Fluorescence Studies With Algae: Changes With Time and Preillumination

GOVINDJEE, JOHN C. MUNDAY, JR., AND GEORGE PAPAGEORGIOU
Department of Botany, and Biophysics Division of Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

Before the discovery of the two light reactions in photosynthesis,[1] pioneer studies in fluorescence induction had been made by Kautsky et al.,[2] Wassink et al.,[3] Franck et al.,[4] and MacAlister and Myers.[5] These investigations have been reviewed at length by Rabinowitch.[6] Several similar studies have been made recently.[7-14] We have reinvestigated some aspects of the fluorescence induction phenomenon (also known as the Kautsky effect) in the millisecond-to-second range and have also followed the slower changes in fluorescence up to one or two hours.

A. SHORT-TIME CHANGES

Our studies of induction are on *Chlorella pyrenoidosa*. After a dark period of a few minutes, fluorescence excited by a wide band (128 mμ) of blue light (maximum at 500 mμ; half-maxima at 558 and 410 mμ) of high intensity (2.6×10^5 ergs cm^{-2} sec^{-1} or 6×10^{16} quanta cm^{-2} sec^{-1}) rises to a hump at 40 msec (O in Lavorel's curves[7]) and a peak at 250 msec (P), shows a second hump at 5 sec, and declines to a steady level (S) after 30 sec. The following experiments deal mainly with the influence of preillumination with far-red light on the shape of the induction curves.

The height of P relative to S is maximal after a 10-min dark period; a 2½-min dark period gives a half-maximum effect. We employ dark periods of 4½ min and light periods of ½ min in most of our experiments; with this protocol we can perform several experiments in a few hours.

The complexity of the steady state fluorescence spectrum has been established by various workers (see review by Duysens[15]). During the induction period, the fluorescence spectrum changes with time. Details have been reported by Lavorel,[8] Duysens and Sweers,[9] and Rosenberg et al.[10] Our experiments confirm their results; at P, the ratio of fluorescence at 685 mμ to that at 705 mμ is higher than at O or at S.

Lavorel[8] has shown that fluorescence yield (F/I) versus I is constant for the level O, whereas F/I for P increases to a saturation value with increasing I. Rosenberg et al.[10] have shown that the ratio of P to S is higher at high excitation intensities. If fluorescence at P contains relatively more 685-mμ light, the fluorescence yield ratios (R) of high to low* excitation intensities measured at 685 mμ are expected to be different at O, P, and S. By varying the intensity of exciting light with Balzer's neutral density filters, we have obtained light curves** of fluorescence at times O, P,

*The "high" incident intensity $\simeq2.6\times10^5$ ergs cm^{-2} sec^{-1}; the "low" incident intensity $\simeq0.2\times10^5$ ergs cm^{-2} sec^{-1}.

**The light curve of fluorescence is defined as $F=f(I)$, where F=fluorescence intensity and I=intensity of incident light.

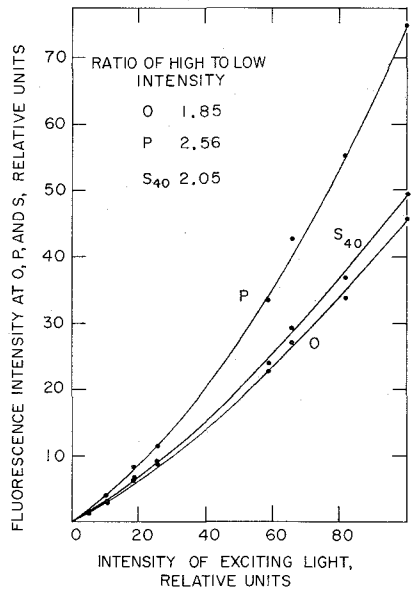

Figure 1. Fluorescence intensity at 685±4 mμ versus exciting light intensity during the induction period (*O*, *P*, and *S* levels) in *Chlorella pyrenoidosa*. Excitation by wide-band blue light. Temperature, 25°C. 5-day-old culture grown in inorganic medium under 16 hr light and 8 hr darkness. 5% CO_2 in air. Cells harvested after 3 hr of light, left in growth medium with added $NaHCO_3$, 0.03 g/2 ml. Absorbance ≃0.04 at 680 mμ for 1.0-mm path length.

and *S* (Figure 1). In this experiment, we found a maximum *R* at *P* (2.56); at *O* it was 1.85, and for *S* at 40 sec it was 2.05, which in general confirms the results cited above.

