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ANALYSIS OF ANAEROBIC FLUORESCENCE DECAY IN *SCENEDESMUS OBLIQUUS*

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SUMMARY

With reduction of System II acceptors during dark anaerobic adaptation in *Scenedesmus obliquus* fluorescence yield rises to a maximum value in two distinct transitions. Subsequent illumination results in a decay of fluorescence yield with the following characteristics:

1. In low intensity light it is independent of temperature and is an expression of light reaction I.
2. In high intensity light it reflects the dark limiting step in the reoxidation mechanism of System II primary acceptors.
3. There is strong inhibition by agents known to block electron transport between the two systems.
4. At light limiting conditions decay kinetics include an initial delay phase and thereafter close to second order behaviour.
5. Following a single brief saturating flash a maximum of 80 % quenching is restored and a second flash yields approx. 95 % restoration.

Comparison with the fluorescence rise in the presence of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea suggests that the decay reflects transfer of one positive charge from System I to the primary System II acceptor with the intermediary carrier pool remaining reduced.

INTRODUCTION

Dark anaerobic storage of *Scenedesmus* cells induces marked changes in the pattern of chlorophyll fluorescence transients. Previously, we have discussed three different phases in the change of the transients [1,2], suggesting different action sites of oxygen in photosynthesis. In the absence of oxygen an endogenous electron donor reduces the intermediates between System II and System I, including the primary System II accep-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, dibromothymoquinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

tor Q. This eventually results in maximal fluorescence yield and a complete deactivation of Photosystem II. Increase in temperature shortens the time required to achieve this state which is approx. 4 h at 30 °C. There is a rapid reactivation of System II centers upon readmission of air or upon illumination. In the light, fluorescence decays rapidly, the initial rate being dependent on intensity and wavelength of the actinic beam. The decay displays a clear System I action spectrum [2]. System I can reoxidize System II acceptors via the electron transport chain and thus mediate regeneration of quenching at System II reaction centers. There is another much slower type of fluorescence decay in anaerobic cells, which occurs even under conditions when System II centers are not functional (*Scenedesmus* mutant MII) or when electron transport between the systems is inhibited [1,3]. In this paper we report more details of the kinetic characteristics of the anaerobic fluorescence decay. This transient provides a unique tool for the study of some controversial points such as: (a) the mechanism of reoxidation of primary System II acceptors; (b) the size of the intersystem equilibrium constant; (c) the action sites and modes of action of System II inhibitors; and (d) the fluorescence quenching mechanisms at System II centers. In principle the reactions reflected in the anaerobic decay are similar to those observed at regeneration of System II centers after preillumination in air. A distinct advantage is, that the anaerobic system is totally dark adapted and there is no interference of the oxidizing side of System II if the decay is mediated by far-red light or a single brief flash. Furthermore molecular oxygen as an uncertain factor in the reoxidation of Q and A [4] is excluded.

MATERIALS AND METHODS

Scenedesmus obliquus was grown as described previously [2]. If not otherwise stated, before the beginning of a series aereated algae were first kept for 30 min in the dark and then flushed with $N_2+4\%$ CO_2 at 30 °C for 3–4 h until maximum initial fluorescence yield was reached. Each curve was recorded with a fresh batch of approx. 1/1000 of a total reservoir of dark anaerobic adapted algae. Cell concentration was in all experiments close to 10^4 cells/μl. Saturating light intensity was provided by a He-Ne laser (Spectra Physics, model 132). Lower intensity narrow band monochromatic light was isolated with a combination of a monochromator (Jarrel Ash, model 82-410) and interference filters (Balzers). Saturating flashes were delivered by Strobotac flash lamps (General Radio, type 1531) with peak intensity after 2 μs and decay to 30 % intensity within 5 μs. Flashes from two lamps were triggered simultaneously and, after passing through blue filters (Corning 9782), were focused on the cuvette by collimator lenses. Triggering occurred approx. 0.5 ms after opening of a measuring beam by an electromagnetic shutter (Compur-electronic-m). The cuvette assembly and fluorescence measuring device were as described earlier [2]. Inhibitors were added in the dark by a syringe, in anaerobic solutions. The DBMIB was a gift from Professor Trebst's laboratory in Bochum.

RESULTS AND DISCUSSION

The development of the anaerobic decay

In low intensity far-red light there is no indication of chlorophyll fluorescence induction in an aerobic sample; with onset of illumination there is an initial fluorescen-

