SLOW FLUORESCENCE FLUCTUATIONS FOLLOWING HIGH LIGHT TO LOW LIGHT OR DARK TRANSITIONS IN *CHLAMYDOMONAS REINHARDI*

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ABSTRACT Slow fluorescence transients in *Chlamydomonas reinhardi* arise after transitions from high light intensities to low light or dark conditions. Characteristics of the newly described transient phenomena include: (*a*) A slow biphasic decrease in fluorescence yield occurs in the dark, followed by an even slower, hour long, increase in fluorescence. (*b*) A similar, but faster, fluorescence yield decrease and subsequent increase also occurs during low intensity illumination periods separating high light intervals, or after transitions from high intensity to low intensity light. (*c*) Short (several seconds) flashes of light given during a dark period have no effect on the dark fluorescence decay, regardless of the flash frequency. Such flash regimes accurately monitor the dark decline of the M_2 level by tracing the parallel decay of flash-generated P_2 (Kautsky) peaks. However, flashes during a low light illumination period do influence the decay kinetics. Frequent flashes allow decay similar to that occurring in dark, but less frequent flashes inhibit the decrease in fluorescence yield.

INTRODUCTION

Slow fluorescence induction transients associated with photosynthesis in whole cells have been investigated for many years, yet no definite explanation or mechanism for these transients have been established (1–4). Our previous work on such transients in the single-celled alga *Chlamydomonas reinhardi* has characterized the basic features of both the slow fluorescence and O_2 evolution transients in this organism (5).

The fast components of fluorescence and O_2 induction (0.01-several s) are explained primarily by changes in the redox state of intermediates in the photosynthetic electron transport chain (6, 2, 3). This accounts for the complementarity which exists between the fluorescence and O_2 induction transients in this time interval. The slow fluorescence and O_2 transients are not the result of redox changes since both fluorescence and O_2 evolution increase simultaneously (4, 5, 7–10).

A typical fluorescence induction transient and the corresponding O_2 transient of *C. reinhardi* are shown in Fig. 1. The faster components of the fluorescence transients are not recorded or discussed in this work. Also, our terminology for the fluorescence transient components is different from the more widely accepted terminology (4). In this work, P_2, M_2, and P_3 correspond respectively to the P, S, and M levels of the more common nomenclature.

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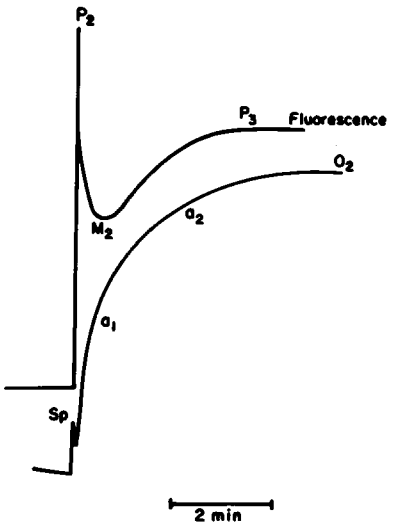


FIGURE 1 Typical induction transients of fluorescence and the rate of oxygen evolution in *C. reinhardi* after a 3-min dark period. Nomenclature of the various components of the fluorescence and O_2 transients are indicated.

Our P_3 is also the steady state level, whereas in much algal work a decay from the M level to steady state (T) is observed.

P_2, the Kautsky spike, is believed to occur as Q and other electron transport intermediates of system II become totally reduced (6, 2, 3). As the fluorescence rises to P_2, the O_2 evolution is inhibited by the reduced state of Q and falls from its initial spike, Sp, to a lower level. The fluorescence decrease from P_2 to M_2 is accompanied by a rise in the rate of oxygen evolution, corresponding to the a_1 phase of the O_2 transient. This decrease from P_2 reflects, in part, the delayed oxidation of Q, probably through the alteration of some step on the system I side of Q (5, 6, 11). The reversal of this step seems to require 30 s of darkness for completion, since P_2 in *C. reinhardi* does not reach its maximum height unless 30 s of dark precedes the illumination period (6, 12, 9, 11). Complicating factors beyond simple redox changes also contribute to the fluorescence decline from P_2. (See Mohanty and Govindjee [11] for a discussion of this transient in algal cells.)

