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FLUORESCENCE AND OXYGEN EVOLUTION FROM *CHLORELLA PYRENOIDOSA*[*,**]

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SUMMARY

The process of photosynthetic energy conversion in *Chlorella pyrenoidosa* was investigated by simultaneous measurement of transient and steady-state rates of O_2 evolution and fluorescence.

1. Alternation or superimposition of light 1 and light 2 illumination induces both fast and slow changes in fluorescence and rate of O_2 evolution. The fast changes are ascribed to changes in conditions of the reaction centers in the context of the HILL–BENDALL[1] model and the kinetic analysis of ELEY AND MYERS[2]. The slow changes are interpreted as adaptations to the intensity and wavelength of illumination. The adaptive mechanism is described in terms of slow variation in fraction (α) of total absorbed quanta delivered to System 2. At low intensities, the calculated value of α for cells adapted to light 2 illumination (light 2 state) is approx. 0.9 of α for cells adapted to light 1 illumination (light 1 state).

2. An increase in fluorescence yield was found to accompany the decrease in O_2 yield at the onset of light saturation with either light 1 or light 2 excitation. Variation in α is proposed to account for the differences between the maximum fluorescence yield observed in steady-state conditions and the 1.5 times higher maximum yield observed in transient conditions or in cells inhibited by 3(3,4-dichlorophenyl)-1,1-dimethylurea. Variation in α can also explain the observation of a higher rate of fluorescence emission with light 1 excitation than with light 2 excitation for a given steady-state rate of O_2 evolution.

3. A model for energy conversion by System 2 is proposed to account for our observations. The model proposes competitive dissipation of absorbed energy by photochemical trapping at reaction centers and by fluorescence and radiationless de-excitation from both the pigment bed and reaction centers of System 2.

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; Fluorescence, denotes total emission and does not imply knowledge of an exponential decay.

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[**] A report, published during preparation of this manuscript, by N. MURATA (*Biochim. Biophys. Acta*, 172 (1969) 242) contains some information similar to that presented here.

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INTRODUCTION

Most current models for photosynthesis require two light-driven reactions sensitized by two different pigment systems[3–5]. Pigment system 1 collects photons and transfers excitation energy to the reaction centers specific for Photoreaction I, and Pigment system 2 collects photons and transfers excitation energy to the reaction centers for Photoreaction II. Any wavelength predominantly absorbed by System 1 is called light 1; likewise any wavelength predominantly absorbed by System 2 is light 2. Photoreaction I generates a weak oxidant and a strong reductant which can reduce ferredoxin and thence pyridine nucleotide. Photoreaction II generates a strong oxidant (leading to evolution of O_2) and a weak reductant. According to the HILL–BENDALL[1] model the oxidant of I and the reductant of II interact *via* an electron transport chain. Specialized chlorophyll molecules revealed by sensitive difference spectroscopy and called P700 are tentatively identified as the reaction centers of Photoreaction I (ref. 6). An absorption change at 690 mμ is tentatively ascribed to Chlorophyll a_{II} (ref. 7). Pending further characterization of this absorption change, we follow JOLIOT's[8] notation for the reaction centers of Photoreaction II; E when oxidized (open) and photochemically active, EH when reduced (closed) and photochemically inactive, and E_{in} for the inactive state which does not lead to O_2 evolution.

Fluorescence comes chiefly from System 2 (refs. 9 and 10). The oxidizing effect of System 1 activity on the reaction centers of System 2 has been invoked to explain the quenching effect of light 1 on fluorescence excited by light 2 (refs. 11–13). The oxidizing effect of System 1 activity is also involved in the enhancement phenomenon; simultaneous illumination by light 1 and light 2 results in a higher rate of O_2 evolution than the summed separate rates[14,15]. This implies a complementarity between rates of O_2 evolution and fluorescence which was suggested by earlier experiments. Roughly complementary induction patterns of O_2 evolution and fluorescence are observed when dark-adapted cells are illuminated[8,16,17]. Also, curves of light intensity *vs.* rates of fluorescence emission and CO_2 fixation[18], O_2 evolution[19] or Hill-reaction rates[20] show increases in fluorescence yield associated with decreases in efficiency of photochemical energy utilization.

When dark-adapted algae are illuminated by light 2, the fluorescence yield exhibits two periods of change. The first wave, completed in seconds, consists of a rise from an initial level (O) to a peak (P) and a decline to an apparently steady level (S). The second wave requires minutes for completion and involves a slow decline to the final steady-state level. The decline may or may not exhibit inflections, depending on culture conditions and exciting intensity. The final steady-state level is lower than "S". DUYSENS AND SWEERS[11] interpreted the first wave as a light-induced reduction and a subsequent reoxidation of System 2 reaction centers *via* System 1 activity. Part of the fluorescence decline observed was attributed to a conversion of some of the reduced reaction centers, denoted by QH, to a hypothetical compound Q′ which quenches the fluorescence and which is slowly converted into the photochemically active form, Q, by a dark reaction. PAPAGEORGIOU[21] reported that the slow decline of fluorescence during the second wave was not directly correlated with the rate of O_2 evolution. He proposed that the decline was due to conformational changes of the lamellar system related to the photophosphorylation process.

This paper reports direct correlations between rates of oxygen evolution and

fluorescence for both fast and slow events. The slow correlated changes of the second wave can be interpreted in terms of a variation in the distribution of quanta to the two pigment systems. The Q' mechanism for fluorescence decay as proposed by DUYSENS AND SWEERS[11] is both unnecessary and inadequate to account for the slow changes observed.

Our experimental design permitted simultaneous recording of fast (msec) and slow (min) changes in rates of both O_2 evolution and fluorescence. Single cell layers were excited at wavelengths 645, 696, or 710 mμ with intensities ranging from below compensation to above light saturation. Fluorescence was measured at 686 or 736 mμ with less than 5 % scattered light contribution.

Curves of light intensity *vs.* steady-state rates of O_2 evolution and fluorescence are presented first to provide a framework for interpretation of further results. Chromatic effects are then considered.

MATERIALS AND METHODS

Cells of the Emerson strain of *Chlorella pyrenoidosa* were grown in a continuous culture chamber[22] under low light intensity at a specific growth rate, k, of 0.7 per day. The cells were washed and placed on a platinum electrode of 2-mm diameter. The electrode was recessed 0.25 mm in black plexiglas. A 10-μ Cuprophan 150 PT dialysis membrane retained the algae within the small chamber and permitted diffusion of O_2, CO_2 and nutrients to the algae from the circulating medium (Knop's medium *plus* 0.01 M NaCl, lacking microelements and citrate, pH 6.8)[22]. The layer of cells which settled upon the electrode was never more than one cell thick, as checked microscopically. The electrode arrangement previously described[23] was modified by use of a chloride-plated silver wire 5 mm from the platinum cathode as a reference electrode and two coils of silver wire as an anode.