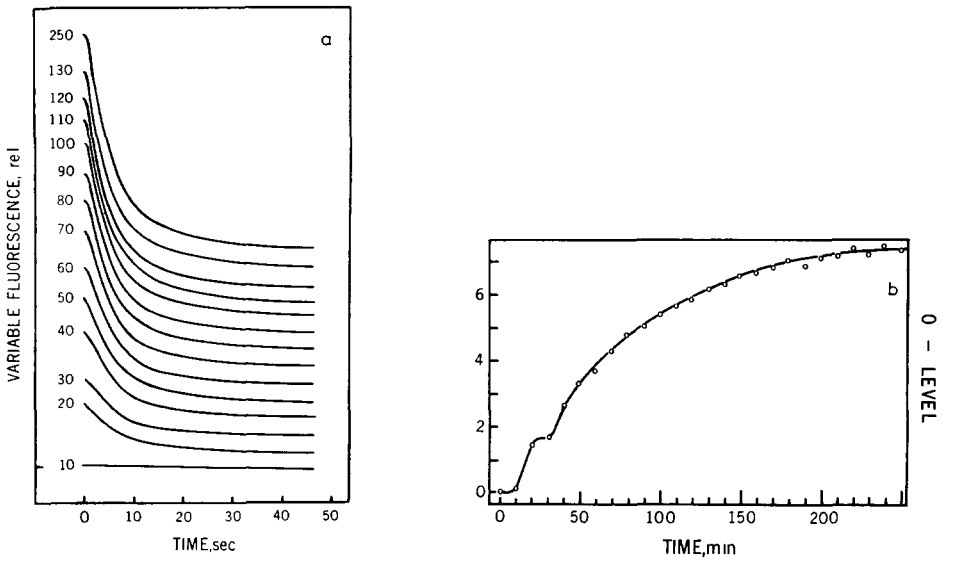

Fig. 1. (a) Development of decay curve with anaerobic dark storage. 701 nm light ($10\,\mu W \cdot cm^{-2}$) serves both as actinic beam and weak fluorescence measuring beam. Fluorescence measured at $\lambda >$ 715 nm with Corning CS 7-69 filter protecting phototube. Each curve is vertically shifted a constant amount relative to the stationary yield, which is practically constant throughout the series. Numbers at left of each curve indicate time (min) of anaerobic dark storage. Temperature 30 °C. Aerobic dark adapted curve is almost identical to 10 min anaerobic curve. (b) Plot of initial fluorescence yield (0-level) versus time of dark anaerobic storage. Same conditions as in (a).

ce yield (defined as O-level) which, under the given conditions, does not change during illumination. The small portion of quanta being distributed to System II does not lead to any appreciable reduction of its acceptors, yet it is sufficient to yield a measurable fluorescence signal. Figs 1a and 1b show how with increasing times of anaerobic dark storage there is a rise in the initial fluorescence yield and the development of a decay curve. Obviously during dark anaerobic storage System II primary acceptors become reduced by some endogenous reductant, and upon System I excitation there is reoxidation via the electron transport chain. In the rise of the O-level with dark anaerobic storage there is a distinct step at approx. 20 % of the maximum yield (Fig. 1b). The cause for this step is not clear. Possible explanations will be discussed in the section on Decay following brief saturating flashes; the phases in the O-level rise appear to be correlated to phases in the decay from maximum to minimum fluorescence following single flashes.

The effect of light intensity and temperature

The fluorescence decay was recorded at different light intensities and temperatures (Fig. 2). Through a wide range of light intensities the decay is independent of temperature and linear with intensity. Eventually the intensity-rate curves separate for different temperatures, linearity is lost and at very high intensities there is saturation and finally even a decrease in rate. Thus over a wide range System I quantum absorption is the limiting step in System II reoxidation, and only at high intensities do the dark steps become visible in the kinetics. The decrease in rate at very high intensities proba-

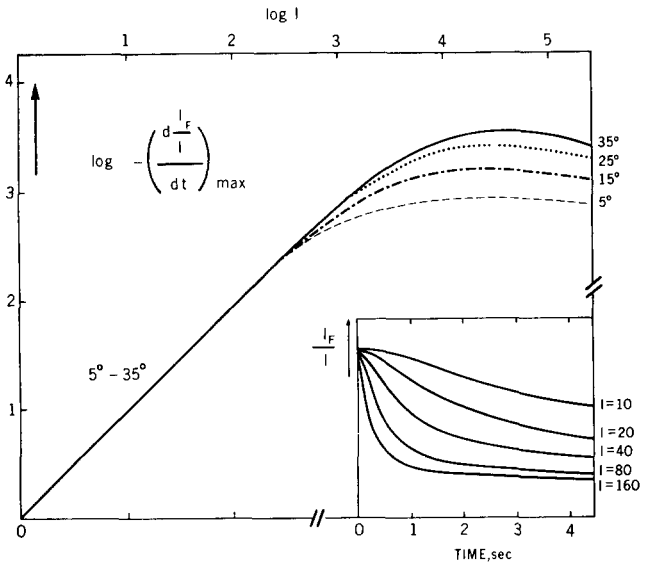

Fig. 2. Light intensity - temperature dependency of maximum decay rate on a double logarithmic plot. $I = 1$ corresponds to 1 $\mu W \cdot cm^{-2}$. Light source, He-Ne Laser, $\lambda = 632$ nm. Inset: Decay in the region of low light intensities. Temperature 35 °C. I_F = relative fluorescence yield.

