The Slow Decline and the Subsequent Rise of Chlorophyll Fluorescence Transients in Intact Algal Cells

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ABSTRACT

The slow decline and the subsequent rise of chlorophyll fluorescence transients in intact algal cells. Mohanty, P. and Govindjee (Dept. of Botany, University of Illinois, Urbana, Illinois 61801, U.S.A.). Plant Biochemical Journal 1(2)78-106, 1974. Dark-adapted algae show changes in chlorophyll *a* fluorescence yield with time of illuminatlon (the Kautsky effect) and there are three rapidly occurring sequential phases in the fluorescence transients. The effect of light, temperature, preillumination, inhibitors of electron flow, anaerobiosis, election acceptors, uncouplers and cofactors of photophosphorylation on the peak (P) to quasi-steady state level (S) fluorescence decay and S to second maximum level (M) fluorescence rise has been compared. On the basis of the data presented, together with the authors' previously published results, it is concluded that the P to S decay, although not directly linked to the oxidoreduction of carriers of electron transport chain, is related to the energy conservation process associated with the non-cyclic electron transport and photosynthetic oxygen evolution. The S to M rise does not need a sustained noncyclic electron flow and is perhaps related to the export of energy from, and import of ions to, the chloroplasts. Both the P to S and S to M changes are suggested to occur through associated microstructural changes of membrane matrix that are caused by ionic flow leading to the changes in the conformation of key protein-chlorophyll complexes.

Index words : *Anacystis / Chlorella/Porphyridium / Chlorophyll fluorescence transients/ conformational changes in membrane structure.*

INTRODUCTION

When a dark adapted aerobic sample of intact algal cells or a leaf-segment is illuminated with saturating intensity of light, the fluorescence of chlorophyll *a*

[1] Abbreviations : CCCP, carbonyl cyanide 3-chlorophenylhydrazone ; Chl *a*, chlorophyll *a*; DCMU, 3-(3, 4 dichlorophenyl)1, 1-dimethyl urea ; FCCP, carbonyl cyanide 4-trifluormethoxy-phenyl hydrazone ; MV, methyl viologen ; PBQ, p-benzoquinone ; PMS, N-methyl phenazonium methyl sulphate ; PS I (II), pigment system I (II) ; Q, primary electron acceptor of PS II ; Tris, tris (hydroxymethyl) amino methane.

(Chl *a*) shows characteristic oscillations (see reference 1 for a review). The floures-cense transient begins from a low origin (0) followed by three rapidly occurring sequential phases within about one second of illumination : an inflection (I), a dip (D), and a peak (P). From P, the fluorescence yield shows a slow decline to a quasi-steady state level (S). After reaching the S level the fluorescence yield slowly rises to a second maximum (M) level and then declines to a level (T) within a few minutes of illumination. The T level represents a terminal level as no significant change in the fluorescence yield occurs thereafter. The fast 0→I rise has been suggested by Joliot (2) to be related to the changes in the redox states of the photoenzyme "S" involved in O_2 evolution (see reference 3). Thus the 0→I rise would reflect the final state change in the charge accumulation process leading from S_3 to S_4 state. Thus, both fluorescence and O_2 evolution rise in parallel during this transient. The subsequent rise to P in the fluorescence transient of Chl *a* is believed to be due to an accumulation of the most of the electron transport carriers of photosynthesis in the reduced state (4). The D→P rise in the fluorescence transient is accompanied by a complementary decline of oxygen evolution (5-8) as would be expected if Chl *a* fluorescence were to compete with the photochemical reactions leading to oxygen evolution. The I→D phase has been suggested to be partly due to the interaction of pigment system I (PS I) with PS II (4,9). Although the P to S decline phase of the fluorescence transient exhibits an antiparallel behaviour with that of oxygen evolution, it does not reflect a simple reoxidation of the primary electron acceptor Q of PS II. This puzzling behaviour was first documented by Duysens and Sweers (10) who observed that if the exciting light was turned off at the S level and then quickly turned on again, the yield of fluorescence remained at the low S level and did not rise up to the P level unless the dark period was long (minutes).

Duysens and Sweers (10) suggested that during the P to S decline phase the primary acceptor of PS II (Q) undergoes a change to a form denoted as Q′ which has a reduced ability to accept electrons but it still quenches fluorescence like Q. In recent years Duysens (11) has modified this hypothesis of the transformation of quencher to accommodate new experimental data (12, 13; see also refrence 1). It has now been suggested that the P to S decline occurs, to a large extent, due to some sort of transformation of "physiological states" of thylakoid membranes facilitating flow of excitation energy from PS II to PS I (see reference 14 for a review) or due to alteration of deexcitation process within (PS II) units (see 7, 15, 16). Furthermore, Papageorgiou (17) has recently suggested that an ultrastructural change of the thylakoid membrane, due to the acidification of the thylakoid interior, facilitates

diffusion of quenchers notably molecular oxygen. In support of this hypothesis is the observation that the P to S decline is absent in anaerobic cells (4, 9, 18).

Krause (19) working with the intact leaves has shown that slow P to S type fluorescence quenching upon prolonged illumination accompanies an increase in absorbance at 540 nm due to shrinkage of chloroplast structure. He has shown that this type of Chl *a* fluorescence lowering depends not on the redox state of the quencher (Q) but on the high energy state of photophosphorylation. Krause (20) and Barber *et al.* (21) have also shown that P to S decline in the fluorescence yield reflects the efflux of cations like Mg^{2+} from grana to stroma region and back influx of the cations into the grana stacks in the subsequent darkness. As cations and proton movements are energy-linked processes, both the hypotheses of Papageorgiou (17) and of Krause (19, 20) would implicate a high energy state to be associated with the fluorescence decline from P to S. Cofactors like PMS (phenanzine methosulphate) appear to cause a P to S type lowering of Chl *a* fluorescence in isolated broken chloroplasts and in algae in the presence of DCMU (22-24). This observation would indicate that some kind of cyclic photophosphorylation may also be involved in P to S decline of Chl *a* fluorescence. Slovacek and Bannister (16) have shown that depletion of CO_2, (a condition which would be expected to induce cyclic photophosphorylation *in vivo*,) does not alter P to S decline of the fluorescence transient in *Chlorella*, althouth S to M rise phase is altered.

In view of these recent developments, we considered it desirable to reinvestigate the P to S decline part of the fast fluorescence transient in three commonly used algae—the green alga *Chlorella*, the red alga *Porphyridium* and the blue-green alga Anacystis. The effects of various treatments including inhibitors of photosynthesis and uncouplers of photophosphorylation on the P to S decline and on the subsequent S to M rise of the fluorescence transient are presented here. The lack of correspondence between the oxidation level of Q (10) and the P to S decline and at the same time the sensitivity of this phase of induction to a variety of uncouplers (see 1,17) indicate that the slow P to S decline may be linked to the development of "energy pressure" associated with photophosphorylation. Similarly, it has been shown that the S to M rise is not directly linked to photochemical reactions, as it is evident from the loss of complementarity between the kinetics of O_2 evolution and fluorescence changes during this period (25). The S to M rise is partly insensitive to inhibitors and partly sensitive to uncouplers ; it exhibits large alterations in the emission characteristics of Chl *a* fluorescence which is indicative of phototransformation of 'states' of thylakoid membranes (see 1,14). These and other results, along with the observations presented here, suggest that the most part of the slow fluorescence change is not linked to the photochemical reactions, but is related to

structural changes of the membrane that may be associated with export and import processes between chloroplasts and cytoplasmic constituents.

MATERIALS AND METHODS

Intact cells of *Chlorella pyrenoidosa*, *Anacystis nidulans* and *Porphyridium cruentum* were used. Cells were grown in continuous light as described by Govindjee and Rabinowitch (26).

Cells were suspended in fresh growth medium or in phosphate or Tris buffer. Fluorescence was measured with a spectrofluorometer described by Shimony *et al.* (27). For the measurement of fluorescence transient, the procedures of Munday and Govindjee (4) and Mohanty *et al.* (8) were followed. All the chemical additions were made at least 10 minutes before measurements. Oxygen measurement was made as described by Mohanty *et al.* (8).