The Far-Red Light Effect

In 1960, we first observed the quenching of fluorescence in *Chlorella* by far-red light.[11] Later, Butler[12] and Duysens and Sweers[9] found antagonistic effects, on the yield of chlorophyll *a* fluorescence, of light absorbed in systems II and I. Pickett and Myers[13] recently suggested that the quenching effect of light I on fluorescence of chlorophyll excited by light II relates not to the steady state but to the induction period. In an independent study, we have found the following results. Preillumination (for 4½ min) with far-red light* (substituted for the usual period of darkness) followed by <1 sec of darkness delays the increase of fluorescence to *O* and to *P*; the delayed peak *P* is decreased in height; however, the level *S* is increased (Figures 2*A* and 2*B*). An additional bump, which we call *N*, appears at 40 msec. Further, we note that after a subsequent dark period, the *O*, *P*, and *S* levels are all higher than

*Far-red light was obtained by filtering white light with a Schott RG-9 filter (10^{-3} transmission at 700 mμ, 0.87 at peak at 820 mμ, and 0.40 at 1000 mμ; over-all incident intensity ≃3.2 $\times 10^5$ ergs cm^{-2} sec^{-1}). The absorbed intensity was estimated to be about 500 ergs cm^{-2} sec^{-1} based on (i) percent absorption of *Chlorella* versus wavelength; (ii) light output by the lamp versus wavelength, and (iii) transmission characteristics of the RG-9 filter.