The optical system is shown schematically in Fig. 1. Excitation by desired wavelengths (645, 696, or 710 mμ) was provided alternately or simultaneously. Deviations from uniform illumination over the area of the electrode did not exceed $\pm$12 %. Fluorescence from the single cell layer on the electrode was measured at

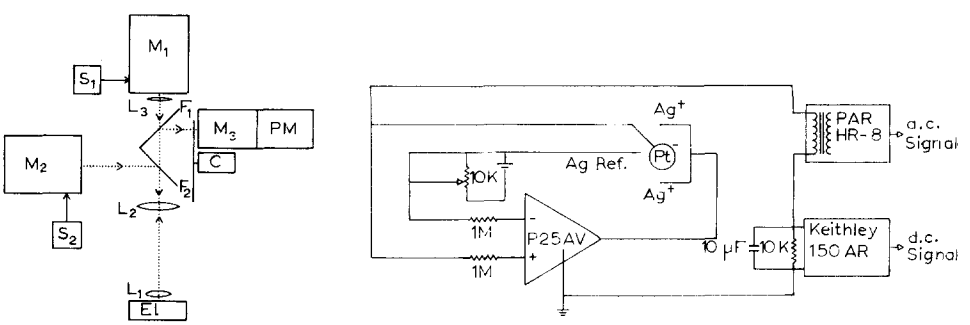


Fig. 1. Schematic representation of optical system. S_1, Osram 2500-W Xenon arc; S_2, DWY quartz-halogen lamp operated at 100-V regulated d.c.; M_1, M_2, and M_3, Bausch and Lomb monochromators; L_1, L_2, and L_3, lenses; F_1 and F_2, beam-splitting mirrors or selected interference filters; C, chopper; PM, an EMI 9558A photomultiplier; El, electrode.

Fig. 2. Electrical circuit. P25AV, Philbrick operational amplifier.

686 or 736 mμ. The chopper, C, could be positioned to interrupt the exit beam (as shown), or one or both of the exciting beams. Chopping either or both exciting beams at 13 or 80 Hz resulted in amplitude-modulated signals of fluorescence[11] and rate of O_2 evolution[24,25].

The potential of the EMI 9558A photomultiplier developed across a 1-MΩ resistor was delivered to a lock-in amplifier (Princeton Applied Research Corp., HR-8, Type A pre-amp). The phase and frequency of this amplifier were adjusted for maximum signal intensity at the beginning of each experiment and changes in illumination were not observed to affect the tuning.

The electrical circuit used for detection of O_2 evolution included a potentiostat arrangement which kept the cathode potential constant at -0.65 V *vs.* the reference electrode (Fig. 2). The classical measure of rate of O_2 evolution from the modified HAXO–BLINKS[26] electrode is referred to as the d.c. signal. The d.c. signal was the current flow from the cathode across a 10-kΩ resistor (R_8) as measured by a Keithley 150 AR millivolt meter. Chopped exciting light results in a modulation of the cathode current with the frequency of the chopped exciting beam. The modulated component, referred to as the a.c. signal, was measured by a second lock-in amplifier (PAR, HR-8, Type B pre-amp) whose phase and frequency were adjusted for maximum signal intensity at the beginning of each experiment. Changes in illumination within the course of an experiment were not observed to affect the tuning. Time constants of the lock-in amplifiers between 30 and 300 msec were selected according to experimental requirements. Outputs of the lock-in amplifiers used to measure the modulated O_2 evolution (a.c.) and fluorescence emission were fed to a two-pen recorder (Texas Instruments, Servoriter II) of 1-sec full-scale response and simultaneously to a two-pen recorder (Brush, Mark 220) with full-scale response up to 40 Hz. Output of the d.c. measuring amplifier was fed to a single-pen recorder (Heath, EUW-20A) with 1-sec full-scale response.

Incident intensity at the electrode was measured by way of a front surface mirror which could direct the beams to the electrode or to the equidistant 2-mm diameter element of a calibrated Clark vacuum thermocouple.

Elimination of scattered exciting light from the fluorescence signal was greatly improved by use of narrow-band interference filters (Baird Atomic) and appropriate cutoff filters on all three monochromators. The beam-splitting mirrors, F_1 and F_2 usually were broad-band interference filters. These measures kept scattered light below 1 % of the fluorescence signal in most optical configurations.

RESULTS

Steady-state measurements of rate of O_2 and fluorescence emission vs. intensity

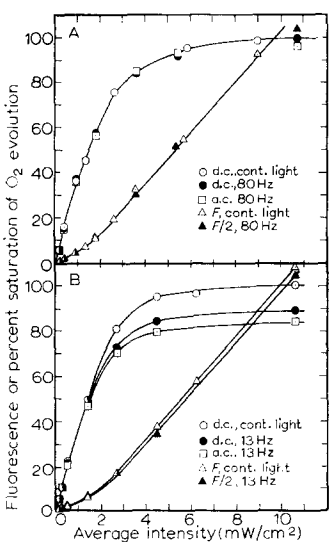
Indirect calibration of the rate-measuring oxygen electrode with 645-mμ excitation was achieved by comparing the relative rates of O_2 evolution to the absolute rates measured with a Beckman concentration-measuring oxygen electrode. Normalized curves of rate of O_2 evolution *vs.* intensity of 645-mμ excitation from the rate- and concentration-measuring electrodes were superimposable when the intensity scale for cells on the platinum electrode was multiplied by 1.85. This adjustment was presumed necessary due to increased intensity by reflection for cells resting on the bright platinum surface. All subsequent intensity measurements with the rate-measuring

electrode were accordingly adjusted to 1.85 times the measured incident intensity.

Effects of intermittent illumination are summarized in Fig. 3. Rates of O_2 evolution and fluorescence emission are shown to have the same relation to average intensity for continuous or 80-Hz illumination. The figure demonstrates equivalence of the saturation characteristics of the unmodulated (d.c.) and modulated (a.c.) signals at 80 Hz. As previously reported[27] illumination at 13 Hz gives a lower average (d.c.) rate of O_2 evolution under light saturation than does continuous illumination. Use of 13-Hz excitation was limited to the low intensity region (less than 1.5 mW/cm^2 of 645 mμ) where illumination with a given average intensity of continuous or intermittent illumination results in the same average rate of O_2 evolution or fluorescence.

Fig. 3 shows an increase in steady-state fluorescence yield in the intensity range where the O_2 yield declines. Fig. 4 shows further that, with 645-mμ excitation, equivalent changes in fluorescence yield are observed with fluorescence emission measured at 686 or 736 mμ. This signifies that with 645-mμ excitation no "dead" or invariant fluorescence emission is measured at 736 mμ that is not equally represented at 686 mμ.