The M_2-P_3 fluorescence rise is the slow component of induction—P_3 corresponding to the steady state level of fluorescence. The a_2 portion of the O_2 curve parallels this fluorescence rise. The M_2 level and the M_2-P_3 rise in *C. reinhardi*, unlike the faster components, are independent of redox changes and unaffected by DCMU (5). (With respect to other algae, DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] has no effect on the slow transients in *Anacystis* [7, 11] but eliminates the slow rise in *Chlorella* [8, 10, 11] and *Porphyridium* [9, 11]). The appearance of the M_2 minimum following dark periods and the M_2-P_3 rise in light are referred to as "slow dark deactivation" and "slow activation," respectively. The terminology, activation/deactivation, is used here in its broadest sense and only serves as a descriptive or working hypothesis since the mechanism causing these transients is not known.

This paper focuses on slow fluorescence changes in the M_2 (and P_2) levels following reductions in light intensities. A biphasic decline of fluorescence from its steady state to the

M_2 level and its subsequent rise back to steady state during dark periods or transitions from high to low light are described. Also, measurements of P_2 levels using a flash regime during dark and low light periods were found to mimic M_2 variations and revealed other transient phenomena resulting from the flash regime itself.

MATERIALS AND METHODS

The organism employed in this work was a strain of *C. reinhardi* from Prof. Paul Levine, Harvard University. The cells were cultured on minimal medium (13) at 30°C with 1% CO_2-enriched air bubbled through the culture flasks. Illumination consisted of ~650-foot candles from incandescent bulbs. At culture densities of 0.5–2.0 μl packed cells/ml, the cells were harvested by centrifugation and resuspended to 5 μl/ml in minimal medium. Several drops of this suspension were mounted directly on a platinum O_2 rate electrode, which was then placed in a special plexiglass chamber containing 100 ml of the minimal medium plus 0.1 N KCl. Temperature of the medium was controlled at 30°C; stirring and gas equilibration were accomplished by constant bubbling of 6% O_2 and 2.4% CO_2 in nitrogen.

Simultaneous measurements of the rate of O_2 evolution and 680-nm fluorescence were made on the sample using the instrumentation and procedures described previously (5). Actinic illumination was provided by either of two Köhler projection systems. In most cases the actinic light used in the experiments was broad blue, generated by two 4303 and one 4308 Corning glass filters (Corning Glass Works, Science Products Div., Corning, N.Y.) in combination with Schott KG-1 and KB-1 infrared absorption filters (Schott Optical Glass Inc., Duryea, Pa.). Weak background or preillumination could be obtained from the second beam with Baird interference filters (Baird Corp., Bedford, Mass.) of 480, 650, or 710 nm in combination with infrared filters. All intensity variations were made by the addition or removal of calibrated neutral density filters. Each shutter was controlled by a programming device which could automatically trigger either shutter, or both simultaneously, at almost any frequency or period.

RESULTS

The Dark Decline and Subsequent Rise of the M_2 Level

The kinetics of the slow decline of the M_2 level (deactivation) were investigated by giving dark periods varying from 5 s up to 8 min or more, interspaced between fixed periods (usually 5 min) of strong actinic light. Actinic light intensities were usually ~5 mW/cm^2, although the range of light intensities varied from 170 μW/cm^2 to 6.8 mW/cm^2. No obvious differences in

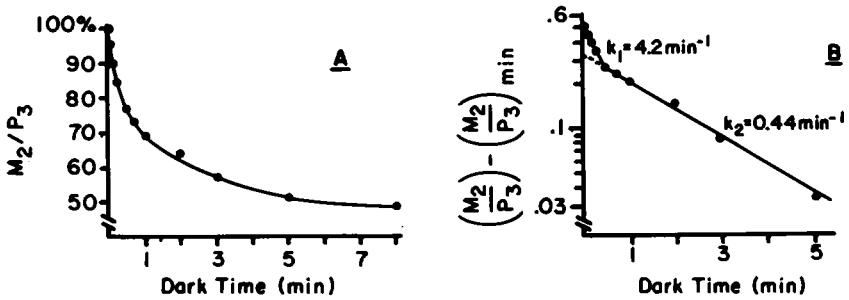


FIGURE 2 Effect of preceding dark time on the M_2 fluorescence level as reflected in the M_2/P_3 ratio. 5.3 mW/cm^2 of broad blue illumination. Curve A, M_2/P_3 (expressed in %) as a function of preceding dark time. Curve B, semilog plot of $(M_2/P_3)-(M_2/P_3)_{minimum}$ as a function of preceding dark time. Rate constants, k_1 and k_2, of the biphasic decay are indicated.

the dark decay kinetics or the slow fluorescence transients themselves resulted over this range of intensities.

