

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

## III. THE DIP AND THE PEAK IN THE FLUORESCENCE

### TRANSIENT OF *Chlorella pyrenoidosa*

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**ABSTRACT** The fluorescence transient of *Chlorella pyrenoidosa*, excited by saturating light absorbed mainly by system II, has a dip *D* between the peak *I* at 75 msec and the large peak *P* at 400 msec (the times depend on light intensity). This dip is observed in aerobic cells and in anaerobic cells where it is prominent. In anaerobic cells, the *I-D* decline is hastened almost equally by absorption of either 705 or 650 nm background light. In aerobic cells, supplementary 700 and 710 nm light given during the transient slightly hastens and heightens *P*. Methyl viologen, an exogenous system I electron acceptor, eliminates *P*. Results suggest that system I action causes *D*, and that *P* is due to reduction of *Q* (fluorescence quencher) and intersystem intermediates caused by development of a block in oxidation of *XH* (*X* being the primary electron acceptor of light reaction I). Mathematical analysis suggests that if only two forms of *Q* participate beyond *I*, then system I action is required for *D*. If three forms participate, then the system  $Q \rightarrow QH \rightarrow Q'$  (see text) may explain *D*. The Malkin model (14), in its present form, does not allow *D*.

### INTRODUCTION

The green alga *Chlorella pyrenoidosa* when illuminated emits fluorescence from chlorophyll *a* (1). If illumination follows a several minute dark period and is sudden and intense, the fluorescence intensity shows a complex transient in the first second (2). (For changes beyond 2 sec, see references 3 and 4.) The problem is to relate the transient to the mechanism of photosynthesis. Currently, photosynthesis is believed to involve two photochemical reactions in series, each reaction using light energy captured by pigment systems (5, 6).

The following simple model of the electron-transport chain in photosynthesis is given to serve as a frame of reference for later discussion.

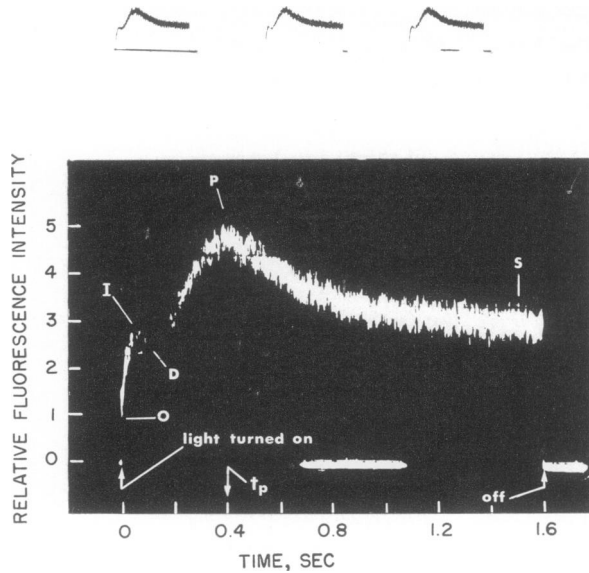
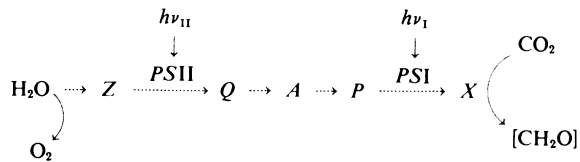


FIGURE 1 The fluorescence transient in aerobic *Chlorella pyrenoidosa*. Wavelength of measurement: 685 nm; half-maximum bandwidth (BW) 6.6 nm. Excitation: 500 nm, BW 120 nm, incident intensity  $1.5 \times 10^4$  ergs/sec-cm<sup>2</sup>. The three small photographs were obtained in one experiment at 4 min dark intervals; the large photograph is an enlargement of the top right-hand photograph. The points *O*, *I*, *D*, *P*, and *S* are clear in each photograph.



where *Z* and *Q* are primary electron donor and acceptor of light-reaction II, *PSII* and *PSI* are pigment systems II and I,  $h\nu_{II}$  and  $h\nu_I$  are quanta absorbed in *PSII* and *PSI*, *P* and *X* are primary electron donor and acceptor of light reaction I.

Most fluorescence is emitted by the chlorophyll *a* in system II, the system which sensitizes the oxygen-evolving reaction (7–14). The factor controlling fluorescence intensity is the redox state of *Q*, the electron acceptor in the system II reaction center (9).

