LIGHT-INDUCED CHANGES IN THE FLUORESCENCE YIELD OF CHLOROPHYLL *a* IN VIVO

II. CHLORELLA PYRENOIDOSA

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ABSTRACT The long-term fluorescence induction in *Chlorella pyrenoidosa* consists of a fast rise of the fluorescence yield from the level S (of the first wave transient) to a maximum M, followed by slower decay to a terminal stationary level T. The maximum M is attained within 40 seconds from the onset of illumination while the decay to the terminal level T lasts for several minutes. The fluorescence rise ($S \rightarrow M$) coincides with an increase in the rate of oxygen evolution, which, however, remains constant during the fluorescence decay ($M \rightarrow T$). Poisons of photosynthesis 3,(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU, *o*-phenathroline) inhibit the fluorescence induction, while uncouplers of photophosphorylation affect the fluorescence time course only when they function at an early stage of the coupling sequence e.g., carbonyl cyanide *p*-trifluoremethoxy phenylhydrazone, (FCCP, atabrin). Phosphorylation inhibitors affecting only the terminal esterification step (phlorizin) have little effect on the fluorescence kinetics. These results suggest that the fluorescence induction requires the operation of a phosphorylating electron transport and that it is possibly related to the light-induced structural changes which accompany photophosphorylation.

INTRODUCTION

The long-term fluorescence induction in *Chlorella pyrenoidosa* (second wave of fluorescence) consists of a rise of the Chl *a* fluorescence yield from the level S (end of the first wave) to a maximum M, followed by a slower decay toward a terminal stationary level T. The $S \rightarrow M \rightarrow T$ change lasts for several minutes and it is reproducible provided that a sufficiently long dark adaptation is interposed between consecutive light periods. In this paper we shall discuss only events that occur after the completion of the first wave.

The induction of Chl *a* fluorescence in *Chlorella* has been studied extensively in the past following Kautsky's (1) original observation of the phenomenon. Comprehensive reviews on the subject have been published by Wassink (2) and Rabino-

witch (3). The correlation of the fluorescence induction with the photosynthetic production of ATP in *Chlorella* was first suggested by Strehler (4) on the basis of the similarity of their time courses. In the accompanying paper (5) we presented evidence that the long-term fluorescence induction in *Anacystis* requires electron transport coupled to phosphorylation. A similar requirement exists for the fluorescence induction in *Chlorella*. Compared to *Anacystis* the kinetics of fluorescence in *Chlorella* appear more complicated, including not only light-induced but light-triggered processes as well.

EXPERIMENTAL

Chlorella pyrenoidosa was grown in inorganic medium over low intensity white light as described by Govindjee and Rabinowitch (6). Samples were prepared by resuspending 3–6 day old cultures in a medium made of 0.02 M tris(hydroxy-methyl)-amino methane and 0.04 M NaCl buffered at pH 8.0. The optical density of the suspension at the red Chl *a* absorption maximum was adjusted to 0.5 for one cm light path. The total chlorophyll content, measured according to McKinney (7) was 18 μg/ml (approximately 2×10^{-5} M). Various additions to the samples were made at least 15 min prior to the fluorescence measurements and a dark adaptation interval of 15 min preceded the recording of the time course of fluorescence.

Optical measurements (absorption and fluorescence) and oxygen evolution measurements were carried out as described in the accompanying paper (5). The time courses of the Chl *a* fluorescence yield are presented after normalization to unity at the *S* level (3 sec of illumination). The fluorescence spectra (fluorescence intensity in quanta per spectral interval) have been corrected for the spectral variation of the photomultiplier sensitivity and the transmission efficiency of the analyzing monochromator. Since only grating monochromators at fixed bandwidths have been used no correction for the bandwidth of observation (8) was required. Also, no correction was made for the "outer" reabsorption and reemission of fluorescence; it is minimal because of the low optical density of our samples (0.05 for 0.1 cm path length in the sample holder) and the "front-face" collection of fluorescence in our instrument (for justification see references 9 and 10). Reabsorption of fluorescence within the individual cell was also not corrected for.

All measurements were carried out at room temperature, 22–25°C. Further experimental details are given in the legends of the figures.

EXPERIMENTAL RESULTS

Fluorescence Induction in Continuous and Flashing Light

Continuous illumination of dark adapted *Chlorella pyrenoidosa* results in a rise of the relative fluorescence yield $f(F_t:F_s'')$ from the initial level *S* to a maximum *M*, within approximately 40 sec. The $S \rightarrow M$ rise is succeeded by a slower decay lasting for several minutes toward a stationary level *T* (Fig. 1, curve *W*). At that stage, the fluorescence yield is considerably lower than the minimum between the first and the second waves of fluorescence induction (*S* level).