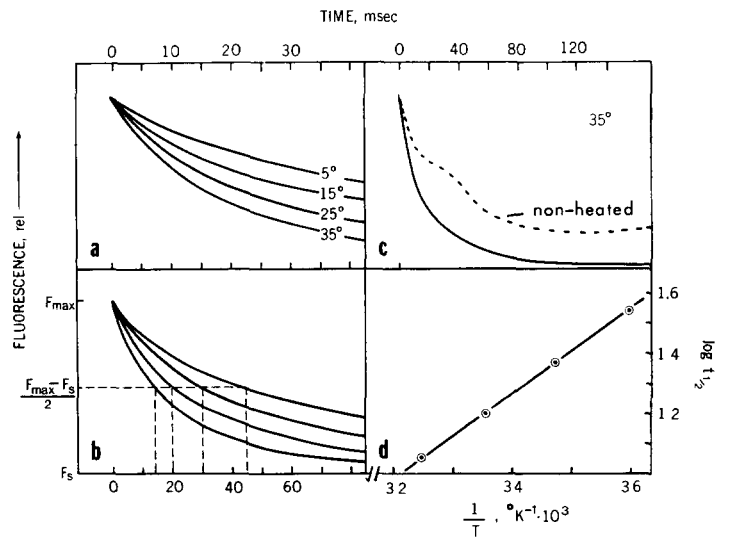

Fig. 3. Decay at high light intensity (0.5 $W \cdot cm^{-2}$). (a–c) Recordings at different temperatures and different oscilloscope sweep rates. F_S is a quasi-stationary level approached by the rapid decay, which is followed by a much slower decay phase not shown here. Light source, He-Ne laser. Sample was heated for 5 min at 45 °C to minimize System II effects; dotted curve in (c) shows non-heated control recorded at 35 °C. (d) Arrhenius plot of temperature dependency of half decay times derived from (b). The slope of the resulting straight line gives $E_a/2.303R$, yielding an activation energy $E_a = 6.7 \cdot 10^3$ cal $\cdot$ mol^{-1}.

bly is due to competitive reduction via Photosystem II. This simultaneous reduction complicates the analysis of the decay at high light intensities. It can be minimized by mild heat treatment (5 min 45 °C), which irreversibly blocks some reaction on the donor side of Photosystem II, without affecting System I activity [5]. In Figs 3a and 3b, decay curves of a heat-pretreated sample are shown for different temperatures. Comparison of control and heat-treated samples at saturating light intensity is given in Fig. 3c. Obviously heat treatment does not inhibit electron transport between the two systems. Temperature dependency of the decay yields with an Arrhenius plot an activation energy of 6.7 Kcal mol^{-1} (Fig. 3d). This value is close to the 7–14 Kcal · mol^{-1} calculated by Rumberg et al. [6] from absorption data for the rate limiting step in plastoquinone reoxidation by System I.

The effect of inhibitors

Addition of DCMU, DBMIB or phenylurethane results in strong inhibition of the decay, while NH_2OH shows practically no effect. This complies with the general assumption that the former three inhibitors block electron transport between the two

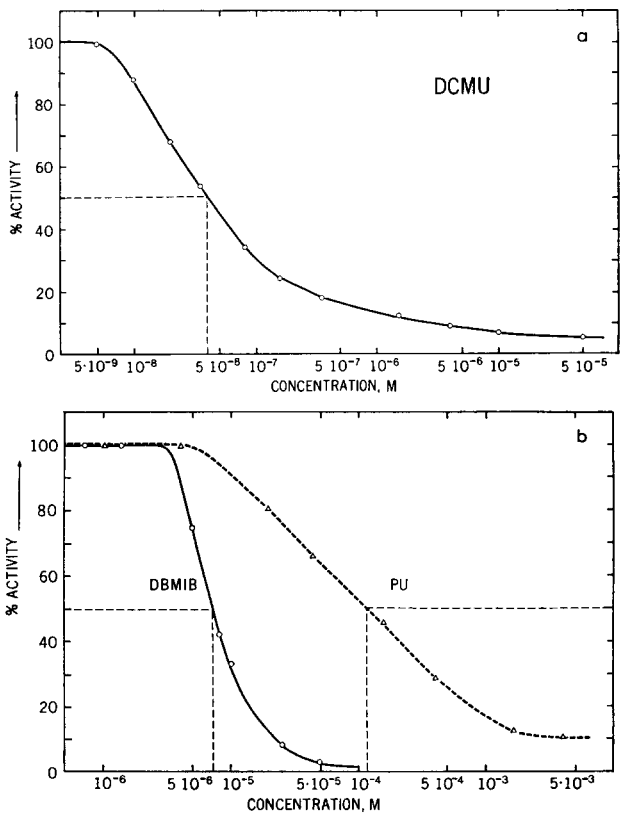

Fig. 4. Effect of inhibitors on maximum decay rate. (a) DCMU. (b) DBMIB and phenylurethane (PU). Percentage of maximum rate in inhibitor free sample plotted versus logarithm of inhibitor concentration. Light intensity, 0.2 mW · cm^{-2}, wavelength 701 nm. Temperature 30 °C. Inhibitors were added O_2-free in the dark. Effect on decay was measured after 20 min incubation. Cell density approx. 10^4 cells/μl.

photosystems, while NH_2OH blocks between H_2O and System II centers. Inhibitor concentration versus activity curves are given in Fig. 4. The order of inhibitor effectiveness is DCMU > DBMIB > phenylurethane; 50 % inhibition concentrations were $4 \cdot 10^{-8}$ M, $7 \cdot 10^{-6}$ M and $1.2 \cdot 10^{-4}$ M respectively. Siggel et al. [7] showed that 50 % -inhibition concentrations for DCMU poisoned spinach chloroplasts differ markedly with light and uncoupling conditions. Their surprisingly low value of $3 \cdot 10^{-8}$ M, which corresponds well with our finding, was only observed for the transport of one electron following a brief saturating flash. The conclusion that a substantial part of the anaerobic decay also reflects transport of just one electron will be supported by flash data presented below.

DBMIB is believed to act as a specific antagonist of plastoquinone [8]. The 175:1 ratio between DCMU and DBMIB effectiveness suggests, at first sight, a much lower affinity of DBMIB to plastoquinone than of DCMU to Q. However, the DBMIB -affinity may be even higher than that of DCMU, if according to Siggel et al [7] there is electron exchange between plastoquinone molecules of different chains and one DCMU molecule blocks two chains.

