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PARALLEL TIME COURSES OF OXYGEN EVOLUTION AND CHLOROPHYLL FLUORESCENCE

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SUMMARY

In strong light, after several minutes darkness, fluorescence induction curves for green algae exhibit the following features: a low initial level O, a first peak P_1 at about 15 msec, a minimum M_1 at about 50 msec, a high second peak P_2 lasting from about 0.2 to about 1 sec, a minimum M_2 at about 30 sec, a third peak P_3 at 60 to 120 sec, and a steady-state level S reached after several minutes. Simultaneously measured induction curves of rate of O_2 evolution indicate a maximum reached in well under 100 msec, a zero rate while fluorescence is at the high P_2 level, a first phase a_1 of accelerating rate complementary to the P_2–M_2 decline of fluorescence, and a slower second phase a_2 during which oxygen evolution rises in parallel with the M_2–P_3 fluorescence climb.

When strong light is given after a preillumination, the P_1, M_1, and P_2 fluorescence levels are substantially heightened. However, the steady-state level is unchanged, and the course of fluorescence from P_2 to S no longer passes through a minimum nor exhibits the M_2–P_3 rise. In addition, the amount of oxygen evolved in the spike is increased, and the acceleration of O_2 evolution during the a_2 phase is faster and kinetically similar to that of the a_1 phase.

The characteristics of fluorescence and O_2 induction, both after darkness and after preillumination, can be explained by assuming a slow activation of System-II units. After darkness, perhaps half of the units would be initially active and capable of fluorescence. As a result, the P_1, M_1, and P_2 levels, and the amount of O_2 in the spike, are relatively low. During the about 30th to about 120th sec of induction, the initially inactive, nonfluorescent units are slowly converted to the active form. The M_2–P_3 rise and the parallel acceleration of O_2 evolution of the a_2 phase are manifestations of this activation. After preillumination, all System-II units would be active. In consequence, the P_1, M_1 and P_2 levels are higher, the M_2–P_3 rise is abolished, and the rate of O_2 evolution rises faster and more nearly continuously with that of the a_1 phase.

The M_2–P_3 fluorescence rise occurs in wild type and System II, but not System-I mutants, and in the presence, as well as the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Furthermore, with monochromatic preilluminations, 708 nm is about three times more effective than 650 nm in maintaining the active form of System-II

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

units. These facts imply that System I sensitizes the slow activation of System-II units. In darkness, inactivation of the units occurs with a half time of about 4 min.

Altogether, three different activations appear to underly induction. One is the fast activation of System II discovered by JOLIOT and accounting for the parallel rises of fluorescence and O_2-evolving activity during the first 10–20 msec. The second is the activation of a dark step which permits regeneration of System II oxidant, and which is manifested in the complementary courses of fluorescence during P_2–M_2 and O_2 evolution during the a_1 phase. The third is the slow activation of System-II units underlying the parallel rises of fluorescence (M_2–P_2) and O_2 evolution (a_2 phase). These three activations appear to provide the basis for a general understanding of the first 2 min of photosynthetic induction.

INTRODUCTION

After several minutes darkness, photosynthesis in strong light begins slowly and reaches a steady state only after several minutes. During this induction period, complex time courses are followed by chlorophyll fluorescence and the rates of O_2 evolution and CO_2 uptake. During the first few seconds, fluorescence and electron transport follow essentially complementary courses. This relationship has been documented both during the first second when fluorescence rises to the 'P' peak and an endogenous oxidant is exhausted[1–5] and also the rate of O_2 evolution (in the O_2 spike) declines to zero[6,7], and in subsequent seconds when fluorescence falls from the P peak and the rate of O_2 evolution rises from zero[8]. The relationship of complementarity suggests that the yield of fluorescence from the pigment-trap sensitizer system is determined by photochemical quenching. Thus, one may suppose that fluorescence, internal conversion, and the photochemical reduction of an oxidant are competing deexcitation steps. The rise of fluorescence to the P peak can then be attributed to the exhaustion of the substrate oxidant, and the subsequent fall from the P peak indicates the starting up of reactions which regenerate the oxidant. Kinetic analyses of fluorescence induction[4,5] and measurements of the amount of O_2 in the spike[9] both indicate that, after several minutes darkness, the exhaustible oxidant is present in an amount of about 1 equiv per 30 moles total chlorophyll.

Some investigations[8,10–16] of fluorescence induction indicate that, after falling to a minimum 3 to 30 sec after the beginning of illumination, fluorescence sometimes rises again (the rise lasting for one to several minutes). WASSINK AND KATZ[16] and VAN DER VEEN[10] termed this rise the 'second wave'. Since rates of O_2 evolution and CO_2 uptake also generally increase during the 1st to 3rd min of illumination, it would appear that fluorescence and electron transport at least sometimes increase in parallel. However, in only a few studies have fluorescence and electron transport been simultaneously measured; thus parallelism is not well documented. One study in which both functions were studied simultaneously was that of MCALISTER AND MYERS[8]. Fig. 1 shows four of their induction curves. They found, both for wheat (Curve B) and Chlorella (Curve D) in high CO_2 concentrations, that fluorescence and CO_2 uptake rose in parallel during the 30th to 90th sec of strong illumination. However, in low CO_2 (Curves A and C), the fluorescence yield remained constant during this period. Recently, PAPAGEORGIOU[13] measured fluorescence and O_2 evolution (in different

experiments, but under similar conditions) and concluded that both functions increase in parallel after about the 3rd sec of illumination; similar results were found with *Chlorella pyrenoidosa* and *Anacystis nidulans*.

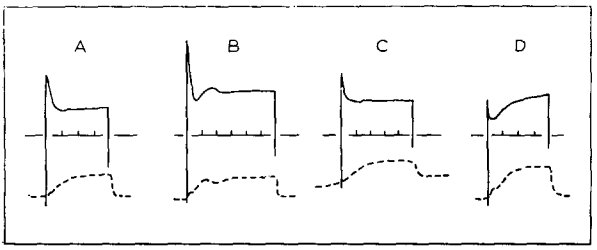


Fig. 1. Induction of chlorophyll fluorescence (upper solid curves) and rate of CO_2 incorporation (lower dashed curves) redrawn from McALISTER AND MYERS[8]. $6 \cdot 10^5$ ergs·cm^{-2}·sec^{-1} blue light. Marks define 1-min intervals. A, B, induction of wheat seedlings grown in air (0.03% CO_2); A, in 0.03% CO_2 after 20 min dark; B, in 0.12% CO_2 in air after 10 min dark; C, D, induction of Chlorella after 10 min dark; C, cells grown in 4% CO_2 in air but measured in 0.24% CO_2; D, cells grown and measured in 0.03% CO_2 in air. (Index to McALISTER AND MYERS: A, curves 1, Fig. 1; B, curves 3, Fig. 5; C, curves 2, Fig. 8; D, curves 10, Fig. 9.)

Parallel changes of fluorescence and electron transport cannot occur in a sensitizer system in which the rate constants for fluorescence, internal conversion, and photochemistry remain constant. Furthermore, no exhaustion of intermediates or activation or inactivation of enzymatic dark steps, can give rise to parallelism of fluorescence and electron transfer, if such processes simply affect regeneration of the primary oxidant. Two possible ways to explain parallelism during induction are the following. First, one can suppose that the fluorescence rate constant increases relative to those of internal conversion and photochemistry. PAPAGEORGIOU[13] and PAPAGEORGIOU AND GOVINDJEE[15] have suggested that slow conformational changes related to swelling and shrinking of chloroplasts may lead to such alteration of the rate constants. Alternatively, one may imagine that after darkness, some of the sensitizer units (*i.e.*, pigment *plus* trap) are in an inactive, non-fluorescent state. In light the inactive units would be converted to the photochemically active form, the fluorescence yield then being determined by chemical quenching. In this hypothesis, the complementarity of fluorescence and O_2 induction in the first seconds would be attributed to the fraction of the sensitizer units which remained active at the end of the dark period, while the parallel phase of induction reflects the slow activation of the initially inactive fraction. Whatever the actual mechanism, the parallelism of fluorescence and electron transport merits careful study from which we may expect to discover new properties of the sensitizer not now accounted for in the chemical quenching hypothesis.

Recent investigations of PAPAGEORGIOU and co-workers[13–15] provide some new information about the slow rise of fluorescence which parallels rising O_2 evolution. Most important, they showed that the fluorescence rise can occur in both unpoisoned and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned cells. (BANNISTER[12] also showed a slow fluorescence rise in the presence of DCMU.) This observation, along with others on the effects of uncouplers and of System-I and System-II illuminations, led PAPAGEORGIOU to conclude that the slow fluorescence rise in inhibited cells results from System-I activity and is, possibly, related to photophosphorylation.

In the studies to be reported here, simultaneous measurements of O_2 evolution and fluorescence establish parallelism beyond any doubt. Observations on wild type and mutant strains of *Chlamydomonas reinhardi*, and on the effects of different pre-illuminations confirm that System I sensitizes the slow fluorescence rise. These and other observations seem to be most easily explained by assuming that System I sensitizes an increase in the number of fluorescent, photochemically active System-II units.

MATERIALS AND METHODS

Most experiments were performed with strains of *Chlamydomonas reinhardi* obtained from Prof. R. P. LEVINE, Harvard University. We are indebted to Dr. TEICHLER-ZALLEN for maintaining the pure strains on slants at Rochester. The wild-type strain was cultured either on minimal medium[17], in 1 % CO_2 in air, about 2000 foot-candles (fluorescent lamps), at 30°, or on minimal medium *plus* 0.3 % sodium acetate, in air, about 300 foot-candles, at about 23°. Mutant strains included Ac-115 and Ac-141 lacking cytochrome *b*-559 and System-II activity[18], Ac-206 lacking cytochrome *f* and System-I activity[17], and Ac-80a lacking P700 and System-I activity[19]. The mutants were grown exactly as was wild type on minimal *plus* acetate. *C. pyrenoidosa* was cultured on the medium of LORENZEN[20], in 1 % CO_2 in air, 2000 foot-candles, at 30°. Samples were taken from liquid cultures when cell density reached about 0.5 to 1 $\mu l \cdot ml^{-1}$. An aliquot of 5 to 10 ml was centrifuged in a hematocrit, the packed cell volume measured, and the required volume of supernatant removed in order to obtain a cell density of about 5 $\mu l \cdot ml^{-1}$. A drop of the concentrated culture was then mounted on the O_2 electrode.