The second wave is reproducible provided that an adequate dark adaptation precedes each light exposure. This observation led us to investigate the decay of the fluorescence yield during a dark interval which follows a light period by monitoring

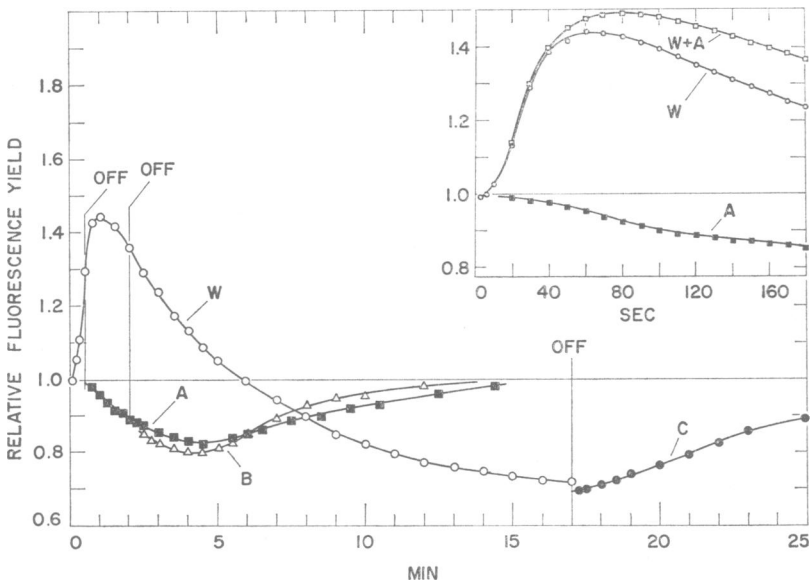

FIGURE 1 Time course of the relative fluorescence yield in continuous and in flashing light in *Chlorella*. In continuous light (*W*); in the dark, measured with light flashes (*A*, *B*, and *C*). Excitation: blue band, Corning filter, C.S. 5–56; half bandwidth, 130 nm; incident intensity, 2.5×10^3 ergs $cm^{-2}sec^{-1}$. Observation: $\lambda = 685$ nm; half bandwidth, 16.5 nm; Corning filter, C.S. 2–60. Insert: Data up to 3 min drawn on an expanded time scale (*W* and *A*); fluorescence induction in the absence of dark changes ($W + A$).

the fluorescence yield with short (2–3 sec) light flashes. The possibility of distortions introduced by the brief light interruptions was examined by varying both the intensity of the flash and the dark spacing between consecutive flashes. Nearly identical fluorescence time courses were obtained in each case, indicating that the dark kinetics of the fluorescence yield are not significantly affected by this procedure.

The time course of the fluorescence yield during a dark interval is a function of the length of the preceding light exposure. Very short flashes given after a long dark adaptation have no influence on the magnitude of the relative fluorescence yield. Shutting off the excitation after 20 sec of illumination (i.e. during the fast rising phase of the second wave) results in an abrupt drop of the fluorescence yield followed by a slower decay to a minimum after which the fluorescence rises slowly approaching the *S* level (Fig. 1, curve *A*). Similar results are obtained when the second wave is interrupted in the decay portion $M \rightarrow T$ (Fig. 1, curve *B*). Since curves *A* and *B* are almost super-imposed, we conclude that a short light exposure (e.g. 20 sec as in *A*) is sufficient to trigger a light independent process which lowers the fluorescence yield. The light-triggered process must be opposed by a second dark change as a result of which the fluorescence yield attains a minimum value in the dark. When

the light excitation is removed at the end of the second wave, the dark change consists of an increase only of the fluorescence yield toward the *S* level (Fig. 1, curve *C*).

These results indicate that the second wave in *Chlorella* originates from both light-dependent and light-triggered processes. It can be argued that the time course of fluorescence in *Chlorella* is the result of a light requiring process which increases the fluorescence yield and a light-triggered process having the opposite effect. This possibility was examined by plotting the sum $W + A$ (Fig. 1, insert) which corresponds to a second wave transient independent of the light-triggered process. The decay of the curve $W + A$ suggests that light not only supports the fluorescence rise but also the fluorescence decay. From the inspection of the time courses of Fig. 1 it appears that the light triggered process accounts only for the decrease of the fluorescence yield from *S* to *T*.

