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RAPID FLUORESCENCE CHANGES OBSERVED IN CHLOROPLASTS: THEIR RELATIONSHIP TO THE O_2 EVOLVING SYSTEM

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SUMMARY

The time course of fluorescence yield was measured either during or after brief flashes given in a sequence to dark-adapted algae or isolated chloroplasts.

(A) The fluorescence kinetics during short (2–12 μs) flashes indicated two light-driven reactions of similar quantum yield, one increasing the fluorescence yield, the other quenching it. The characteristics of the quencher were consistent with those of a carotenoid radical or triplet.

(B) The oscillations of fluorescence yield attained in each flash in a sequence given to dark-adapted chloroplasts were eliminated by 100 mM methylamine, which does not alter O_2 production during the flashes. This indicates that the fluorescence yield during a flash, although influenced by the S states, is not directly controlled by them.

(C) Measurements of the fluorescence yield from 80 to 1000 μs after individual saturating flashes in a sequence indicated the following:

1. A previously unseen 35-μs rise occurs after the flash. The rise correlates with a lag in System II recovery. It is not eliminated by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), is accentuated by NH_2OH and is only a little dependent upon the S states.

2. For the first two flashes in a sequence the kinetics of fluorescence recovery correspond to the kinetics of relaxation of the O_2 system. The recovery after the first flash was faster than after the second. The recoveries were not changed by the addition of 10 mM NH_2OH.

3. The recovery kinetics are complex (at least biphasic). Under all conditions studied, including a varying degree of reduction of the acceptor Q, there appeared to be a significant, 200–250-μs component.

(D) The results are interpreted in terms of a single type of System II trap. The oscillations of the fluorescence yield and the 35-μs rise are governed by movement of charge on the donor side of System II. Fluorescence behavior at longer times is governed primarily by the state of the acceptor Q.

INTRODUCTION

Fluorescence has proven to be a useful parameter for describing the state of

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

trapping centers in photosynthetic organisms. In principle photochemistry competes with fluorescence for quanta: when the traps are closed, fluorescence is high; when the traps are open, quanta are converted and unavailable for fluorescence. This principle, while applicable to reaction centers of bacteria and Photosystem II of green plants and algae, apparently is not applicable to System I of green plants, as evidenced by the failure to observe fluorescence changes within System I. In green plants a high fluorescence yield has generally been attributed to the reduction of Q[1], the presumed primary acceptor of the System II trap. The donor presumably reacts very rapidly so that for most measurements it is in its open, reduced state.

A number[2,3] of recent observations indicate that the fluorescence characteristics of green plants at short times are too complex to be explained by the behavior of Q alone: Mauzerall[2] found that with completely dark-adapted *Chlorella*, the fluorescence following a 2-ns flash was initially low and then increased *via* two dark steps with half-times of 25 ns and about 3 μs, respectively. The 25-ns rise was not seen after subsequent flashes. Delosme[3] observed an oscillation of the fluorescence yield during each flash in a sequence of flashes, correlated with the momentary state of the four step O_2-yielding mechanism[4].

Here we report on further investigations of fluorescence changes of chloroplasts and green algae during short saturating flashes and in dark periods following such flashes. We will consider the types of interactions involved and try to interpret the fluorescence changes in terms of those due to changes in Q and those having other origins.

MATERIALS AND METHODS

Chloroplasts and algae were prepared as described previously[5]. No acceptor was used unless indicated. The sample chamber was 1 cm of a 2-mm diameter Teflon tubing imbedded in a rectangular piece of plexiglass to lessen focussing by the tubing. This resulted in illumination which was still not completely homogeneous. The absorbance was in all cases less than 0.2.

Fluorescence during 2-μs flashes was observed by means of an S 20 surface photomultiplier. The preamplifier signal was fed into a Biomation 610 transient recorder operating at 0.1 μs per advance with a frequency response of 2 MHz. The signal was then fed into a signal averager to average noise due to flash fluctuations. Each curve was the average of between 32 and 128 observations, each on a fresh sample. An automatic sample changer was used to replace the sample on command with a fresh, dark-adapted one. The chloroplasts were dark-adapted at least 20 min before measurements commenced. Complementary filters (Corning 2-64 and 4-96) were used to separate fluorescence from measuring light.

