparently biphasic. In Fig. 4B the redox level of Q (calculated from the fluorescence yield) and that of the total acceptor pool are plotted *vs* each other. The results are in fair agreement with the assumption that there is only one quencher (Q) which is in equilibrium with the

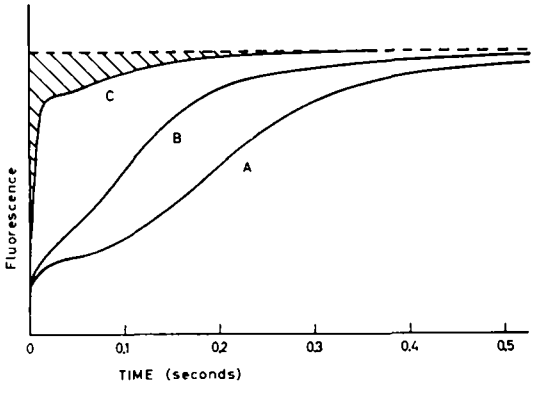

Fig. 3. Fluorescence rise curves obtained in absence and presence of dithionite. The fluorescence rise curves were measured with strong blue actinic light (2 mW/cm²). Curves B and C were recorded after incubation of dark-adapted chloroplasts with 10 mM dithionite for 1.5 and 22 s, respectively. Curve A was recorded 1.5 s after mixing with buffer instead of dithionite solution.

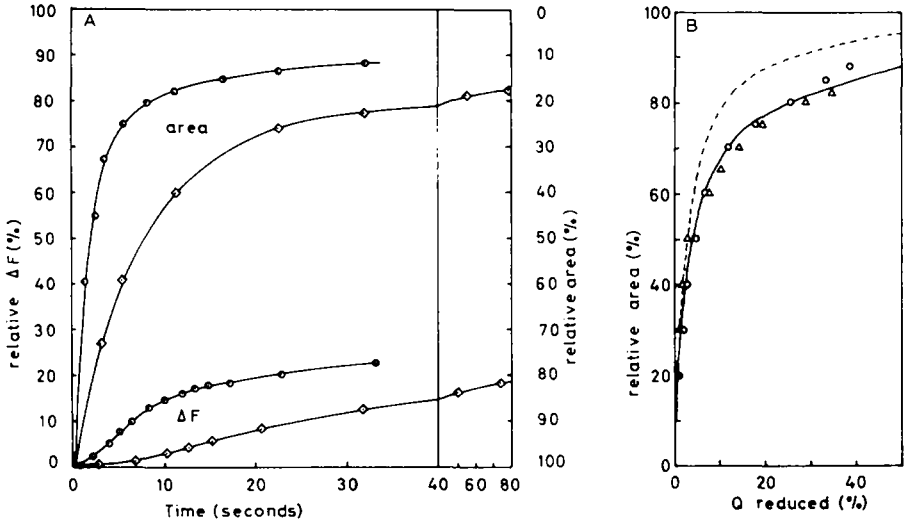

Fig. 4. (A) Lower curves (ΔF): time courses of dithionite-induced fluorescence yield increase of dark-adapted chloroplasts, at two different concentrations of dithionite. The fluorescence yield increase is measured in relative units, taking the fluorescence yield increase obtained by illuminating the chloroplasts with strong blue actinic light (see Material and Methods) as 100%. Upper curves (area): Time courses of dithionite-induced decrease of area over fluorescence rise curves, at two different concentrations of dithionite. Fluorescence rise curves were measured with strong blue actinic light (2 mW/cm^2) at various times after the addition of dithionite to dark-adapted chloroplasts. The measured values for the area over the rise curves are normalised with respect to the area over the fluorescence rise curve obtained with chloroplasts to which buffer was added instead of dithionite solution. Curves for different dithionite concentrations were measured with different preparations of chloroplasts. Dithionite concentration (final concentration): ○—○, 10 mM; ◇—◇, 2 mM. (B) Reduction level of Q *vs* area over the fluorescence rise curve during reduction by dithionite, at two different concentrations of dithionite. The data of (A) are used for this figure (○, 10 mM dithionite; △, 2 mM dithionite). The reduction level of Q was calculated from the fluorescence yield according to an equation introduced by Joliot and Joliot[16], assuming an "energy transfer probability" between photosynthetic units of 0.55 (ref. 17). Two theoretical curves are given, calculated with the following assumptions: (1) The relative area over the fluorescence rise curve is proportional to the degree of reduction of the total pool (P) of acceptors of Photosystem II; (2) P consists of two one-electron acceptors, Q and A; (3) Q and A are in equilibrium, with an equilibrium constant $K=40$; (4) broken line: Q accounts for 5% of P, solid line: Q accounts for 20% of P.

secondary acceptor pool (with an equilibrium constant K approx. 40) if it is assumed that Q accounts for about 20% of the total pool (see Discussion). The latter assumption would also explain why only about 80% of the total pool is rapidly reduced.