Fig. 1 shows typical fluorescence transients in *Chlorella*. *O*, *I*, *P*, and *S* are features labeled by Lavorel (15) and Joliot and Lavorel (16); we also see a dip *D* between *I* and *P*. A dip was first seen by Kautsky and Franck (17) in 1943 in anaerobic cells. Only recently has a dip been seen in aerobic cells by Delosme (18) and Bannister.<sup>1</sup>

Kautsky, Appel, and Amann (19) concluded in 1960 that the dip in anaerobic cells results from the interplay of two photochemical reactions. They suggested that

<sup>1</sup> T. T. Bannister. Personal communication.

one reaction reduces a fluorescence quencher to a nonquenching form, thus causing the fluorescence rise from *O* to *I*, and that the second reaction oxidizes an intermediate which in turn reoxidizes the quencher, thus causing the decline from *I* to *D*. The rise from *D* to *P* was thought to result from the exhaustion of a substrate for the second photoreaction. In terms of the series hypothesis, this explanation suggests that the rise from *D* to *P* is caused by a block in oxidation of *XH*, the reduced system I electron acceptor. Investigators since 1960 have not mentioned *X* in connection with the *D*-*P* rise, believing apparently that the *D*-*P* rise involves only the reduction of *Q* and an intersystem intermediate denoted *A* (13, 14, 16, 18, 20). The regular occurrence of *D* in aerobic samples raises again the question whether the *D*-*P* rise involves *X* in addition to *Q* and *A*. We conclude from our study that *D* reflects a dynamic balance of the reduction of *Q* by system II and the oxidation of *QH* by system I, and that the *D*-*P* rise parallels reduction of *X*, and hence of *A* and *Q*.

## METHODS

*Chlorella pyrenoidosa* (Emerson strain 3) was grown in inorganic medium (21) at 20°C with light intensity (40 watt tungsten) of 3750 ergs/sec-cm<sup>2</sup>. Aliquots from 2 to 5 day old cultures were centrifuged and resuspended in NaHCO<sub>3</sub>-K<sub>2</sub>CO<sub>3</sub> buffer (85/15 mixtures of 0.1 molar stock, pH 9.2). Transients at pH 9.2, 10.2, and 10.7, and in growth medium at pH 5.5 were indistinguishable. In normal cultures, *P/S* was about 1.5 and the *P-S* decline was completed by 1.5 sec (at our light intensity; see below). Abnormal cultures were rejected.

A 3 cc sample was placed in a Dewar flask (H. S. Martin & Co., Evanston, Ill.) with illumination and fluorescence collection through a clear flat bottom. The liquid depth was 0.374 cm and the optical density before the cell settling was 0.15 at 678 nm. After settling, the cells did not overlap. About  $5 \times 10^5$  cells were illuminated.

Absorption was measured with a Bausch and Lomb, Inc. (Rochester, New York) Spectronic 505 spectrophotometer equipped with an integrating sphere. Absorbed intensities were calculated from incident light intensities measured by a thermopile, and absorption measured by a spectrophotometer. Despite an integrating sphere, the absorption spectrum showed apparent absorption beyond 740 nm, where photosynthetic pigments have negligible absorption. Based on the work of Latimer (22-24), we conclude that the apparent absorption is non-selective scattering with negligible wavelength dependence between 650 and 740 nm. Therefore, we adjusted upward the absorption spectrum baseline at 650 and 705 nm by the amount of apparent absorption at 740 nm. Aside from approximations inherent in this procedure, error in absorbed intensities depends on the spectrophotometer and is at most a few per cent.

Light intensity was measured with an Eppley Laboratory, Inc. (Newport, R.I.) Bi/Ag thermopile and a Keithley Instruments, Inc. (Cleveland, Ohio) 605A Microvoltammeter. Intensity was varied by Balzers (Geraetebauanstalt, Balzers, Fürstentum, Leichtenstein) neutral density filters.

Fluorescence was measured by a spectrofluorometer described previously (25) but modified for measurement of transients (Fig. 2). The exciting light came from a Radiant Lamp Corporation (Newark, N.J.) DDY 750 watt projection lamp, with 120 v AC from a variac and a Sola Electric Co. (Elk Grove Village, Ill.) line voltage regulator. A 5 cm water bath absorbed heat. Blue light was obtained by Corning Glass Works (Corning, New York) C.S. 3-75 and 4-96 filters (maximum 500 nm; half-maximum bandwidth (BW) 120 nm). This blue band pref-