RESULTS

It is now well known that the fast OIDPS transient is seen in almost all types of intact cells including those of blue green algae (28). Compared to other algal cells, the OIDPS transient in blue green algae is somewhat lower in magnitude than the SMT transient. Fig. 1 shows the IDPS transient in two different cultures of *Anacystis* ; the magnitude of P to S decline depends very much on the culture conditions. Munday (29) observed similar dependence of P to S decline on the culture conditions in *Chlorella*. Fig. 2 shows both the fast (IDPS) and the slow SM transients in another unicellular blue-green alga *Microcystis*. It has been shown earlier by Papageorgiou and Govindjee (25) that blue green algae exhibit a pronounced S to M rise and a very slow M to T decline. The S to M rise is resistant to DCMU treatment in *Anacystis* (see 24, 28).

1. *Intensity dependence*

In *Porphyridium*, D to P rise and P to S decline portions of the fast transient saturate at different light intensities (8). Fig. 3 shows a plot of the amplitudes of O, P and S as function of exciting light intensities in *Porphyridium*. Except for the level at O, the fluorescence intensities, at P and at S, do not show linear rise with increase in intensity of light. As the slope of the fluorescence versus intensity curve is proportional to the quantum yield of fluorescence, the yield at O does not change with intensity as shown earlier by Lavorel (30) and Govindjee *et al.* (31). The plot of relative yields at P and S represented as (P-O)/O and (S-O)/O, indicates that the variable yield at S tends to saturate at much lower intensity than

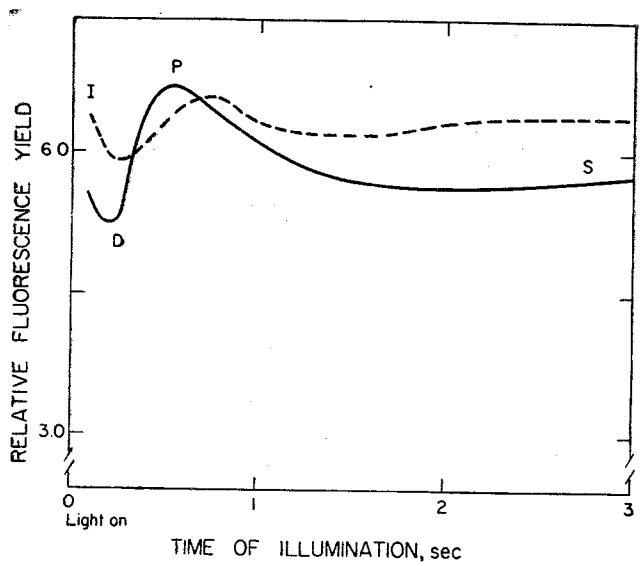

Fig. 1. Time course of Chl *a* fluorescence of two different cultures of *Anacystis*. λ excitation, 633 nm ; intensity, 26 K ergs cm^{-2} sec^{-1} ; λ observation, 685 nm ; C.S. 2-61 before the analyzing monoehromator. Dashed line, cells grown in high light (100 watts, 13 cm away) ; solid line, cells grown in low light (40 watts, 13 cm away) ; 6-day-old samples suspended in growth medium ; both samples were adjusted at the "red" absorption peak of Chl *a* to 0.3 optical density for 1 cm path length ; OI rise not recorded.

at P. Fig. 4 is a similar graph showing the intensity dependence of the variable fluorescence yield at P, (P-O)/O, and at S, (P-S)/S, in *Chlorella*. Here again, the variable fluorescence at S is saturated at relatively lower intensity than at P. This observation suggests that the P to S decline is not directly related to the development of the D to P phase ; the latter has been shown to be due to accumulation of reduced carriers (9).

2. *Temperature dependence*

Rabinowitch (32) has reviewed early observations on the temperature dependence of fluorescence transients. Franck *et al.* (33) have shown that the P to S decline is inhibited at low temperatures. Fig. 5 shows the IDPS transient measured at 5° and 23°C in *Porphyridium*. Here we observe that both D to P rise and the

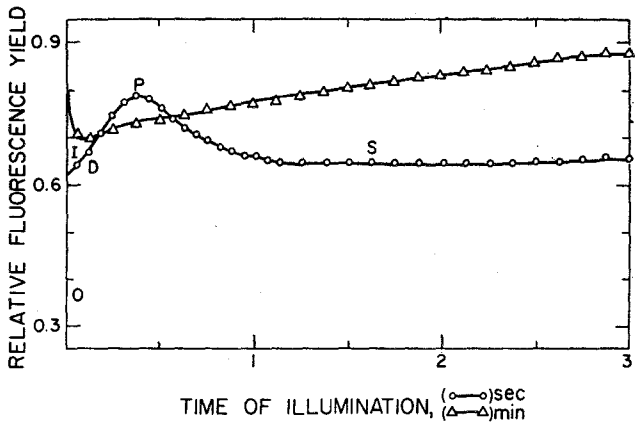

Fig. 2. Time-course of Chl *a* fluorescence transient in *Microcystis*. λ excitation, 633 nm ; λ observation, 685 nm (half band width, 6.6 nm) ; C.S. 2-58 before the analyzing monochromator ; open circles, fast transient ; open triangles, slow transient ; 6-day-old cultures used. Note that the abscissa has two time scales ; 15 min dark period between each measurement ; the fast OI not recorded.

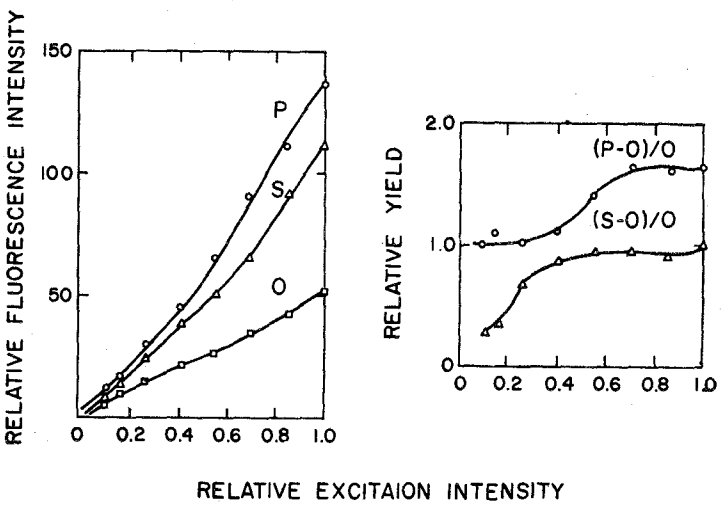

Fig. 3. The amplitude of Chl *a* fluorescence yields at 0, P and S (left) and the relative variable yield (right) versus excitation intensity in *Porphyridium*. Intensity 1.00 = 14 Kergs cm^{-2} sec^{-1}. Relative yields were expressed as (P-O)/O or (S-O)/O ; 8-day-old cells suspended in carbonate-bicarbonate and NaCl buffer (pH 8.5).

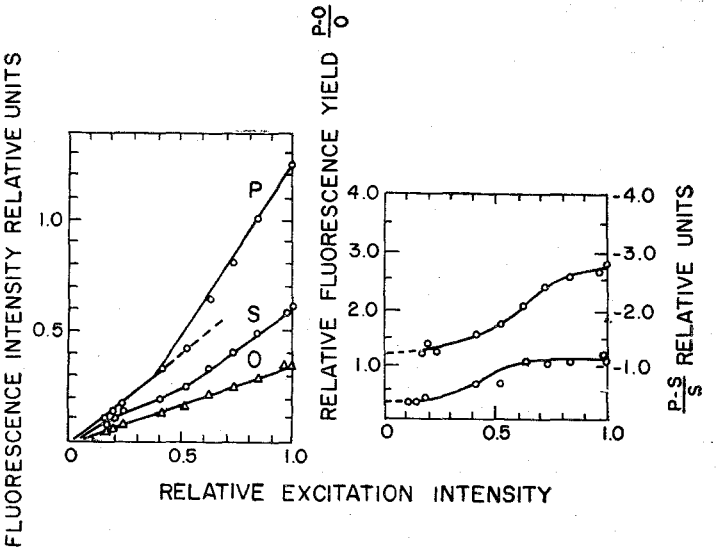

Fig. 4. Relative variable fluorescence yield at P represented as (P-O)/O (right, upper curve) and at S as (P-S)/S (right, lower curve) in *Chlorella* as function of excitation intensity. Left: fluorescence intensities at O, P, and S as function of exciting light intensity. Intencity 1.00=12 K ergs cm^{-2} sec^{-1}. Excitation blue light, white light filtered through C.S. 4-72 and C.S. 3-73 filters.