Rates of O_2 evolution were measured with a horizontal platinum rate electrode having an area of about 2×12 mm^2 (refs. 21, 22). The platinum surface was recessed 0.20 mm below the cellulose acetate dialyzing membrane. The electrolyte outside the membrane was minimal medium *plus* 0.1 M KCl; usually the medium was equilibrated and stirred with 2.4 % CO_2–6 % O_2 in N_2. In most experiments, the electrode was thermostated at 30°. Knowing (1) the volume of algal suspension confined over the platinum, (2) the cell density of the suspension, and (3) that about 99 % of evolved O_2 is reduced at the electrode, and, furthermore, assuming four electrons per O_2, rates of photosynthesis (in μl O_2 per μl cells per h) could be calculated from the equation $r = 48\ i/d$. Here, i is the net photocurrent in μAmperes and d is the cell density in μl/ml. Typical rates for wild type cells were usually 30–50 $\mu l \cdot \mu l^{-1} \cdot h^{-1}$, while with DCMU-poisoned wild type and System-II mutants, a photoinhibition of respiration of 1 to 2 $\mu l \cdot \mu l^{-1} \cdot h^{-1}$ was regularly observed. The platinum electrode was polarized at —0.55 V. Electrode current was either amplified by a Keithley 150 A microammeter and recorded on one channel of a two-pen potentiometric recorder, or the current was passed through a 1 to 3 kΩ resistance and the potential displayed on a high sensitivity (up to 200 $\mu V \cdot cm^{-1}$) oscilloscope (Tektronix type 502). In the latter case, the scope display was photographed.

Illumination of the sample on the electrode was accomplished with a double Köhler projection system previously described[22]. The apparatus consisted of 1000 W tungsten projection lamps, infra-red filters, electrically operated shutters, and lenses. The two beams were combined by a partially reflecting, partially transmitting

element so that both beams shared a single projection lens, and a single optical axis from the projection lens to the sample on the electrode. By turning a prism, the actinic beams could be directed through an identical optical path to a thermopile, which with a galvanometer had been calibrated against a NBS tungsten–iodine standard lamp. Strong blue actinic illumination was employed in all experiments; it was obtained by filtering the beam through (a) a 1.5-cm layer of water and a Schott KG-1 glass filter to absorb infra-red, and (b) blue Corning glass filters (two 4303 and one 4308). The tungsten lamp was operated at about 100 V, the exact setting being chosen to give a standard illumination of 5.1 $mW \cdot cm^{-2}$ on the algae or, in some of the experiments with a more reflective combining plate, 2.2 $mW \cdot cm^{-2}$. In the pre-illumination experiments, the second beam was also employed to give monochromatic light. Baird Atomic type B-2 interference filters, with suitable infra-red blocking filters, were employed to obtain 480-, 650-, or 708-nm illuminations. Calibrated neutral filters were used to obtain monochromatic illuminations too small to measure directly with the thermopile.

A third Köhler illumination system, having the platinum electrode as source, was used to collect fluorescence. The fluorescence was collected by a lens mounted a few inches above the light pipe of the electrode, and a 3 to 4 times magnified image of the electrode was focused on an appropriately shaped diaphragm. Immediately behind the diaphragm, a second lens cast an image of the first lens on the photocathode of an EMI 9558 B photomultiplier. Two Corning red cut-off filters (No. 2403, 2404) and a special Baird Atomic type B-1 680 interference filter isolated the fluorescence. In a few of the early experiments, a type B-2 696-nm filter was used instead. The photomultiplier was operated at 700 V from a regulated supply. The anode current was either amplified by a picoammeter (Keithley Model 417) and recorded on the potentiometric recorder, or was led directly to the second channel of the oscilloscope (1 MΩ input). Typical fluorescence signals (with 5.1 $mW \cdot cm^{-2}$ incident light) were about 1 μA. When white $CaCO_3$ powder was substituted for algae, the signal due to stray light was less than 1 nA.

In general, after polarizing the electrode, cells were automatically exposed to illumination cycles of 5 min 5.1 $mW \cdot cm^{-2}$–3 min dark for 1 h, or until reproducible rates of oxygen evolution and intensities of fluorescence were observed. Measurements were then begun. Poisons when used were added to the electrolyte solution; responses to DCMU and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) could be detected within a few seconds after adding poison and full inhibition was realized within 10 min. Freshly made, ethanolic stock solutions of FCCP were employed. DCMU stock solutions (in ethanol or buffer) were stored at 5°. The combined volumes of DCMU and FCCP solutions added never exceeded 2 % of the electrolyte volume.

RESULTS

Examples of the slow fluorescence rise

Fig. 2 shows fluorescence and O_2 induction of *C. reinhardi* wild type. The curves have been selected from the records of many experiments in order to illustrate variation in the importance of the slow fluorescence rise. Following a system to be described shortly, we identify the features of fluorescence induction, seen in Fig. 2, as follows: P_2, the initial peak; M_2, the minimum after 3–30 sec of illumination; P_3,

the maximum reached after 1–3 min; and S, the final steady-state value. The rise of fluorescence M_2–P_3 we term the 'slow rise' or 'third wave'. Examination of the curves in Fig. 2 shows that the M_2 and P_3 features vary both in the exact time of occurrence after the start of illumination and in amplitude relative to the initial fluorescence

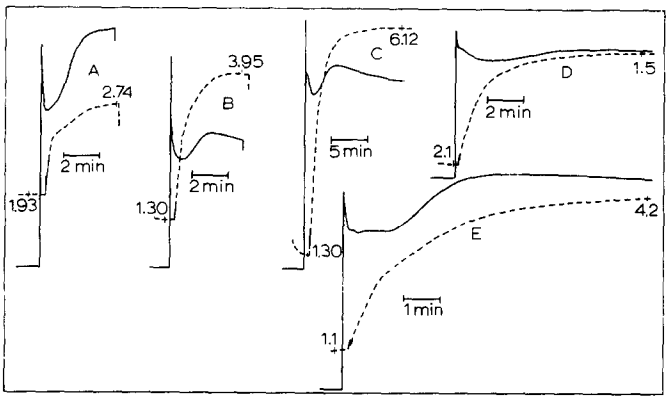


Fig. 2. Induction of fluorescence (solid curves) and rate of O_2 evolution (dashed curve) in wild type *C. reinhardi*. These examples, all recorded after 3 min dark periods, have been selected to show the greatest variation. A, B, 2.4 % CO_2–6 % O_2 in N_2, 30°, 2.4 mW·cm^{-2}. C, D, E, 3.6 % CO_2–6 % O_2 in N_2, 5.1 mW·cm^{-2}, 22–24°.

peak (P_2). Variation is also observed in the time course of fluorescence after P_3. Sometimes the P_3 level is the steady-state level. More commonly, there is a slow decline to the steady-state (S) level. Occasionally (as in B and C) the decline is comparatively rapid.

O_2 induction curves frequently display features which appear to be correlated with the third wave of fluorescence. Such a correlation is especially obvious in examples A and E of Fig. 2. In these two cases, the time course of the rising rate of O_2 evolution is obviously biphasic. In the earlier phase, O_2 evolution accelerates rapidly at the same time that fluorescence declines from P_2 to M_3. In the later phase, O_2 evolution accelerates more slowly while, concurrently, the fluorescence increases from M_2 to P_3. Usually, the biphasicness of O_2 induction curves is not as pronounced as in A and E. However, when carefully examined, most O_2 induction curves, recorded with a suitable chart speed, show signs of a discontinuity, which frequently occurs when the rate reaches about half the steady-state value. We are thus led to consider that biphasicness is a general property of O_2 induction.

In experiments with *C. reinhardi* wild type, fluorescence induction is more commonly like that of A, B, and C of Fig. 2. Only infrequently are cultures encountered in which the third wave is either very weak, or in which O_2 induction is very markedly biphasic. To what extent culture and measurement conditions may affect the appearance of the third wave has not been systematically studied. McAlister and Myers[8] observed a third wave only when CO_2 levels were higher during measurements than during culture. The presence of the third wave in our experiments is consistent with their finding. Our cultures were grown either in minimal medium *plus* 1 % CO_2 in air, or in minimal *plus* 0.2 % acetate in air, but measurements were made in the presence of 2–3 % CO_2. However, with Chlorella grown in 1 % CO_2 and measured in 3 % CO_2, the third wave is usually (but not always) weak. Interestingly,

with Chlorella grown synchronously in cycles of 11.5 h light–12.5 h dark, WANG[23] in our laboratory, has shown that the third wave is most prominent 3–5 h after the beginning of the light period and is absent during the dark period.

Denomination of fluorescence and O_2 induction features

In the following section we consider the relation between the slow fluorescence rise and other earlier transients of fluorescence and O_2 induction. For this discussion, a system of identifying the various transient features is needed. Heretofore systems of identifying induction features have either been hard to remember or else have dealt with only a limited portion of induction. We find convenient the following denominations of the features of fluorescence induction: O, the initial level (hidden by slow shutter opening in our experiments). P_1, the first peak; commonly seen under anaerobic conditions and sometimes under aerobic conditions (P_1 is the 'oscillation' of DELOSME[24], '1' of MUNDAY[14,25], and '2' of KAUTSKY, APPEL AND AMANN[1]). M_1, first minimum ('D' of MUNDAY, '4' of KAUTSKY, APPEL AND AMANN,' O' of LAVOREL[26]). Under aerobic conditions, P_1 and M_1 often merge and appear as a single shoulder or plateau. P_2, second peak ('P' of LAVOREL, MUNDAY and others; '6' of KAUTSKY, APPEL AND AMANN). M_2, second minimum occurring after 3–30 sec of illumination; sometimes taken as the steady-state level in studies restricted to the fast transients. The P_2–M_2 decline is sometimes complex and occasionally a shoulder or even an additional peak is observed. (See Curve B, Fig. 4 and Curve D, Fig. 5.) P_3, third peak; reached typically in the 2nd or 3rd min. The M_2–P_3 rise was called the 'second wave' by WASSINK AND KATZ[16], VAN DER VEEN[10], and PAPAGEORGIOU[13]. We prefer 'third wave' since two preceding waves (O–P_1 and M_1–P_2) occur. S, the final steady-state level, which may take 10 min to reach.