Excitation by varied intensities of light 1 (as 696 mμ) and light 2 (as 645 mμ) alter the rates of O_2 evolution and fluorescence emission as shown in Fig. 5. Fig. 5 shows clearly that an increase in steady-state fluorescence yield accompanies the



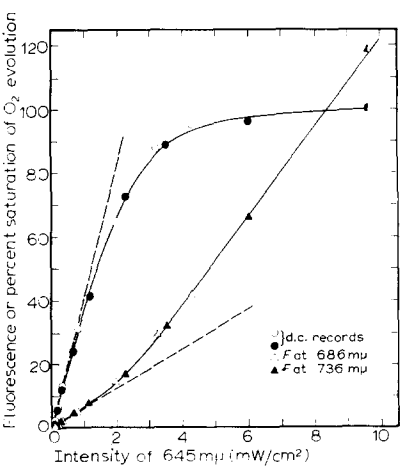


Fig. 3. Relative rates of steady-state O_2 evolution and fluorescence *vs.* intensity of chopped or continuous excitation. Chopped excitation at 645 mμ provided at 80 Hz (A) and at 13 Hz (B). The a.c. curves are normalized to match the initial slopes of the d.c. curves. Fluorescence (F), measured at 686 mμ, was chopped at exit monochromator M_3 when cells received continuous light. Fluorescence levels observed with chopped exciting light were multiplied by sector transmission of 0.5 for comparison with the levels observed when fluorescence was chopped at the exit monochromator.

Fig. 4. Steady-state rates of O_2 evolution (d.c.) and relative fluorescence emission (F) *vs.* intensity of 645-mμ excitation. The actinic light was continuous, and the fluorescence beam was chopped at 480 Hz at exit monochromator M_3. Fluorescence measurements made at 686 and 736 mμ have been normalized at 9.6 mW/cm^2 of 645 mμ. The dashed lines are linear extensions of the initial slopes.

decrease in O_2 yield with either 645-mμ or 696-mμ excitation. This is contrary to the observation by GOVINDJEE[28] that in the red alga *Porphyridium cruentum* an increase in fluorescence yield is seen only with light 2 excitation.

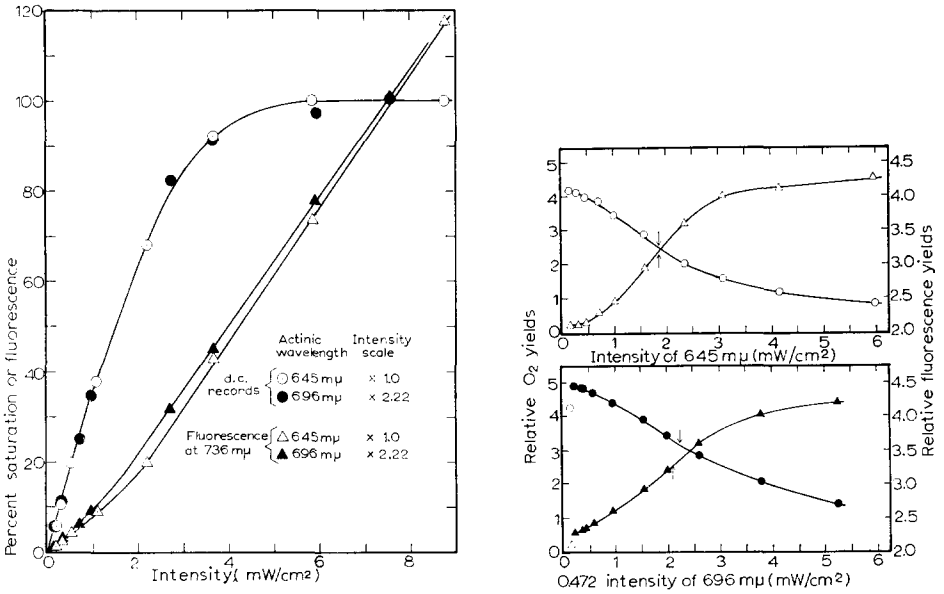


Fig. 5. Steady-state rates of O_2 evolution and relative fluorescence *vs.* intensity of 645- and 696-mμ excitation. Fluorescence was measured at 736 mμ by chopping at 480 Hz at exit monochromator M_3. Excitation by 645 or 696 mμ was provided alternately with intervening dark periods. The maximum rate of O_2 evolution (d.c.) was the same for both 645- and 696-mμ excitation.

Fig. 6. Typical plots of steady-state relative yields of O_2 evolution (⊙, ●) and fluorescence (△, ▲) for light 2 *vs.* intensity of 645 or 696 mμ background. The measuring beam was 150 μW/cm^2 of 645 mμ chopped at 13 Hz. The abscissa plots total intensity, *i.e.*, 150 μW/cm^2 *plus* indicated background intensity. The arrows indicate intensities which induced half of the observed changes in O_2 and fluorescence yields. Fluorescence was measured at 736 mμ.

Since the detecting system (for a.c. and F) responds only to modulated signals, it was possible to make direct measurements of the dependence of steady-state O_2 and fluorescence yields on the intensity of 645-mμ or 696-mμ background (unchopped) illumination. Typical results of such experiments are shown in Fig. 6. The background intensities which produce one-half the observed change in O_2 yield also produce approximately one-half the observed change in fluorescence yield (as predicted in ref. 20). Fig. 6 clearly illustrates the interdependence of steady-state O_2 and fluorescence yields and is a basis for further experiments in which correlated changes in O_2 and fluorescence yields are used to gain insight into photosynthetic energy conversion.

Enhancement

Isolated chloroplasts with no added electron acceptor exhibit a fluorescence rise on illumination following a dark period. The rise is ascribed to a reduction of members of the electron transport chain which links Photoreaction I and II (ref. 29). A small amount of added ferricyanide will temporarily quench the fluorescence and a larger

amount of the oxidant added prior to illumination will prevent the occurrence of the normal fluorescence rise[29]. Fig. 7 shows analogous changes induced by an unchopped light 1 background for fluorescence from Chlorella excited by light 2. Fluorescence quenching by light 1 under similar conditions has been previously reported[11–13]. Fig. 7 shows clearly that the quenching effect of light 1 is related to an enhancement of O_2 evolution, inferentially ascribed to an oxidation of System 2 reaction centers.