Our results, therefore, indicate that a minimum of four processes participate in the fluorescence induction of *Chlorella*. Two of them are light-dependent and have opposing effects on the fluorescence yield. A third process requires a minimum of light dose but not simultaneous excitation and it is opposed by a fourth dark process. The triggering of the dark induction is reminiscent of the postirradiation overshoot of the pH of the suspension medium (11) and of the light scattering increase in chloroplast preparations (12).

Changes in the Emission Spectrum During the Flouresence Induction

Contrary to our results with *Anacystis* (5, 13) no significant changes were observed in the emission spectrum of *Chlorella* during the fluorescence induction. Fig. 2 shows the emission spectra recorded at the *S*, *M*, and *T* stages of the second wave. The spectrum at *T* was recorded by automatic scanning while the spectra at *S* and *M* from the fluorescence induction curve observed at different wavelengths after an adequately long dark adaptation. All spectra in Fig. 2 are typical Chl *a* in vivo emission bands, peaked at 686 nm and having a half bandwidth of 22 nm. The same peak location and half bandwidth characterize the difference spectra *S-T*, *M-T*, and *M-S* given in the insert of Fig. 2. Apparently, the fluorescence induction is evenly distributed throughout the Chl *a* emission envelope.

Fluorescence Induction and the Intensity of Excitation

The fluorescence induction is light intensity dependent as illustrated in Fig. 3. Very weak excitation causes no appreciable change in the relative fluorescence yield. The fluorescence induction at weak light intensities is characterized by a biphasic rise which becomes monophasic as the intensity is increased. An *optimum* excitation intensity for the second wave amplitude is apparent in the time courses depicted in Fig. 3. A similar response to the intensity of excitation is exhibited by the rise and decay rates of the second wave.

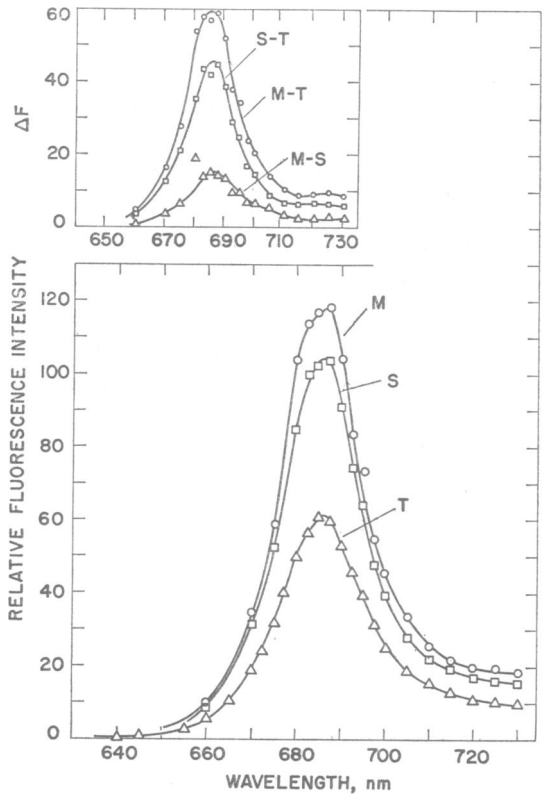

FIGURE 2 Emission spectra of *Chlorella* at 3 sec (*S*), 40 sec (*M*), and 12 min (*T*) of light exposure. Excitation: $\lambda = 480$ nm; half bandwidth, 10 nm; incident intensity, 1.4×10^{3} ergs cm^{-2} sec^{-1}. Observation: half bandwidth 6.6 nm; Corning filter, C.S. 2–63. Insert: Difference emission spectra (*M-S*; *M-T*; *S-T*).

Since the individual processes participating in the second wave cannot be studied separately we chose to examine only the effect of the intensity of excitation on the terminal change $f_3'' - f_T$ (i.e., the difference in the relative fluorescence yields at the states *S* and *T*). In Fig. 4, $f_3'' - f_T$ is plotted against the incident excitation intensity (curve *A*) together with the stationary state fluorescence intensities (light curve of fluorescence; curve *B*). The latter can serve as an indicator of the light saturation of the rate of the photosynthetic electron transport. In the region of saturating excitation (beyond the break point of the fluorescence light curve), $f_3'' - f_T$ declines so that the light curve shows an intensity *optimum*.

The *optimum* intensity relationships described above resemble similar observations on the second wave rise (14) and of the steady-state ATP content in *Chlorella* (4). It appears, therefore, that a low terminal fluorescence yield (f_T) is related to an increased concentration of ATP or an immediate precursor of it. It is also significant that $f_3'' - f_T$ is variable even in the range of excitation intensities where photosynthesis is saturated.