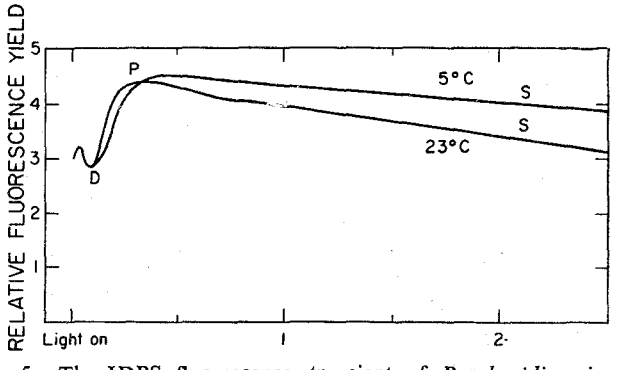

Fig. 5. The IDPS fluorescence transient of *Porphyridium* incubated at two different temperatures during dark adaptation; other details as in Fig. 3.

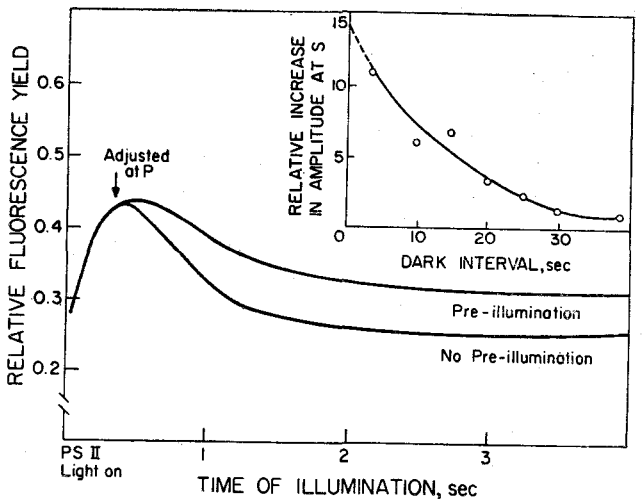

Fig. 6. The effect of 60 second system I preillumination on the decline in the fluorescence transient of *Porphyridium*. Preillumination, 435 nm light (half band width, 10 nm); intentisy, 0.8 K ergs cm^{-2} sec^{-1}. Insert: dark decay of the enhancement of S level by PS I preillumination.

P to S decline are slowed down at 5° C; the P to S decline seems to be more affected than the rise from D to P. If the slower P to S decline at 5° C, as compared to the one measured at 23° C, indicates a slower reoxidation of reduced Q by the pool of intersystem carriers, a faster D to P rise should have been observed, as this rise is associated with the accumulation of reduced Q (QH) (see 4, 9, 10). But this does not seem to be the case (Fig. 5). It has been shown (see 6, 7, 8), that the rise in the rate of O_2 evolution after the initial O_2 'gush' (spike) and the slow P to S decline of Chl *a* fluorescence are antiparallel. This antiparallel (or complementary) behaviour of O_2 evolution and Chl *a* fluorescence would indicate that the Chl *a* fluorescence during this induction (P to S decline) period is linked to the redox state of Q, the quencher of fluoescence. We have observed, however, that the rate of rise of O_2 evolution after the initial O_2 'gush' does not differ appreciably when measured at 5° C and at 25° C, although the P to S decline is significantly altered at these two temperatures (see fig. 5). This again suggests that P to S decline of Chl *a* fluorescence is not solely governed by the oxidoreduction state of Q. It is also clear from Fig. 5 that P to S decline is not "affected" by the extent of D to P rise.

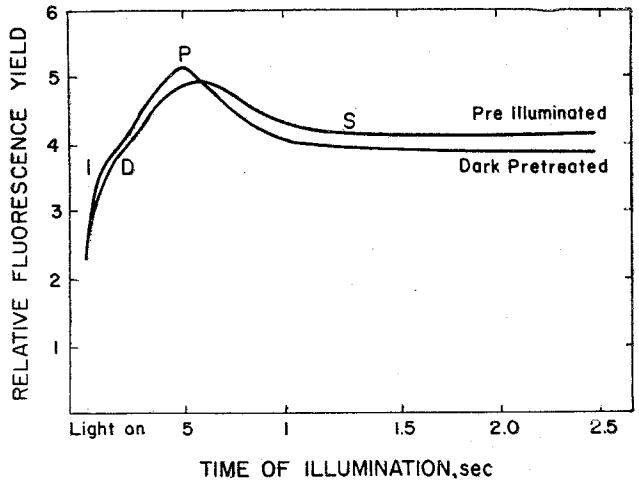

Fig. 7. Fluorescence induction in *Chlorella* cells. Note enhancement of the amplitude of S level fluorescence by system I preillumination. Dark time, 7 min; preillumination, 710 nm light (1.3×10^2 ergs cm^{-2} sec^{-1}) for 60 seconds. Transient was measured 2 seconds after preillumination. Data obtained at room temperature.

3. *Preillumination effects*

Munday and Govindjee (9) have shown that P to S decline and the final S level in *Chlorella* is influenced by the wavelength of the preilluminating light. A preillumination of the sample with light absorbed mainly by PS I slowed down the P to S decline and raised the level of S (9, 28, 31). Fig. 6 shows similar results on the effect of preillumination for one minute with 435 nm light (mainly PS I) on the P to S decline in *Porphyridium*. (In this figure we have adjusted the fluorescence yield at P to read the same value as we assume that at P almost all the Q are reduced to QH.) The half-time for the decay of the preillumination effect was found to be approximately 12-15 seconds. Fig. 7 shows a similar transient obtained with *Chlorella*. Although a two-second dark-time intervened between preillumination (PS I) and excitation (PS II) light, it is nevertheless clear that the PS I-light preillumination slowed down both the D to P rise and the P to S decline. The slower D to P rise must be due to the accumulation of the intersystem carriers (A pool) in an oxidized state by the preilluminating PS I light, but the slower P to S decline cannot be explained by the oxidation state of the electron transport carriers (9, 29, 34).

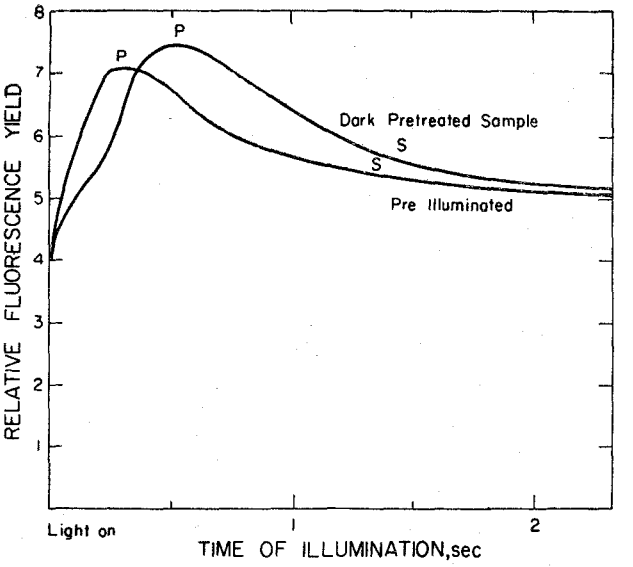

Fig. 8. Effect of brief system II preillumination on the Chl *a* fluorescence transient in *Chlorella*. Dark pretreatment, 7 min ; preillumination with blue-green exciting light until P was reached, the light was turned off, and, then turned on again. The two transients are adjusted at "O". Cells were suspended in 0.05 M phosphate buffer (pH 8.0) plus 0.01 M NaCl. Intensity of exciting blue light, 14 K ergs cm^{-2} sec^{-1}.