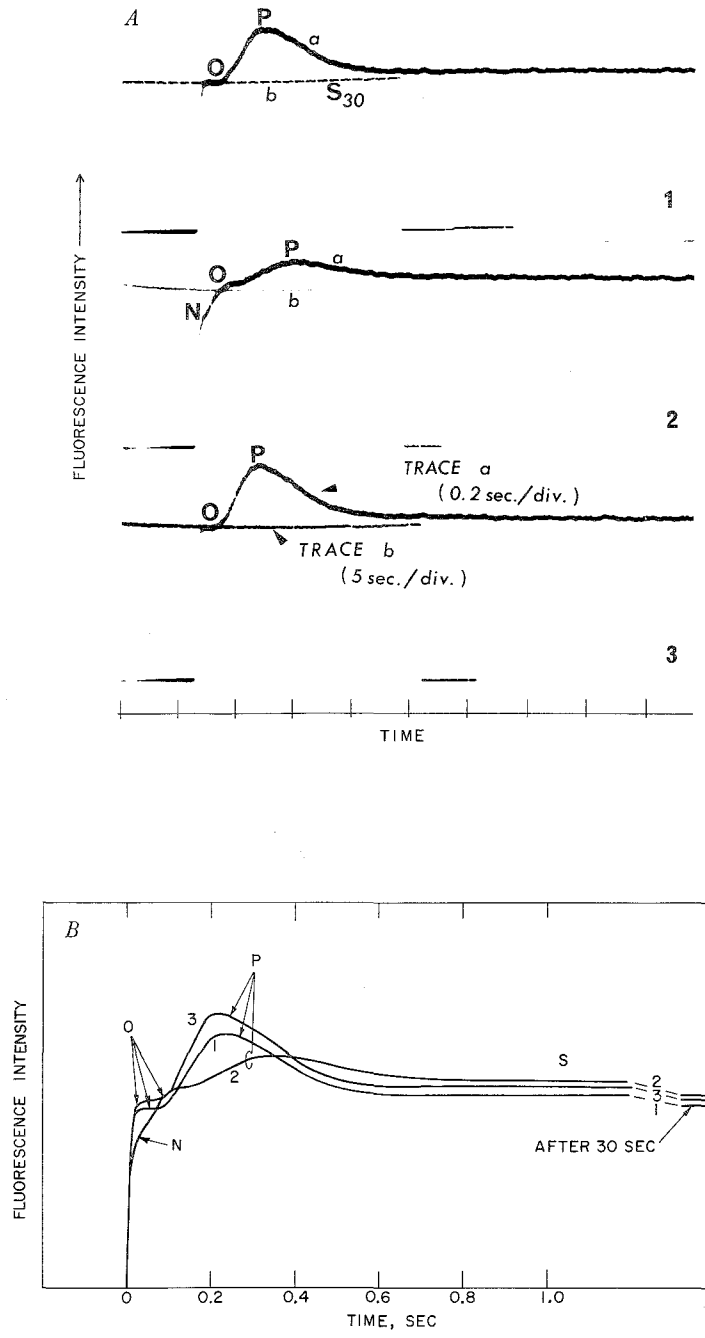

Figure 2. Fluorescence induction in *Chlorella pyrenoidosa*. Fluorescence intensity (at 685 mμ) excited by blue light was displayed on a Tektronix type 502A dual-beam oscilloscope and photographed with an oscillograph camera (model 1458 Cossor Instruments). *A*: (1) After 4½ min dark; (2) after 4½ min illumination by far-red light; (3) after subsequent 4½ min dark; *B*: superimposed drawings from the photographs.

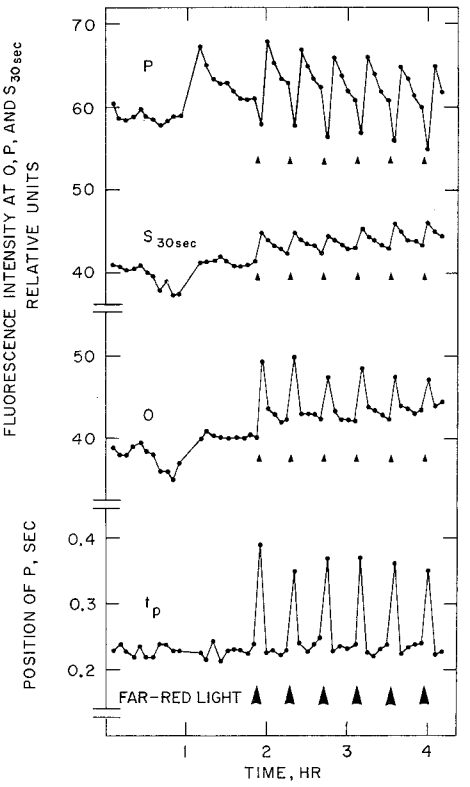

Figure 3. Effect of far-red preillumination on fluorescence induction in *Chlorella pyrenoidosa*. Levels at *O*, *P*, and *S* at 40 sec and time position of $P(t_p)$ versus time. 30 sec blue light alternated with 4½ min darkness or far-red light. Application of far-red light is indicated in the figure.

they were before the far-red exposure. This course of events can be repeated over and over (Figure 3), although, with the protocol employed, the raised levels gradually decline over a half-hour period.

The above results can be explained in a qualitative way by the "series" model of photosynthesis and also by another model that will be discussed in section C. Perhaps the "parallel" model could explain them too.