Fluorescence decay after flashes was measured in an apparatus described previously[5]. Saturating actinic flashes (approx. 2 μs long) were given to the suspension, and the fluorescence yield monitored by a weak, 14-MHz, modulated measuring light. The measuring light was on only during the time of measurement. A rotating mechanical shutter protected the photomultiplier from the flash and from the flash-induced fluorescence. The electronic time response of the system was adjusted to 5 μs. Each presented curve is the average of between 256 and 1024 observations, each made with a fresh sample. The photomultiplier shutter opened completely 80 μs after each flash. Flashes were given 1 s apart for chloroplasts and 0.2 s for algae.

RESULTS

Fluorescence during the flash: kinetics

Delosme[3] observed that the fluorescence during brief xenon flashes first rose and then declined. We did similar experiments to investigate the cause of the decline and determine whether the decline represented a dark step or a light-driven reaction. A typical example of this rise and decline is seen in Fig. 1(a), where the variable part of the fluorescence (*i.e.* total fluorescence observed less F_0, the initial fluorescence for dark adapted chloroplasts) is plotted against quanta absorbed during the fifth flash of a sequence given to dark adapted chloroplasts. Similar results were obtained for other flashes. When the traps are kept closed by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and weak background light, only the decline is seen (Fig. 2). The results of Figs 1 and 2 (open and closed traps, respectively) both show a decline in fluorescence. We conclude from these experiments that light produces a quencher of fluorescence and that the production of the quencher does not require traps to be open.

A number of observations, to be described below, suggest that the quencher is identical to carotenoid radicals or triplets, which are formed with high quantum yield (close to unity) when traps are closed[6–9]. Mathis and Galmiche[9] found that these radicals recover in about 3 μs and 0.6 μs in air and O_2, respectively. Wolff and Witt[8] suggested that this reaction serves as an outlet for excess energy.

By using a longer stretched (12 μs) flash, we observed that the extent of the decline in the presence of DCMU and weak background light at a flash intensity I_0, which produced a half time of 0.25 μs for the decline, was the same as the extent seen at 2.5 times the intensity. The extent at 0.25 I_0 was only 75% of that seen at I_0. Equilibrating the suspension with air at 0.25 I_0 further decreased the decline to 50%. From this we estimate, assuming a first-order light-driven quenching reaction followed by a first-order back reaction, the lifetime of the quencher to be about several μs in

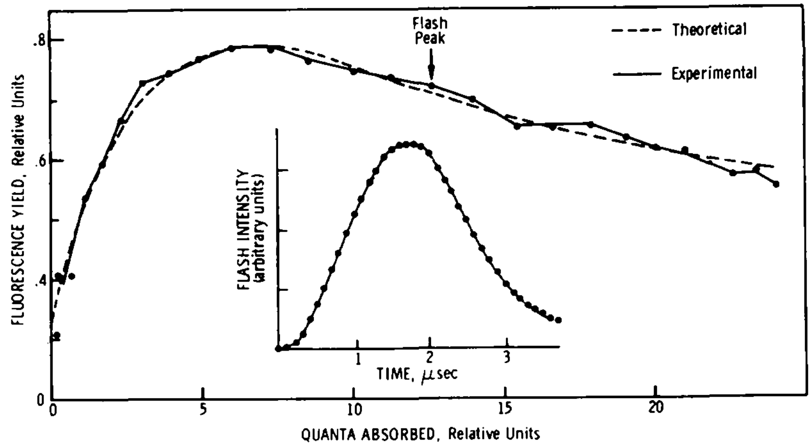

Fig. 1. (a) ●—●, variable part of the fluorescence yield as a function of quanta absorbed during the fifth, brief (2 μs) flash given to dark-adapted chloroplasts. The quanta absorbed at time t was calculated from the time course of the intensity of the flash (insert) assuming that quanta are proportional to $\int_0^t I\,dt$, where I is flash intensity. The peak of the flash is indicated by the arrows (b) -------, theoretical curve as described in the text.