The denominations we employ for the induction features seen in time courses of rate of O_2 evolution are: sp, the maximum rate during the O_2 spike. With high light, sp is registered with a rate electrode at about 100 msec. Since diffusion from the site of O_2 production within cells to the platinum surface where reduction occurs undoubtedly causes a lag in registration, we suppose that sp actually occurs substantially earlier (see JOLIOT[27]). The sp spike apparently corresponds to the 'pre-a' or 'a_1' transient described by VIDAVER[28]. z, the minimum rate following the spike. a_1, a first period of accelerating rate of O_2 evolution typically ending at about 30 sec and concurrent with the P_2–M_2 fluorescence decline. Apparently, the 'a-gush' of VIDAVER[28] is an additional transient which sometimes appears superimposed on the a_1 rise. In the experiments described in this paper, VIDAVER's 'a'-transient was not seen. a_2, a second period of acceleration concurrent with the M_2–P_3 fluorescence rise, and ending in the steady state. f, final steady-state rate.

Relation of the third wave of fluorescence and the a_2 phase of O_2 evolution to earlier features of induction

In order to establish these relationships, oscilloscopic and recorder data were replotted on a log time base, so that all features of fluorescence and O_2 induction from about 15 msec to 200 sec could be viewed at one time. Results of two experiments with Chlorella appear in Figs. 3 and 4, of an experiment with Chlamydomonas wild type in Fig. 5. Since we wished to examine fluorescence and O_2 time courses for complementarity and parallelism and also to see the effects of other parameters (*e.g.*,

illumination in Fig. 3, O_2 tension in Fig. 4, and pre-illumination in Fig. 5), four or more curves have had to be plotted in each figure.

Fig. 3 shows induction curves for Chlorella after 81 sec darkness, in the presence of 6 % O_2, in 1/12 ×, 1/3 ×, and 1 × saturating light. Fluorescence (actually relative yield of fluorescence) induction is shown at all three illuminations, O_2 induction in the two higher illuminations. In 1 × saturating light fluorescence (Curve A) displays obvious P_1–M_1, P_2, M_2, P_3 and S levels. Fluorescence in 1/3 × illumination (Curve B) shows these same features but the P_1–M_1 level is lower, the rise to P_2 is later, and the M_2 and P_3 features occur somewhat earlier. In the lowest light, fluorescence (Curve C) is essentially featureless. Curves A′ and B′ show rate of O_2 evolution measured simultaneously with the fluorescence of Curves A and B, respectively. In A′, there is an O_2 spike sp at about 100 msec, a minimum z at about 2 sec, and and readily distinguished a_1 and a_2 periods of accelerating rate. In Curve B′, obtained with 1/3 × illumination, the O_2 spike is lower and occurs later (at about 300 msec); the a_1 and a_2 phases, like the related features of fluorescence, occur somewhat earlier than in 1 × illumination.

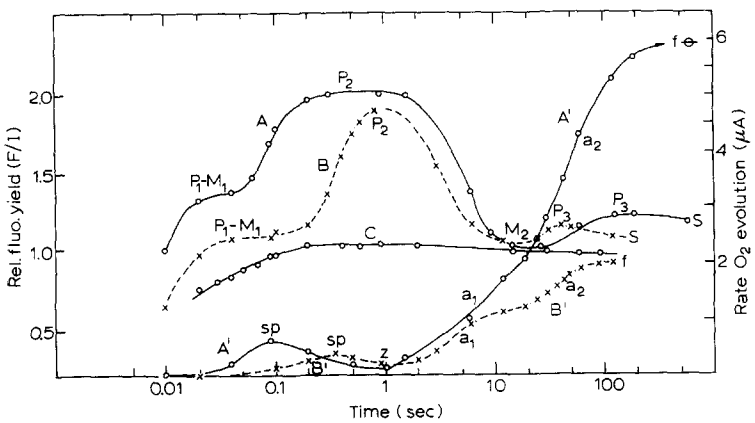


Fig. 3. Induction of fluorescence and rate of O_2 evolution in *C. pyrenoidosa*. 21 Feb. 1967. After 81 sec dark, 3.6 % CO_2–6 % O_2 in N_2, 25°. A, A′, 6.8 mW·cm^{-2} incident blue illumination; B, B′, 0.8 mW·cm^{-2}; C, 0.19 mW·cm^{-2}. Fluorescence induction features, P_1, M_1, P_2, M_2, P_3 and S and O_2 induction features sp, z, a_1, a_2 and f are identified in the A, B and A′, B′ curves.

In Fig. 4 one sees the effect of anaerobic conditions on fluorescence and O_2 induction, in Chlorella in saturating light. Curves A and A′ show fluorescence and O_2, respectively, with 30 % O_2. Fluorescence induction (Curve A) shows distinct P_1–M_1, P_2, M_2, and P_3 levels; however the amplitude of the M_2–P_3 rise is rather small in this experiment. O_2 induction (Curve A′) displays especially obvious a_1 and a_2 phases which occur simultaneously with the respective P_2–M_2 and M_2–P_3 phases of fluorescence. In comparison with aerobic induction, anaerobic induction shows the following differences. With regard to fluorescence (Curve B), the P_1 and M_1 levels are much elevated, the P_2 level is relatively unaffected, and the P_2–M_2 decline is greatly slowed. An M_2 minimum and a P_3 maximum remain evident. With regard to O_2 induction (Curve B′), the spike sp is much diminished, the a_1 phase is essentially abolished, and the entirety of the acceleration of O_2 evolution follows a_2-phase kinetics. Evidently

anaerobic conditions greatly delay the process governing the P_2–M_2 phase, but have little effect on the mechanism underlying the M_2–P_3 phase.

In Fig. 5 simultaneously measured fluorescence and O_2 induction of Chlamydomonas wild type, in two different illuminations, in 6% O_2, are seen. Curves A,A′ and

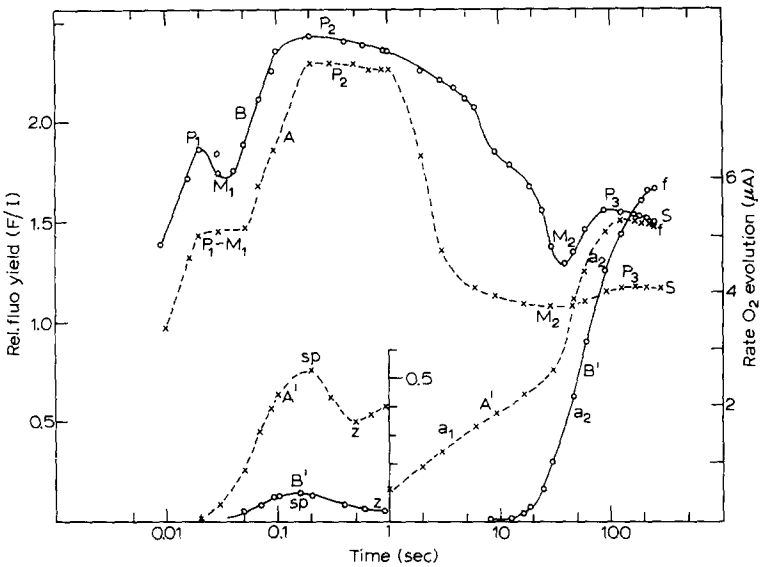


Fig. 4. Fluorescence and O_2 induction in *C. pyrenoidosa*. 22 March 1967. After 1.5 min dark, 2.4% CO_2, 25°. A, A′, 30% O_2, 6.8 mW·cm^{-2}; B, B′, 0% O_2, 6.8 mW·cm^{-2}. O_2 induction prior to 1 sec is plotted on an expanded scale.

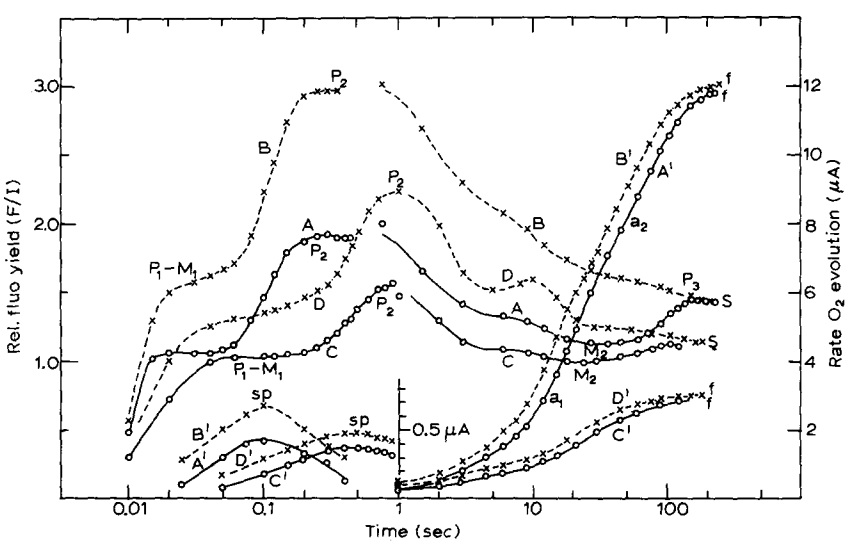


Fig. 5. Effect of 710 nm preillumination on fluorescence and O_2 induction in wild type *C. reinhardi*. 22 March 1968. After 3 min of darkness or preillumination. 2.4% CO_2–6% O_2 in N_2, 30°, cell density, 9 μl ml^{-1}. A, A′, 5.1 mW·cm^{-2}, after darkness. B, B′, 5.1 mW·cm^{-2}, after preillumination. C, C′, 0.62 mW·cm^{-2}, after darkness. D, D′, 0.62 mW·cm^{-2}, after preillumination. Preillumination (3.1 mW·cm^{-2} of 710 nm) gave steady-state rate of O_2 evolution of about 0.1 μA. O_2 evolution prior to 1 sec is plotted on an expanded scale.