B. LONG-TIME CHANGES

Steady state fluorescence of photosynthetic systems has often been used for the investigation of energy transfer between pigment components. Our earlier work[16] on steady state fluorescence was concerned either with the fluorescence properties of pigment complexes prepared from chloroplasts[17–19] or with the fluorescence characteristics of cells exposed to high light intensity,[20] subjected to low temperatures[19,21,22] or to DCMU,[21] or grown in D_2O.[23] The quantitative evaluation of these experiments, however, must be carried out cautiously because of fluorescence changes over long periods of time. Time-course studies of fluorescence have been restricted to

short times after the onset of illumination. Furthermore, the changes in steady state fluorescence vary with the light history of the organism. Brody and Brody[24] have shown that, in *Porphyridium cruentum* whose pigment compositions have been changed by growing the cells in blue or green light, preillumination with either blue or green light for one hour causes changes in the fluorescence spectra; the direction of these changes depends on the spectral quality of the light used for culturing and preillumination. Culturing *Anacystis nidulans* in weak and strong lights of different color (white, red, and orange) gives results similar to those obtained with *Porphyridium cruentum*. Ghosh and Govindjee[25] concluded that *Anacystis* grown in light of high intensity shows a decreased energy transfer from phycocyanin to chlorophyll a_2 accompanied by an increased transfer to chlorophyll a_1, as compared with cells grown in light of low intensity.

We have now investigated the effects of preillumination with blue or orange light on the fluorescence intensity and the shape of the emission spectra of normal and DCMU-poisoned *Anacystis nidulans* grown in weak white light. We have found that preillumination modifies the emission spectrum, and that the direction of the changes depends on the quality of the light to which the cells have been exposed. The changes in spectra occur around 685 mμ (the usual peak) and around 695 mμ (a new difference fluorescence peak).

Changes in Intensity of Chlorophyll *a* Fluorescence

When orange light (590 mμ; intensity, 2.3×10^{15} quanta cm^{-2} sec^{-1}) served both as the preilluminating and the exciting light, fluorescence intensity at 685 mμ initially increased with time (5 to 10 min) and then decreased. In most cases the decrease went below the levels recorded at 1 min. DCMU-poisoned cells exhibit similar changes 2 to 4 times as large. This result was confirmed in 8 experiments. When blue light (436 mμ; intensity, 1.3×10^{15} quanta cm^{-2} sec^{-1}) served both as the preilluminating and the exciting light, the fluorescence intensity of the poisoned cells increased with time, and that of the normal cells decreased with time. We have found that *Anacystis* cultures show individuality, especially when the light history and thus the phycocyanin-to-chlorophyll *a* ratio is different. In one experiment, when this ratio was very low (<1), preillumination with blue light led to an increase of fluorescence with time in both the normal and poisoned cells; in normal cells, however, fluorescence declined after 50 min. In the same experiment, orange light treatment gave the usual results (see above).

Changes in Fuorescence Spectra

Difference spectra were obtained by plotting $\Delta F(=F_{t_2}-F_{t_1})$ versus wavelength, where t_1 and t_2 indicate time after the beginning of illumination. A negative value corresponds to a decrease of fluorescence with time, a positive to an increase (Figure 4). With orange preillumination, comparison of the spectrum at short time with that at long time reveals a decrease of fluorescence in the region of 650 to 720 mμ; the differences are centered around 684 and 690 mμ for both the normal and the poisoned samples. In some normal samples the 690-mμ hump was more pronounced than shown here. The two maxima in the difference spectrum (Figure 4) were observed in 8 experiments. (In some experiments the difference spectrum showed an