The ratio M_2/P_3, indicating the extent of the M_2 height, is plotted as a function of preceding dark time in Fig. 2. M_2/P_3 decreases from 100% at time 0 to a minimum of 50% after ~8 min of darkness. Thirty such "dark deactivation" curves were generated using unpoisoned and DCMU-poisoned *C. reinhardi*. Qualitatively, the results from this series of experiments were the same. After 8–10 min of darkness, M_2 fell to a minimum that was typically 50–60% of the P_3 level, with extremes of 38 and 82%.

The dark decay was found to occur in two phases (Fig. 2 *B*). An initial, faster phase accounts for ~50% of the total decay in the first ~30 s. From the slopes of the decay curves during the fast and slow phases first order rate constants, k_1 and k_2, can be calculated. The values for k_1 ranged from 1.4 to 7.9 min^{-1} with the average k_1 being 4–5 min^{-1}. k_2 ranged from 0.25 to 1.1 min^{-1} with an average value of 0.5 min^{-1}.

As dark times are extended beyond 10 min, the decline of the M_2 level reverses and the M_2 fluorescence slowly rises toward the steady state level. Sixty min or more of continuous darkness are required for M_2/P_3 to attain 100%. Also, following such a "long dark reactivation," M_2/P_3 tends to remain elevated for an extended period, with little dark deactivation occurring in the next light-dark cycle. Only during several shorter cycles (3 min dark/5 min light) does M_2 gradually fall to the level associated with the shorter dark periods.

Although DCMU-poisoned cells show the same biphasic dark decay of the M_2 level, DCMU prevents the long dark reactivation found in unpoisoned cells. During long darkness, the M_2 component remains constant at the low level achieved after the initial 8 min of dark.

The Decline and Subsequent Rise of the M_2 Level following Transitions from High to Low Light Intensities

The substitution of weak preillumination for darkness in a repeated regime of 5 min strong light/3 min dark will eliminate or greatly reduce the M_2-P_3 rise in each subsequent high light period (5, 11, 12). This investigation shows that deactivation and subsequent reactivation occur as a function of preillumination time. Five-min actinic light periods were separated by different durations of preillumination, and the M_2 and P_3 levels were measured after each preillumination. Fig. 3 shows the resulting preillumination decay curves along with one for darkness. A slow decline begins immediately after the actinic-to-preillumination change, and this leads to a minimum. Thereafter, the M_2 level rises steadily toward complete activation. For all levels of preillumination, the initial decay rates appear to be identical. However, the stronger the preillumination the shallower the M_2/P_3 minimum, the sooner it is reached, and the greater is the rate of reactivation.

M_2 fluctuations during preillumination periods also can be observed by increasing the amplifier sensitivity and directly monitoring the fluorescence transients during the preillumination period. The transition from high actinic light to weak illumination induces slow fluorescence transients: the fluorescence falls to a minimum and then rises again to the steady state level (1). These high-low light transients show complete correspondence to the M_2/P_3 curves representing the changes in the M_2 level after varying preillumination times. Likewise, the M_2 level accurately reflects the point within the preillumination transient at which the high light was turned on.