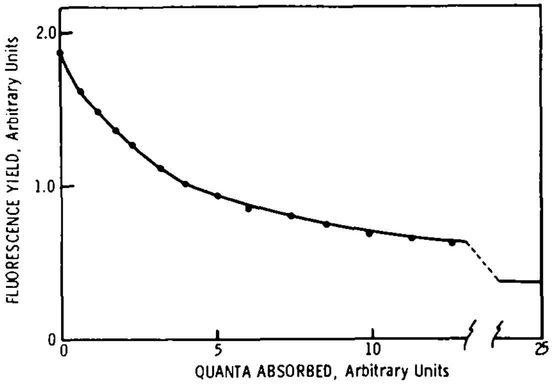

Fig. 2. Variable part of the fluorescence as a function of quanta absorbed during a brief 2-μs flash given to chloroplasts containing 1 μM DCMU in the presence of weak background light. Flash intensity and ordinate same as in Fig. 1 (a).

air, and about three times faster in O_2. The inhomogeneity of the beam, the changes in flash intensity with time, and the possible production of more than one quencher per trap, precluded very accurate measurements of the quencher lifetime. The influence of these interferences is indicated by deviations from first order for the decline (Fig. 2). Still, our lifetime estimates based on first order assumptions are good enough to warrant comparison with those previously measured for carotenoid radicals and to conclude that carotenoid radicals or triplets are responsible for the quenching. A similar conclusion has been reached independently by Duysens *et al.*[10].

If we assume that at higher flash intensities for which the back reactions are small the light-induced quenching is produced only after the traps are closed and that the quantum yield for quencher production is the same as that for closing the traps, and use for the quantum yield and for the final quenching those values obtained in the presence of DCMU (Fig. 2) we predict the fluorescence kinetics shown in Fig. 1(b). The ultimate height that would have been obtained without quenching and the yield at $t=0$ were adjustable parameters. The prediction shown in Fig. (1b) agrees well with the measurement (Fig. 1, a). Both indicate that the maximum yield (of the variable fluorescence) reached is only 40 ± 15% of that which would have been reached in the absence of the second quenching reaction.

Fluorescence maximum during the flash

Delosme[3] showed that in dark-adapted chloroplasts and algae the peak of the fluorescence yield oscillates with a period of four. The results of similar measurements made by us are shown in Fig. 3(a). These results are quite similar to those reported by Delosme, namely, high yields during Flashes 1, 5, 9 and low yields during Flashes 3 and 7. Delosme[3] related the oscillatory behavior of fluorescence to the S states[4] of O_2 evolution. Indeed, we found that elimination of O_2 by 10 μM DCMU or 5 mM NH_2OH (Fig. 3, b) eliminated the oscillations of fluorescence. In the presence of NH_2OH, not only were the oscillations eliminated but also the fluorescence yield was greatly diminished.

We observed small (approx. 10%) variations from one chloroplast preparation to another leading us to believe that the S states alone are insufficient to define the

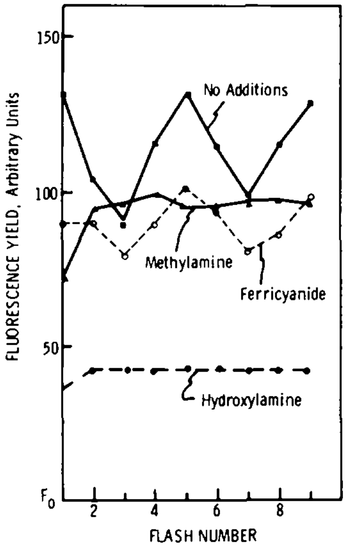

Fig. 3. Variable part of the fluorescence yield at the maximum yield during each of a series of nine flashes given to dark-adapted chloroplasts as a function of flash number, (a) with no additions, (b) with 5 mM NH_2OH, (c) with 100 mM methylamine, (d) with 200 μM $Fe(CN)_3$. All chloroplasts were from the same preparation. The maximum fluorescence yield obtained with DCMU and steady light is approx. 300. All yields expressed in arbitrary units.

oscillatory pattern. This belief is strengthened by the observation that the addition of methylamine which did not alter the oscillatory behavior of O_2 (these O_2 measurements were kindly performed by Marion McGloin Smyth using apparatus described in ref. 4), caused rather distinct changes in the oscillations of fluorescence (Fig. 3,c). It decreased the fluorescence yield during the first flash and abolished the normal oscillatory behavior. These effects of methylamine were only observed with concentrations greater than approx. 30 mM. We previously noted[5] that similar, high, concentrations of methylamine altered the 35-μs component of delayed light such that the normal oscillations were eliminated. However, these concentrations of methylamine do not alter the 200-μs delayed light component[5]. We therefore conclude that neither the oscillation of the fluorescence in the flash (Fig. 3, a) nor that of the 35-μs delayed light are correlated in a simple manner with the S states of O_2 evolution.