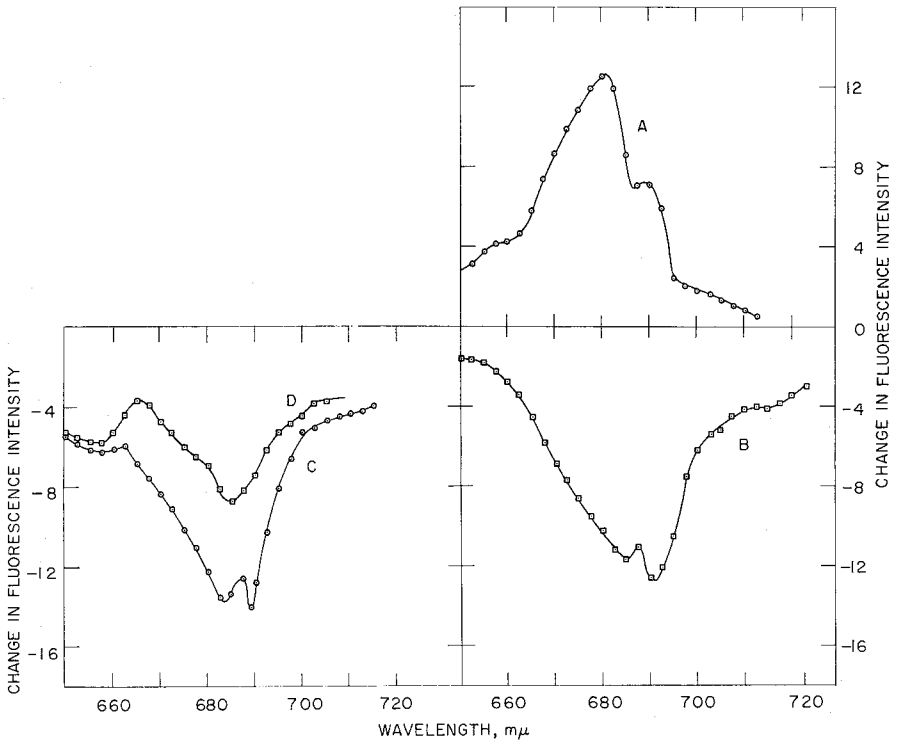

Figure 4. Right: Steady state difference fluorescence spectra ($\Delta F = F_{60} - F_0$) for normal (*A*) and DCMU-poisoned (*B*) *Anacystis nidulans*. Excitation and preillumination at 440-mμ light (half-band width $\simeq$15 mμ). Half-band width of analyzing monochromator = 3.5 mμ. F_0 indicates fluorescence measurements at about 1 min. Left: Steady state difference fluorescence spectra ΔF ($= F_{120} - F_0$) for normal (*C*) and ΔF ($= F_{120} - F_{15}$) for DCMU-poisoned (*D*) *Anacystis nidulans*. Excitation and preillumination at 590 mμ (half-band width $\simeq$15 mμ). Half-band width of analyzing monochromator = 2.5 mμ. The negative ΔF values indicate decrease of fluorescence. *Anacystis nidulans* suspended in Warburg's carbonate-bicarbonate buffer #9. Absorbance $\simeq$0.03 at 680 mμ for 1-mm path. DCMU concentration = 60 μ*M*. Spectrofluorometer used has been described elsewhere.[16,19,21]

additional hump around 675 mμ.) Difference spectra obtained with blue preillumination also showed two maxima, around 685 and 695 mμ. This result was observed in three experiments with normal and poisoned cells.

The hump produced around 690 to 695 mμ is of special interest to us since Krey and Govindjee[20] have observed in *Porphyridium* cells a band at 693 mμ. This band appeared in the difference fluorescence spectrum obtained by subtracting the spectrum obtained at low excitation intensity from that obtained at high excitation intensity after normalization at 685 mμ. We have observed two bands (without normalization) – one at 685 mμ and another at 691 mμ – in similar experiments with *Anacystis* and *Plectomena* (unpublished). However, we have failed so far to observe the 691 to 693-mμ band in *Chlorella*.

We suggested earlier that the 693-mμ difference band in *Porphyridium* originated in the energy trap of pigment system II. This explanation may apply also to the 691-

mμ difference band in *Anacystis* and *Plectonema*. The lack of a similar band in *Chlorella* may mean that the traps and the bulk pigments have nearly coincident fluorescence maxima.

We are now confronted with a different situation because a band around 690 to 695 mμ occurs even at low intensities, in normal cells and more clearly in poisoned cells, when emission spectra are compared at different times of illumination with blue or green light. Under these circumstances, too, the trap II may become less or more fluorescent, depending upon how the energy is channeled at different times.