Phenylurethane is only about 1/3000 as effective as DCMU in inhibiting the decay. Even at saturation concentration the decay persists with 10 % of its maximum rate. Whether this relatively low effectiveness is due to lower affinity or nonspecifity remains uncertain. Additional information about the effect of these inhibitors is given in Fig. 5. Even with DCMU and DBMIB slow decay curves persist at saturation concentrations. Lower concentrations of DBMIB tend to inhibit only the slower part of the decay (Fig. 5a); at higher concentrations a very slow but still remarkable part (approx. 50 % of maximum amplitude) remains. In addition there is a strong static quenching of fluorescence (Fig. 5b), a well-known property of quinones [9]. With DCMU (see Fig.

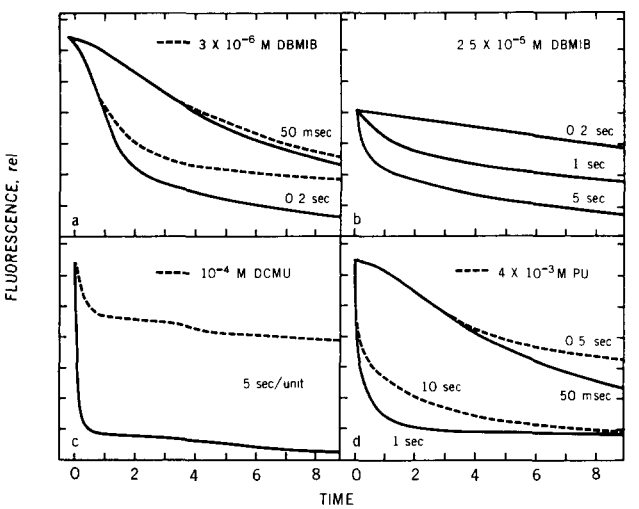

Fig. 5. Effect of inhibitors on decay curve characteristics. Same conditions as in Fig. 4. Time units, differing for different recordings, are indicated on curves. (a) Low concentration of DBMIB. Full lines without inhibitor. (b) High concentration of DBMIB. (c) Saturating concentration of DCMU. Full line without inhibitor. (d) Saturating concentration of phenylurethane. Full lines without inhibitor.

5c) approx. 35 % of the decay remains, although strongly reduced in rate. In Fig. 5d time courses of the decay are compared for saturation concentration with phenylurethane and without phenylurethane: The initial rate is reduced by a factor 10 and finally the same stationary yield is reached with and without the inhibitor. What the remaining decays are indicative of remains to be considered. It was recently shown that DCMU is practically ineffective during the 16th hour of synchronous growth of *S. obliquus* [10]. According to this, presence of 10^{-5} M DCMU should leave a small part of the decay, corresponding to the 16 h cells in our batch culture, with approx. 80 % of the maximum rate. As we in fact find a 35 % amplitude at only 10 % of the maximum rate, this explanation does not not seem satisfactory. Possible explanations for the inhibitor resistant parts of the decay curves are: (1) Intrinsic quenching of fluorescence by the plastoquinone-pool which may persist if electron transfer from System II to plastoquinone is blocked. (2) Quenching of fluorescence by slow α-changes [11], resulting in a more effective utilization of the absorbed energy by System I.

The fact that slow, pronounced decays were also observed in the System II deficient *Scenedemus* mutant M11 [3] agrees with these two explanations, but not with an explanation based on inhibitor inefficiency.

In the experiments described above, inhibitors were added to algae samples in a completely dark and anaerobic adapted state. In the case of DCMU under these conditions the same high inhibition efficiency was observed as by Siggel et al. [7] in an aerobic sample, illuminated by repetitive flashes. This observation appears important as it rules out the hypothesis that DCMU acts on a substance in the oxidized state [12], as well as the hypothesis that DCMU acts on a molecule in an excited state [13].

Comparison with the DCMU rise curve

As shown above (see Fig. 2) the decay is independent of temperature over a wide range of light intensities. In this range it can be considered as indicator for light reaction I, which is the limiting step in the reoxidation of System II electron acceptors, reflected in the decay. A reduction of System II acceptors is practically excluded by the use of low intensity far-red light. The inverse analog of the decay is the rise of fluorescence in presence of DCMU, with System II acceptors being reduced by a light reaction and System I being cut off by the inhibitor. Thus it appears reasonable to compare the DCMU rise curve and the anaerobic decay. In Fig. 6 this is done for equal, low intensities of 680 nm light, which is assumed to be absorbed by both photosystems at the same rate [14]. To minimize regeneration of System II centers via backreaction [15], the experiment was carried out at rather low temperature (15 °C). Under these conditions the maximum yield reached in the DCMU-rise is almost identical to the initial yield from which the anaerobic curve decays. The two curves seem to follow almost antiparallel time courses. An analysis of their kinetics shows, though, that the decay is second order (see following section), while the rise is closer to first order. Despite this difference in kinetics it appears that the two curves reflect respectively restoration and removal of quenching at approximately the same rate. Thus reoxidation of the hypothetical quencher Q proceeds about as fast by 680 nm quanta absorbed by System I, as its reduction by 680 nm quanta absorbed by System II. This is surprising since any concomitant reduction of Q by Photosystem II in the 680 nm light did not affect the decay appreciably. It can be concluded that the intersystem equilibrium constant is very high at the given conditions. The pool of electron carriers between System I and Q obviously is not sub-