C,C′ were obtained after 3 min darkness. Curves B,B′ and D,D′, obtained after pre-illumination, will be discussed in the next section. With Chlamydomonas somewhat higher illumination was required to saturate steady-state O_2 evolution than was the case with Chlorella. In this experiment the higher illumination (5.1 $mW \cdot cm^{-2}$) was only about 80% saturating, the lower illumination was about 20% saturating.

In Fig. 5 curves A,A′ (high illumination) and C,C′ (low illumination) show the same relations seen in Fig. 3 for Chlorella. In the fluorescence Curves A and C, the P_1–M_1, P_2, M_2, P_3 and S levels were clearly seen. The M_2–P_3 rise is particularly strong at the high illumination (Curve A). Unlike Chlorella, both high and low illumination gave identical P_1–M_1 levels. As with Chlorella the O_2 spike and the correlated M_1–P_2 rise occur later with the lower illumination. In Curve A′ (O_2 induction in high light) the a_1 and a_2 phases of O_2 induction are relatively poorly resolved. However, careful examination of the curve shows a discontinuity at about 30 sec (the same time as the M_2 fluorescence minimum) which we believe demarcates the two phases. Two phases are evident in O_2 induction in the lower illumination (Curve C′).

From the results of Figs. 3, 4, and 5, a number of conclusions can be drawn.

(i) If it is accepted that the actual maximum rate of O_2 evolution during the spike precedes the recorded maximum sp, then the M_1–P_2 rise and the P_2–M_2 decline of fluorescence can be regarded as essentially complementary to the sp–z decline and the a_1-phase rise of O_2 induction, respectively. This confirms previous work[1–8] and allows the fluorescence yield changes to be attributed to chemical quenching. Presumably oxidants are exhausted during M_1–P_2 and then regenerated (after activating an enzymatic dark step) during P_2–M_2. Consistent with this view are the observations that the time to reach P_2 is longer in low light (when exhaustion of oxidant is more slowly achieved) and shorter under anaerobiosis (when presumably some of the oxidant is initially in the reduced state).

(ii) The data of the figures provide additional evidence of parallelism of the M_2–P_3 fluorescence rise and the a_2 phase of accelerating O_2 evolution. Typically, the discontinuity in O_2 evolution which demarcates the a_1 and a_2 phases occurs when the rate of evolution is about half the final steady-state value.

(iii) The P_1–M_1 and M_2 levels of fluorescence appear to define a lower limit of fluorescence yield. In Fig. 3, the same low yield of fluorescence pertains for M_2 in the highest illumination, for P_1–M_1 and M_2 in the intermediate illumination, and for the entirety of induction in the lowest illumination. In the next section we will show that the M_2–P_3 rise is associated with a rise in this lower limit of yield.

(iv) In strong light, the Curves A in the three figures all show that the fluorescence yield remains constant at the P_2 level from about 200 msec to about 1 sec. This relatively long period of constancy is consistent with a static condition, not a dynamic transient one. We believe that the P_2 level in strong light is an upper limit corresponding to complete exhaustion of oxidants. The regeneration of the oxidants (reflected in the P_2–M_2 decline) apparently does not begin until some time after complete exhaustion.

(v) That the P_2 level in strong light is a fixed upper limit is also indicated by two other observations. One is that the P_2 level is independent of illumination over a considerable range of high illumination. For example, the P_2 peak in intermediate illumination (Curve B, Fig. 3) is at almost the same level as that in saturating light (Curve A, Fig. 3). The other observation is that approximately the same P_2 level is

reached under anaerobic and aerobic conditions (Curves A, B, Fig. 4), even though the P_1–M_1 level, the time to reach P_2, and the time of the onset of the P_2–M_2 decline are all noticeably dependent on oxygen tension. It may be further noted (Curves C of Figs. 4 and 5) that in illumination low enough so that the P_2 level does not reach the upper limit, then P_2 peak is transitory. Apparently when exhaustion of oxidant is not completed by the time regeneration begins, then the P_2 maximum is dynamically determined.

(vi) Evidence that different mechanisms govern the P_2–M_2 and M_2–P_3 phases of induction can be seen not only in the fact that O_2 induction is complementary in the first and parallel in the second, but also in the much stronger effect of anaerobiosis on the P_2–M_2 phase. As Fig. 4 shows, anaerobiosis greatly slows the decline of fluorescence from P_2, at the same time eliminating the a_1 phase in O_2 evolution. In contrast, the times of the M_2–P_3 fluorescence rise and of the a_2 phase of O_2 induction differ little under anaerobic and aerobic conditions.

Effects of preillumination

If, after the completion of the M_2–P_3 rise in a period of strong illumination, weak preillumination is given in place of darkness, then induction in a second period of strong light differs from that after darkness. In Fig. 5, Curves B,B′ (strong illumination) and D,D′ (moderate illumination) show fluorescence and O_2 induction after 710 nm preillumination (3.1 $mW \cdot cm^{-2}$). (Preilluminations with 650 and 480 nm give similar results.) For the case of strong illumination, in comparison with induction after darkness (Curve A), fluorescence induction after preillumination (Curve B) shows (i) P_1–M_1 and P_2 levels elevated about 50 %, (ii) no changes in the times of the P_1–M_1 shoulder, M_1–P_2 rise, and prolonged P_2 level, (iii) a decline from P_2 not passing through a minimum M_2 but finally ending at the same P_3 and S levels of induction after darkness.

Preillumination also generally causes the disappearance of the a_2 phase in O_2

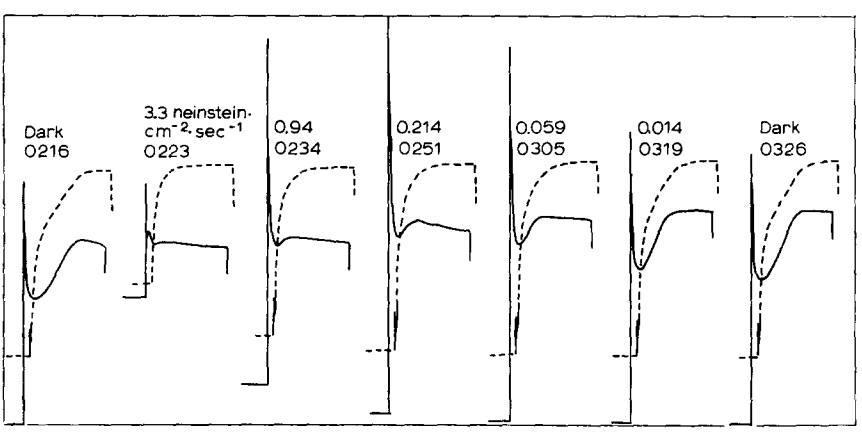


Fig. 6. Effects of 650 nm preillumination on fluorescence and O_2 induction in half-saturating blue light. 5 Oct. 1967. *C. reinhardi* wild type. Measured in 2.4 % CO_2–6 % O_2 in N_2 at 30°. Illumination cycle: 4 min broad blue band (2.4 $mW \cdot cm^{-2}$, 9.5 $nEinsteins \cdot cm^{-2} \cdot sec^{-1}$), 3 min dark, 4 min broad blue band, 3 min 650 nm preillumination. Preillumination intensities and times at beginning of induction are indicated in figure. A gradual increase (about 15 %) in the steady-state fluorescence level occurred in the 70 min during which these curves were recorded.

induction. In the experiment of Fig. 5, this effect is poorly shown, the a_2 phase being relatively obscure in induction after darkness (Curve A'). An indication of this effect is the significantly faster rise of O_2 evolution after preillumination. Noting that log of time is plotted on the abscissa, the two nearly parallel O_2 curves (A', B' of Fig. 5) are seen to be separated by only about 3 sec early in the a_1 phase of the rise, but by about 30 sec toward the end of the a_2 phase.

The elimination of the a_2 phase is much more obvious in Fig. 6 which shows portions of the record of an experiment in which wild type cells were subjected to repeated cycles consisting of 3 min darkness, 5 min strong blue light, 3 min monochromatic (650 nm) preillumination, and 5 min strong blue light. The 5 min periods were long enough so that O_2 evolution and fluorescence attained essentially steady-state levels. After darkness, O_2 induction clearly shows an a_1 phase of more rapid acceleration followed by an a_2 phase of more slowly accelerating evolution. Fluorescence induction after darkness displays a pronounced M_2–P_3 rise associated with the a_2 phase of O_2 induction. With increasing intensity of preillumination, the a_2 phase is gradually eliminated and the level of the M_2 minimum rises. With strong enough preillumination, the a_2 phase and the M_2 minimum are abolished entirely. Fig. 6 also shows that the amplitude of the P_2 fluorescence maximum is affected by preillumination: the P_2 level at first increases markedly with increasing preillumination intensity (as was shown in Fig. 5), then declines somewhat at the highest intensities.

Fig. 6 shows that comparatively weak preillumination is required for the effects on P_2, a_2, and the third wave. Maximal effects were obtained with 0.94 nEinstein$\cdot cm^{-2}\cdot sec^{-1}$ of 650 nm preillumination. Comparison of the steady rates of O_2 evolution in the period of preillumination which preceded, and in the dark period which followed, the high light period shows that 0.94 nEinstein gave only about 10 % of the rate of O_2 evolution as did the high light. Since the high light itself produced only about 50 % of the light-saturated rate, we may conclude that a preillumination giving about 5 % of light-saturated O_2 evolution suffices to maximize both the M_2 and P_2 levels and to abolish the third wave of fluorescence and the a_2 phase of O_2 induction.

It should be emphasized that the effects of preillumination in these experiments was to maintain an activation accomplished in the preceding period of strong light. Investigations in progress indicated that considerably stronger preillumination is needed to carry out the activation in dark-adapted cells.