Hydroxylamine is supposed to reduce S_3, S_2 and perhaps S_1 (ref. 18). This is also in agreement with the effect on luminescence. Fig. 5 shows the effect on the luminescence induced by dithionite. In this experiment, hydroxylamine (10 mM) was added 7 s after the addition of dithionite. The luminescence intensity was reduced to half the control level in less than 0.5 s; the quenching was complete after 3 s. Therefore, one would expect hydroxylamine to abolish the effect of flash number on the dithionite-induced fluorescence increase.

This prediction appeared to be correct, when we compared the dithionite-induced fluorescence increase after one and three flashes. An addition of hydroxyl-

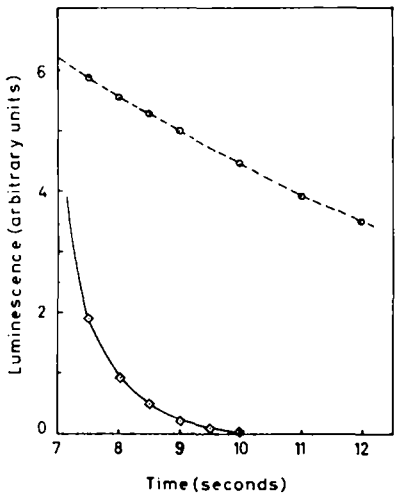

Fig. 5. Time course of dithionite-induced luminescence of preilluminated chloroplasts after an addition of hydroxylamine to a final concentration of 10 mM. Solid line: same conditions as for Fig. 1B, except that 7 s after the addition (at $t=0$) of dithionite an equal volume of hydroxylamine solution has been added. Broken line: no hydroxylamine added (buffer added instead). Preillumination: 2 flashes.

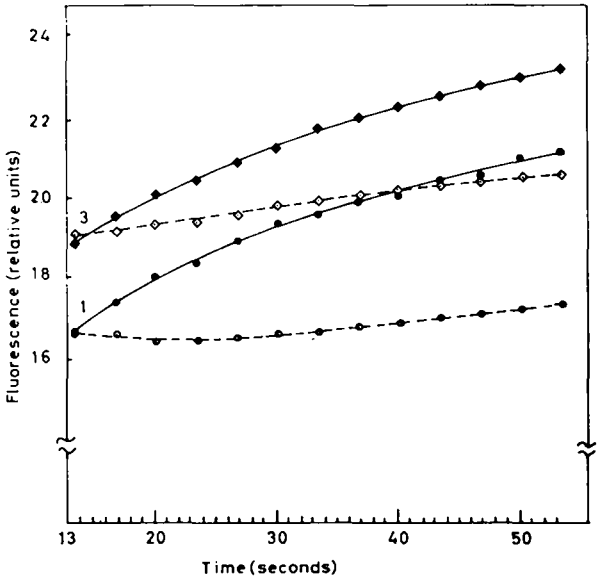

Fig. 6. Time course of dithionite-induced increase in fluorescence yield of preilluminated chloroplasts upon addition of hydroxylamine. Same conditions as for Fig. 1A except that 13 s after the addition (at $t=0$) of dithionite an equal volume of 20 mM hydroxylamine was added. Number of preilluminating flashes: ○---○ and ●—●, 1 flash; ◇—◇ and ◆—◆, 3 flashes. Broken curves: no hydroxylamine added (buffer added instead). Same units for Fig. 1A; maximal fluorescence yield: 4.0.

amine (10 mM) after the last flash, 10 s before the addition of dithionite, caused the fluorescence kinetics to be identical (not shown). Remarkably, hydroxylamine did not abolish differences in fluorescence when added after the addition of dithionite. Fig. 6 shows an experiment in which hydroxylamine was added during the "slow phase" of the fluorescence increase. The rates of fluorescence increase were stimulated by its addition and became identical, and the differences in fluorescence level remained the same.

DISCUSSION

The easiest way to explain the biphasic kinetics of the increase in fluorescence observed in Fig. 1A (broken line), is perhaps by the assumption of two quenchers, one rapidly reducible by dithionite and another which is more slowly reduced. The same assumption was made by Joliot and Joliot[4] on basis of experiments in which the pool of "primary" quencher was determined in the presence of DCMU after prior addition of dithionite. The rapidly reducible quencher was called Q_2, the other one Q_1 by these authors.

The results obtained after preillumination of the chloroplasts (solid lines in Fig. 1A) might be taken as further evidence for the heterogeneity of the quencher pool, and then would seem to suggest that the reduction rate of Q_2 is independent but that of Q_1 is dependent upon the S state. The kinetics of luminescence then would indicate that it is caused by a back reaction of Q_2^- with an oxidized product of Photosystem II.