Fig. 8 shows fluorescence transients from a dark adapted sample and a sample of *Chlorella* preilluminated with predominantly PS II light. In the latter case the light was on until the P level was reached and turned off for 2 sec, and then the same light was turned on again and the complete transient was recorded. The short term preillumination with PS II light caused, as expected, a rapid D to P rise owing to an accumulation of reduced "A" pool caused by the PS II preilluminating light. However, the faster P to S decline in the preilluminating sample, as compared to the dark-adapted one, cannot be easily explained on the basis of a simple reoxidation of electron carriers.

In summary, the PS I preillumination retards the P to S decay while PS II preillumination hastens it. This behaviour is contrary to what one would expect from the known antagonistic effect of PS II and PS I light on the redox state of Q. We also note that in *Chlorella*, there is no quenching of fluorescence by PS I

light at the S level, and the extent of quenching brought about by PS I light during the S to M rise portion of the transient is less than during the subsequent M to T decline phase (35).

4. Decay of P to S in light or in dark

Heath (36) reported that the dark decay of maximal fluorescence (F_{max} in isolated chloroplasts, without any exogenous acceptor) is very complex and the yield falls to the initial level only after 1 to 2 min. of darkness. The decay of P level in *Porphyridium* was found to be much faster than in broken isolated chloroplasts. Fig. 9 shows that in *Porphyridium* the yield at P declines much faster in

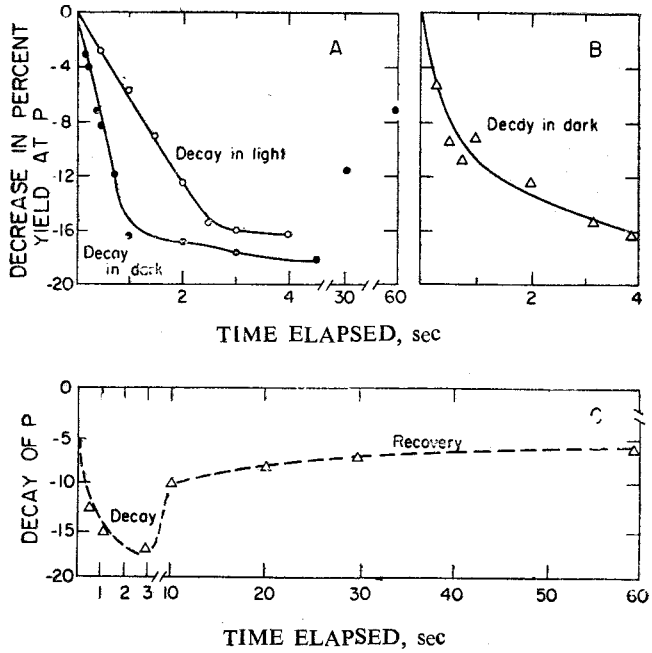

Fig. 9. The dark decline of the P level of Chl *a* fluorescence in *Porphyridium*. After dark adaptation, the system II light was turned on until P level was reached and then it was turned off. After dark interval, as shown on the X axis, the light was turned on again and the complete transient was recorded. The decrease in the amplitude of P by the second illumination is plotted as percentage decay. A and B, dark decay of P from two different cultures ; for comparison, the P to S decay in light is also plotted as percented decay with time in A. C, rapid decay and slow restoration of P in darkness.

darkness than in light ($t_{1/2}$ = 0.25-0.5 sec in dark ; 1.5 sec in light). One may expect the oxidation of QH to be faster in light than in dark as part of the exciting light is absorbed by PS I. However, the results shown in fig. 9 are contrary to this expectation. Again, although fluorescence decays to a low level fairly rapidly in dark (1-3 sec), yet the restoration of P level in subsequent illumination can be achieved only after longer (>10 sec) dark adaptation (fig. 9c). These results clearly show that P to S decline does not reflect the redox state of Q and other processes regulate the fluorescence yield during this period of induction.

5. Effects of inhibitors

While the preceding observations indicate that the P to S decline does not seem to directly reflect the oxidation of reduced primary acceptor (QH), nevertheless, the photosynthetic noncyclic electron flow may be thought to be necessary for the P to S decline. This is because of the fact that DCMU abolishes this decline. It is known that DCMU blocks the electron flow very close to Q, inhibiting the flow of electrons from Q to the pool of intersystem intermediates (A) (37). Unlike DCMU, hydroxylamine (NH_2OH) has been shown to inhibit oxygen evolution but not the electron transport between two photosystems (see 38,39). DCMU accelerates the D to P rise and NH_2OH slows it down. Fig. 10 shows that like DCMU, NH_2OH, at a high concentration (10mM), also inhibits the characteristic P to S decay in *Porphyridium*. Mohanty *et al.* (39) have shown that at this concentration NH_2OH feeds electrons to photosystem II and a noncyclic electron flow exists between the two photosystems. It is likely that, with NH_2OH as electron donor for PS II, the photophosphorylation ability of cells is reduced and this may have indirectly caused the suppression of P to S decay. This would suggest that P to S decline is indeed complex. [In *Anacystis*, unlike the case in *Porphyridium*, both NH_2OH and DCMU together do not suppress S to M rise but abolish P to S decline (28).]

6. Effect of anaerobiosis

Kautsky *et al.* (18) reported that under anaerobic conditions there is no P to S decline. Munday and Govindjee (4) showed that in *Chlorella* under anaerobic condition the P to S decline was abolished, although the IDP phase of the fast transient was quite prominent under this condition. Franck and Hoffman (40) and Schreiber *et al.* (41) have also reported a progressive inhibition of the P to S decline with increasing anaerobiosis. One may expect anaerobiosis as well as DCMU treatment to induce a cyclic flow of electrons that supports photophosphorylation (42). If this view is correct, the prevention of P to S decline by anaerobio-

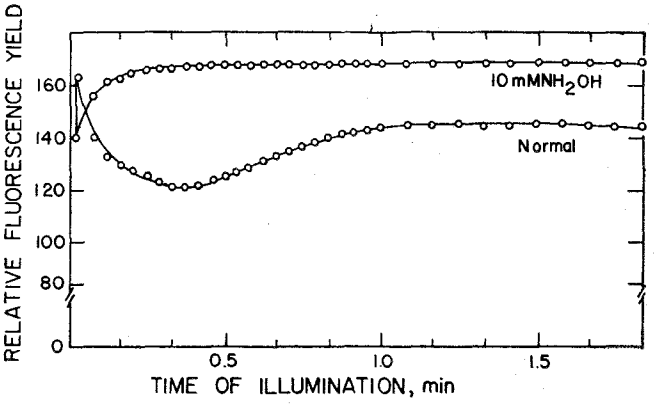

Fig. 10. The slow fluorescence transient in normal and NH_2OH (10 mM)-treated *Porphyridium*.

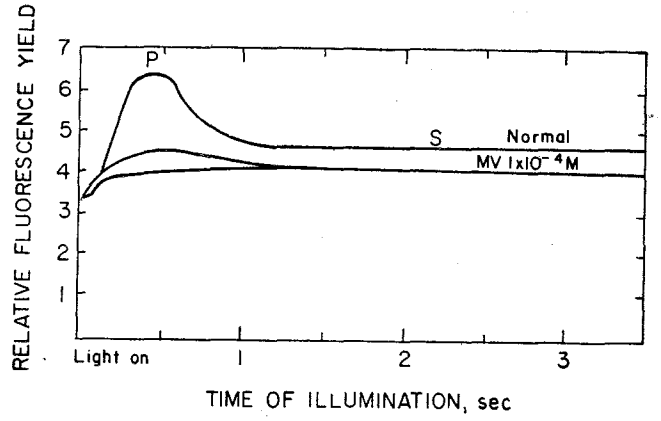

Fig. 11. Effect of methyl viologen on the DPS transient in *Chlorella*. Cells suspended in carbonate-bicarbonate buffer (0.1 M, pH 9.2). Upper trace, untreated sample; middle trace, 1 minute after addition of 0.1 mM MV; lower trace, 10 min. after the addition of MV. In each case sample was kept 7 min in darkness before measurement.

sis or by DCMU poisoning would indicate that the cyclic electron flow is not directly linked to P to S decline. Papageorgiou *et al.* (43) showed that the slow decline of Chl *a* induced by reduced PMS ($PMSH_2$) in DCMU-treated chloroplasts is also susceptible to oxygen tension suggesting that the P to S decline may include quenching by molecular oxygen.