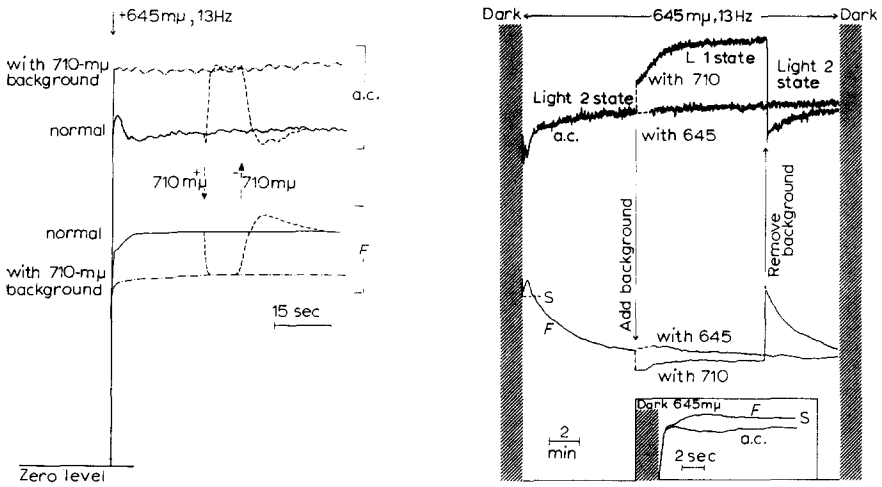


Fig. 7. Fluorescence quenching and enhancement of O_2 evolution induced by light 1 background. "Normal" tracings are records of O_2 evolutions (a.c.) and fluorescence (F) for an average intensity of 150 μW/cm^2 of 645 mμ at 13 Hz turned on after a 5-min dark period. Unchopped background of 700 μW/cm^2 of 710 mμ preceded (30 sec) and accompanied the 645-mμ excitation (–·–·–) or was added for 10 sec (– – – –). Fluorescence was measured at 686 mμ.

Fig. 8. Development of the light 2 state after darkness, slow transition to the light 1 state induced by a light 1 background, and slow return to the light 2 state upon removal of light 1 background. An average intensity of 300 μW/cm^2 of 645 mμ was interrupted by 8 min of darkness before recording the changes in O_2 evolution (a.c.) and fluorescence (F) which accompany the dark-to-light transition. Fluorescence was measured at 686 mμ. The inset shows the dark-to-light transition as simultaneously recorded on a fast Brush recorder with the fluorescence channel set at twice the normal sensitivity. The apparently steady level of fluorescence shown in the inset "s" is shown to be far from the final steady level. The time constants of the lock-in amplifiers were set at 0.1 sec for the interval shown in the inset and then changed to 0.3 sec. The light 1 and light 2 backgrounds were matched to give approximately equal rates of O_2 evolution (d.c.) at 50 μW/cm^2 of 645 mμ and 572 μW/cm^2 of 710 mμ.

Slow changes: The light 1 and light 2 states

Fig. 7 illustrates fluorescence quenching by light 1 after a few seconds of light 2 illumination. A much smaller effect of light 1 is observed in steady-state conditions, achieved after 6–10 min of light 2 illumination. The slow correlated changes which lead to steady-state conditions are wavelength- and intensity-dependent and represent a phenomenon not previously studied.

It is convenient to introduce the terms light 1 state and light 2 state to denote adaptation to light 1 or light 2 illumination. Transition between light 1 and light 2 states is a slow process requiring 6–10 min. The differences in light 1 and light 2 states are illustrated in Fig. 8. It shows the slow development of a light 2 state after a long dark period, the slow transition to a light 1 state induced by unchopped light 1 back-

ground, and the slow return to a light 2 state upon removal of the light 1 background. Addition or removal of a light 2 background is shown to have little effect. Fluorescence quenching by added light 1 is not extensive for cells in the light 2 state.

We necessarily make a distinction between fast events which occur within a few seconds and slow events that require minutes for completion. The addition of light 1 background causes a rapid increase in O_2 yield and complementary decrease in fluorescence yield. Removal of light 1 background causes a rapid decrease in O_2 yield and increase in fluorescence yield. These fast transitions are considered to be the consequence of shifts in the concentration of open reaction centers of System 2 induced by the addition or removal of the oxidizing effect of Photoreaction I.

The inset of Fig. 8 shows on a faster time scale the events which accompany the transition from dark to light. The fluorescence level "s" appears steady on the fast record but is clearly far from the final steady-state level. Both fast and slow recordings are presented to permit comparison of these results to previous reports.

Darkness is shown to induce some of the characteristics of the light 1 state, in that similar slow changes are seen with light 2 illumination after a dark period or after exposure to light 1. The complexities in a.c. and fluorescence records observed in the first seconds of light 2 after darkness but not after light 1 are attributed to complexities of the activation process proposed by JOLIOT[30].

The existence of distinct states associated with adaptation to light 1 and light 2 illumination is supported by the results of Figs. 5 and 9A. Fig. 5 shows that a higher rate of fluorescence emission is associated with light 1 excitation than with light 2 excitation for a given rate of O_2 evolution. This is shown again in Fig. 9A which presents the data of Fig. 6 as relative fluorescence yields (ϕ_f) *vs.* relative O_2 yields (ϕ_{O_2}). The plots are linear over a wide range of intensities with either 645-mμ or 696-mμ background illumination. Extrapolation of the linear portions of the ϕ_f *vs.* ϕ_{O_2} plots to the intercept at $\phi_{O_2} = 0$ (complete saturation) gives the same value of ϕ_f with either background. For all other values of ϕ_{O_2}, the light 1 background produces a higher ϕ_f than the corresponding light 2 background. This is true whether fluorescence

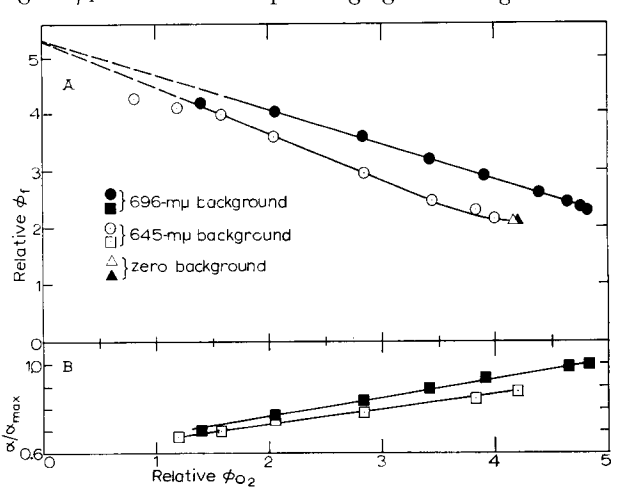


Fig. 9. A. Data of Fig. 6 plotted as ϕ_f *vs.* ϕ_{O_2}. The dashed lines are linear extrapolations. B. Predicted values of α/α_{max} *vs.* ϕ_{O_2} with 645- or 696-mμ background. Data from Fig. 6. See DISCUSSION for details.

is measured at 736 mμ, as in Fig. 9A, or at 686 mμ with 710-mμ excitation as light 1. The non-linearity of the plots in the low ϕ_{O_2} region is presumed to be due to photoinhibition, a poorly understood phenomenon which results in lowered yields of fluorescence and O_2 evolution at high excitation intensities.