Ferricyanide (200 μM) lowered the fluorescence yield of the first flash (Fig. 3, d). We have no ready explanation for this interesting observation. The slight lowering on subsequent flashes is probably due to screening of the actinic light by the ferricyanide.

Fluorescence rise after the flash

In addition to the 3-μs recovery of the carotenoid quencher described above, we observed a second, previously unseen, increase of the fluorescence yield which occurred after termination of the flashes. This second dark rise was most readily observed after the first flash given to dark-adapted chloroplasts containing DCMU which prevents the subsequent dark decay of the yield (Fig. 4). Although measure-

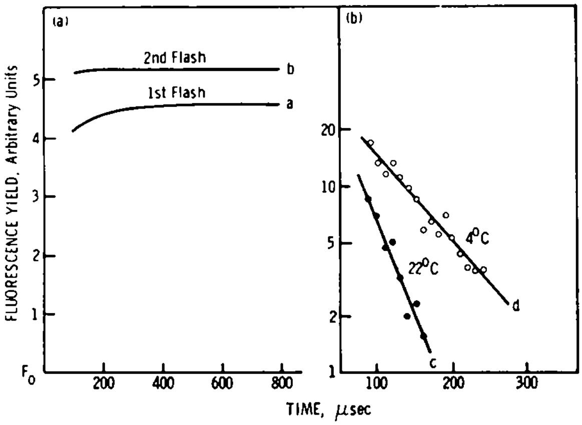

Fig. 4. The variable part of the fluorescence (*i.e.* measured from F_0) as a function of time after (a) the first flash, (b) the second flash given to dark-adapted chloroplasts at 4 °C containing 10 μM DCMU. Measurements were taken at 10-μs intervals. All measured points lie within the solid lines. (c) The log of the difference between the fluorescence yield at time t after the first flash and the average yield between 700 and 800 μs as a function of t for chloroplasts at 22 °C containing 10 μM DCMU, (d) at 4 °C.

ments were limited to times greater than 80 μs after the flash, the data were precise enough (Fig. 4,d) to compute the half time of an assumed first-order rise to be 65 ± 15 μs at 4 °C. Raising the temperature to 22 °C decreased the half-time of this rise to about half (Fig. 4,c) and thus decreased our ability to measure it. Contrary to the light-induced quenching and the subsequent 3-μs rise, this rise is not seen after a flash given when the traps are closed by either weak background light or prior flashes in the presence of DCMU.

An indication of the rise can also be seen in the absence of DCMU, especially in the presence of NH_2OH (Fig. 5). A rise which precedes the decay can be seen after all flashes but the first. The inability of our instrument to measure fluorescence at times less than 80 μs after the flash prevented adequate observation of the rise after

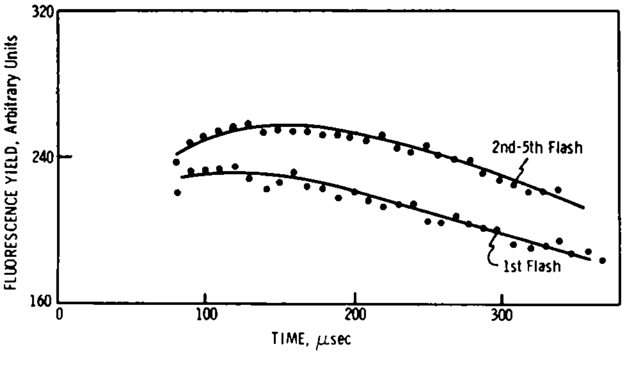

Fig. 5. The variable part of the fluorescence yield as a function of time after each flash given to dark-adapted chloroplasts at 4 °C containing 5 mM NH_2OH. (a) Lower curve, after the first flash, (b) upper curve, after subsequent flashes.