7. Effect of system I electron acceptor (methyl viologen)

Munday and Govindjee (9) showed that methyl-viologen (MV) quenches the fast (DP) Chl *a* fluorescence rise in *Chlorella*. Mohanty (44) confirmed these results in *Chlorella, Porphyridium and Anacystis*. Fig. 11 shows that in the presence of 0.1 mM MV, the OI phase was not affected but MV suppressed the development of P and lowered the S level in *Chlorella*. Fig 12 shows our results obtained with *Anacystis* cells. We have observed that 0.1 mM MV gradually inhibits O_2 evolution with time of incubation in dark and finally O_2 uptake is observed in lieu of O_2 evolution. Also, in *Chlorella* the extent (amplitude) of cytochrome f oxidation by system I light was found to be roughly 2.5 times greater in MV-treated samples than in the untreated samples. These results indicate that MV accelerates the flow of electrons in the intact algal cells as it does in isolated chloroplasts. Thus, the suggestion of Munday and Govindjee (4,9) that the D to P rise is brought about by the transitory

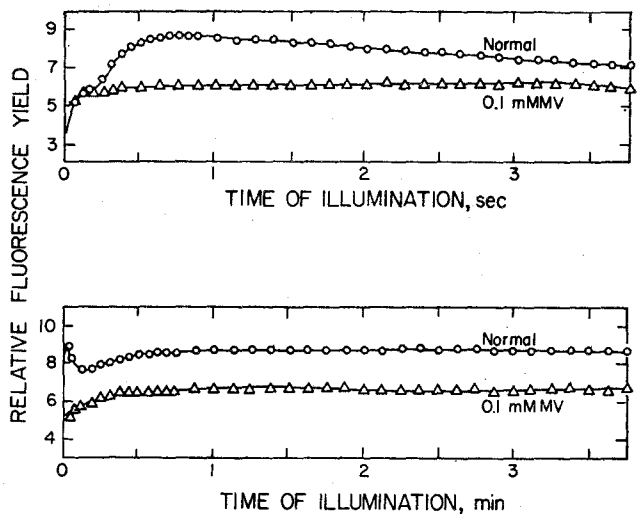

Fig. 12. Time course of Chl *a* fluorescenee in normal and methyl viologen treated *Anacystis*. Methyl viologen, 0.1 mM ; λ observation, 684 nm ; intensity, $\approx$ 26 K ergs cm^{-2} sec^{-1}.

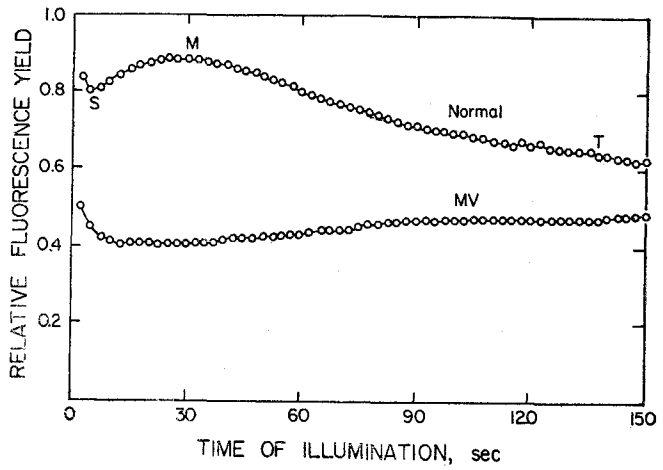

Fig. 13. The slow transient in Chl *a* fluorescence yield with and without Methyl viologen (MV) in *Chlorella*. Upper curve, untreated cells; bottom curve, with 1 mM MV. Fluorescence was measured at 685 nm; λ excitation, blue light (C,S. 4-72+C.S. 3-73); intensity, $\approx$10 K ergs cm^{-2} sec^{-1}.

accumulation of most of the electron transport carriers in reduced state seems to be consistent with our observations. As noted above, MV eliminates most of the D to P rise and lowers the S level; this may indicate that an accumulation of reduced Q is a prerequisite for and is linked to the P to S decay, although the latter does not merely reflect the oxidation of QH.

Besides the effect on ODPS transient, MV also arrests most of the development of the slow SMT type of fluorescence change in *Chlorella* (see fig. 13). We do not know the reason for the absence of the S to M rise. This may be due to the fact that MV in light might have caused an 'over oxidation' of carriers and, thereby, perhaps imposed some irreversible alteration of structure. It may be added that we could not remove the effect of MV from the treated cells by washing the samples with buffer. MV, thus, in intact cells also may bring about some irreversible structural change.

8. Effect of system II acceptor (quinone)

Figure 14 illustrates the alteration of the time course of fluorescence in *Chlorella* by 0.1 mM benzoquinone (PBQ) a well known Hill oxidant (see 45).

PBQ suppressed the P level and lowered the amplitude of S (PBQ also affected slightly the O level, not shown). Since PBQ acts as a Hill oxidant for algal cells, we conclude that the suppression of the variable fluorescence (D to P phase) is due to the oxidation of QH by quinones. The slow rise that persists the quinone treatment is reminiscent of the S to M rise although this rise was slower here than in the normal cells. We note that a slow rise of fluorescence yield was invariably seen with PBQ-(fig. 14) but not with MV-treated *Chlorella* (fig. 13).

We do not know why the slow S to M rise in Chl *a* fluorescence is resistant

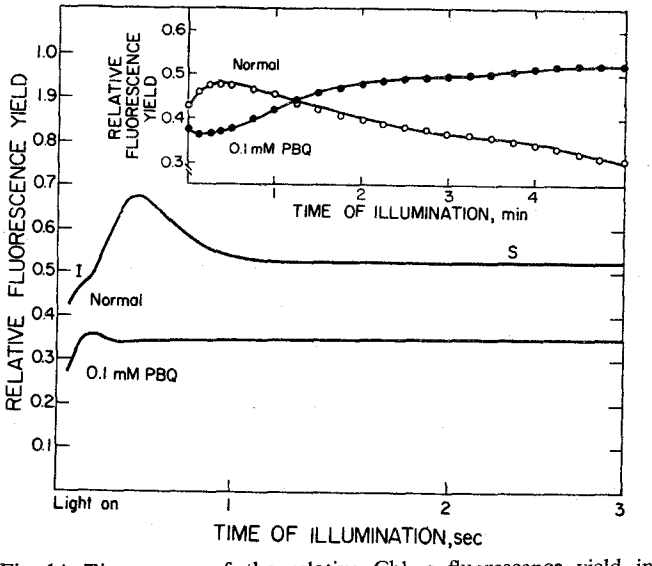

Fig. 14. Time course of the relative Chl *a* fluorescence yield in *Chlorella* with and without *p*-benzoquinone (PBQ). Main figure shows the fast transient, and the insert the slow transient.

to PBQ but not to MV. It may be possible that cyclic photophosphorylation is suppressed in the presence of MV, but not in the presence of PBQ. This would suggest that the S to M fluorescence rise is linked to cyclic electron flow-mediated phosphorylation. However, one difficulty in assuming this is the fact that no alteration in Chl *a* fluorescence yield is observed in *Chlorella* in the presence of DCMU (44,46). The slow and the persistent S to M rise observed in the presence of PBQ may be related to the change in the so-called "constant" yield fluorescence.

Amesz and Fork (47) have shown that the quenching of Chl *a* fluorescence by quinones may be of two kinds: (i) a dynamic physical quenching similar to what has been seen in case of Chl *a* solution and (ii) quenching due to oxidation of QH. Our results indicate that the main cause of lowering of DP transient by PBQ is due to its action as a Hill reagent. However, it appears that the slow S to M rise is not at all linked to oxidation state of Q ; also, this slow rise does not seem to depend on the development of the fast (OIDPS) transient.