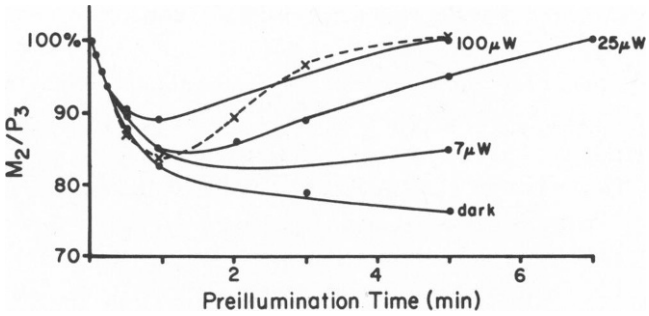


FIGURE 3 The effect of preillumination time and intensity on the extent of deactivation and reactivation as reflected in the M_2/P_3 ratio. Five-min periods of actinic light (3.4 mW/cm^2 of broad blue) were interspaced with varying periods of lower intensity broad blue preillumination. The M_2/P_3 value observed in the high light was plotted against the duration of the preillumination period. The numbers on the curves indicate the intensity of the preillumination. Total reactivation during the 100 μW/cm^2 preillumination may have been achieved before 5 min; readings between 1 and 5 min were not made at this preillumination. The dark decay for these cells and the dashed curve representing the deactivation/reactivation in the presence of 35 μW/cm^2 of 710-nm preillumination (a different sample of cells) are shown for comparison.

DCMU poisoning prevents any reactivation during the preillumination period. Thus there is no subsequent rise in either the high-low light transient or in the M_2 level after extended preillumination times. The decay component does occur in the presence of DCMU but the extent of the decay for a specific preillumination intensity tends to be less with DCMU than in the unpoisoned algae.

Flash Experiments: Slow Dark Decay Monitored by P_2 Levels

Although the P_2 component of fluorescence results, in part, from the redox state of the photosynthetic intermediates, the level of the P_2 fluorescence should also reflect the degree of slow activation or deactivation (11). The P_2 height increases to a maximum as preceding dark periods lengthen to ~30 s, due to the dark alteration of an electron transport step. With further lengthening of the dark period (>30 s) the P_2 height declines and reaches a minimum at 8–10 min. This decline of P_2 parallels the decline of M_2 and can be taken as another measure of the extent of slow deactivation. Since the P_2 level can be determined by a 1–3 s actinic flash, measuring P_2 height changes with a series of short flashes is a convenient means of monitoring the slow deactivation over a single 8–10 min dark period. Such flashes have no effect on the slow deactivation (7). However, the P_2 method of following slow deactivation has the shortcoming that the initial P_2 level (at 0 dark time) is not known and must be estimated by extrapolation. Also, the redox alteration of Q which raises the P_2 height during the first 30 s of darkness must be considered. In DCMU-poisoned cells, however, both of these problems are eliminated.

Fig. 4 shows decay curves obtained as the envelopes of P_2 height, measured during 3-s flashes that were repeated after 6-, 30-, and 57-s dark intervals. Notice that the curves are the same regardless of the flash frequency. Semilog plots indicate a first order decay from ~1 to 8 min and a faster component at shorter times. The decay kinetics of P_2 (Fig. 4) and the M_2/P_3 kinetics obtained from the same cells (Fig. 2) are similar. DCMU-poisoned cells gave P_2 decay kinetics which were equivalent to those of unpoisoned cells.