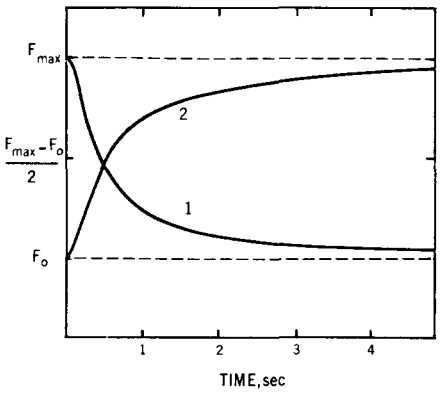

Fig. 6. Comparison of anaerobic decay curve and rise curve in presence of DCMU. Curve 1, decay after 90 min anaerobic dark storage at 35 °C. Curve 2, rise after 30 min dark incubation in $5 \cdot 10^{-5}$ M DCMU in air. Both curves recorded at same intensity of 680 nm light ($50\,\mu W \cdot cm^{-2}$), and same temperature (15 °C).

stantially reoxidized before all Q is reoxidized. This conclusion agrees with Joliot's [16] results which suggest a high K in the dark and a change to a low value only with the accumulation of positive charges on the donor side of System II. The accumulation of positive charges is impossible before Q is reoxidized. Furthermore it was shown that after prolonged anaerobic treatment the electron donor side of System II is reversibly inhibited [2]. It appears difficult to fit our data to Radmer and Kok's model [17] of Q being one molecule of a homogenous pool A_2, reacting at high K with a secondary pool A_1. Such a scheme would predict 4–5 times longer times for Q-A_2 reoxidation than for Q reduction in the presence of DCMU.

Diner and Mauzerall [4] derive from repetitive double-flash experiments in anaerobic *Chlorella* that A and Q are related by a K close to unity. These authors did not observe any increase in oxygen yield in a second flash as consequence of one oxidizing equivalent generated by Photosystem I during a first flash. We can reconcile their data with ours, if we consider a change from high K to low K, as a consequence of the repetitive flash preillumination. As we will show below, a single brief saturating flash leads to significant reoxidation of Q under the conditions used in our experiments.

Amesz et al. [18] reported a fast phase in Q-reoxidation not paralleled by simultaneous plastoquinone-oxidation, which is discussed by these authors as being due to back reaction at System II centers. It appears that a more likely explanation is the mechanism discussed above with high K between Q and plastoquinone, such that the positive charges originated on the System I side of the electron transport chain will first tend to reoxidize Q. Reoxidation of Q via a backreaction in approx. 200 ms also appears rather fast, if compared to Bennoun's data [15].

Decay kinetics at light limiting conditions

In Fig. 7, a typical decay curve at low intensity 701 nm illumination and its reciprocal plot are shown. Except for some deviation at the beginning and at the end, the decay follows closely second-order kinetics. This second-order behaviour is not affected by assumption of interunit energy transfer [19], as one can see from an easily derived

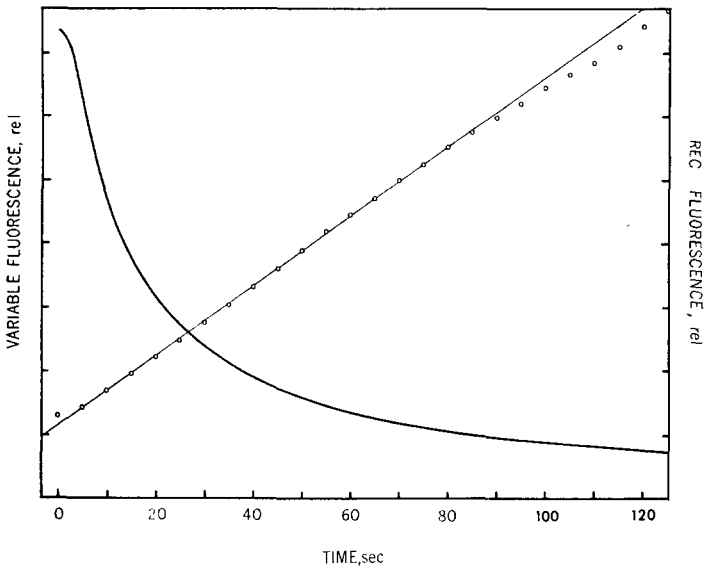

Fig. 7. Decay at light limiting conditions and reciprocal plot to demonstrate second-order behaviour. Light intensity, 5 μW · cm^{-2} at 701 nm; temperature, 30 °C.