The preillumination experiments permit the following conclusions. First, the results confirm that different mechanisms underlie the complementary and parallel phases of induction. This is evident in that preillumination eliminates the parallel phase of induction (M_2–P_3 and a_2) but not the complementary phases (P_1–M_1–P_2 and P_2–M_2).

Secondly, the fact that the times of the P_1–M_1 shoulder, the M_1–P_2 rise, the prolonged P_2 level, and the onset of the decline from P_2 are the same after preillumination and darkness indicates that the nature and kinetics of the processes underlying the early complementary phases of induction are not changed by preillumination and are independent of the changes occurring in the M_2–P_3 phase.

Thirdly, the facts that the rate of O_2 evolution approximately doubles during the a_2 phase, and that after preillumination O_2 evolution rises to steady state faster (and with a_1-phase kinetics) indicate that the M_2–P_3 phase reflects an increase in

photosynthetic capacity, and furthermore, that this increased capacity is maintained by preillumination.

Fourthly, the fact that preillumination markedly elevates both the lower (P_1–M_1) and upper (P_2) limits of fluorescence yield, along with the parallelism of fluorescence and O_2 inductions in the M_2–P_3 phase, is most simply accounted for by supposing that the increase of photosynthetic capacity comes about from an activation of System-II units. This hypothesis will be considered further in the DISCUSSION.

Third wave in mutants; effects of DCMU and FCCP

Previously, BANNISTER[12] reported induction curves for wild type cells poisoned with DCMU and DCMU *plus* FCCP. It was shown that, in the presence of DCMU (10 μM), fluorescence, after rising to the high P_2 level in the first second, does not fall thereafter to a lower M_2 level, but rather undergoes a second slower rise lasting 2–3 min. At the same time, DCMU eliminated the O_2 spike and most of the steady-state O_2 evolution. However, a very small apparent evolution (1–2 μl O_2 per μl cells per h), saturated by about 200 $\mu W \cdot cm^{-2}$, persisted in the presence of DCMU. We believe this small apparent evolution actually results from a photoinhibition of respiration sensitized by System I. HOCH, OWENS AND KOK[29] have previously described a photoinhibition of respiration in other algae and shown that the effect is far-red sensitized, DCMU-resistant, and saturated by weak light.

Figs. 7–10 show induction of the four mutant strains of *C. reinhardi*, in the absence of poisons, after addition of DCMU, and after addition of FCCP. Induction in the absence of poisons is as follows. With the System-II mutants Ac-115 and Ac-141, which are deficient in cytochrome *b*-559 and System-II activity[18], fluorescence induction is similar to that of DCMU-poisoned wild type. There is no P_2–M_2 fluorescence decline, but there is a several minute-long fluorescence rise. In contrast, with the System-I mutants Ac-206 lacking cytochrome *f* (ref. 17) and Ac-80a lacking

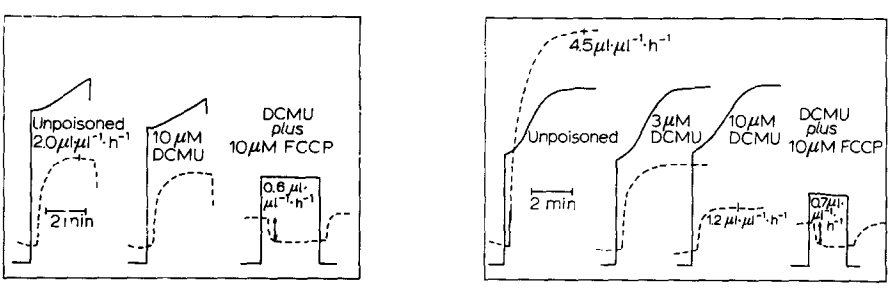


Fig. 7. Induction in *C. reinhardi* mutant Ac-115. 13 May 1967. Transients in 5.1 $mW \cdot cm^{-2}$ broad blue, after 2 min dark periods. 2.4 % CO_2–6 % O_2 in N_2, 30°. No O_2 evolution was ever observed with this strain. Photoinhibition of respiration saturated at about 0.15 $mW \cdot cm^{-2}$, and a slow rise of fluorescence yield was always observed.

Fig. 8. Induction in *C. reinhardi* mutant Ac-141. 22 March 1967. Transients in 5.1 $mW \cdot cm^{-2}$ broad blue. After 2 min dark periods. 2.4 % CO_2–6 % O_2 in N_2, 30°. In all experiments with Ac-141, a small capacity (about 10 % of wild type) for photosynthesis has been observed. Evidence of this is seen in the presence of a weak O_2 spike, a steady-state O_2 production 2–4 times larger than the photoinhibition of respiration, the inhibition by DCMU, and the saturation of O_2 production at illuminations typical of saturation in wild type. With 10 μM DCMU, only the photoinhibition of respiration occurs as indicated by saturation at about 0.15 $mW \cdot cm^{-2}$. A slow fluorescence yield rise also persists. FCCP (10 μM) added to the DCMU-poisoned cells reduces the fluorescence yield and leads to a small photoconsumption of O_2.

P700 (ref. 19), the slow fluorescence rise is absent. Differences in O_2 induction are also evident. With Ac-115, no O_2 spike is observed, but there is a small apparent O_2 evolution of about 1–2 $\mu l \cdot \mu l^{-1} \cdot h^{-1}$ which is saturated at low light and which can be reasonably attributed to a photoinhibition of respiration. With Ac-141, a very small O_2 spike is observed along with an apparent steady-state evolution of 4–5 $\mu l \cdot \mu l^{-1} \cdot h^{-1}$.

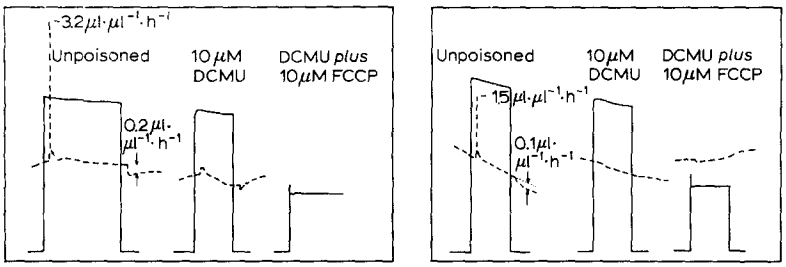


Fig. 9. Induction in *C. reinhardi* Ac-206 lacking cytochrome *f*. 10 May 1967. Transients in 5.1 $mW \cdot cm^{-2}$ broad blue after 2 min dark periods. 2.4 % CO_2 in N_2, 30°. A normal O_2 spike is seen. However, there is no steady-state O_2 evolution, no significant photoinhibition of respiration, and no slow increase of fluorescence yield. DCMU eliminates the O_2 spike but has no other effect. FCCP lowers the fluorescence yield; the O_2 curve was 'noisy' but showed a small photoconsumption in light.

Fig. 10. Induction in *C. reinhardi* Ac-80A (lacking P700). 11 May 1967. Conditions and results same as in preceding figure.

Light curves of O_2 evolution show two components: one of (about 1–2 $\mu l \cdot \mu l^{-1} \cdot h^{-1}$) which is saturated in low light and can be attributed to photoinhibition of respiration, and a second (of 2–3 $\mu l \cdot \mu l^{-1} \cdot h^{-1}$) which is only saturated at high light. The latter component and the small O_2 spike indicate that Ac-141 retains a small capacity for normal photosynthetic O_2 evolution. With the System-I mutants, O_2 induction is quite different. Both Ac-206 and Ac-80a exhibit normal O_2 spikes, but no significant photoinhibition of respiration nor O_2 evolution.

After addition of DCMU, the transients appear as follows. With the System-II mutants, the slow fluorescence rise and the photoinhibition of respiration persist unaltered. As expected, the small photosynthetic activity of Ac-141 is abolished. With the System-I mutants, DCMU eliminates the O_2 spike but has no other effect; just as in the absence of DCMU, there is no slow fluorescence rise. Figs. 7–10 show that the fluorescence yield of all the strains is very markedly diminished when FCCP (10 μM) is added to the already DCMU-poisoned cells. Thus the effect of FCCP, previously reported for wild type[12], occurs also with System-I and System-II mutants.

The fact that the slow rise of fluorescence, seen in wild type and System-II mutants, with or without DCMU, all occur in the same period of induction—*i.e.*, starting some tens of seconds after the start of illumination and continuing for about 1 to 3 min, suggests a common origin in all cases. One may conclude that the third wave fluorescence rise, originally defined for unpoisoned wild type, also occurs in the presence of DCMU and in mutants which retain System-I activity. Evidently System-II activity is not required for the third wave.

The figures indicate furthermore that the process underlying the slow rise of fluorescence takes place whether the oxidants of System II are in the oxidized or reduced state. In unpoisoned wild type, in which rapid reoxidation of the reduced

oxidant presumably is the cause of the P_2–M_2 fall, the third wave begins at the low M_2 level corresponding to oxidant mainly oxidized. In contrast, in DCMU-poisoned wild type, the third wave rise commences at the high P_2 level, corresponding to the oxidant mainly (or completely) reduced. With the mutants, we presume that the initial fluorescence yield (after a few seconds of illumination) is also high corresponding to complete reduction of oxidant. (This presumption is supported by the fact that FCCP markedly lowers the fluorescence of mutants just as it does the high fluorescence in DCMU-poisoned wild type.) We conclude from these observations that the process causing the third wave rise is at least qualitatively independent of the redox state of System-II oxidants.

Finally, the low level to which fluorescence falls in the presence of FCCP suggests that both the chemical quenching and third wave mechanisms of fluorescence yield increase are abolished by FCCP.