The high intensities of excitation needed for these experiments were achieved through a wide blue band (Corning glass filter C.S. 5–60). Narrow band (half band-

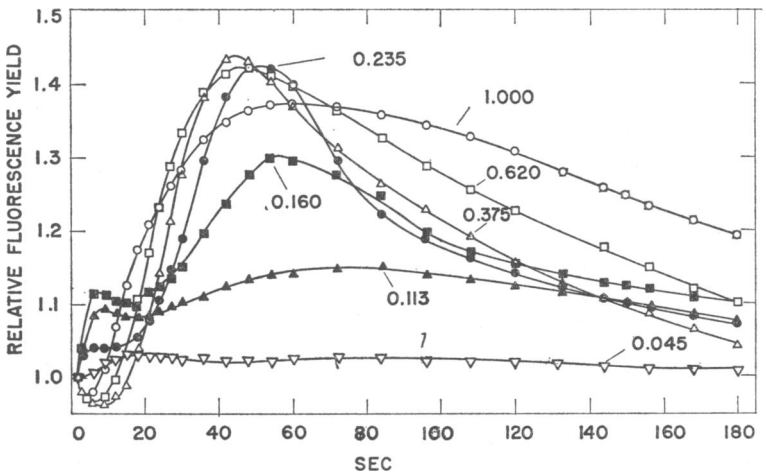

FIGURE 3 Time course of the relative fluorescence yield ($f = F_t:F_3''$) at different exciting intensities. Intensity (indicated on the graph), 1.00 = 6.6×10^5 ergs $cm^{-2}sec^{-1}$; Excitation: blue band, Corning filter, C.S. 5–60; half bandwidth, 100 nm. Observation: λ = 685 nm; half bandwidth, 16.5 nm; Corning filter, C.S. 2–60.

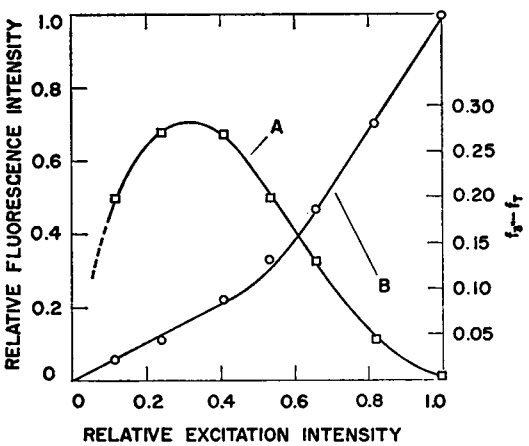

FIGURE 4 The fluorescence change $f_3'' - f_T(A)$ and the fluorescence intensity at $T(B)$ as a function of the incident light intensity in *Chlorella*. Intensity 1.00 $\equiv$ 25.5×10^5 ergs cm^{-2} sec^{-1}. Excitation: blue band, Corning filter, C.S. 4–72; half bandwidth, 150 nm; Observation: λ = 685 nm; half bandwidth, 16.5 nm; Corning filter, C.S. 2–60.

width 10 nm) excitations at 436 nm and 480 nm gave similar results as the polychromatic one, although the correspondence was limited to the range of low intensities. To answer the question whether both system I and system II can support the second wave change, we constructed the action spectrum of the terminal change ($f_3'' - f_T$) observed at 720 nm (Fig. 5). Since $f_3'' - f_T$ is not linearly proportional to the intensity of excitation we maintained the latter constant at each wavelength. This was achieved by aligning the two monochromators and replacing the sample by a reflector so that we could use the photomultiplier signal (corrected for spectral sensitivity) as a measure of the excitation intensity. By regulating the current through the light source the excitation could be adjusted to a constant intensity.