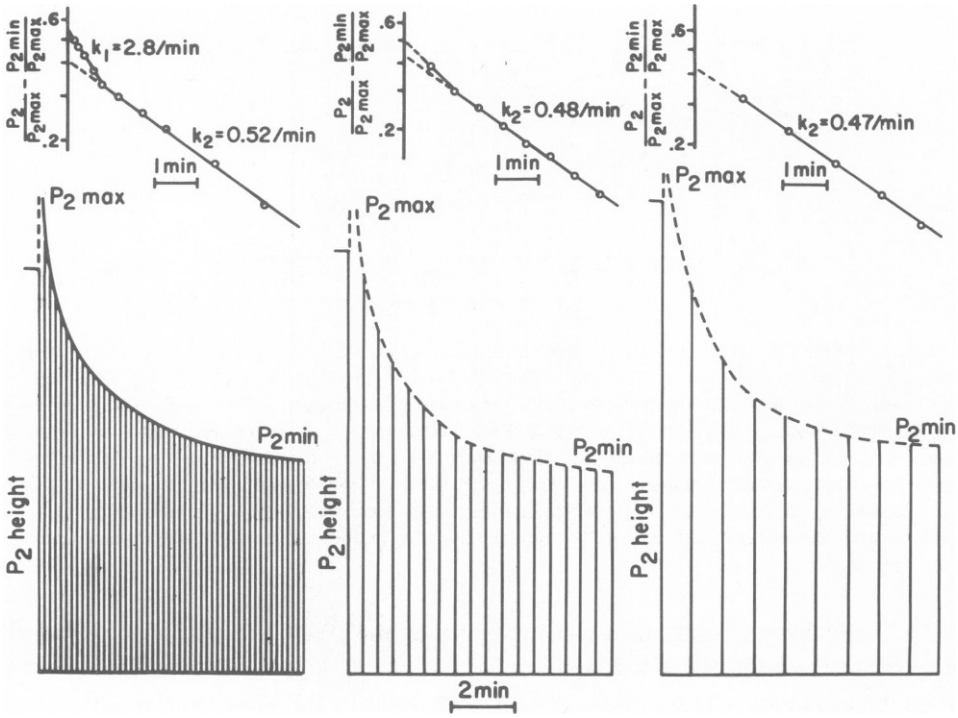


FIGURE 4 Dark deactivation monitored by P_2 height. P_2 levels monitored by 3-s light flashes (5.3 mW/cm^2 of broad blue light) interspaced by 6-, 30-, and 57-s dark periods are shown in three separate curves. All indicate a steady decline in P_2 height analogous to the dark decline of the M_2/P_3 ratio. Semilog plots of the dark deactivation based on P_2 levels are shown above each of the respective P_2 decay curves. The rate constants for each of these decays are also indicated.

It is particularly interesting that the 3-s flashes separated by 6-s dark periods gave as rapid a decay as the less frequent flash regimes. While a continuous illumination one-third the strength of the actinic illumination would have maintained the fully active state, the flash regime, providing the same time-average illumination, permitted decay at the same rate as in continuous darkness. Evidently, the slow activating mechanism involves the formation of an element or state that disappears in darkness and whose generation in light requires >3 s.

Flash Suppression—Interrupted Preillumination

The flash experiments show that the P_2 and M_2 dark decay kinetics are the same, with flash frequency having no affect on the dark decay. In the preillumination investigations, an initial, partial decay and following reactivation occurred when strong actinic light was turned off and weak preillumination was continued. However, when flashes are used to assay the P_2 level in the presence of weak illumination, the flashes themselves influence the state of activation. Frequent flashes allow the slow deactivation to proceed as it does in the dark, with no prevention or reversal of the decay as normally occurs during weak illumination. However, widely separated flashes (57 s) on the illumination background maintain the completely active state as indicated by constant P_2 levels. This effect, which we term flash suppression, is

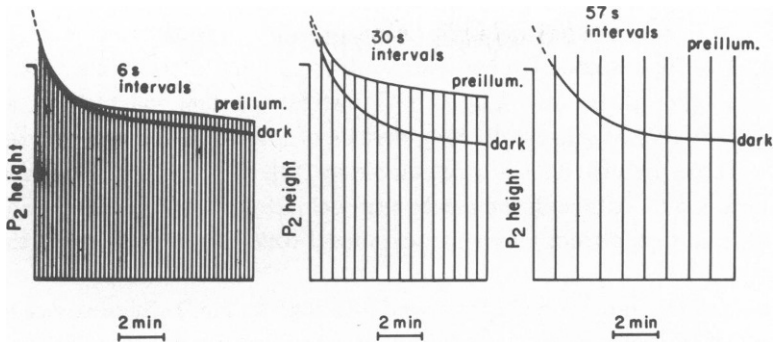


FIGURE 5 Effects of weak illumination on dark deactivation monitored by P_2 height. P_2 levels monitored by 3-s light flashes of 5.4 mW/cm^2 of broad blue light interspaced by 6, 30, and 57 s of 650-nm low light illumination (20 μW/cm^2) are shown. The more frequent flashes in the presence of illumination allow the P_2 level to decay; less frequent flashes prevent significant decay. Superimposed on each curve is the P_2 decay obtained when equivalent intervals of dark replace the weak illumination periods.

illustrated in Fig. 5 for three flash frequencies. Also shown for comparison is the decay in darkness, as determined with flashes. Flash suppression is also seen with DCMU-poisoned cells (Fig. 6). Here, a preillumination, which prevented deactivation in the absence of flashes, had no preventive effect in the presence of 1-s flashes repeated every 10 s.