9. Effect of uncouplers of phosphorylation

The effect of uncouplers of photophosphorylation on the fluorescence yield changes has been reported by many investigators (see 1). One of the most studied uncoupler is carbonylcyanide hydrazone (CCCP or FCCP). We have recently reported the effect of CCCP on the IDPS transient of *Porphyridium* and *Chlorella* (24). FCCP at a relatively high concentration (10-30 μM) was found to suppress the development of P. The P to S ratio (measured after 3 sec of illumination) was also lowered. Although FCCP lowered the P to S decline, the slow S to M type rise of Chl *a* fluorescence was not completely inhibited by this uncoupler. Fig. 15 shows the slow S to M rise of Chl *a* fluorescence in *Chlorella* in the presence of 10 μM FCCP. Note that the rise of fluorescence from low S level is very slow and there is no M to T decline The steady state level of fluorescence is also high. This slow fluorescence rise in the presence of FCCP was significantly more at a lower pH (6.0) of the suspending medium than when the pH was high (8.0). This supports the earlier observation of Papageorgiou and Govindjee (46) that FCCP stimulates slow S to M rise in *Chlorella* although it suppresses both the P to S and the M to T decline in this alga.

Fig. 16 (top) shows traces of the fast fluorescence transient of *Anacystis* with and without CCCP. At a low concentration of CCCP (5 μM) the amplitude of P is low and the P to S decline is also lowered, but the slow fluorescence rise is not altered at this concentration. However, with 10 μM of FCCP, the P level was further depressed and the S to M rise was also arrested (also see 25, 28, 46). Fig. 16 (bottom) shows the effect of 5 and 10μ M CCCP on the slow fluorescence changes in DCMU-poisoned *Anacystis* cells. 10μ M CCCP did not cause the same extent of suppression of S to M rise in the presence of DCMU as in its absence. Papageorgiou and Govindjee (46), however, have observed a more dramatic differential effect with FCCP in normal and DCMU-treated *Anacystis* cells.

Fig. 17 shows the effect of the uncoupler FCCP (5 μM) on the rate of O_2 evolution in *Anasystis*. FCCP depressed the O_2 spike and caused a transitory O_2

uptake. With time, the rate of O_2 evolution increased although at a slower rate than in the control. It seems FCCP accelerated O_2 uptake in these cells and slightly suppressed the net O_2 production. This inhibition of O_2 production may be either

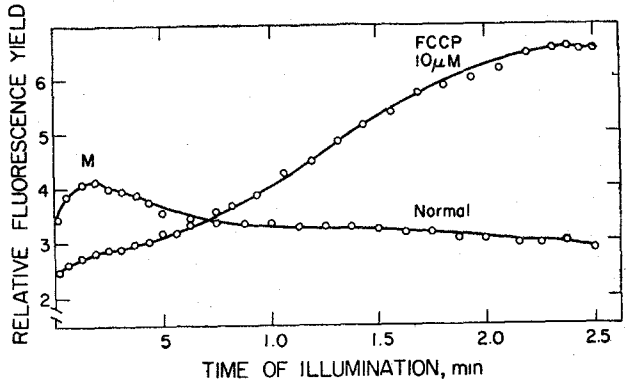

Fig. 15. Time course of slow changes in Chl *a* fluorescence yield in *Chlorella* with and whithout 10 μM FCCP. Buffer, phosphate and NaCl.

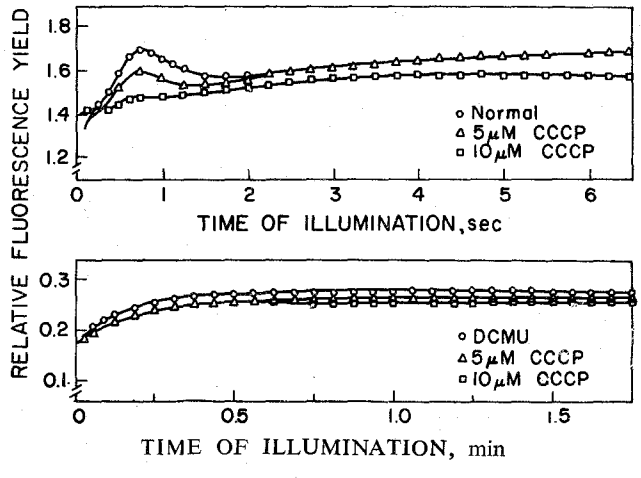

Fig. 16. Effect of the uncoupler of phosphorylation (CCCP) on the fluorescence yield of Chl *a* in normal and DCMU poisoned *Anacystis*. Top figure, fast fluorescence changes in normal cells. Bottom figure, slow fluorescence changes with 15 μM DCMU added. Buffer, 50 mM Tris-Cl plus 1 mM NaCl, pH 7.8.

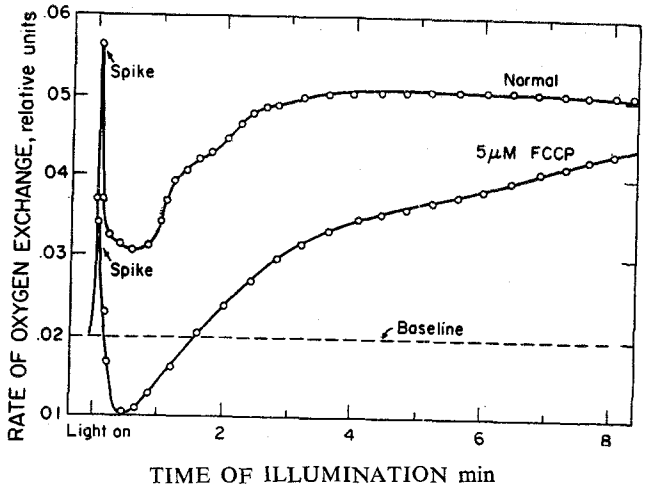

Fig. 17. Time course of O_2 exchange in *Anacystis* in the presence of FCCP.

due to the uncoupling of photophosphorylation or to the photoinduced O_2 uptake. Unlike CCCP, NH_4Cl did not seem to alter the characteristic pattern of OIDPS (48) in *Chlorella*. In some cultures, however, we observed a depression of the P to S decline ; in others, it remained unaltered.

Slovacek and Bannister (49) have shown that NH_4Cl enhances the slow S to M rise in the *Chlorella* depleted of CO_2 supply. These cells show no S to M rise in the absence of NH_4Cl although one would expect that these CO_2 depleted cells of *Chlorella*, like normal cells, possess the ability for cyclic photophosphorylation. Hence, the enhancement of S to M rise by the addition of NH_4Cl to these CO_2-starved cells cannot be related to the uncoupling effect of this chemical.

Slovacek (unpublished results) has also observed that depletion of CO_2 increases the ratio of System I to System II fluorescence and NH_4Cl reverses this effect and causes an increase in PS II fluorescence. This is in confirmity with the results of Mohanty *et al.* (8) that in the S to M rise the M level exhibits more PS II fluorescence than the S level. Unlike the case in *Chlorella*, the S to M rise in *Anacystis* is suppressed by uncouplers, notably salicylanalides and FCCP ; these uncouplers, also, arrest the macroscopic structural alteration as measured by change in optical density at 540 nm (50). Furthermore, the glutaraldehyde fixed cells do

not show S to M fluorescence rise (28,39). This suggests that the S to M rise of fluorescence may be linked to some structural alterations of the thylakoid membranes.

10. *Effect of cyclic electron transport confactor (PMS)*

Phenanzine methosulfate (PMS) is known to accelerate cyclic flow in isolated chloroplasts. In intact cells of *Anacystis*, reduced PMS ($PMSH_2$) abolishes the P to S decline (24). However, the fluorescence yield slowly increases from a low level to a level approximately that of the P level and then remains unaltered. There is no P to S decline, although fluorescence level is high. The slow change in fluorescence persists in $PMSH_2$-treated cells ; in some cases it is enhanced. This may suggest that cyclic electron flow induces S to M rise but this does not seem to be the case, as $PMSH_2$ quenches S to M rise in the presence of DCMU. It is possible that the $PMSH_2$-induced slow fluorescence rise in *Anacystis* is linked to some sort of non-cyclic electron flow mediated via PS I.