equation: A second-order reaction between equal molecules is characterised by the rate equation $dx/dt = k\,(a-x)^2$, with the second-order rate constant k, initial concentration a mol and x mol reacted at time t. Integration leads to $a-x=1/(kt+1/a)$ where $(a-x)$ can be substituted by $[Q^-]$. Following Joliot [19] there is non-linearity between fluorescence yield ϕ and $[Q^-]$, due to interunit energy transfer: $\phi = [Q^-]/(1-p[Q^-])$ or $[Q^-]=\phi/(1+p\phi)$, with p representing transfer efficiency. Applied to the integrated second order rate equation this yields $\phi/(1+p\phi)=1/(kt+1/a)$ or $1/\phi=kt+1/a-p$. As one can see, unaffected by $1 \langle p \rangle 0$, a reciprocal plot of fluorescence yield with time will always yield a straight line for a second-order reaction. On the other hand no satisfactory fit to first order kinetics was obtained with a great variety of assumed values for p and the "true" baseline. The most simple explanation for this behaviour is that regeneration of quenching at System II involves a bimolecular reoxidation mechanism. Initially this reaction shows a characteristic delay. At low light intensities (linear range in Fig. 2) the duration of this "quadratic" delay phase is proportional to light intensity and practically independent of temperature. It therefore appears to reflect some peculiarity of Photoreaction I. At medium high intensities, where dark reactions become limiting, temperature increase hastens the whole decay thus also shortening the delay, but without eliminating it (Fig. 8, curves 1 and 3). Only at saturating light intensities does the decay appear free of the delay phase and this even at low temperatures, where the rest of the decay is rather slow (Fig. 8, curve 2). A reasonable explanation is suggested by the fact that the reoxidation mechanism involves oxidation of the two-electron carrier PQ^{2-} (plastohydroquinone) by some one-electron carrier, which we can call P, at the System I side of PQ (plastoquinone): If two positive charges have to accumulate per chain, this explains a delay at low light intensities. At saturating light intensity generation of positive charges is faster than the consecutive thermal

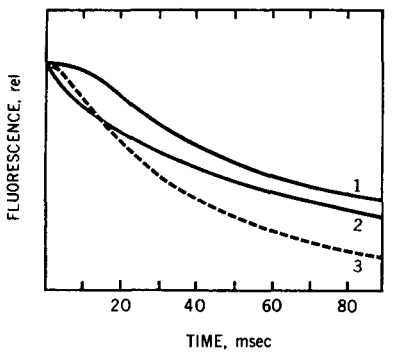

Fig. 8. Effect of light intensity and temperature on initial behaviour of decay. Curve 1, $I = 1$, 5 °C; curve 2, $I = 100$, 5 °C; curve 3, $I = 1$, 25 °C. Conditions as in Fig. 3. $I = 1$ corresponds to 5 mW · cm^{-2}.

reactions, which explains the lack of a delay. Experiments with brief saturating flashes (reported in next section) argue for possible charge cooperation of different P^+ molecules. Such charge cooperation will not eliminate the delay phase at low intensities, but will reduce it at higher intensities. A particular situation is given at medium high intensities [see Fig. 8, curves 1 and 3] where there is clearly less delay at higher temperature. One may conclude that, while a light reaction is prerequisite for the overcoming of the delay, a dark reaction is also involved.

At present, no model is available to explain the bimolecular decay kinetics at light limiting conditions. One should expect the following reaction sequence:

$$P \xrightarrow[h\nu]{\lambda} P^+ \tag{1}$$

$$2P^+ + PQ^{2-} \xrightarrow{k_1} 2P + PQ \tag{2}$$

$$PQ + 2Q^- \xrightarrow{k_2} PQ^{2-} + 2Q \tag{3}$$

But for $\lambda < k_1 \leqslant k_2$ a kinetic analysis yields a zero-order decay of Q^- except for the very last part of the curve. Overall rate is determined by the constant λ and independent of concentrations until $[Q^-]$ becomes very small. An obvious shortcoming of such a treatment is the implicit assumption that the molecules involved behave like molecules in solution, while in reality they probably have only limited degrees of freedom in some membranous matrix. In any discussion of the analogous DCMU-rise curve kinetics at light limiting conditions [see also Fig. 6] the situation is more simple due to the lack of intermediates between the photoreaction and reduction of the quencher. In general, for this case strict separation of chains is assumed, each photocenter having access to only one molecule of Q. Deviation from expected first order kinetics are then explained by non-linearity between $[Q^-]$ and fluorescence yield due to interunit energy transfer [19]. In the above reaction sequence cooperation of two chains is assumed in steps 2 and 3 to explain reaction of the two electron carrier PQ with the one-electron carriers P and Q. No such cooperation is needed with a modification of step 3, which was suggested recently by Bouges-Bouquet [20] and Velthuys and Amesz [21]:

$$PQ + R^{2-} \cdot Q^- \rightarrow PQ^{2-} + R \cdot Q^- \leftrightarrow R^- \cdot Q \tag{3'}$$

and an analogous, up to now merely speculative modification of steps 1 and 2:

$$P \cdot U \xrightarrow[h\nu]{\lambda} P^+ \cdot U \leftrightarrow P \cdot U^+ \xrightarrow[h\nu]{\lambda} P^+ \cdot U^+ \leftrightarrow P \cdot U^{2+} \quad (1')$$

$$P \cdot U^{2+} + PQ^{2-} \xrightarrow{k_1} P \cdot U + PQ \quad (2')$$

where U is some unknown intermediate like R. In such a 'separate chain model' one would expect that reoxidation kinetics start out with a lag due to the consecutive first order light reactions and then proceed close to first order, as only $[R^{2-} \cdot Q^-]$ will determine the reaction rate.

Thus neither a 'separate chain model' nor a 'random solution model' explain the observed second-order decay. Assumption of electronically connected PQ pools [7] and limited cooperation of Q^- molecules may yield a kinetic model describing the observed characteristics. Analog computer simulation studies will be helpful in clarifying this problem.