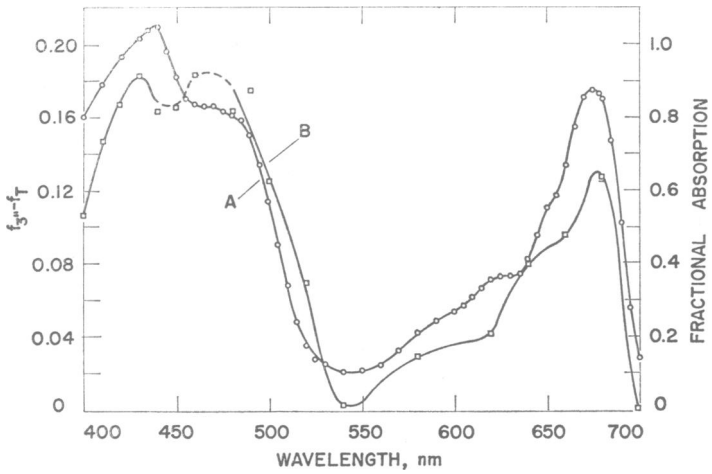

FIGURE 5 Fractional absorption spectrum (*A*) and the action spectrum of the change $f_3'' - f_T(B)$ in *Chlorella*. Excitation: half bandwidth, 6.6 nm. Observation: λ = 720 nm; half bandwidth, 10 nm; Corning filter, C.S. 7–69.

The action spectrum (together with the fractional absorption spectrum of *Chlorella*) shown in Fig. 5 indicates that the second wave can be excited in the entire blue absorption band of *Chlorella*. The effective overlap of the absorption bands of system I and system II does not allow for a clear differentiation of their influence on the induction. However, the sharp drop of the effectiveness at wavelengths longer than 680 nm (where the red absorption band of system I is presumably located) may indicate the necessity of system II activation of the induction. This is also supported by the fact that the ratio of the fractional absorption at 480 nm (mainly Chl *b*) to that at 440 nm (mainly Chl *a*) is 0.76, while the same ratio in the action spectrum is approximately 1.0 suggesting increased effectiveness of Chl *b* (mainly system II).

Fluorescence Induction and the Rate of Oxygen Evolution

The possibility of an inverse relationship between the fluorescence yield and the rate of oxygen evolution was examined by recording the time courses of the rate of oxygen evolution and fluorescence in two identically prepared samples. The samples were prepared from the same stock culture and they were subjected to identical light conditions. A stream of 5% CO_2 in air was passed through the electrolyte medium in the polarograph but not through the suspension in the spectrofluorometer.

Fig. 6 shows that the rate of oxygen evolution and the fluorescence yield increase is parallel during the rising phase of the second wave. With continuing illumination the rate of photosynthesis tends to a high sationary level while the fluorescence yield experiences a typical second wave decay. This decay proceeds even after the rate of oxygen evolution has attained a constant value. The absence of complimentarity between oxygen evolution and fluorescence during the second wave rise has been con-

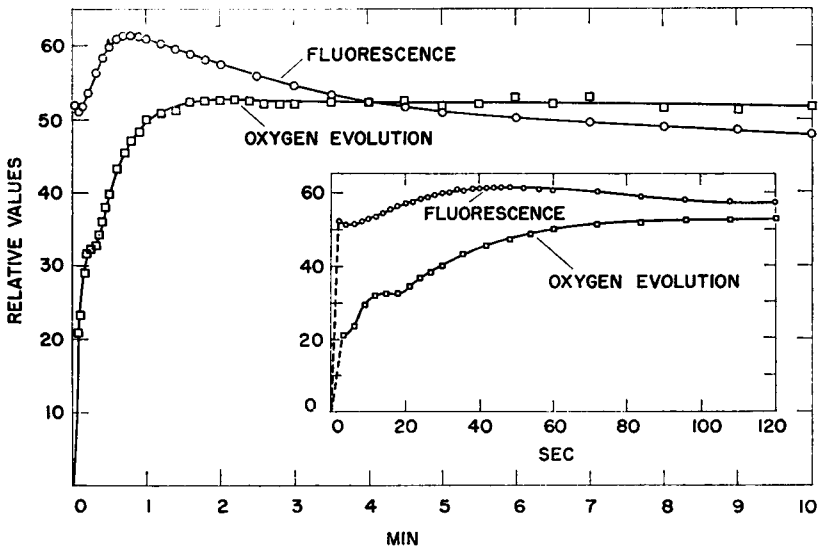

FIGURE 6 Time course of the fluorescence intensity and of the rate of oxygen evolution in *Chlorella*. Excitation: λ = 480 nm; half bandwidth, 16.5 nm; incident intensity, 3.1 $\times$ 10^3 ergs $cm^{-2}sec^{-1}$. Observation: λ = 685 nm; half bandwidth, 16.5 nm; Corning filter, C.S. 2–60. Insert: Data up to two min on an expanded time scale.

firmed recently by Bannister and Rice for *Chlamydomonas reinhardi* and *Chlorella pyrenoidosa*.[1]

Effects of Inhibitors of Photosynthesis and of Uncouplers of Photosynthesis on the Fluorescence Induction

The action spectrum of the fluorescence induction in *Chlorella* (Fig. 5) suggests an increased effectiveness of system II and consequently a possible dependence of the induction on the photosynthesis electron transport. Additional evidence is provided by the inhibition of photosynthesis with DCMU and *o*-phenanthroline which also obliterate the second wave change (Fig. 7). In the presence of these poisons the relative fluorescence yield rises slowly to a stationary level. The absolute magnitude of this change is much smaller than that observed with photosynthesizing *Chlorella*, and it is too slow to be accounted for by the accumulation of the reduced form of the primary system II oxidant.