However, the long-wave difference bands around 691 to 693 mμ may have other explanations. For example, the following possibility cannot be excluded. Accumulation or disappearance of some intermediates causes a change in the "environment" or the medium in which the fluorescing pigments are located, and thus a broadening or shift in the spectra of the bulk pigments of system II may occur. The half-band widths of the 685-mμ band, however, remain unchanged, suggesting that there is no broadening of the spectra. Also, the possibility cannot be excluded that the 695-mμ band is due to the bulk pigments of system I. But it is then difficult to imagine how (aqueous) photosynthetic intermediates will accumulate in a phase which may be lipoidal (Rabinowitch has suggested that pigment system I is in lipoidal phase). Brody and Brody[24] ascribed the changes in chlorophyll *a* emission in *Porphyridium* to modifications of the energy transfer efficiency from phycoerythrin to chlorophyll *a*. According to them, light treatment may cause reorientation of the pigments and thus affect the transfer of excitation energy between them. The DCMU-poisoned cells showed a greater decrease than normal cells in fluorescence with orange light treatment. Spatial reorientations may not be the only explanation for this effect, since it is unlikely that the low concentration of DCMU used can affect the orientation of molecules.

We wonder whether the 695-mμ difference band in *Porphyridium* and the 691-mμ band in the blue-green algae obtained upon excitation with high light intensities, and the difference bands observed in the present study, originate from the same or different causes. We cannot, as yet, answer this question.

C. REMARKS ON A TWO-LIGHT-REACTIONS MODEL OF PHOTOSYNTHESIS

The series model for hydrogen transfer[1] has influenced the thinking of most workers, and everything seems to fit nicely in this scheme. Franck,[26] Rabinowitch,[6] and Gaffron[27] have discussed at length how respiration (in particular ATP produced during respiration) may influence the measured quantum yield of photosynthesis. *In vivo* photosynthesis is thus constantly under the influence of other reactions of the cell (see French[28]).

As another alternative to the series[1] and the parallel[29] models, we propose, to provoke discussion, a model derived from ideas of Rabinowitch,[30] R. Govindjee,[31] W.A. Arnold,[32] Goedheer,[45] and in particular Hoch and Owens.[33] In this model (Figure 5) the sole purpose of light reaction I is to produce a high energy intermediate which we call HEI; it may be Jagendorf's[34] X_e or some energy-rich compound. HEI is not a reductant. It is utilized to supply the needed energy for the reduction of ferredoxin (F_d) by the reduced Y (produced by light reaction II):

$$Y^- + F_d + HEI \rightarrow Y + F_d^- + I\,.$$

Light absorbed in pigment system I oxidizes P 700 and reduces X. The reduced X returns its electron to P 700$^+$ via plastoquinone (P_q), plastocyanin (P_c), and cytochrome f (C_f). The production of HEI is coupled to the transfer of electrons from X to P 700$^+$. The light reaction II makes a reductant (Y^-); this reductant cannot reduce ferredoxin directly because of the potential barrier – the Y/Y^--couple is suggested to have an E_0 value of -0.2 eV. HEI (which may have as much energy as ATP) can provide the energy for the reaction (see equation above) in which ferredoxin is reduced. Light reaction I overcomes a potential of 1.0 eV from P 700 ($E_0 = +0.4$ eV[35]) to X ($E_0 = -0.6$ eV[36]). Light reaction II in this scheme also overcomes a potential of 1.0 eV from H_2O/O_2 ($E_0 = +0.8$ eV) to Y/Y^- ($E_0 = -0.2$ eV). Kok and Datko[37] suggest that Y/Y^- has a potential of $+0.2$ eV. However, they have left other possibilities open in the discussion to their paper.