Altering the frequency of the flashes on a weak illumination background causes an abrupt change in the decay rate. If one allows the P_2 decay to proceed to a low level with a 6-s flash interval and then switches to a 57-s flash interval, the P_2 level increases with each subsequent flash. This increase in P_2 height during the longer interval will be reversed if the 57-s illumination period is interrupted by several equally spaced 3-s dark intervals. With several 1-s dark interruptions, the P_2 level stops rising and may fall back slowly. However, with several 0.5-s dark interruptions, the P_2 level continues rising. These short dark interruptions are probably another example in which a light-to-dark transition triggers inactivation and suggest that the critical dark duration to initiate deactivation is ~1 s.

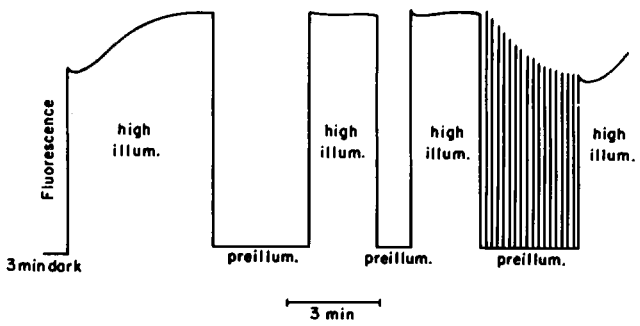


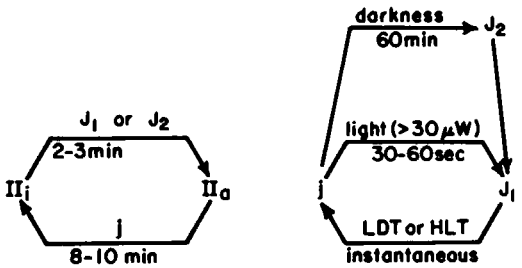
FIGURE 6 The effects of preillumination on deactivation in the presence and absence of flashes in DCMU-poisoned *C. reinhardi*. 650-nm preillumination preceding 3 mW/cm^2 of broad blue light for periods of 3 and 1 min allows no deactivation, while the same preillumination over 3 min in the presence of 1-s flashes (3 mW/cm^2 broad blue) given every 10 s permits deactivation.

DISCUSSION

Our observed slow fluorescence fluctuations result from light intensity changes. Any abrupt transition from high light to low light or dark instigates a slow inactivation; activation or reactivation occurs only in reasonably long periods of uninterrupted light or over very long dark periods. These perturbations are detectable in both the M_2 and P_2 components of the fluorescence transients. Although the mechanism behind these fluorescence transients is not known, it is helpful to construct a hypothetical model which attempts to unify the individual observations.

We define an active state (II_a) characterized by a high system II fluorescence yield, and an inactive state (II_i) characterized by a low fluorescence yield. II_i and II_a could be inactive and active system II units, or different energy distribution states (following Bonaventura and Myers [14]). In the latter case, states II_a and II_i would have respectively large and small fractions of absorbed energy passing to photosystem II. Either hypothesis could explain parallel changes in oxygen evolution rate and fluorescence yield.

Either continuous light stronger than ~30 $\mu W/cm^2$ and lasting 3–4 min, or continuous darkness of 60 min, leads to a maximum extent of activation. Only after a light-dark transition (LDT) or a high to low light transition (HLT), does a transitory inactivation occur. We suggest that the LDT and HLT quickly generate an inactivating condition or state (designated *j*), which then over the course of minutes converts $II_a \rightarrow II_i$. Activation or reactivation would then entail the conversion of the *j* state to an activating form *J*. Because of differences between the active states in the light and after long darkness, we will consider two different activating states—J_1 arising in light and J_2 arising in long darkness. J_2s initially unresponsive nature to a LDT following long dark reactivation indicates that J_2 returns only slowly to the J_1 (or *j*) state. (A second II_a state incapable of immediately inactivating could also be postulated instead of J_2). These points are summarized in the following diagrams:



When the actinic light is extinguished, inactivation begins immediately and maximum inactivation is reached after 8–10 min of darkness (Fig. 2). Two points bear emphasis. First, the rate of deactivation—as indicated by the M_2 decline—is maximum initially, and the rate gradually declines in accordance with two first order processes. Also, actinic flashes given during the decay in darkness do not alter the kinetics (Fig. 4). From these observations, one infers that the initiating LDT instantaneously (within 1 s) generates the inactivating state *j*. If the *j* state arose gradually after the LDT, then the rate of inactivation would have increased with time. Flashes evidently cannot cause a significant reversion of *j* to an activating state, J_1 or J_2, during this 8–10 min of dark deactivation.