DISCUSSION

Table 1 summarizes some of the general effects of various treatments on the different phases of the fluorescence transients. The observations presented earlier and listed in table 1 amply illustrate some of the difficulties encountered in explaining the slow changes in Chl *a* fluorescence. This is because these slow changes reflect many complex physiological processes associated with photosynthesis. In spite of these complexities, however, some general trends seem to be clear. Thus, several physical and physiological processes seem likely to be associated with the different phases of the transient in intact cells; these are listed in Table 2.

The P to S Decay

There is ample evidence (see 1) that the P to S decline is not linked to the redox state of Q (also see Table 1, items 4 and 5). The effect of preillumination (Figs 6-8) on the P to S decline clearly indicates that the latter is not directly linked to the redox state of Q. However, P to S decay is somewhat dependent on the flow of electrons between the two photosystems as DCMU and *o*-phenanthroline completely suppress it (Table 1, 6a). The inhibitor NH_2OH at high concentrations, but not at low concentrations, suppresses the P to S decline (39). The absence of P to S decline under anaerobiosis (Table 1 ; see also 4, 9, 18, 40, 41) indicates that the P to S decline may also be susceptible to quenching by molecular oxygen (17).

The P to S decline is extremely vulnerable to uncouplers (Table 1, 8a),

TABLE 1

A Summary of the Effects of various Treatments on Chlorophyll Fluorescence Transients in Algae

Treatment	Algae used	Induction phase				References
		O to P	P to S	S to M	M to T	
(1) Anaerobiosis	Chlorella	Pronounced	Absent	—	—	4, 9, 18, 33, 41
(2) CO_2 depletion	Chlorella	Present	Present	Absent	Absent	16, 49
	Chlamydomonas	Enhanced	Enhanced			
(3) pH	Chlorella	Remained unaltered between pH 6 to 8	No Significant change	No chage	Abolished	16, 52
(4) Low Temperature (5°C)	Porphyridium	Slow rise	Slow decline	—	—	33, 42, 44, this paper
(5) Preillumination by : (a) PS I light	Chlorella Porphyridium	(a) Delayed low P level	Slow decline	—	—	PS I : 4, 9, 34; PS II : 34, this paper
(b) PS II light	—do—	(b) Fast rise	Fast decline	—	—	
(6) System I light	Chlorella	Quenching at P	—	Less quenching	More quenching	11, 35
(7) Inhibitors of electron flow a) DCMU, and o-phenanthroline	Several	(a) Very fast rise to maximal level	Absent	Absent in Chlorella & Porphyridium but present in Anacystis	Absent	DCMU : 25, 28, 39, 58

TABLE 1 (Contd.)

1	2	3	4	5	6	7
b) NH_2OH		b) DP rise present	Absent	Slightly suppressed or not at all (Anacystis)	Absent	NH_2OH : 39 this paper
(8) Uncouplers of photophosphorylation a) CCCP/FCCP	Chlorella	a) Lowers P; slow rise	Suppressed	Enhanced in Chlorella but not in CO_2-depleted Anacystis	Abolished	24, 25, 28, 46 this paper.
b) NH_4Cl	Chlorella	b) No change	No change	Stimulated in CO_2-depleted Chlorella	Absent	16, 48
(9) Electron Transport Carriers	Chlorella Porphyridium					
a) MV		a) Low P level; only OI rise	Absent	Suppressed	Absent	4, 9, this paper
b) PBQ	Chlorella	b) Low	Insignificant; absent	Slow rise	Absent	44, this paper.
c) $PMSH_2$	Anacystis	c) High P level	Absent	Persists	Absent	17, 24 this paper.
(10) High salt (KCl, $MgCl_2$ or K acetate)	Chlorella	Quenching at P	—	Less quenching	More quenching	48.

TABLE 2

Possible Relationship of various Physical and Physiological Process with different Phases of Chlorophyl Fluorescence Transient

Induction Phase	Process linked to the transient phase	Reference
(1) O-I rise	Primary photochemical reaction at the reaction centre II ; redox state governs the fluorescence yield	2, 6.
(2) I-D decline	Interaction of Q^- with pool of acceptor A	4, 9.
(3) D to P rise	"Traffic jam" due to a bottleneck on the acceptor side of pigment system I ; accumulation of reduced carriers	4, 9.
(4) P to S decline	Lowring of yield due to changes in "phosphate potential" or proton gradient or membrane potential leading to structural changes facilitating decrease in fluorescence yield; seems to be linked to loss of Mg^{2+} ions; may be affected by molecular oxygen	17, 20.
(5) S to M rise	Independent of redox state of Q ; regulated by structural changes of the membrane facilitating increase in fluorescence yield; may be linked to export of energy and uptake of materials between chloroplasts and their surrounding cellular constituents	28, 35, 50.
(6) M to T decline	Indirectly linked to synthesis and utilization of ATP in CO_2 fixation	8, 25. 46.

which indicates that this slow decline is somehow regulated by phosphorylation. One would expect that a cyclic electron flow supported photophosphorylation to be stimulated by anaerobiosis and DCMU poisoning. However, under these conditions no P to S decline is observed, at least, in *Chlorella* (46) and *Porphyridium* (8) which indicates that the P to S decline may be linked not to a cyclic electron flow, but to non-cyclic photophosphorylation in these algae. Experimental support in favour of this hypothesis may be taken from the results of Krause (19) who has shown that the slow P to S decline in intact chloroplasts is suppressed by uncouplers

of phosphorylation. Krause (20) and Barber *et al.* (21) have further demonstrated that the P to S decline is linked to the loss of Mg^{2+} ions from the grana stacks to the stroma region. Krause (20) concluded (also see 1) that a "phosphate potential" is responsible for a structural alteration of the membrane leading to the P to S decline. Although Krause's experiments indicate that loss of Mg^{2+} like cations may be counterbalanced with the exchange of protons, it is not certain if the loss of Mg^{2+} by grana into the stroma region is compatible with the H^+ uptake. Hind *et al.* (51) have shown that the uncoupler CCCP, but not NH^+_4 ions, inhibits Mg^{2+} efflux to the medium in isolated chloroplasts which lends support to the hypothesis (see Table 1, 8b) that loss of Mg^{2+} is linked to the P to S decline. In the blue green algae there is no clear demarcation of stroma and grana regions but these algae also exhibit the P to S decline (28). We do not yet know if there is any compartmentation in the lamellar regions of the blue-green algae.

The hypothesis of Krause (19) for the P to S decline differs from that of Papageorgiou (17) in that the latter proposed collisional-quenching of Chl *a* fluorescence by molecular oxygen. Papageorgiou (17) suggests that acidification of the inside of the thylakoid membranes makes the pigment bed more susceptible to quenching by O_2. This hypothesis is supported by the observation that $PMSH_2$ induced lowering of Chl *a* fluorescence in DCMU-treated isolated chloroplasts is suppressed at low O_2 tension (43). A difficulty in this hypothesis is the fact that during the P to S decline phase, fluorescence and oxygen evolution are complementary. This relationship would not be retained if substantial amount of O_2 would be needed to quench Chl *a* excitation. Also, under anaerobiosis the O_2 evolution is not insignificant although the P to S decline is totally arrested (Table 1, 1). Furthermore, it has been shown that during the P to S decline the ratio of System I to System II emission is altered. No such alteration is seen in PMS-induced lowering of Chl *a* in isolated chloroplasts (22, 23) ; this indicates that the PMS-induced lowering in isolated chloroplasts may not be quite analogous to the P to S decline in algae. In spite of these objections the suggestion that the P to S decay may be partly due to quenching by molecular O_2 remains feasible.

Slovacek and Bannister (16) suggested that the P to S decline reflects an activation of some dark enzymatic step in the electron transport chain. Such activation would accelerate electron flow and reactivate PS II. However, this proposition cannot easily explain the initial observation of Duysens and Sweers (10) and the data shown in Fig. 9C that a dark period is necessary to restore the high level fluorescence. Thus, it appears that the most attractive theory for the P to S

decline would involve the postulation of a build-up of a "phosphorylation potential" due to non-cyclic electron flow : the fluorescence decline must occur because this potential creates structural changes on the membrane which lead to an increase in the rate constant of internal conversion and / or transfer to weakly fluorescent Chl *a* molecules.