The fluorescence induction kinetics are also sensitive to the presence of uncouplers of photophosphorylation, suggesting a dependence of the phenomenon on the phosphorylating electron transport. Addition of FCCP results in retardation of the second wave decay (Fig. 8). Thus, the relative fluorescence yield of the control sample at the end of the induction is significantly lower (~40%) than the yield of the

[1] T. T. Bannister and G. Rice. Submitted for publication.

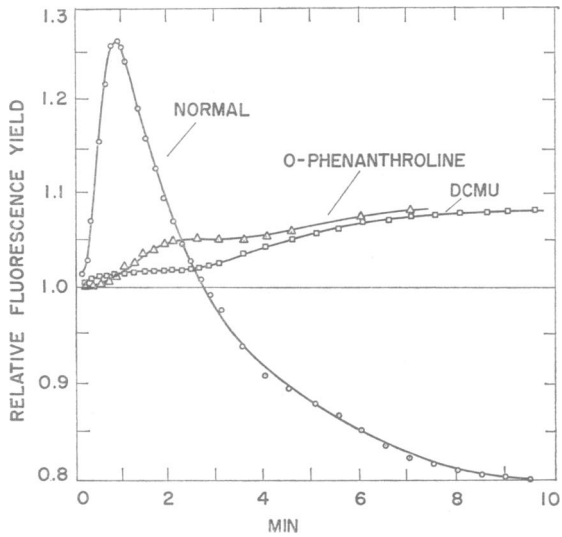

FIGURE 7 Time course of the relative fluorescence yield ($f = F_t:F_3''$) in *Chlorella*. Control; with DCMU, 5×10^{-5} M; with *o*-phenanthroline, 5×10^{-4} M. Excitation: $\lambda = 436$ nm; half bandwidth, 16.5 nm; incident intensity, 2.1×10^3 ergs $cm^{-2}sec^{-1}$. Observation: $\lambda = 685$ nm; half bandwidth, 16.5 nm; Corning filter, C.S. 2–60.

FCCP containing one. On addition of 10^{-3} M cysteine, the FCCP inhibition is removed and the second wave change is reinstated (Fig. 8). Half-maximal inhibition of the second wave decay requires a FCCP concentration of 4.5×10^{-6} M, close to 1×10^{-6} M required for half-maximal inhibition of the photophosphorylation in chloro-

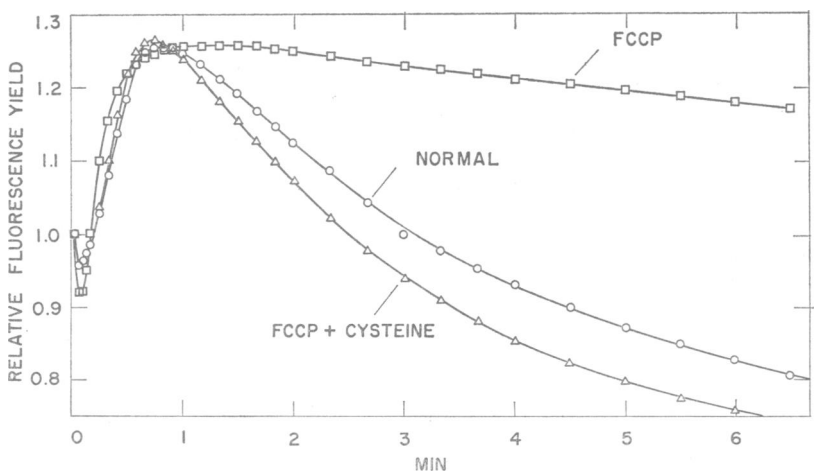

FIGURE 8 Time course of the relative fluorescence yield ($f = F_t:F_3''$) in *Chlorella*. Control; with 10^{-5}M FCCP; with 3×10^{-5}M FCCP, and 10^{-3}M cysteine. Excitation: $\lambda = 480$ nm; half bandwidth, 10 nm; incident intensity, 1.4×10^3 ergs $cm^{-2}sec^{-1}$. Observation: $\lambda = 685$ nm; half bandwidth, 11.6 nm; Corning filter, C.S. 2–60. (Additions were made at least 30 min prior to illumination.)

plast preparations (15). The fluorescence time course in the presence of FCCP is reproducible in consecutive light and dark cycles.