D. SOME IMPLICATIONS OF THE PRESENT MODEL

The major evidence for the series model is the oxidation of intermediates by light I and consequent reduction by light II. In the present model these results are

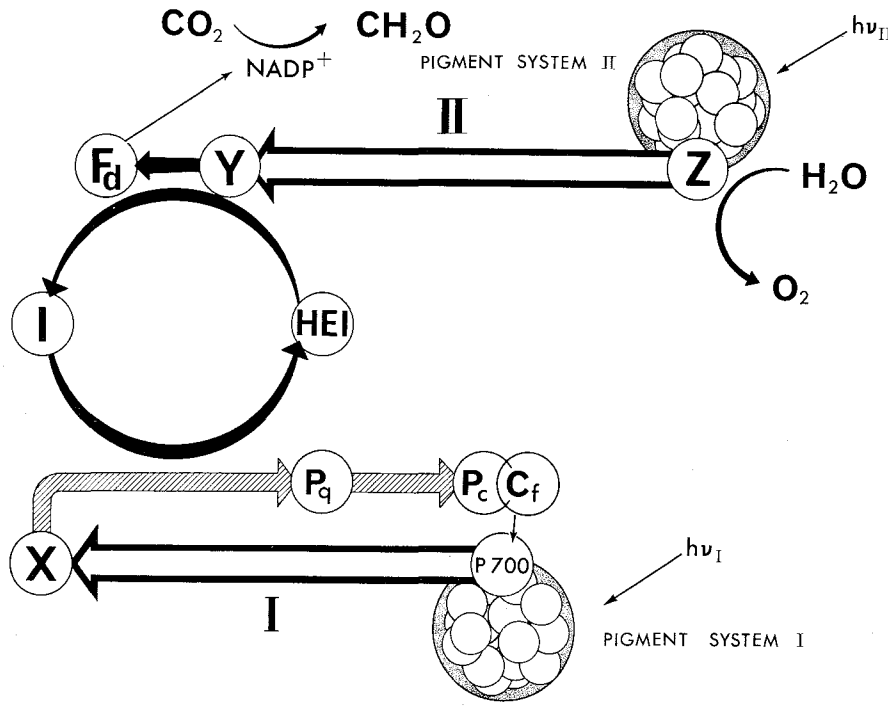

Figure 5. A model of photosynthesis. Z = primary oxidant made in light reaction II, reduced Y = primary reductant made in light reaction II, F_d = ferredoxin, $NADP^+$ = nicotinamide adenine dinucleotide phosphate, P 700 = pigment 700, X = primary acceptor of electron in photoact I, P_q = plastoquinone, P_c = plastocyanin, C_f = cytochrome f, I = some intermediate, and HEI = high energy intermediate.

explained as follows. Light I causes an oxidation of the intermediates, and consequently the HEI pool is filled. Light II, on the other hand, depletes the HEI pool, and this change in the pool size indirectly influences the redox rates of the intermediates.

Another implication of the model is the physical separation of X and ferredoxin – otherwise ferredoxin should compete with intermediates in the chain. Perhaps they are in a different environment. In addition to the HEI which is used to boost the electron from Y^- to ferredoxin, more HEI is made from the energy available from the back reaction of X with P 700^+, and this may be used to produce ATP (noncyclic phosphorylation). Cyclic phosphorylation can occur if almost all the energy available in light reaction I is used to produce ATP.

Absence of Emerson Enhancement

For some time we have been concerned with reports[38–40] of failures to observe Emerson enhancement. Recently Bedell[41] (in our laboratory) found that *Chlorella vulgaris* grown in high light intensity showed the absence of Emerson enhancement when 650-mμ light was added to 714-mμ light. Furthermore, the high-light cells had a respiration rate 4 times as high. These experiments, done by manometry, should be checked by mass spectrophotometry before any definite conclusions can be drawn. However, for the sake of our discussion, we assume that the absence of the Emerson enhancement in high-light cells is not due to the effect of light on the O_2 uptake. The series model can explain this result if a pool of an intermediate (of the electron transport chain) is postulated that somehow influences the two light reactions. We believe our alternative model explains the results equally well. If a pool of HEI* exists, it can be depleted by energy-requiring reactions (other than in the main chain of photosynthesis) and may be filled by reactions (e.g., respiration) other than light reaction I (cf. Goedheer[45]). It may thus be possible to change the size of the pool by having algal cultures with widely different metabolic conditions. Under conditions of a filled-up pool (highly respiring cultures), light reaction I should not be required, and therefore the Emerson enhancement would be absent, as shown by Bedell. The same culture should show the absence of an antagonistic effect of light absorbed in systems I and II on the redox level of cytochromes and other intermediates, and should also show the absence of a quenching effect of light I on chlorophyll a_2 fluorescence. Further, the time course of fluorescence of such cells should resemble the time course of fluorescence of normal cells preilluminated with system I light. We plan to check some of these predictions.