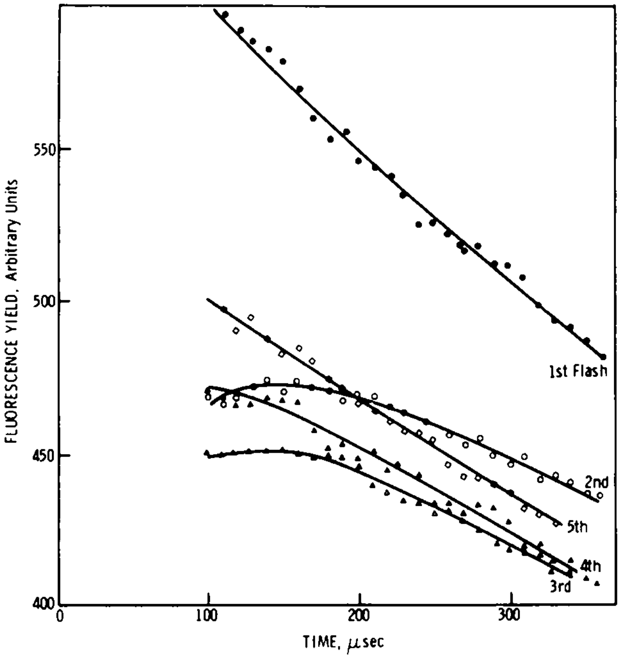

Fig. 6. The variable part of the fluorescence yield after the first five flashes given to dark-adapted *Chlorella* at 4 °C as a function of time after each flash.

the first flash, so that we can only speculate about its presence. In this case, we observed a delay in the fluorescence decay which may result from a 65-μs rise.

A similar situation exists for untreated *Chlorella* (Fig. 6) where the effect is seen after the second, third and fourth flashes. A similar, less pronounced effect is observed with untreated chloroplasts.

Fluorescence decay after the flash: System II trap recovery

Previous information concerning the time for relaxation of the O_2 system comes from experiments which measure the O_2-producing ability of a flash at varying times after a previous flash[4]. The second flash in the pair is only effective at times long enough to permit the system to recover from the first flash. The relaxation time of the O_2 system obtained in such a manner gives the maximum possible time for trap recovery. (The term O_2 relaxation will be used to indicate the relaxation of the O_2 system as measured by O_2 during flash pair experiments. Trap recovery will be used to describe relaxation of the primary donor and acceptor). Conceivably the traps may recover more rapidly, but the electron hole pair produced may be lost (recombine) because some subsequent step has not yet recovered. O_2 relaxation measured by paired flashes given to dark-adapted chloroplasts indicated a distinctly faster recovery after the first than the second flash[4].

Our previous fluorescence measurements[5] indicate decay times comparable to those for O_2 relaxation. The time course of the fluorescence yield at 22 °C after the first five flashes is shown in Fig. 7. The initial fluorescence recovery after the first

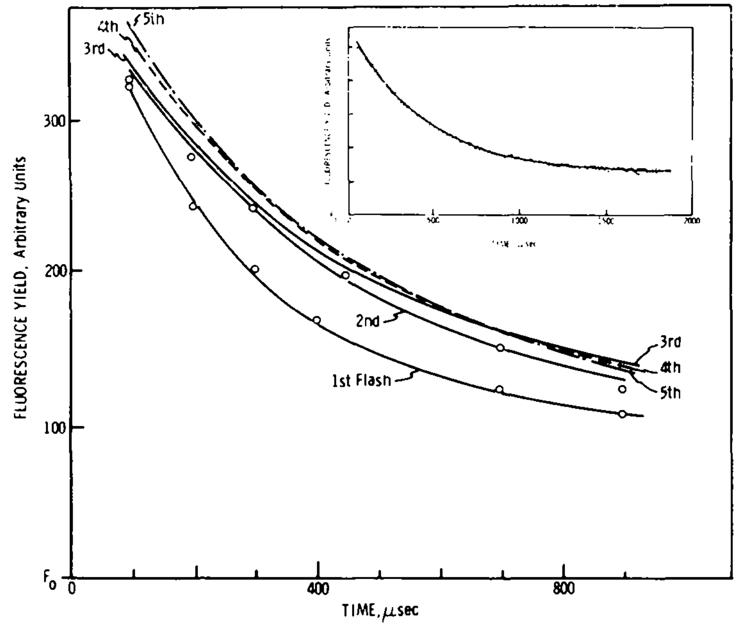

Fig. 7. The variable part of the fluorescence yield after the first five flashes given to dark-adapted chloroplasts at 22 °C as a function of time after each flash. The points indicate System II relaxation as measured previously by Kok *et al.*[5] from flash pairs. These points were normalized to the curves at 100 μs and 1 ms. The insert gives the change of the fluorescence yield as a function of time after each of repeated flashes spaced 1 s apart given to chloroplasts containing 100 μM methylviologen and 10 mM NH_4Cl. The change is measured from the steady state yield F_s just prior to each flash. Measurements were started after 30 flashes given to each sample. The sample was changed after 128 subsequent flashes. Four samples were used. The deviations of points from a smooth curve is typical for other curves in the figure.