Inhibition of the second wave decay with FCCP is particularly severe at low light intensities, as it is also the inhibition of chloroplast photophosphorylation (16). This is illustrated in Fig. 9 (left), where the relative fluorescence yield at 4 min illumination (f_4') is plotted against the incident exciting intensity. Very low light intensities cannot support the second wave rise so that the f_4' value is low. At high light intensities, the low f_4 values indicate an accelerated decay of the second wave and consequently a reduced inhibition by FCCP. Finally, the emission spectra of the control and the

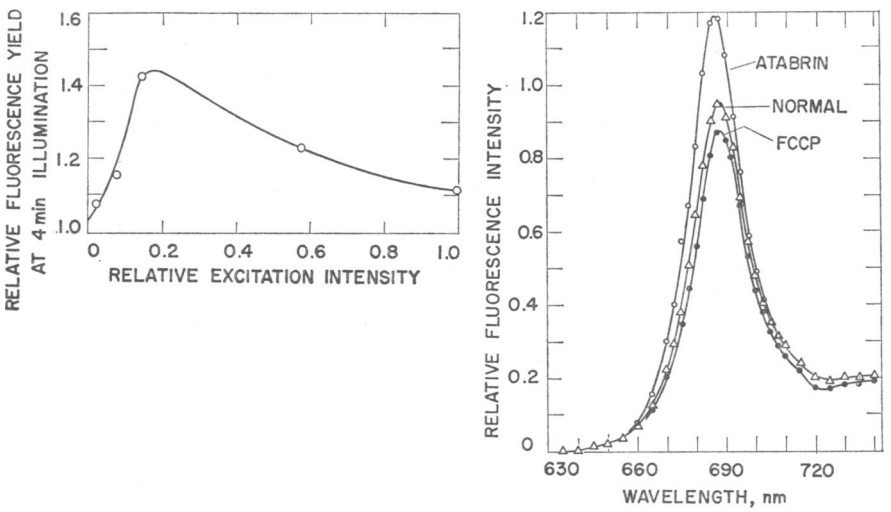

FIGURE 9 Left: Relative fluorescence yield at 4 min of light exposure in FCCP (10^{-5} M) treated *Chlorella* as a function of the incident light intensity. Intensity 1.00 ≡ 9.4×10^3 ergs $cm^{-2}sec^{-1}$. Excitation: blue band, Corning filter, C.S. 5–56; half bandwidth, 130 nm. Observation: λ = 685 nm; half bandwidth, 10 nm; Corning filter, C.S. 2–60. Right: Emission spectra of *Chlorella*. Control; with 10^{-5} M FCCP; with 3×10^{-5} atabrin. Excitation: λ = 485 nm; half bandwidth, 10 nm; incident intensity, 1.4×10^3 ergs $cm^{-2}sec^{-1}$. Observation: half bandwidth, 6.6 nm; Corning filter, C.S. 3–69.

FCCP-treated samples recorded at the stationary states are nearly identical (Fig. 9, right).

Addition of the uncoupler atabrin eliminates almost completely the fluorescence induction in *Chlorella* (Fig. 10). A small, slow increase by about 5% occurs which is reversed in a subsequent dark period. Low atabrin concentrations ($\ll 10^{-6}$ M) result in a second wave transient of smaller amplitude and of higher terminal stationary level as compared to normal samples. The stationary state emission spectrum of *Chlorella* treated with atabrin is nearly identical to the spectrum of untreated cells (Fig. 9, right).

Contrary to the effects of FCCP and atabrin, the phosphorylase inhibitor phlorizin does not prevent the fluorescence induction (Fig. 10). Phlorizin accelerates both the