Decay following brief saturating flashes

In a saturating flash of approx. 5 μs duration each P700 is oxidized in a single turnover [22]. In the dark time following the flash re-reduction of P700 takes place, which is coupled to oxidation of components of the electron transport chain and eventually of System II primary acceptor Q. Fig. 9 shows the decay of fluorescence in a relatively weak measuring beam following a flash. Fluorescence decays in two distinct phases from its maximum value to a minimum value, close to the stationary yield in far-red light. The first rapid phase restores about 70 % of the quenching with a half-de-

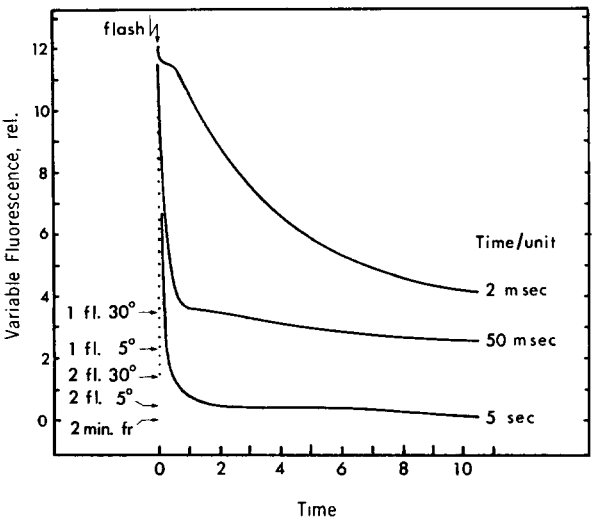

Fig. 9. Decay phases following single saturating brief flashes. Recordings at three different rates. Measuring, beam He-Ne laser, 0.1 mW · cm^{-2}. Flash triggered 500 μs after full opening of measuring beam. Flash intensity saturating; half intensity yields same decay rate. Temperature, 30 °C. Levels indicated by arrows, recorded as initial yield in measuring beam (time resolution 100 μs) after 2 s dark following a flash or 2 min far-red (701 nm, 10 μW · cm^{-2}). Second flashes were triggered 2 s after first flashes.

cay time of approx. 5 ms at 30 °C. This time corresponds well with the 4.3 ms for the rate limiting step in P700 re-reduction after a saturating flash as measured by Haehnel [23] under conditions of a highly reduced electron transport chain. The second, much slower phase reflects further reoxidation of System II acceptors in the "measuring beam". If 2 s dark are given after a flash before the measuring beam is switched on, the initial fluorescence yield in the measuring beam is close to the level separating the two phases. A second flash given 2 s after the first causes fluorescence to decrease to approx. 10 % of its maximal value. Additional flashes yield a further decrease of fluorescence close to the minimal level measured with far-red light in the steady state. There is a brief lag of about 1.5 ms after the flash before the decay starts. Despite its resemblance to the shoulder observed in the low intensity curves (see Fig. 8) this lag appears to be of different origin. As the light reaction is restricted to the duration of the flash, the lag must be due to consecutive dark reactions between $P700^+$ and Q^-. As shown in Fig. 10 the length of the lag phase is much shorter at higher temperatures and the rate of the decay is higher. There is more restoration of quenching after single flashes and 2 s dark time at lower temperature: at 5 °C approx. 80 % after 1 flash and 95 % after 2 flashes, as indicated in Fig. 9. Supposedly this is due to suppression of temperature dependent competitive dark reduction of quenchers. The reductant may be the same which causes the rise in O-level (see Fig. 1) or some substance at the acceptor side of System I, cycling electrons back to System II [24,25]. The fact that the measuring beam is relatively strong impedes the analysis of the decay kinetics following a flash. Neither a logarithmic nor a reciprocal plot yielded a satisfactory linear relation. Use of lower measuring light intensity was not possible in the present study because of luminescence and photomultiplier artifacts due to the saturating flash.

The fact that a single brief flash can yield appreciable reoxidation of Q is very important. It shows that (a) there is charge cooperation at the System I side of the electron transport chain to oxidize PQ^{2-}; (b) electron 'holes' produced at P700 in a flash end up with high probability at the primary System II acceptor, which argues for

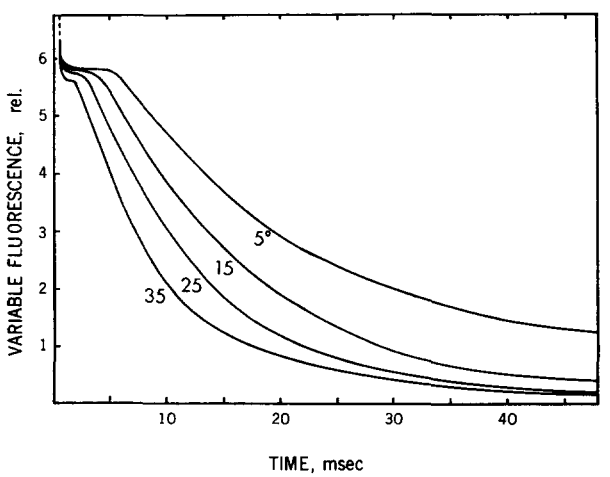

Fig. 10. Temperature effect on decay curve following saturating brief flash. Only rapid first phase of decay shown. Conditions as in Fig. 9.

a high intersystem equilibrium constant under the given conditions. In the model of Stiehl and Witt [26] for the mechanism of electron transport from System II to System I a PQ-PQ pair, connected to 2 Chl a_{II} serves as primary System II acceptor, and the probability for the location of PQ^{2-} is the same at all positions within the plastoquinone pool. It appears that such a model can not explain the high restoration of quenching with one flash.