flash has a distinctly shorter half time than that of the others, indicating some special event after the first flash. There are some slight differences between the others, the fluorescence after the third recovering a little slower and after the fifth a bit faster.

The fluorescence decay for the first two flashes is compared in Fig. 7 to O_2 relaxation measured by Kok *et al.*[4]. There appears to be good corrlation between fluorescence and O_2 recovery; both O_2 and fluorescence show an initial faster recovery after the first flash than after the second. A correlation is also evident when comparing fluorescence with O_2 data by Bouges[11]. The lag observed in the relaxation of the O_2-evolving system after the second flash appears to correspond to the lag in fluorescence recovery discussed in the previous section. Both lags have the same temperature dependence. Bouges-Bocquet, B., (personal communication) computed the rate of relaxation of the O_2 system after the third flash ($S_3 \xrightarrow{h\nu} S_4 \xrightarrow{h\nu} S_0 + O_2$) and found it to be considerably slower than what we observe. If this result is correct, the relaxation of the O_2 system is limited in this case by a step subsequent to and independent of trap recovery.

To eliminate the influence of the S states on the fluorescence yield, the decay after individual flashes was also measured using a high concentration of NH_2OH instead of water as the electron donor. The measurements (80–1000 μs after the flashes) were made at room temperature to eliminate effects of the lag (Fig. 5) which is quite pronounced in NH_2OH. We could not distinguish any effect of NH_2OH on the fluorescence decay for the first two flashes. In both cases the kinetics after the first flash were easily distinguished from those after the second.

Since high concentrations of NH_2OH as used here are known to inhibit O_2, while acting as a donor and permitting electron transport from the acceptor to proceed unimpaired[12], it appears that the fluorescence kinetics during the first 35 μs are governed by the donor side of System II, and the decays 200 μs and longer by the acceptor side. DCMU, acting on the acceptor side inhibits the decay but not the 35-μs rise. The decay did not depend on the presence of 100 mM methylamine, indicating that methylamine, also, did not affect oxidation.

After each flash in a sequence given to dark-adapted chloroplasts, the decay of the fluorescence could be interpreted as resulting from a component having a half-time of approximately 200–250 μs and slower components with half-times of ms (ref. 5 and Fig. 7). A similar interpretation can be given to the decay observed after each of a series of repetitive flashes given long enough to attain steady state conditions. The results for chloroplasts containing NH_4Cl as an uncoupler and methylviologen as an electron acceptor (Fig. 7, insert) indicate a first-order recovery of about 2/3 the amplitude with a half time of 200–250 μs with the other third decaying in a complex manner with a half time of several ms. The redox state of Q before each flash could be altered by omitting methylviologen, and either changing the flashing rate or adding background light. The decay after each flash contained a significant approx. 200-μs component even when much of Q was reduced prior to each flash.

Mauzerall[2] reports that O_2 relaxation as measured in repetitive flash pair experiments is best fit by two exponential decays having half times of 200 μs and 2 ms indicating a correlation of O_2 relaxation and fluorescence decay for steady-state conditions. Vater *et al.*[13], however, found O_2 relaxation as measured by repetitive flash pairs to occur in a 600-μs, first-order process.

DISCUSSION

The fluorescence changes discussed above are summarized in Fig. 8. The 25-ns rise, seen by Mauzerall[2] after the first flash given to dark-adapted algae, is not shown in this figure. A number of observations indicate that the behavior of the first flash given to dark-adapted systems is anomalous: The appearance of the 25-ns rise[2], the abnormally low value of fluorescence after long dark[14], and the peculiarity of the decay kinetics. The reasons for this anomalous behavior are not known and will not be discussed further.