Dark time for maximum third wave amplitude

With wild type cells, the M_2 fluorescence minimum appears in induction curves only after dark periods longer than about 30 sec. As the dark period is lengthened up to at least 4–5 min, the level of the minimum deepens and the amplitude of the third wave increases. Such behavior has already been documented[8,12]. With the System-II mutant Ac-115 (Fig. 11), in which the P_2–M_2 fall of fluorescence is absent, one finds that the longer the preceding dark interval (up to about 10 min), the lower the initial fluorescence level and the larger the amplitude of the third wave rise. An identical dependence of initial fluorescence yield and third wave amplitude was also observed with wild type cells poisoned with DCMU. Evidently, in all cases the high fluorescence yield condition developed during the third wave decays slowly in darkness, and the M_2 level in wild type and the initial levels in DCMU-poisoned wild type and System-II mutants are determined by the extent of the dark decay. The half-

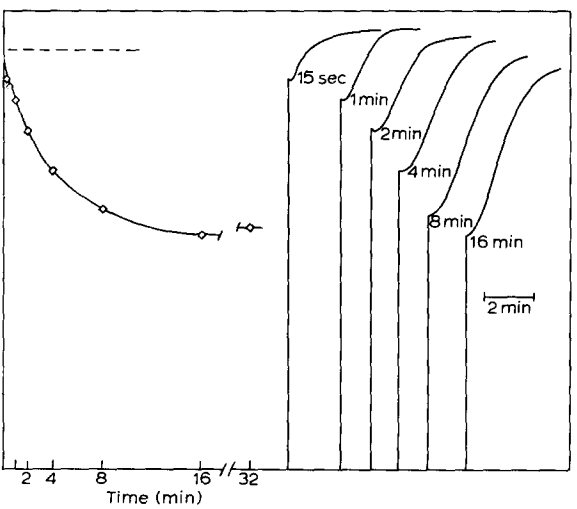


Fig. 11. Initial fluorescence yield as a function of preceding dark time. 30 Nov. 1967. Ac-115, 2.4 % CO_2–4.3 % O_2 in N_2, 30°. Periods of illumination (4 min 2.38 mW·cm^{-2} broad blue) separated by dark intervals lasting 15 sec to 32 min. At the right are fluorescence induction curves, at the left a plot of initial fluorescence intensity against dark time.

time of dark decay appears to be about 4 min; however, data are too incomplete to prove that the decay times are exactly identical in all cases.

The slow dark decay, which leads to lower M_2 levels in wild type and to lower initial levels in DCMU-poisoned cells and in System-II mutants can be distinguished from another faster dark reaction which affects the P_2 level of unpoisoned cells. When measured after varying dark times, the P_2 level of unpoisoned wild type is found to rise to a maximum as dark times increase to about 30 sec, then to decline gradually as dark time increases to several minutes (refs. 8, 12). The faster dark process, we think, reflects the inactivation of a dark step which regenerates System-II oxidants. When the dark period is less than 30 sec, inactivation is incomplete and the maximum P_2 level (corresponding to complete exhaustion of oxidants) is not attained. The much slower decline of P_2 with dark times increasing beyond 30 sec, we think, is attributable to the same slow dark process which lowers the M_2 level of wild type and regenerates the capacity for the third wave.

Spectral sensitization of the slow fluorescence rise

The evident correlation (in the mutant experiments) between the occurrence of the third wave and the presence of an active System I made it desirable to see if the process underlying the third wave rise is sensitized more efficiently by far-red light. Because the process appeared to be light-limited at illuminations too low for convenient measurement of fluorescence, we did not attempt to measure directly the time course of third wave in weak monochromatic light. Instead, we determined the efficiency with which different monochromatic preilluminations diminished the third wave in strong light.

As in the preillumination experiment of Fig. 6, cells were subjected to automatically repeated cycles, each cycle consisting of 3 min darkness, 5 min strong blue light, 3 min weak monochromatic preillumination, and 5 min strong blue light. The intensity of preillumination was varied over an approx. 5000-fold range using calibrated neutral filters. For the upper portion of this range, the steady-state O_2 evolution maintained by preillumination could be measured easily, and it was possible to plot light curves of O_2 evolution as a function of the intensity of preillumination. (Actually plotted was the ratio of the O_2 evolution in preillumination to that in the constant high light; in this way any gradual change in O_2 evolving capacity during the several hour-long experiment was corrected for.)

In the lower range of preilluminations, the elevation of the M_2 level (in the induction curves in high light) was seen as the intensity of preillumination increased. As a quantitative measure of the extent to which preillumination maintained the activated state, we computed the 'fluorescence loss'

$$\text{F.L.} = \frac{(f_d - i_d) - (f_l - i_l)}{f_d - i_d}$$

Here f_d and f_l are the final steady-state levels of fluorescence in high light, after darkness and preillumination, respectively. Similarly, i_d and i_l are the M_2 levels of fluorescence induction in high light, after darkness and preillumination, respectively. Essentially, F.L. measures the extent to which preillumination dimishes the amplitude of the third wave; as the preillumination intensity increased, so F.L. increased. Since the loss of amplitude is expressed relative to the amplitude after darkness, any

slow change of fluorescence during the experiment is corrected for. It was found that, within experimental error, F.L. was proportional to preillumination intensity in the range 0 % < F.L. < 70 %. After the strongest preillumination, the M_2 level (i_1) was sometimes slightly higher than the steady-state level (f_1); in these cases, F.L. is somewhat greater than 100 %. This property does not appear to affect the usefulness of F.L. as a quantitative measure of the extent of activation maintained by preillumination.

In each experiment, light curves of fluorescence loss and relative O_2 evolution were measured with 480-, 650- and 708-nm preilluminations. The curves were plotted on log–log paper. Straight lines of 45° slope (all curves which are linear and pass through the origin in a rectilinear plot are straight and have a 45° slope in a log–log plot) were drawn and found to be 'good fits' considering the relatively large experimental errors. Using the lines drawn, the preillumination ($i_\lambda^{O_2}$) which gave a relative O_2 evolution of 0.5 (actually about 25 % of the light-saturated rate) and the preillumination (i_λ^F) giving a fluorescence loss of 0.5 were read from the graph. The efficiency (R_λ) of light of wavelength λ, relative to that of 650 nm, was calculated as

$$R_\lambda = \frac{i_\lambda^{O_2}/i_\lambda^F}{i_{650\,nm}^{O_2}/i_{650\,nm}^F}$$

Basically, R_λ compares wavelengths λ and 650 nm in their relative efficiencies in sensitizing fluorescence loss and O_2 evolution. Values in excess of unity indicate that, compared with 650 nm, wavelength λ sensitizes fluorescence loss more effectively than O_2 evolution. If the fluorescence loss is sensitized primarily by System I, and if 650 and 480 nm are both primarily absorbed by System II, we anticipate that, $R_{480} = R_{650} < R_{708}$.

Altogether seven experiments were performed, four with unpoisoned wild type, one with DCMU-poisoned wild type, and two with the System-II mutant Ac-115. The illumination curves for one of the experiments with wild type are shown in

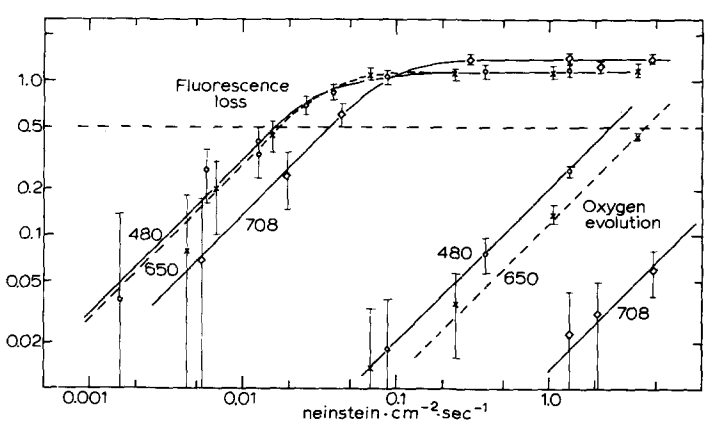


Fig. 12. Log–log plot of fluorescence loss and rate of O_2 evolution as functions of the intensity of monochromatic preillumination. 27 July 1967. *C. reinhardi* wild type. Measurements in 2.4 % CO_2–6 % O_2 in N_2, 30°. Illumination cycle (4 min 2.2 mW·cm^{-2} or about 8.8 nEinsteins·cm^{-2}·sec^{-1}, broad-band blue, 3 min dark, 4 min broad blue, 3 min monochromatic preillumination). Relative O_2 evolution of 0.5 corresponds to about 25 % of the light-saturated rate. Bars show estimated maximum possible errors.

TABLE I

ILLUMINATIONS (i_λ), ILLUMINATION RATIOS, AND RELATIVE EFFICIENCIES (R_λ) WITH WHICH 480, 650 AND 708 nm SENSITIZE THE LOSS OF THE SLOW FLUORESCENCE TRANSIENT

All illuminations in nEinsteins·cm^{-2}·sec^{-1}.

Expt.:	*26 Jul*	*27 Jul*	*5 Oct*	*2 Nov*	*2 Nov*	*8 Oct*	*17 Nov*	*Average*	*Average*
Cells:	*Wild type*	*Wild type*	*Wild type*	*Wild type*	*Wild type + DCMU*	*Ac-115*	*Ac-115*	*Wild type*	*Wild type + DCMU and Ac-115*
$i^{O_2}_{650nm}$	4.0	4.2	4.1	3.4	—	—	—	3.9	—
i^{F}_{650nm}	0.0085	0.018	0.046	0.015	0.027	0.096	0.038	**	**
$i^{O_2}_{480nm}$	2.9	2.5	3.0	2.5	—	—	—	2.7	—
i^{F}_{480nm}	0.011	0.017	0.056	0.017	0.025	0.089	0.038	**	**
$i^{O_2}_{708nm}$	33.0	40.0	37.0	16.0	—	—	—	37.0*	—
i^{F}_{708nm}	0.033	0.038	0.013	0.047	0.069	0.26	0.13	**	**
$i^{O_2}_{480nm}/i^{O_2}_{650nm}$	0.72	0.60	0.72	0.72	—	—	—	0.70	—
$i^{F}_{480nm}/i^{F}_{650nm}$	1.3	1.1	1.2	1.1	0.94	0.92	0.99	1.2	0.94
$i^{O_2}_{708nm}/i^{O_2}_{650nm}$	8.4	9.8	9.1	4.5	—	—	—	9.1*	—
$i^{F}_{708nm}/i^{F}_{650nm}$	3.9	2.5	2.7	3.2	2.6	2.7	3.4	3.1	2.9
R_{650nm}	1.0	1.0	1.0	1.0	—	—	—	1.0	—
R_{480nm}	0.56	0.57	0.60	0.66	—	—	—	0.60	—
R_{708nm}	2.2	3.9	3.3	1.4	—	—	—	3.1*	—

* Discrepant datum of 2 Nov not included in average.