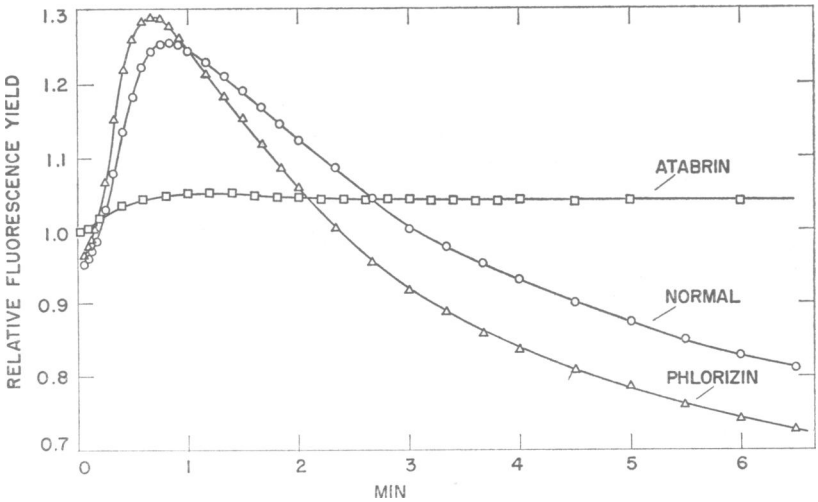

FIGURE 10 Time course of the relative fluorescence yield ($f = F_i:F_s''$) in *Chlorella*. Control; with 3×10^{-6}M atabrin; with 2×10^{-3}M phlorizin. Excitation: $\lambda = 480$ nm; half bandwidth, 10 nm; incident intensity, 1.4×10^3 ergs $cm^{-2}sec^{-1}$. Observation: $\lambda = 685$ nm; half bandwidth, 11.6 nm; Corning filter, C.S. 2-60.

rise and the decay of the second wave. It appears, therefore, that the fluorescence induction is affected only when the inhibition of the photophosphorylation takes place at an early stage of the coupling mechanism.

DISCUSSION

The participation of nonphotochemical components in the second wave of fluorescence induction in *Chlorella pyrenoidosa* rules out its complete dependence on the rate of the photosynthetic electron transport. Support for this argument is provided by the absence of complimentarity in the time courses of fluorescence and the rate of oxygen evolution as it is required on the basis of direct competition between fluorescence and photochemistry. In addition, strong excitation has an effect on the magnitude of the fluorescence induction although the rate of the photosynthetic electron transport is constant.

The fluorescence induction, however, is not completely independent of the photosynthetic electron flow since both processes are inhibited in the presence of DCMU and *o*-phenanthroline and since system I excitation ($\lambda > 680$ nm) does not support it. It appears, therefore, that the photosynthetic electron transport affects the fluorescence yield by an indirect process which is in all likelihood related to the phosphorylation coupling mechanism.

Powerful uncouplers of photophosphorylation, such as FCCP and atabrin, exert a prominent influence on the second wave kinetics. FCCP, which functions near the electron transport chain, delays the second wave decay while atabrin, which may uncouple at a later stage, inhibits the second wave completely. The inhibition by FCCP is light intensity dependent, being less pronounced at high light intensities.

Bannister (17) reported that FCCP prevents both the rise and the decay of the second wave in *Chlorella* which is not in accordance with our observations. It is possible that the discrepancy originates from the greatly different excitation intensities employed by Bannister ($\sim 50 \times 10^3$ ergs cm^{-2} sec^{-1}) and by us ($\sim 5 \times 10^3$ ergs cm^{-2} sec^{-1}).

Inhibition of the ADP phosphorylation by the phosphorylase inhibitor phlorizin does not affect the second wave except for an acceleration of the rise and decay phases. These results appear to "localize" the origin of the fluorescence transient at a site between the noncyclic electron pathway and the terminal phosphorylation step. Accordingly, the second wave may indeed be related to the accumulation of the phosphorylation potential X_E (18) and the conformational changes of the lamella associated with it (19). This interpretation correlates the low terminal fluorescence yield to an increased content of X_E. A relationship between the second wave in *Chlorella* and the light driven ATP synthesis has been suggested also by Strehler (4) on the basis of the similarity of their respective kinetics.

The parallel increase of the fluorescence yield and the rate of oxygen evolution, in the time interval of the induction, were interpreted by Bannister and Rice[1] as a light activation of Chl *a*. Phenomenologically, this activation appears as a conversion of a nonphotoactive and nonfluorescent portion of Chl *a* to the photoactive and fluorescent form. In a kinetic interpretation, the activation is equivalent to a reduction of the rate of internal conversion and a concomitant increase in the rate of photochemistry and fluorescence (9).

A modification of the arrangement of the Chl *a* molecules imposed by the variable conformation of the supporting lamella, may in principle alter the fluorescence yield. For example, shrinkage or distension of the quantasome may form or dissociate nonfluorescing Chl *a* aggregates which drain the electronic excitation from the photosynthetic unit. Changes also will occur in the rate of the resonance migration of the excitation to the reaction center, which will affect the thermal quenching via the nonfluorescent species.

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