Weak light inhibits the inactivation process ($II_a \rightarrow II_i$) and/or accelerates the reactivation ($II_i \rightarrow II_a$). After an HLT, inactivation begins immediately with the same initial rate as in darkness. However, the stronger the weak light, the sooner the inactivation is replaced by a reactivation (Fig. 3). In explanation, we suppose that light slowly converts j into the J_1 state. The rate of this photoconversion would be light-saturated at ~30 $\mu W/cm^2$ and, in this illumination, complete conversion would require 30–60 s. The sigmoidal character of the M_2-P_3 fluorescence induction also is consistent with this model. The initial lag of 30 s or more reflects the generation of J_1 and the following 2–3 min rise to the P_3 level reflects J_1s conversion of II_i to II_a.

The LDT or HLT accompanying flashes seems to trigger an inactivation. Flashes on a dark background have little effect on the inactivation triggered by an LDT. However, on a low light background frequent flashes appear to inhibit the $j \rightarrow J_1$ transformation by regularly instigating inactivation ($J_1 \rightarrow j$). Only during less frequent flash regimes allowing constant low light durations approaching 60 s can significant reactivation occur. This is reflected in enough reactivation to maintain high P_2 levels.

A problem exists with our observations involving DCMU-poisoned cells. DCMU was found to have no effect on some of the slow fluorescence transients. Unpoisoned and DCMU-poisoned cells gave identical results for the M_2-P_3 rise, its dark decay, and all of the flash phenomena. However, reactivations occurring after long dark or in high light-low light transients in unpoisoned cells are eliminated in DCMU-poisoned cells. The significance of these observations is not known, but they suggest that a more complicated mechanism may be required.

RELATED WORK

Most work of late has concentrated on explaining slow fluorescence fluctuations in terms of α changes or redistributions of energy between system I and system II after the model of Bonaventura and Myers (14). The majority of this work has been accomplished by alternate or simultaneous exposure with system II and system I light to induce the slow α changes (14–18). Our slow transients in *C. reinhardi* are initiated merely by intensity changes, independent of wavelength. Our transients could also be explained by α changes if we assume that energy shifts or redistributions between the photosystems occur with induction or intensity changes. Such α changes must, however, be considerably different in nature from those described by Bonaventura and Myers and must account for changes in fluorescence yield of considerably greater magnitude than demonstrated in their transients.

Ried and Reinhard (18), working on α changes in red algae, have presented observations on short flashes and long dark times which seem similar to some of our induction phenomena. A 2-s flash of system I light is enough to initiate the transition from state II to state I, which then fully develops over a 1-min dark period. Ried and Reinhard attribute this to a fast light reaction triggering a slow dark reaction. Their data also show further fluctuations in the fluorescence yield as dark times are extended beyond 1 min. For instance, after 1 min of dark the fluorescence is at its maximum or 100% state I level (the level in constant system I light); by 10 min the fluorescence yield decreases to 50% of the state I level, and after hours of dark the yield is twice the maximum height attained with system I illumination. This oscillation over extended dark periods with the final fluorescence yield being well above any values

previously associated with α changes may be a consequence of our slow dark deactivation and reactivation.

No distinction between α changes, activation of units, or other mechanisms involving shifts in the relative rates of the deexcitation processes can be made at this time with respect to slow fluorescence changes. However, numerous investigators (7–9, 19–22) have suggested the involvement of conformational changes in slow transients. Cation concentration and ion fluxes also have been linked to changes in fluorescence yield (22–24) as well as to conformational changes (20, 22, 24–26). Also, data involving uncouplers (7–9, 19, 21) and *C. reinhardi* mutants lacking system I (5, 3) suggest that phosphorylation is involved in slow fluorescence changes. A rationalization of these transients in terms of energetics seems reasonable because ATP or energy requirements may be more variable during transitions than during steady state photosynthesis, and ATP concentration changes during induction in *Chlorella* supports this idea (27, 28). Generation of the much slower J_2 state may reflect influences of respiration or other metabolic processes on the ATP or energetics of the chloroplast (29).

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