** Values of i^F_λ vary widely in different experiments; thus average is without meaning.

Fig. 12. In Table I, values of $i_\lambda^{O_2}$, i_λ^F, and R_λ are presented for all of the experiments. In the cases of DCMU-poisoned wild type and Ac-115, there is no O_2 evolution and R_λ cannot be computed. For comparison of the wild type and DCMU and mutant results, values of various illumination ratios are also tabulated.

Examination of Table I leads to the following conclusions:

(i) For each wavelength, values of $i_\lambda^{O_2}$ are reproducible among the four experiments with wild type cells. Moreover, the relative values are reasonable: only 0.7 as many of the more strongly absorbed 480-nm quanta are required as of 650 nm, while the very weakly absorbed 708-nm quanta are needed in nine times the number.

(ii) The illuminations i_λ^F for a fluorescence loss of 0.5 are much more variable among the different experiments. However, very little variation occurs in the ratios $i_{480\,nm}^F/i_{650\,nm}^F$ and $i_{708\,nm}^F/i_{650\,nm}^F$. Evidently, the same relative (but not absolute) illuminations of the three wavelengths produced equal fluorescence losses in the different experiments.

(iii) The same relative 708- and 650-nm illuminations produced identical fluorescence losses in cells with and without O_2 evolving ability. A slightly smaller relative 480 nm illumination sufficed with cells not evolving O_2 than with cells which did evolve O_2.

(iv) In general, i_λ^F values are 0.01 to 0.001 of $i_\lambda^{O_2}$ values. For a fluorescence loss of 0.5, only 1/400–1/4000 of a photosynthesis-saturating illumination is needed.

(v) The relative efficiency ($R_{708\,nm}$) of 708-nm illumination is 3-fold greater than that of 650-nm light. Unexpectedly, $R_{480\,nm}$ of 480-nm light is only 0.6 that of 650 light. The high efficiency of 708-nm light supports the hypothesis that System I sensitizes the fluorescence loss; the low efficiency of 480-nm is anomalous.

(vi) Since $i_{708\,nm}^F/i_{650\,nm}^F$ is the same for unpoisoned wild type and for Ac-115 and DCMU-poisoned wild type, and since $R_{708\,nm}$ for wild type indicates System-I sensitization, we conclude that in Ac-115 and DCMU-poisoned cells the slow fluorescence rise is also primarily sensitized by System I.

DISCUSSION

Slow activation of units, a hypothesis

The parallelism of fluorescence and O_2 evolution during the M_2–P_3 and a_2 phases implies that the rate constant for fluorescence increases relative to those of internal conversion and primary photochemical step. If we accept the widely held view[30] that fluorescence is emitted primarily from System II, we conclude that the nature of System-II units changes. One conceivable change, leading to higher fluorescence levels after preillumination, would be an increase in the amount of active, fluorescent chlorophyll serving each photochemical center. However, the observation that the P_2 level is reached at the same time (at about 0.2 sec in strong light), both after darkness and after preillumination, argues against such an increase. Were there an increase, the time to reach P_2 should be shortened. This same observation also argues against a change in the amount of oxidant per center, assuming that chlorophyll per center is constant. Only, in the unlikely event that both chlorophyll and oxidant increase together, could a change in composition of units be reconciled with identical times to reach P_2, after darkness and after preillumination. Another argument against a change in the amount of oxidant per unit is that such a change

should not affect the yield of fluorescence when the oxidant is exhausted. Yet preillumination elevates the P_2 level. We think the P_2 level corresponds to exhaustion of oxidant since, both after darkness and after preillumination, fluorescence remains constant at the P_2 level from 0.2 to about 1 sec.

If compositional changes within units are unlikely, then the parallelism of fluorescence and O_2 evolution must be attributed to different forms of whole units of fixed composition. The simplest hypothesis is that whole units exist in either of two forms, one photochemically active and fluorescent, the other inactive and non-fluorescent. We may suppose that after darkness, roughly half the units are in the inactive form and are slowly converted to the active form during the M_2–P_3 phase of induction in strong light. Once activated in strong light, relatively weak illumination (like the preilluminations used in our experiments), would suffice to maintain the units in the active form. This hypothesis appears to explain, at least qualitatively, all the observed effects. First, with regard to the early features of induction, both the fluorescence levels and the maximum rate of O_2 evolution during the spike will be higher after preillumination, because more active units are present. However, the time coordinates of the induction features will remain unchanged, because the composition of the units is not affected by activation. Secondly, after darkness, the M_2–P_3 fluorescence rise and the distinct a_2 phase of O_2 evolution are explained as manifestations of the slow activation of units. After preillumination, which maintains all units in active form, neither the M_2 minimum nor the M_2–P_3 rise are observed, and O_2 evolution, instead of exhibiting the slow a_2 phase, rises faster and more nearly continuously with the a_1 phase.

Sensitization of the slow activation

The occurrence of the slow fluorescence rise in DCMU-poisoned cells and in mutants with System-I but not System-II activity, and the 3-fold greater efficiency of 708 compared to 650 nm in maintaining activated units, form strong evidence that the slow activation of System-II units is sensitized by System I. (A similar activation of System-II fluorescence by System I was considered previously in order to account for the marked lowering of fluorescence by FCCP[12]; however, new data (to be published) indicate that FCCP can act directly on System II as well as on System I.)

The kinetics of the slow activation appear to be complex. The often sigmoidal time course of the third wave fluorescence rise (see Figs. 2E, 7, 8 and 11), indicates that the slow activation starts up only after a delay of 10–20 sec. The back reaction in darkness is very slow, the half-time for inactivation being about 4 min. There is some evidence that the back reaction is also complex—an initially faster phase being followed by a slower one. Detailed studies of the kinetics of both the forward light-dependent reaction and the dark back reaction are currently in progress.

Comparison of PAPAGEORGIOU's *and our results*

PAPAGEORGIOU[13] found that, with Chlorella, fluorescence increased from a minimum (M_2) at about 3 sec to a maximum (P_3) at about 1 min, then declined in about 10 min to a steady-state level lower than that of M_2. The earlier times at which M_2 and P_3 occurred in his experiments may be due to the lower illumination (about 10^3 ergs·cm^{-2}), the higher O_2 level (air-equilibrated samples), and the longer (15 min)

dark periods which he employed. At lower illuminations, we also observe (Figs. 3 and 5) that M_2 and P_3 are reached somewhat earlier than in high light. With Anacystis[13–15], measured at room temperature in $2 \cdot 10^3$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}, PAPAGEORGIOU found that fluorescence increased from 3 sec (M_2) to a very high maximum (P_3) at 2–3 min, and then remained constant at the high level. In our limited work with Anacystis, photosynthesis appears generally strongly inhibited at room temperature and only becomes fast at 35–40°. Possibly, the high, constant fluorescence observed by PAPAGEORGIOU was due to an inhibition of photosynthesis.

The most important discrepancy between PAPAGEORGIOU's and our work is concerned with sensitization by System I. With Chlorella, PAPAGEORGIOU concluded that DCMU inhibited the third wave fluorescence rise. However, Fig. 17 of his thesis[13] shows that there is, in fact, a slow fluorescence rise in the presence of DCMU. In this as well as his other induction curves, PAPAGEORGIOU plotted fluorescence intensity at time t relative to that at 3 sec; thus absolute differences in the amplitudes of the fluorescence of poisoned and unpoisoned cells were largely hidden. If one takes into account that the initial fluorescence level at 3 sec is probably about 2-fold higher in DCMU-poisoned cells than in unpoisoned cells, then the amplitude of the third wave rise in poisoned cells would be about 2/3 that in unpoisoned cells. In unpoisoned Anacystis, PAPAGEORGIOU found a strong third wave in System-II light, but not in blue System-I light. On the other hand, both lights gave a third wave in cells poisoned with DCMU. The absence of the third wave in System-I light might be due, not to the ineffectiveness of System-I light in accomplishing the activation, but rather to a different character of fluorescence induction when System I is sensitized faster than System II. If PAPAGEORGIOU had examined induction in System-II light, after darkness and after far-red preillumination, we suppose he would have observed a System-I sensitization of the slow activation. Certainly, his results with DCMU are consistent with this prediction. In summary, we think PAPAGEORGIOU's results, like our own, are consistent with System-I sensitization of the slow activation, both with and without DCMU.

Low efficiency of 480 nm in sensitizing the slow activation

The unexpectedly low value of $R_{480\,nm}$ cannot be due to inactive absorption by carotenoids. Such an inactive absorption should increase $i^{O_2}_{480\,nm}$ and $i^{F}_{480\,nm}$ in the same proportion thus leaving the ratio $R_{480\,nm}$ unaffected. Nor could a specific stimulation of respiration by blue light[31] cause the low efficiency. In this case, $i^{O_2}_{480\,nm}$, and hence $R_{480\,nm}$, would be anomalously high, not low. Finally we considered whether the especially strong System-II character of 480 nm, indicated by enhancement studies with green algae[32,33] could lead to low efficiency. To explore this possibility we employed equations previously described[22] for the rate of O_2 evolution in System-I and -II lights, and we assumed that fluorescence loss (F.L.) could be described by related equations. Thus for separate package

rate of O_2 evolution = constant =

$$a_{480nm}f_{480nm}i^{O_2}_{480nm} = a_{650nm}f_{650nm}i^{O_2}_{650nm} = (1-a_{708nm})f_{708nm}i^{O_2}_{708nm}$$

F.L. = constant =

$$a_{480nm}f_{480nm}i^{F}_{480nm} = a_{650nm}f_{650nm}i^{F}_{650nm} = a_{708nm}f_{708nm}i^{F}_{708nm}$$

Here f_λ is the fraction of incident quanta absorbed and a_λ is the fraction of the absorbed quanta entering System I directly. Expressions for R_λ can be derived by eliminating $i_\lambda^{O_2}$ and i_λ^F. It is found that identical efficiencies should be obtained with all System-II lights. Analysis of the 'spill-over' case leads to the same result. The low efficiency of 480 nm could only be explained if, in contradiction to information for other green algae[32–34], 650 nm were a System-I light in *C. reinhardi*. For the present the low efficiency remains an unexplained anomaly.