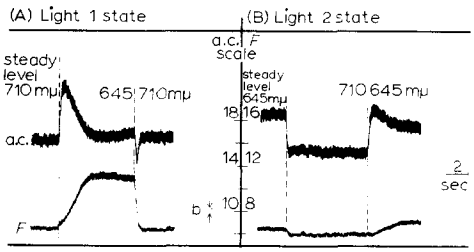


Fig. 10. Chromatic transients of O_2 evolution (a.c.) and fluorescence (F) for cells in the light 1 state (A) and the light 2 state (B). Both light 1 (710 mμ) and light 2 (645 mμ) were chopped at 13 Hz. The average intensities of excitation in A and B were 273 μW/cm^2 of 645 mμ and 3850 μW/cm^2 of 710 mμ. The time constant of both amplifiers was 0.03 sec. With this filtering some 13-Hz modulation is followed by the fast Brush recorder resulting in the wide tracings shown. More extensive filtering was found to misrepresent fast events. Fluorescence was measured at 686 mμ with the scattered light contribution negligible for 645-mμ excitation and indicated (b) for 710-mμ excitation.

Chromatic transients in light 1 and light 2 states

Wavelength-induced transients in rate of O_2 evolution and fluorescence are shown in Fig. 10. The chromatic transients of Fig. 10A occurred when the cells were adapted to light 1 illumination. The transients of Fig. 10B occurred on switching between the same intensities of light 1 and light 2 as in Fig. 10A but after the cells were adapted to light 2 illumination. The transients are clearly different in the two cases. The light 1 state is characterized by a low O_2 yield and high fluorescence yield for light 2 and a high O_2 yield and high fluorescence yield for light 1. The light 2 state is characterized by a high O_2 yield and low fluorescence yield for light 2 and a low O_2 yield and low fluorescence yield for light 1. The extent of the slow changes leading

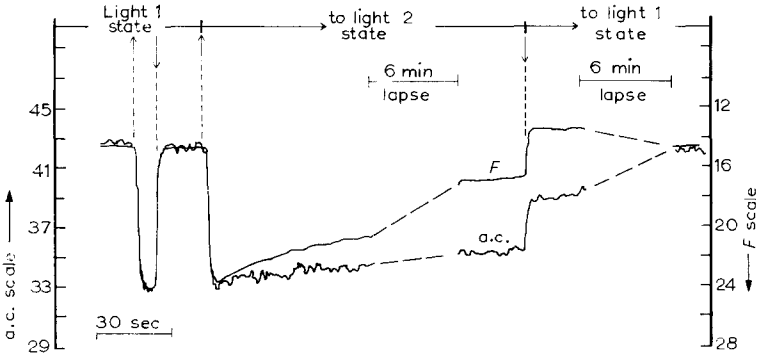


Fig. 11. Fast and slow changes of O_2 evolution (a.c.) and fluorescence (F) showing complementary relations between a.c. and F for the fast changes only. The light 1 state is initially assumed in response to modulated light 2 (13 Hz, 645 mμ, 300 μW/cm^2) with light 1 background (710 mμ, 572 μW/cm^2). Removal of light 1 background is shown by upward arrows, addition by downward. Fluorescence was measured at 686 mμ.

to a light 1 or a light 2 state determine the magnitude of the fast complementary transients in O_2 evolution and fluorescence.

Fast and slow changes

Changes in System 2 reaction center conditions are expected to cause changes in O_2 and fluorescence yields which are opposite in direction and proportional in magnitude. In Fig. 11 this complementarity is shown to exist for the fast but not the slow changes which accompany addition or removal of a light 1 background. The tracings of Fig. 11 are the a.c. and fluorescence records obtained in response to illumination by modulated light 2. The cells are initially in a light 1 state due to a 10-min exposure to light 1 background. The recording of the fluorescence signal was inverted and its sensitivity adjusted to give a fluorescence yield increase equal in magnitude to the O_2 yield decrease which accompanies removal of a light 1 background. The same time constant (0.3 sec) was used with both lock-in amplifiers. Complementary changes in rate of O_2 evolution and fluorescence emission will result in superimposable records under these conditions. The tracings of Fig. 11 show the complementarity of the rapid transients in a.c. and fluorescence induced by removal and prompt readdition of a light 1 background. Removal of light 1 background permits the light 2 state to develop. The magnitude of the fluorescence decrease exceeds that of the a.c. increase during the slow transition to the light 2 state. Subsequent addition of light 1 background results in rapid complementary shifts of a.c. and fluorescence levels which are much smaller than the rapid shifts observed when light 1 background was added to cells in the light 1 state. The records again show slow non-complementary changes as the light 1 state is restored.

The rapid complementary changes are readily understood in terms of changes in System 2 reaction center conditions[2,25]. The slow changes require some other explanation. Fig. 12 shows both fast and slow changes induced by a light 1 background. The cells are initially in a light 2 state brought about by about 10 min of exposure to

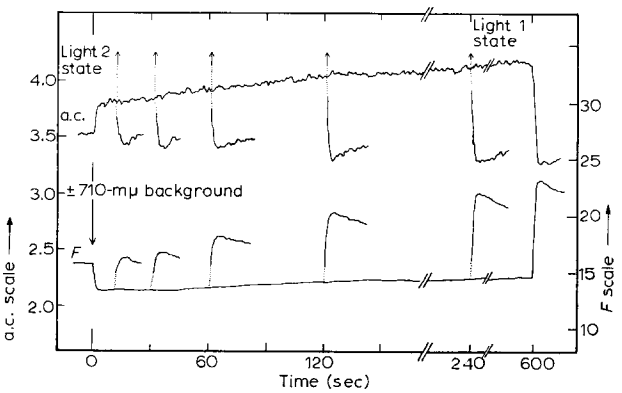


Fig. 12. Slow light 1 induced transition from the light 2 state to the light 1 state and consequences for rapid transients in O_2 evolution (a.c.) and fluorescence (F) when light 1 is removed. Prolonged exposure to modulated light 2 (645 mμ, 13 Hz, 300 μW/cm²) brought the cells to the light 2 state. Light 1 background was 572 μW/cm² of 710 mμ. Tracings begun with dotted lines are from consecutive experiments in which the length of exposure to 710 mμ was limited as indicated on the abscissa. The recorder used has a 1-sec full-scale response characteristic. The time constants of both lock-in amplifiers were set at 0.3 sec. Fluorescence was measured at 686 mμ.

modulated light 2. Addition of unchopped light 1 causes rapid changes, presumably by oxidation of system 2 reaction centers. A slow parallel increase in both O_2 and fluorescence yields then brings the cells into the light 1 state. Fig. 12 shows further that the extent to which the light 1 state is established determines the magnitudes of the rapid changes which accompany removal of light 1 background.