However, as stated already, the assumption of two quenchers may not be necessary to explain our results; they may be explained in terms of only one Q, if it is assumed that the midpoint potential of Q is significantly lower than that of the secondary acceptor A, and that Q is reduced by dithionite *via* A. The latter assumption is supported[2] by the inhibition by DCMU of the reduction of Q by dithionite. Assuming that Q and A are in equilibrium according to:

$$K = \frac{[Q][A^-]}{[Q^-][A]}$$

then $[Q^-]/[Q] = (1/K) \cdot ([A^-]/[A])$, where K is the equilibrium constant. The rate of increase in $[A^-]/[A]$ will be dependent on the concentration of A and therefore may be expected to slow down at high reduction levels of the A pool. If K is large, the corresponding decline in the rate of increase of $[Q^-]/[Q]$ will occur at rather low reduction levels of Q, giving rise to an apparent biphasic increase in the fluorescence yield. The results of Fig. 4 are in agreement with these assumptions; a difficulty is, however, that one apparently has to conclude from Fig. 4 that the size of the Q pool is about 20% of the total acceptor pool. Estimates of Q by other methods[15,19] indicate that Q accounts for only about 5% of the acceptor pool.

This apparent discrepancy can be resolved by assuming that the 20% of the acceptor pool which is indirectly reduced not only contains Q but also some small secondary acceptor pool, with a midpoint redox potential equal to that of Q. The existence of a secondary acceptor pool with the same midpoint potential as Q is also indicated by other evidence[20–22].

To explain the preillumination dependence of the kinetics shown in Fig. 1A

(solid lines) in terms of a homogeneous Q pool, we can make use of the results of the luminescence experiments. The stimulation of delayed fluorescence upon the addition of dithionite is apparently correlated with the reduction of the rapidly reducible part of Q, as is indicated by the correspondence observed between the kinetics of luminescence and fluorescence after various numbers of preilluminating flashes and with different concentrations of dithionite. This result is in accordance with the concept that luminescence is a result of a reversal of the primary reaction of Photosystem II (ref. 23). This model implies that luminescence is correlated with a reoxidation of Q^- by oxidized photoproduct of Photosystem II. From Fig. 1B one would expect the reoxidation to occur especially after one and two flashes. The retardation of the "slow" fluorescence increase, which is strongest after one and two flashes, could now be explained by reoxidation of Q^- which occurs simultaneously with the slow reduction of Q by the A pool. This would also explain why in some cases, as in the experiment with 20 mM dithionite of Fig. 2, a temporary decrease in fluorescence was observed after the first rapid rise.

Cramer and Butler[24] showed the redox titration curve of fluorescence to have two successive "waves". This result may be interpreted as to agree with the hypothesis that there are two Q's, with different redox potentials. However, there is a rather large discrepancy between the relative amplitude of the rapid phase of the fluorescence increase and the amplitude of the fluorescence increase accompanying the reduction of Cramer and Butler's[24] high-potential component. The former is less than 20% of the maximum attainable fluorescence increase (also in refs 2 and 4) while the latter was reported to be 40–50% of the maximum increase. It might be that the high-potential wave of the fluorescence titration curve was in part or wholly due to an artifact. A continuous exciting beam was used to record the fluorescence changes due to changes of redox potential[24]. Although the intensity of this detecting beam was very low it may have affected the results significantly. The fluorescence increase caused by the beam is expected to be dependent on redox potential. At decreasing redox potential the rate of reoxidation of photochemically reduced Q will decrease, due to chemical reduction of A and of oxidants produced by Photosystem II.

On basis of the assumption that the action of dithionite revealed the presence of two Q's, a model was proposed by Joliot and Joliot[4], where the presence of two primary acceptors, differing in redox potential, was used to explain a number of puzzling phenomena, among them the apparently high redox potential of Q in the light[15,25,26] and the S-state dependence of fluorescence induction by flashes[27] and at -40 °C[4]. According to this model[4] both Q's act alternately as primary acceptors: the low potential one (Q_1) in States S_0 and S_1, the high potential one (Q_2) in States S_2 and S_3. Because of this the fluorescence yield would not only be dependent on the reduction state of the different Q's, but also on the S state. In particular, reduction of Q_2, the quencher easily reducible by dithionite, would be of much more effect on fluorescence in the S_2 and S_3 state than in the S_0 or S_1 state.

This hypothesis is apparently contradicted by our results, in particular those of our Fig. 1A which showed that the rate of increase of fluorescence upon addition of dithionite is initially almost independent of the S state. Moreover it appears from Fig. 5, that a change in the S state, brought about by hydroxylamine, did not have any direct effect on the fluorescence yield.

The dependence of the luminescence yield upon the number of preilluminating

flashes is similar to that observed by Barbieri *et al.*[10] for "normal" luminescence and by Mayne and Hobbs[11] and Hardt and Malkin[12] for luminescence stimulated by acid–base transition and some other treatments. This suggests that the presence of S_2 and S_3 is essential for all these kinds of luminescence. It does not necessarily imply that S_2 and S_3 are reactants in the luminescence reaction: luminescence may be the result of a back reaction of some other more primary oxidized product which reduced Q. As a matter of fact, the almost identical rates of decay of luminescence after various flashes suggest that the reactants involved in the luminescence reaction, and their redox potentials, are the same in all cases. The difference between the yield after 1 or 2 flashes may be caused by a difference in the "exciton yield" of the back reaction. The "exciton yield" is defined here as the number of excited chlorophyll molecules produced (by the back reaction) divided by the number of reactant molecules converted during the same time interval. It was suggested by van Gorkom and Donze[28] that its value may be dependent on the S state.

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