The S to M rise

The S to M rise part of the transient seems to be more independent of the redox state of Q than the P to S decline phase (Table 1, 6, 7). This is evident from the facts that (a) the S to M rise is resistant to DCMU and *o*-phenanthroline (Table 1, 7) in *Anacystis* (50, 52) and (b) a development of the fast fluorescence transient, which is associated with redox reactions of the electron transport chain (see 1) is not a prerequisite (see Fig. 14) for the development of the S to M rise (Table 1, 9b & c). The S to M transient is not much affected by the pH of the surrounding medium (Table 1, 3) (52) ; also, the effect of system I light to quench the fluorescence evoked by PS II light is comparatively less in this phase than in the M to T phase (Table 1, 6) (35).

Uncouplers like CCCP and FCCP have somewhat different effects (Table 1, 8a) on the S to M rise phase in *Chlorella* and in *Anacystis*. In *Chlorella*, although CCCP and FCCP suppress both the P to S and the M to T declines, they do not affect S to M rise as shown in Fig. 15 (also see 46). In *Anacystis*, the S to M transient is suppressed at a low concentration of FCCP (25, 50). FCCP at high concentrations and salicylanalides at fairly low concentrations suppress the S to M rise in DCMU-treated *Anacystis* (50). We have also shown that the S to M rise is associated with structural alteration in this alga as measured by changes in optical density at 540 nm. In spite of the differential behavior of the S to M phase of the transient to DCMU (25, 28, 46, 50) (also see Table 1, 7a, b) and FCCP (Table 1, 8a) in *Chlorella* and *Anacystis*, it is reasonable to assume that some structural changes are associated with this S to M change in *Chlorella* as we have shown in the case of *Anacystis* ; in both cases the fluorescence yield changes are not linked to Q.

Slovacek and Bannister (49) have shown, as noted earlier, that in CO_2-depleted cells of *Chlorella*, the S to M phase is suppressed ; however, the addition of NH_4Cl to CO_2-depleted samples revives a large S to M rise and also increases the steady state fluorescence level. These authors argue that such large fluorescence yield changes observed by the addition of NH_4Cl cannot be accommodated by the

change in distribution of quanta between the two photosystems, i.e., the so called-α-change hypothesis, but can be explained by activation of PS II units.

The stimulation of S to M rise in *Chlorella* in the presence of FCCP (Fig. 15) is somewhat similar to the effect of NH_4Cl on the CO_2-depleted *Chlorella*. Also the S to M rise in *Chlorella* is practically unaffected by pH of the medium (52). Slovacek and Bannister (49) have suggested that there is possibly no large proton gradient in CO_2-supplemented whole cells of *Chlorella* during steady state photosynthesis as fluorescence level remains unaltered with or without NH_4Cl. Thus the S to M rise may not be linked to any proton gradient, and the uncouplers must be acting on the structure of chloroplasts in some indirect way. The stimulation of S to M rise in the presence of FCCP in *Chlorella* and the inhibition of the S to M rise in DCMU-treated *Anacystis* by high concentrations of FCCP and salicylanalides indicate that the S to M rise may be related to structural organization of the thylakoid membrane. It seems to us that the S to M rise may be similar to the cation-stimulated (23, 53, 54) fluorescence rise in isolated chloroplasts which is not directly linked to the energy conservation process. However, in intact cells, the export of energy from the chloroplast to the cytoplasm or the cell may be accompanied by some slow active import of Mg^{2+} from the cytoplasm (55). Such active import of materials like sugars (e.g., glucose) or anion would be expected to be affected indirectly by uncouplers. Although some of the experimental data cannot be adequately explained by the above suggestion, it seems very clear that the S to M rise originates from structural alteration that facilitates transformation of photostates in favour of the so-called state I (see 1, 14, 17).

The M to T decline

The M to T decline phase has been shown to be very susceptible to growth conditions, uncouplers of phosphorylation (25), CO_2 content (16) and energy transfer inhibitors (25). The slow M to T decline in *Porphyridium* is also suppressed by an energy transfer inhibitor like Phlorizin (8, 25). The slow M to T decline thus, seems to reflect the slow processes of ATP synthesis and utilization during photosynthesis. It is clear from our past work and that of others that the major slow fluorescence yield changes in intact cells are not linked to the photochemical reaction but associated with the energy conservation and other physiological processes : they are linked to phosphorylation in the form of proton gradient or membrane potential and are associated with the structural alteration of the membranes linked with photo-transformation of states.

Concluding remarks

The P to S decay is retarded by PS I preillumination and is accelerated by PS II preillumination, while the D to P rise exhibits opposite effects under these conditions. The P to S decay is slowed down at 5°C as compared to 23°C while the D to P rise does not show significant change at these two temperatures. The P to S decay is abolished by inhibitors of electron flow (*e.g.* DCMU, *o*-phenanthroline), inhibitors of O_2 evolution (high concentrations of NH_2OH) and anaerobiosis. Under these conditions the fluorescence level usually remains high and does not show P to S decay. Also, no P to S decline is observed in the presence of the terminal PSI acceptor methyl viologen. Methyl viologen keeps the Chl *a* fluorescence at a low steady S level—mostly due to the oxidation of carriers in the electron transport chain. The above results suggest that a normal photosynthetic O_2 evolution may be necessary for the development of the P to S decline although it does not reflect oxidation-reduction of Q. The P to S decline is extremely sensitive to uncouplers like CCCP and FCCP. This suggests that the P to S decline may be linked to "energy conservation" process coupled to non-cyclic electron flow. However, the results obtained with methyl viologen (MV) and NH_2OH cannot be easily explained on the basis of this hypothesis. One must assume that in the presence of NH_2OH cells lose their ability to phosphorylate, and in the presence of MV, the oxidation level of Q and the build up of "phosphate potential" keep the yield of fluorescence at a minimum level.

The slow S to M rise is also not associated with the non-cyclic electron flow. The S to M rise is insensitive to DCMU. Although MV arrests the S to M rise, *p*-benzoquinone (PBQ), which suppresses the P to S decline, has no or slightly stimulatory effect. The S to M rise is not as sensitive to uncouplers of phosphorylation as the P to S decay is. In *Chlorella*, the S to M rise is resistant to low concentrations of CCCP and FCCP ; at high concentrations, they stimulate the S to M rise. In *Anacystis*, high concentrations of FCCP inhibit the S to M rise in presence of DCMU. Furthermore, the S to M rise is resistant to NH_2OH in DCMU-treated *Anacystis*. PMS suppresses the S to M fluorescence rise in DCMU-treated cells of *Anacystis* ; it does not affect the S to M rise in untreated cells, although it abolishes the P to S decline. In untreated cells, PMS seems to act as a Hill oxidant like PBQ. The above results are complex and somewhat variable with organisms, yet some generalizations can be made : (i) The S to M rise does not need sustained non-cyclic electron flow : (ii) it is partially resistant to uncouplers of phosphorylation and (iii) it exhibits large alterations in the emission characteristics of Chl *a* fluorescence (8, 15). These results, together with our earlier observation that the

S to M rise is arrested by glutaraldehyde fixation, indicate that this slow change in fluorescence yield may be analogous to the cation-induced fluorescence yield changes seen in isolated broken DCMU-treated chloroplasts (23, 54). Thus the S to M rise seems to reflect phototransformation of 'states'. We assume that the S to M changes in whole cells may be linked to export of energy from the chloroplasts and import of ions (or sugars) to the chloroplasts.

The ultimate causes of fluorescence yield changes must be in changes in either (a) rate of photochemical reactions or (b) rate constant of excitation energy transfer among strongly fluorescent and weakly fluorescent Chl *a* molecules, or (c) rate of internal conversion. Both the P to S and the S to M changes seem to occur partially through associated microstructural changes of membrane matrix that lead to changes in (b) or (c).

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