Variations in ϕ_{fmax}/ϕ_{fmin}

Many of the quantitative ideas about the kinetics and yields of photosynthetic reactions are based on the concept that fluorescence is a simple reflection of unused light energy through direct competition with other photoprocesses. On this basis the ratio of maximum to minimum fluorescence yield, ϕ_{fmax}/ϕ_{fmin}, is a measure of photosynthetic efficiency. Fig. 13 presents measurements of initial and steady-state fluorescence and O_2 yields obtained when algae were subjected to rapid changes in intensity of excitation. The ratio of maximum to minimum fluorescence yield (ϕ_{fmax}/ϕ_{fmin}) observed in steady-state measurements is about 2.5 (Figs. 9 and 13). The maximum fluorescence yield is much higher in initial measurements, resulting in a ratio of ϕ_{fmax}/ϕ_{fmin} of 3.8. The discrepancy between transient and steady-state values of the ratio is an indication of variability in the energy conversion process.

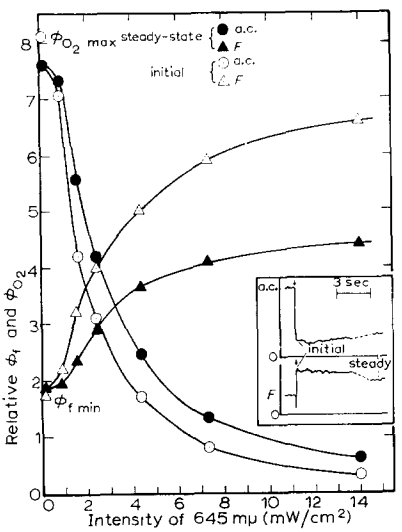


Fig. 13. Initial and steady-state yields of O_2 (⊙, ●) and fluorescence (△, ▲) on addition of a background of 645 mμ of varied intensity. The measuring beam was 645 mμ chopped at 13 Hz with an average intensity of 150 μW/cm². Fluorescence was measured at 686 mμ. The method of measurement for each intensity is shown in the inset for 4.2 mW/cm² (the down-transient in fluorescence is a spurious instrumental transient). The time constants of the amplifiers were set at 0.1 sec. $o_{2\,max}$ and $\phi_{f\,min}$ were the initial values observed on addition of 710-mμ background.

The steady-state fluorescence yield of cells inhibited with $3 \cdot 10^{-5}$ M DCMU when compared to the minimum yield from uninhibited cells exposed to a 710-mμ background gives ratios of ϕ_{fmax}/ϕ_{fmin} of 3.5–3.7, similar to those obtained in transient measurements. Progressively lower values of ϕ_{fmax} (with DCMU) $/\phi_{fmin}$ were found when the intensity of the chopped exciting beam was decreased below 1 mW/cm²,

e.g. ratio 2.1 at 10 μW/cm^2. However, the ratio becomes maximum and independent of exciting intensity below 1 mW/cm^2 if an unchopped background illumination (as 1 mW/cm^2 of 645 mμ) is added during measurements of $\phi_{f\max}$. The intensity dependence of the ratio is considered a consequence of the occurrence of a nonfluorescent and inactive form of the System 2 reaction centers, denoted E_{in} (ref. 8), at intensities where the rate of back reaction to the inactive form is appreciable compared to the rate of photoactivation. The activation and inactivation reactions are apparently not blocked by DCMU. This is indicated also by the observation that in DCMU-treated cells the changes in fluorescence yield brought about by background illumination are reversible. As a check on the measurements it was found that the intensity of light emission from a fluorescence standard of black paper was unaffected by addition or removal of high intensity background illumination.

DISCUSSION

It has been shown that rapid (msec–sec) transients in both O_2 and fluorescence yields can be induced by shifts in exciting wavelength. The fast transients can be accounted for if fluorescence is considered to reflect the state of the System 2 reaction centers.

Slow fluorescence changes have been known for years[16–18]. To account for the slow changes DUYSENS AND SWEERS[11] proposed Q′ as a nonfluorescent and inactive form of System 2 reaction centers. Simultaneous slow changes in rate of O_2 evolution are less pronounced than the slow fluorescence changes. As shown in Fig. 10, the slow changes in rate of O_2 evolution represent an adaptation to the wavelength of excitation. The adaptation which makes light 2 utilization more efficient (by 9% in Fig. 10) simultaneously decreases the efficiency with which light 1 can be utilized and *vice versa*. The slow adaptation to exciting wavelength decreases the wavelength dependence of the quantum yield for O_2 evolution. As shown in the preceding figures, the slow changes in O_2 evolution are consistently correlated with slow changes in fluorescence. The Q′ explanation for fluorescence changes cannot account for the observed correlations. It is the main point of this paper that the correlated slow changes can be accounted for by variation in the distribution of quanta to the two photosystems.

Variable α: An explanation of slow changes in O_2 evolution

The wavelength-dependent parameter α denotes the fraction of absorbed quanta of a given wavelength delivered to System 2. The fraction delivered to System 1 is $1-\alpha$. It is proposed that a change in exciting wavelength is accompanied by an instantaneous change in α and a subsequent slower adaptive change. Light 1 illumination delivers more quanta to System 1 than to System 2. In the light 1 state (cells adapted to low intensity, light 1 illumination) the slow adaptive changes bring α to its maximum value, $\alpha_{\max}$, for the specific light 1 excitation employed. The light 1 and light 2 states can be described in terms of $\alpha/\alpha_{\max}$.

The equation used to describe the rate of O_2 evolution is a modified form of that used by ELEY AND MYERS[2] and JOLIOT *et al.*[25].

$$v_\lambda = (\text{const.})\,(\alpha/\alpha_{\max})\,\frac{\alpha_{\max\lambda} I E}{1 - pEH} \qquad (1)$$

where v = rate of O_2 evolution, $\alpha_{max\lambda}$ = maximum fraction of absorbed quanta of wavelength λ delivered to System 2, I = absorbed intensity, E and EH denote fractions of System 2 reaction centers open and closed respectively, and p is assigned the value of 0.55 as estimated by JOLIOT *et al.*[25]. The wavelength dependence of E and EH is built into the equation by inclusion of the term $\alpha_{max\lambda}$. Illumination with 710 mμ with a low value of $\alpha_{max\lambda}$ (ref. 2) is believed to open all System 2 reaction centers (EH = 0).

The differences between light 1 and light 2 states are shown in detail in Fig. 10. A slow decrease of α/α_{max} from a value of 1.0 to 0.9 in the slow transition from the light 1 state to the light 2 state can account for the results shown. It would increase the fraction of quanta to System 1, increase the rate of Photoreaction I, open more of the System 2 reaction centers, and consequently cause a slow increase in rate of O_2 evolution. The larger fluorescence decay may be ascribed to the combined effects of decrease in α/α_{max} and decrease in the number of closed System 2 reaction centers.