The fluorescence level reached by a single flash at optimum conditions is close to the level reached after the first transition in the O-level rise with dark anaerobic storage (see Fig. 1). It is tempting to see a correlation between these two things: The second transition in the rise could reflect the inverse reaction of the first decay phase, and the first transition in the rise consequently could correspond to the second decay phase. In the following, possible explanations for this behaviour are given and discussed:

(1) Inhomogeneity of System II primary acceptors with one fraction more easily reducible than the other [16,27]. In Joliot's terminology [16] each System II center is connected to one molecule of Q_1 with low potential and to one molecule of Q_2 with higher potential. With dark anaerobic storage one would expect a first phase in O-level rise due to Q_2 reduction parallel to reduction of the secondary A-pool; with practically all A and Q_2 reduced, in a second phase Q_1 becomes reduced. After total reduction of Q_1 and Q_2 in the dark a first flash will cause reoxidation of Q_1, and further flashes reoxidation of Q_2 along with A. This model fits our results well, if Q_1 quenching constitutes approx. 80 % of the total quenching, a value in the range proposed by Joliot [16].

(2) Inhomogeneity of fluorescence quenching [28], with Q_F quenching involving Q_1 or Q_2, Q_R quenching intrinsically involving the oxidized plastoquinone pool, and Q_S quenching involving the oxidative side of System II. From our data we conclude that Q_R quenching does not constitute more than approx. 5 % of the total quenching, as after two flashes 95 % quenching is restored, with plastoquinone still being reduced. Following this new model by Joliot [28] the first decay phase corresponds to restoration of Q_F quenching, with Q_1 connected to the center and the second phase to restoration of Q_S quenching with Q_2 connected to center.

(3) A two step redox mechanism $Q \rightleftarrows Q^- \rightleftarrows Q^{2-}$, with only Q^{2-} being reoxidized by the A pool [29,30]. If Q^- shows approx. 80 % quenching efficiency as compared to Q, this would explain the phases in O-level rise and decay.

(4) In a variation of (3) a two step redox mechanism involving an intermediate R between Q and A [21]:

$$Q \cdot R \rightarrow Q^- \cdot R \leftrightarrow Q \cdot R^- \rightarrow Q^- \cdot R^- \leftrightarrow Q \cdot R^{2-} \rightarrow Q^- \cdot R^{2-}$$

where only R^{2-} is reoxidized by A.

Both explanations 3 and 4 appear only possible, if at the same time, with high interunit transfer efficiency [19], non-linearity between Q and fluorescence yield is assumed: With one flash only 1/2 PQ will be produced per chain, giving an optimum of 1/2 Q or 1/2 $Q \cdot R^-$ per chain. If this is to correspond to 80 % restoration of quenching, transfer efficiency p has to be approx. 0.75, a value close to that discussed recently by Joliot et al. [31]. This argument is only valid with the assumption of a 1:1 ratio between System I and System II centers.

CONCLUSIONS

Due to dark anaerobic reduction of the electron carriers between System I and System II by an endogenous electron donor a unique state of the photosynthetic apparatus is achieved, which includes maximal fluorescence yield and total inhibition of System II centers. Upon illumination this inhibition is completely overcome by System I activity via the electron transport chain and reoxidation of System II acceptors is paralleled by restoration of fluorescence quenching. When monitored under light limiting conditions the resulting fluorescence decay yields valuable information about Photosystem I primary reactions; if measured under dark limiting conditions it is an indicator of intersystem electron transport. It thus complements the information gained from the fluorescence rise curve in the presence of DCMU, which relates to the Photosystem II primary reactions and exhaustion of fluorescence quenchers.

The rates of the DCMU rise and the anaerobic fluorescence decay, under light-limiting conditions and comparable active absorption, are approximately equal. From this it may be concluded that the decay also represents a single electron transfer. Restoration of as much as 80 % of fluorescence quenching by a single flash supports this conclusion, as do the DCMU-inhibition characteristics [7]. These findings are in accord with the concept of a large electron carrier pool between the photosystems, only if the primary System II acceptor is clearly distinguishable from the main plastoquinone pool and related to this by a high K, under conditions of the decay.

As does the DCMU-rise [28,30] the fluorescence decay consists of two phases, which possibly reflect heterogeneity in quenching at System II centers. This heterogeneity is also expressed in the phasic rise of fluorescence yield with dark anaerobic adaptation.

The kinetics of the decay display remarkable characteristics: (a) with light limiting conditions there is an initial "quadratic" phase; (b) the rest of the curve follows close to 2nd order kinetics; (c) with dark limiting conditions there is a lag, which is more apparent at low temperature, but one flash is sufficient to initiate a pronounced decay. While (a) seems to indicate some priming reaction, (c) excludes this. Therefore we conclude that, while there is necessity of cooperation between positive charges formed at Photsystem I, separate chains can join to oxidize PQ^{2-}. No current model of Photosystem I initiated electron transport is adequate to explain the apparent second order decay under light limiting conditions. Kinetic analysis of fluorescence transients is complicated by uncertainty about non-linearity between quencher concentration and fluorescence yield. However a second order reaction will yield linear reciprocal plots regardless of interunit energy transfer efficiency.

As yet only part of the information inherent in the decay has been collected. More insight into the mechanisms involved are expected from simultaneous absorption measurements of P700, cytochrome *f*, PQ and C550 under the conditions of the anaerobic decay studies.

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