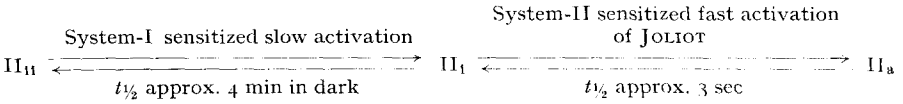
Definition of three activation steps

Besides the slow activation postulated to account for the third wave, two others appear to have roles in induction. One is the fast activation of the O_2 evolving capacity of System II which JOLIOT[9,27] demonstrated. This activation is completed when about 1 quantum is absorbed per unit of 200 chlorophyll molecules (in about 15 msec in our experiments with 5 mW·cm^{-2} of blue light). The fast activation is sensitized by System II, and the back reaction in dark has a half time of about 3 sec (ref. 9).

A second activation (we call it the '5-sec activation') must be postulated to explain the P_2–M_2 fluorescence decline and the essentially complementary a_1 phase of accelerating O_2 evolution. Because of the complementarity, this activation can be supposed to occur at a dark step in electron transport. After darkness, the step would be inactivated, and it is this inactivity which leads to exhaustion of oxidant and the M_1–P_2 fluorescence rise. When steady-state photosynthesis is interrupted by increasingly long dark intervals, one finds that the P_2 level (in subsequent strong light) reaches a maximum after about 30 sec; we may suppose that this is the time for complete inactivation to be achieved. In strong light, activation of the step commences at about 1 sec (judging from the onset of the P_2–M_2 decline and the starting up of O_2 evolution) and continues at least up to M_2 and perhaps beyond. The activation is slower at low O_2 and may be complex.

The location of the blocked step affected by the 5-sec activation is not known. However, the fact that an essentially normal O_2 spike is seen in the mutants lacking System-I activity implies that the amount of oxidant lying between the activatable step and System I is small, compared with the amount (about 10–20 equiv per System-II unit of 200 chlorophylls) exhausted during P_1–M_1–P_2. Apparently, the step is close to System I; it could be between Systems I and II, or beyond I.

Both the fast and slow activations appear to involve System-II units. As a consequence, three forms of System-II units must be postulated: (i) a twice inactive Form II_{11} resulting from the slow ($t_{1/2}$ approx. 4 min) back reaction. After several minutes darkness, roughly half of System-II units are in the 'II_{11}' form; (ii) Form II_1, which is the product of the slow activation, but in which the fast activation has not been accomplished; after several minutes darkness, the remaining half of System-II units are in this form; (iii) finally, after both fast and slow activations are achieved, the photochemically active Form II_a is obtained. The following diagram summarizes the relationships:



Implicit is the assumption that the fast activation occurs only after the slow activation is accomplished.

General theory of induction

Together, the fast, 5 sec, and slow activations, along with their back reactions, provide the basis for a general understanding of induction. In conjunction with information on the sizes of System-II units and their oxidant pools, it appears possible to account (at least qualitatively) for the occurrence of observed induction features, for their amplitudes and times of appearance, and for the effects of preillumination, and varying preceding dark times. Our understanding may be summarized as follows.

After about 10 min darkness, roughly half of System-II units are in the 'twice-inactive' Form II_{II}, the remaining half in the 'once-inactive' Form II_I. When saturating light is turned on, five phases of induction are observed:

O–P_1. During this phase, fast activation converts the Form II_I into II_a. Parallel increases in fluorescence and in O_2 evolution capacity attend this activation. In 5.1 mW·cm^{-2} of blue light, P_1 is reached in about 15 msec, the time for absorbing 1 quantum per unit of 200 chlorophylls.

P_1–M_1–P_2. During this phase of complementarity, fluorescence at first remains at the P_1–M_1 level, then rises to P_2 as the initially available oxidant is exhausted. Concurrently, the rate of O_2 evolution declines from a maximum early in the period to zero at the end. The oxidant pool amounts to about 10–20 equiv per System-II unit of 200 chlorophylls, as indicated by JOLIOT[9], MALKIN AND KOK[4] and MALKIN[5] and our data as well.

P_2–M_2. Fluorescence remains at the P_2 level from about 0.2 sec to about 1 sec, then declines to M_2. As the fluorescence declines, the rate of O_2 evolution rises from zero to about 1/2 of steady-state value. During this phase the approximately complementary courses of fluorescence and O_2 evolution are governed by the activity of a dark step—the activity being zero at P_2 and gradually increasing at least up to M_2. The '5-sec activation' is responsible for the increasing activity.

M_2–P_3. During this phase, fluorescence and rate of O_2 evolution increase in parallel, as the slow activation converts inactive units (II_{II}) into active ones (II_a). The rising time course (a_2) of O_2 evolution is kinetically different from that of the preceding period. During a_1, O_2 evolution follows the growing activity of the dark step, whereas, in a_2, it is limited by the rate of increase of active units.

P_3–S. In the final phase, fluorescence often declines somewhat from P_3, while O_2 evolution rises the remaining few percent of steady-state value. The mechanisms of these changes remain to be elucidated.

Preillumination, which converts System-II units into the active form, elevates all except the steady-state fluorescence levels, and in addition permits the rate of O_2 evolution, from z to f, to follow the increasing activity of the dark step undergoing the 5-sec activation. When induction in strong light is observed after dark periods varying from a few seconds up to several minutes, the following behavior is seen. After a few seconds, only the back reaction of the fast activation has time to occur, and O_2 evolution and fluorescence return to steady levels within seconds. As dark times increase to about 30 sec, the 5-sec activation is reversed. As a result, P_2 levels increase to a maximum, and a marked O_2 induction following a_1-phase kinetics is observed. With dark times increasing above 30 sec, the slow inactivation of System-II

TABLE II

COMPARISON OF MUNDAY'S AND OUR FINDINGS ON THE EFFECTS OF 710 AND 650 NM PREILLUMINATIONS (ABOUT 0.2 nEINSTEIN INCIDENT·cm^{-2}·sec^{-1}) ON INDUCTION IN STRONG BLUE LIGHT

Effects expressed as % increase (+) or decrease (—) of magnitude in induction after 3–4 min darkness.

Illumination cycle	BANNISTER-RICE	MUNDAY	
	3 min dark, 4–5 min strong blue (preillumination replaced dark)	*4 min dark, 2 sec strong blue (preillumination replaced dark)*	
Preillumination	*710 or 650 nm*	*710 nm*	*650 nm*
P_1–M_1 level	+50% or more	−10%	−10%
P_2 level	+50% or more	−30%	−30%
M_2 level	+50% or more	+<10%	−15%
Time to P_2	Effect, if present, <10%	+10–20%	−10–20% (very weak 650 gave 10% increase)
Duration of preillumination to produce above effects in dark-adapted cells	Several minutes (stronger preillumination may be required)	About 3 sec	
Half time of dark decay of preillumination effect	3–4 min	About 15 sec	

units becomes important. As the fraction of initially active units declines, the P_2 level falls, the M_2 minimum becomes progressively deeper, and the a_1 and a_2 phases of O_2 induction become distinguishable.

Comparison of MUNDAY's *and our findings*

Recently, MUNDAY[25,14] described effects of preillumination on induction during the first seconds of strong illumination. As Table II shows MUNDAY's effects differ markedly in magnitude, direction, and time constant from those observed by us. Furthermore, whereas we attribute our observations to effects of preillumination on the distribution of units between active (II_1, II_a) and inactive (II_{11}) forms, MUNDAY concluded that his effects are solely due to shifts in the redox states of oxidants Q, A, and X.

We think that MUNDAY's and our results are not contradictory, but arise from the different conditions of measurement. Under MUNDAY's experimental conditions (see Table II), we think that the cells were maintained permanently in a dark-adapted state and that there was never any appreciable slow activation of II_{11} units. Our reasons for thinking this are the following. First, the short 2-sec flashes of strong blue light were far too brief to accomplish the slow activation. Secondly, investigations in progress indicate that a preillumination of 0.2 nEinstein·cm^{-2}·sec^{-1}, although able to prevent the inactivation of units previously activated in strong light, is not strong enough to bring about any appreciable activation in dark-adapted cells. Thus MUNDAY's preilluminations probably did not activate any II_{11} units. From these considerations, we can agree with MUNDAY that his effects concern solely induction

in II_1 units and that the effects of preillumination are mainly due to shifts in the redox states of Q, A, and X.

In contrast our conditions tend to maximize the effects of preillumination on the slow activation system. Thus our preillumination maintained all units active whereas, after darkness, only roughly half the units were active. The large changes of fluorescence yield associated with the slow activation, we believe, 'swamp out' the smaller effects described by MUNDAY.

Final remarks

While our conception accounts satisfactorily for most characteristics of induction, many details and relations need further investigation. Among these may be mentioned the dependence of P_1 and M_1 levels on dark time, the uncertain location of the dark step acted upon in the '5-sec activation', the unknown nature of the final phase of induction, the quantitative kinetics of the 5 sec and slow activations, and the quantitative relations between oxidant concentration and O_2 and fluorescence yields.

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A recent paper of L. N. M. DUYSENS AND A. TALENS (presented at the International Congress of Photosynthesis Research, Freudenstadt, in June 1968) reports a slow increase in the yield of System-II fluorescence caused by System-I illumination, in a blue-green alga. Thus, three groups (PAPAGEORGIOU, BANNISTER AND RICE, and DUYSENS AND TALENS) have independent evidence of a slow activation of System II sensitized by System I. Adopting a previous hypothesis of BANNISTER[12], DUYSENS AND TALENS discussed the possibility that the slow activation of System II is brought about by ATP generated by System I.

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