The maximum rate of O_2 evolution in the light 2 state with 645-mμ excitation following 710-mμ excitation is predicted to be 0.9 of its value in the light 1 state. This is observed, with a difference of about 1 %. Instrumental time resolution was inadequate for quantitative analysis of the minimum rate of O_2 evolution with 710-mμ excitation following 645-mμ excitation in light 1 and light 2 states. The observations do show, however, a lower minimum rate in the light 1 state than in the light 2 state as predicted.

The rate of O_2 evolution with 710-mμ excitation after the rapid down transient is seen to be lower in the light 2 state than in the light 1 state. This is ascribed to a lower value of α/α_{max} in the light 2 state than in the light 1 state. The extent of the rapid down transient below the following level with 710-mμ excitation decreases as the light 2 state develops. This is an indication of a decrease in the EH level which would be a consequence of decreased α/α_{max}. The slow increase in rate of O_2 evolution and fluorescence emission with 710-mμ illumination (shown in Figs. 8, 10–12) is ascribed to an increase in α/α_{max} from 0.9 to 1.0.

A mathematical model

We have searched for an expression of the relationship between O_2 evolution and fluorescence which would allow us to make a quantitative analysis of the observations reported here. The model selected has features of both the LUMRY *et al.*[20] and VREDENBERG AND DUYSENS[31] models and the model proposed by JOLIOT[8, 30] and JOLIOT AND JOLIOT[32]. The model proposes that fluorescence emission in Chlorella may originate in both the closed reaction centers and the light-harvesting pigment bed. It proposes that fluorescence competes with photochemical trapping and radiationless de-excitation as a means of dissipating absorbed energy.

Consider a system with 1 mole of System 2 reaction centers ($e_t = 1$) and a much larger concentration, C_t, of associated System 2 chlorophyll *a* molecules. Energy delivered to System 2 is expressed by αI where I represents the intensity absorbed within the system and α represents the fraction of absorbed quanta of a chosen wavelength which are delivered to System 2.

The probability density of absorbed excitation in a photosynthetic unit is probably equilibrated in a few psec[33]. After equilibration the concentration or probability density of excitation in the pigment bed, C^*, may be considered to have a constant

relation to the concentration or probability density of excitation in System 2 reaction centers, e^*. Then $e^* = K_t C^*$ where K_t depends on the depth and abundance of the reaction centers relative to the molecules of the pigment bed. It is assumed for simplicity that equilibration is independent of System 2 reaction center conditions (E_{in} or E or EH).

The following events are considered.

$C \xrightarrow{aI} C^*$	excitation
$C^* \xrightarrow{k_f} C + h\nu$	fluorescence from pigment bed
$C^* \xrightarrow{k_h} C +$ heat	heat from pigment bed
$e^* \xrightarrow{kE_{in}} e$	activation reaction
$e^* \xrightarrow{kE} e + 1/4\ O_2$	Photoreaction II
$e^* \xrightarrow{k_{fc}EH} e + h\nu$	fluorescence from reaction centers
$e^* \xrightarrow{k_{hc}EH} e +$ heat	heat from reaction centers

E_{in}, E, and EH are the fractions of e_t which are inactive, open and closed, respectively. C and e represent unexcited molecules of the pigment bed and reaction centers, respectively.

The approximation $dC^*/dt = 0$ may be used for intervals long compared to the lifetime of the excited state (estimated at about 1 nsec). Accordingly, for purposes of this analysis the formation and dissipation of excited states are assumed to occur at equal rates. The resulting equation describes the proposed model,

$$aI = k_f C^* + k_h C^* + kE_{in}K_t C^* + kEK_t C^* + k_{fc}EHK_t C^* + k_{hc}EHK_t C^* \quad (2)$$

$$v = kEK_t C^* = \text{rate of Photoreaction II } (O_2 \text{ evolution}) \quad (3)$$

$$F = k_f C^* + k_{fc}EHK_t C^* = \text{rate of fluorescence emission} \quad (4)$$

The expressions for fluorescence and Photoreaction II may be rewritten in the form introduced by JOLIOT[25,30]. Then

$$v = c_1 aIkK_t \left(\frac{E}{1 - pEH}\right) \quad (5)$$

and

$$F = c_1 aI \left(\frac{k_f + k_{fc}K_t EH}{1 - pEH}\right) \quad (6)$$

where

$$c_1 = \frac{1}{kK_t + k_h + k_f} \quad (7)$$

and

$$p = \frac{K_t(k - k_{fc} - k_{hc})}{kK_t + k_h + k_f} \quad (8)$$

The transformed expressions are useful since the average value of p has been experimentally estimated to be 0.55 (see refs. 25 and 32).

The slow parallel increase in fluorescence and O_2 evolution in the transition to

the light 1 state (with E = 1) shown in Figs. 8, 10–12 does not permit the total emission to be regarded as a variable part from closed reaction centers and an invariant part from chlorophyll molecules not associated with reaction centers. Further, the ratio of $\phi_{f\max}/\phi_{f\min}$ of 3.8 (Fig. 13) and JOLIOT's estimate of 0.55 for p of Eqn. 1 are inconsistent with the pigment bed acting as the sole source of emission. If k_{fc} and k_{hc} of Eqn. 8 are taken equal to zero (no fluorescence or radiationless de-excitation from closed reaction centers) then the resulting expression for p describes the maximum O_2 yield,

$$p = \frac{K_t k}{kK_t + k_h + k_f} = \phi_{II\max} \tag{9}$$

The quantum yield for Photoreaction II must be greater than the estimated value of $p = 0.55$ to account for measured quantum yields of O_2 evolution (8–12 quanta per O_2). Similarly, if $p = 0.55$ and is expressed by Eqn. 9, the ratio of maximum to minimum fluorescence which is given by

$$\frac{\phi_{f\max}}{\phi_{f\min}} = \frac{kK_t + k_h + k_f}{k_f + k_h}$$

can be no larger than 2.2, and larger values are observed.

The "hybridization" of models which is proposed here makes possible an understanding of observed ratios of $\phi_{f\max}/\phi_{f\min}$ in light of the estimated value of p, predicts value of about 0.74 for $\phi_{II\max}$, and makes further quantitative analysis possible.

The model's predictions of changes in yields of fluorescence and O_2 evolution *vs.* E and EH are shown in Fig. 14. The model predicts that fast changes, which quickly change the state of the reaction centers, should proceed along the curves for fixed α/α_{max} and result in complementary changes in fluorescence and O_2 evolution. This is experimentally observed (Figs. 10–12).