The Far-Red Light Effect on Fluorescence

Preillumination with far-red light causes a strong decrease of the *P* level and an increase of the *S* level (Figures 2 and 3). This result can be explained in both the series model and our alternative model. In both models, one has to postulate, first, a pool of an intermediate that has a long lifetime (seconds) and, second, a back reaction. In the series model, it is postulated that far-red light creates a pool of an oxidant (A^+) which survives a few seconds and is used up by a product made in light

*We assume that HEI is a long-lived intermediate and can last for seconds – like X_e of Jagendorf, or the far-red product of French, which has a half-life of 18 to 20 sec.

reaction II. Far-red light preillumination should thus allow an increased rate of oxygen evolution under light II. French[42] (also see ref. 43) has observed such a "result": in *Porphyridium* a green flash given within several seconds of a red flash causes an increased rate of oxygen evolution. A high rate of oxygen evolution leads to a low fluorescence yield at *P*, since fluorescence and photosynthesis have a complementary relationship.[14] That the final *S* level is higher with far-red preillumination may be due to a back reaction of the primary reductant of photosynthesis with some left-over A^+, causing a reduction in photosynthesis and thus increased fluorescence. (For an explanation of the preillumination effect on the induction phenomena in relation to oxidation of P 700 and cytochrome *f*, see Vredenberg and Amesz.[44])

In sum, the interplay of the two reactions which use up A^+, a back reaction of photoact I, and light reaction II, may be responsible for changes in photochemical reactions and for initially decreased and subsequently increased fluorescence.

The HEI model can also explain the fluorescence data shown in Figures 2 and 3. Far-red light preillumination produces HEI, which persists. When blue light follows far-red light, it uses the HEI pool, photosynthesis is increased,[cf.42,43] and fluorescence decreases. As excess HEI is used up by system II operation and by back reaction of photoact I, photosynthesis decreases and fluorescence is increased. If there are other biosynthetic reactions that may utilize HEI in darkness and thus deplete the pool, the next illumination with blue light will show an increased *P* peak – which is observed (Figure 3).

SUMMARY

Preillumination with far-red light causes a strong decrease of the *P* peak and an increase in the *S* level in *Chlorella*. After a subsequent dark period, the *P* level is higher than before. To explain these results we postulate the existence of a pool of an intermediate – produced by far-red light – and a back reaction.

Normal and DCMU-poisoned *Anacystis nidulans* cells show first an increase followed by a decrease of fluorescence with time when both the preilluminating light and the excitation light are orange (590 mμ). Poisoned cells show changes 2 to 4 times as large as normal cells show. The decrease is not uniform at all the wavelengths. A difference spectrum between the fluorescence spectrum taken when illumination begins and that obtained after one hour (or so) shows two bands – one around 685 mμ (the usual band) and another around 695 mμ (a new band). Blue-light preillumination causes usually an increased fluorescence with time, and here again difference fluorescence spectra show two bands (around 685 and 695 mμ).

An alternative model of photosynthesis is discussed. In this model, the sole purpose of light reaction I is the production of a high energy intermediate (HEI) which is not a reductant. It permits an electron transfer from reduced Y (the primary reductant made in reaction II) to ferredoxin. This model can explain the often noted absence of Emerson enhancement. It also explains the changes in the time course of fluorescence observed with far-red preillumination.

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