The fluorescence rises (A of Fig. 8) during the flash in a time inversely proportional to the flash intensity. Before the rise is complete, subsequent light produces a quencher, driving the fluorescence down (Fig. 8, B). The maximal level reached is only about 40% of what would have been reached in the absence of the quencher. The fluorescence then rises in a dark step of about 3 μs (Fig. 8, C) as the light-induced quencher decays. There is, under certain conditions, a further rise (Fig. 8, D) in the dark with a half time of approx. 35 μs at 22 °C and approx. 65 μs at 4 °C. This rise was most clearly seen in chloroplasts containing 10 mM NH_2OH. The rise appears to be related to a lag in trap recovery. The rise is, in turn, followed by a complex dark

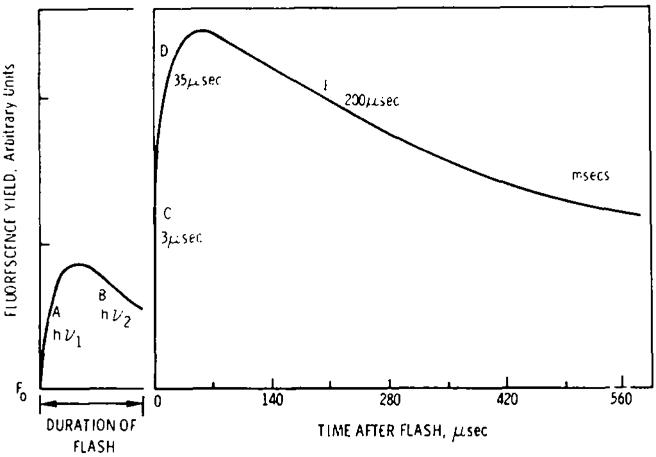

Fig. 8. A typical time course of the fluorescence yield during and after a flash.

decay (Fig. 8, E) having half-times on the order of 200 μs and several milliseconds. The decays are not affected by NH_2OH. The decay, but not the rise, is eliminated by DCMU.

The rise (Fig. 8, A), appears to correspond to the closing of the traps by light. The light-induced quenching (Fig. 8, B) appears to be caused by a carotenoid radical or triplet. Its occurrence may be the reason Mauzerall[2] did not see a large rise until 3 μs after the flash. It appears that energy in excess of what can be used for photosynthesis goes into the production of such quenchers. The 3-μs rise (Fig. 8, C) is the relaxation of the quenchers. As will be described below, the decay (Fig. 8, E) can be attributed to the recovery of Q. The phenomena seen at shorter times, the oscillations and 35-μs rise (Fig. 8, D), can be attributed to additional quenching by positive charges on the donor side of System II.

Joliot and Joliot[15] attributes the fluorescence oscillations and differences in O_2 relaxation after different flashes to two different acceptors, Q, having different quenching properties. Our data are more conveniently summarized by a model containing only one type of System II trap and acceptor. In the model the acceptor Q and some neighbor A′ come into equilibrium in about 200 μs. Due to a low equilibrium constant between Q and A′, Q is not completely oxidized. A′ and the other intermediates A between Q and System I take 1 ms or longer to interact, depending on the state of the intermediates. $Q \rightleftarrows A' \leftrightarrows A$. This produces, through the interaction of Q and A′, the slower components of fluorescence decay.

The behavior on the oxidizing side of the trap appears to be too complex to completely disentangle with the data available. We will attempt to explain only part of this behavior. The transfer of equivalents appears to depend on the S states and chemical additions such as NH_2OH or high concentrations of methylamine. It appears that at least in some instances Z is reduced in about 35 μs by some intermediate B which quenches less than Z. $Z \rightarrow B \rightarrow S$. This accounts for the 35-μs fluorescence rise and also for the 35-μs component of delayed light[5]. The delayed light produced by recombination of Z^+ with Q^- has a half time determined by the reduction of Z^+. We previously concluded[5] on the basis of delayed-light measurements a 1-ms transfer of charge between Z and S, the S states of Kok[4], suggesting that B recovers in about 1 ms. A faster alternative reaction between Z and S may account for the variability in the fluorescence rise and an about 10-μs component of delayed light[5]. The 1-ms component of delayed light and the small differences in fluorescence decay (Fig. 7) seen after different flashes (other than the first, which appears to be a special case) can be explained by assigning different quenching properties and luminescence yields to B and the various S states.

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