Variable α: An explanation of slow changes in fluorescence and O_2 evolution

Fig. 14 shows values of α/α_{max} from 0.6 to 1.0. The slow changes shown in Figs. 10–12 may be quantitatively accounted for in terms of transitions between the different α/α_{max} curves of Fig. 14. Specifically, in the transition to the light 1 state in Fig. 12 both the O_2 and fluorescence changes may be described by a transition from the curve for α/α_{max} of 0.91 to the curve for α/α_{max} of 1.00. Values of E after removal of light 1 background may be calculated from the value of the subsequent fluorescence maximum or O_2 minimum by considering α/α_{max} fixed during the fast changes. The values of E after removal of light 1 background agree within 5 % when calculated independently from the O_2 or fluorescence measurements of Fig. 12. An alternate statement in support of the proposed variability of α/α_{max} is that the fluorescence peaks of Fig. 12 predicted from the O_2 rate measurements differ from the peak observed by less than 2 %[34].

A reversible change from 1.0 to 0.9 in α/α_{max} was invoked to account for slow wavelength-induced changes in oxygen evolution and fluorescence observed at low intensity. Although we can present no experimental test of the proposal, the differences between initial and steady-state yield measurements (Fig. 13) can also be accounted for in terms of the model with greater variation of α/α_{max}. The calculated values of intensity-induced variations in α/α_{max} are plotted *vs.* ϕ_{O_2} in Fig. 9B. The calculated

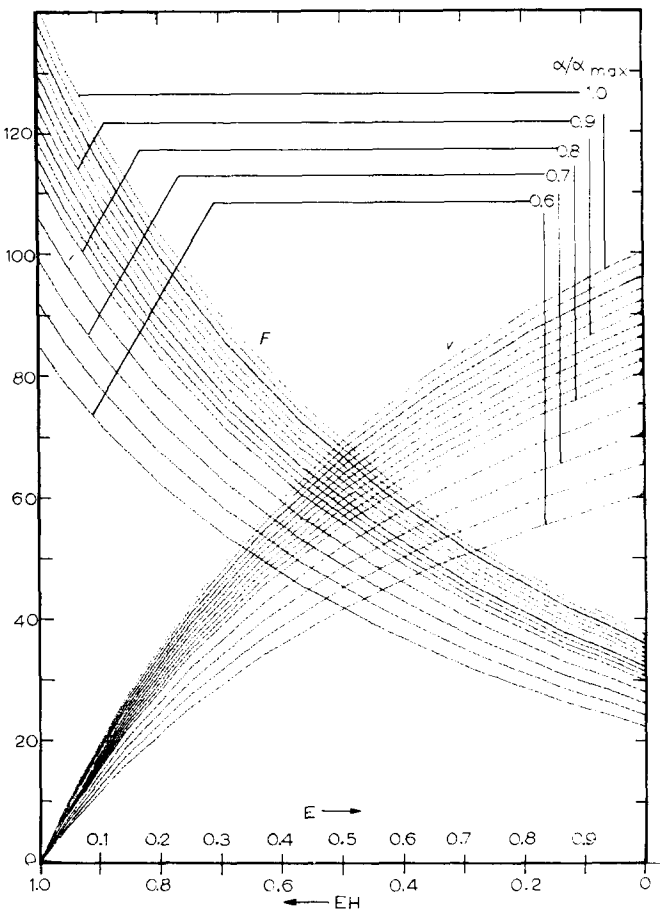


Fig. 14. Calculated yields of photoreaction II (v) and fluorescence (F) *vs.* fraction of system 2 traps open (E) or closed (EH) with values of α/α_{max} from 0.6 to 1.0. The functions plotted are

$$v = (a/a_{max})\ (100)\left(\frac{E}{1 - 0.55\ EH}\right)$$

and

$$F = (a/a_{max})\ (37.5)\left(\frac{1 + 0.71\ EH}{1 - 0.55\ EH}\right)$$

See text for details.

values plotted in Fig. 9B do not constitute a test of the proposed variability of α/α_{max} but are presented to illustrate a consequence of the proposal.

Fig. 9A shows that a light 1 background causes a higher fluorescence yield than a light 2 background when the backgrounds are matched to induce the same rate of O_2 evolution in response to the chopped light 2 beam. The proposed variability in α can account for this by ascribing a higher α and lower concentration of open reaction centers to the steady-state condition induced by the light 1 background. The prediction of lower E with the light 1 background can be checked experimentally. A low intensity chopped beam (as 645 mμ) can be combined with light 1 or light 2 backgrounds to give equal steady-state O_2 yields. The fluorescence yield observed

with light 1 background will exceed the fluorescence yield observed with light 2 background (Fig. 9A). A measure of the E concentration can then be accomplished in the manner of JOLIOT AND JOLIOT[32]. A single, brief (0.1 msec), and saturating flash produces an O_2 yield which is considered to be proportional to the concentration of open reaction centers (E). Preliminary experiments done in this way support the hypothesis of variable α. The flash yield we observed with light 1 background was lower than the flash yield with light 2 background in the special case where the backgrounds induced equal rates of O_2 evolution in response to the chopped beam.

The proposed variation of α/α_{max} has been shown to account operationally for the slow changes observed. Other characteristics of the photosynthetic apparatus which could conceivably give rise to slow changes in the energy conversion process have been considered. Among those considered which cannot account for the slow changes are (a) a nonfluorescent and inactive Q′ form as proposed by DUYSENS AND SWEERS[11], (b) variation in the equilibrium constant between the System 1 and 2 reaction centers, and (c) variation in the relative turnover rates of the two photoreactions. Some special simultaneous variations of several parameters might give rise to the observed slow changes. The parameter α is the only single parameter considered which can account for them.

We call attention to two important consequences of our hypothesis: (1) since darkness induces the salient features of the light 1 state (Fig. 8), the α change in adaptation to the light 2 state is expected to make an important contribution to the slow events generally observed in induction; (2) if, indeed, the light 2 state is developed at high light intensities (Fig. 9), then the more severely decreased α provides the same kind of protection against high light intensity which DUYSENS AND SWEERS[11] attributed to their Q′. That α/α_{max} should be both intensity and wavelength dependent is understandable provided that it is controlled by the extent of reduction of electron transport intermediates or some concomitant variable.

There is no obvious and simple explanation of a physical mechanism which would induce changes in α, *i.e.* changes in distribution of absorbed quanta to the two photosystems. However, the following observations may be pertinent.

The lamellar chloroplast structure has been shown to shrink reversibly or to swell in response to changes in either wavelength or intensity of illumination[35,36]. NOBEL[37] recently reported that illumination of intact pea plants prior to chloroplast isolation resulted in a decrease of chloroplast volume and an increase in rate of photophosphorylation over that seen without pre-illumination. Half-times for the changes in volume and phosphorylation (2–5 min) are similar to those of the slow changes reported here. It is suggested that such conformational changes may control the distribution of quanta to the two systems by altering the proximity of pigments to reaction centers.

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