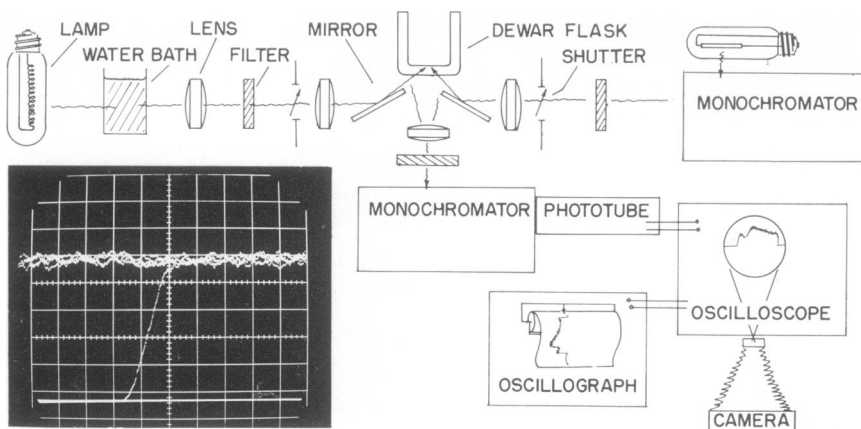


FIGURE 2 Block diagram of the Spectrofluorometer (see text). Bottom left—an oscillograph of the phototube signal during a shutter opening. Horizontal scale, msec/division. The sloping vertical line denotes the opening; the opening is complete in 2 msec.

erentially excites Chlorophyll *b*, and thus mainly but not exclusively system II. The intensity was  $1.5 \times 10^4$  ergs/sec-cm<sup>2</sup>; absorbed intensity was roughly  $4 \times 10^{14}$  quanta/sec-cm<sup>2</sup>. This intensity was twice that causing a change in slope in measured fluorescence vs. light intensity curves (11, 26–30). In some experiments a rotating sector disk produced alternating light and dark periods, each 40 msec long.

Supplementary illumination and preillumination was obtained from a General Electric Co. (Cleveland, Ohio) microscope illuminating lamp (tungsten ribbon filament, 18a/TID/1–6v) run at 20 amps and a Bausch and Lomb monochromator (600 grooves/mm; 3.3 nm BW/mm slit; blaze 300 nm). Wavelengths between 640 and 725 nm were used to compare effects of absorption in systems II and I (13, 31–33). (It is recognized that the shorter wavelengths excite both systems.) A Corning C.S. 3-69 filter eliminated scattered blue light from the monochromator output. The maximum intensity was 2600 ergs/sec-cm<sup>2</sup>. Both illuminations reached the bottom of the Dewar flask. Compur shutters from Burke and James, Inc. (Chicago, Ill.) intercepted each beam; the exciting light shutter opened in less than 2 msec.

Fluorescence passing through the Dewar flask bottom traversed a lens and was deflected into a second Bausch and Lomb monochromator (blaze 750 nm; BW 6.6 nm) set at 685 nm, the chlorophyll *a* fluorescence peak *in vivo* (34). A Corning C.S. 2-60 filter absorbed scattered exciting light. Monochromator output was measured by an Electra Magadyne, Inc. (New York) 9558B photomultiplier tube operated at 1050 v from a regulated supply. Tube output was amplified and displayed by a Tektronix, Inc. (Portland, Oreg.) 502A oscilloscope, and the trace photographed on Eastman Kodak Co. (Rochester, New York) 35 mm linagraph ortho film. Alternately, the oscilloscope output was recorded with a Midwestern Instruments, Inc. (Tulsa, Okla.) 801B oscillograph. For steady-state fluorescence measurements, tube output was fed to a Keithley 150A Microvolt-ammeter, and recorded on a Minneapolis-Honeywell Reg. Co. (Philadelphia, Pa.) Brown recorder.

Gas composition of the sample was varied by capping the Dewar flask and passing gas mixtures through a hole in the cap. The surface to volume ratio in the flask was 7 cm<sup>-1</sup>; this high ratio permitted rapid equilibration of gases between the liquid and the air above. The transient was repeatable for several hours in stagnant air, in flowing air, and in flowing CO<sub>2</sub> in air, allowing the conclusion that CO<sub>2</sub> and O<sub>2</sub> are not limiting for several hours. Under

argon, fluorescence yield was higher than normal, and the transient was modified (17). Temperature of the sample was 25°C.

The standard procedure was a sequence of 2-sec light exposures separated by 4 min dark intervals. These intervals allowed *P* to rise to more than 95% of its fully dark-adapted height. After the initial 8 cycles, the transient was repeatable, and various tests were then begun.

## RESULTS AND DISCUSSION

### *I. The Anaerobic Transient*

The use of anaerobic cells necessitated a check of the features of the anaerobic transient. Fig. 3 shows the change in shape of the transient as 2% CO<sub>2</sub> in air is replaced by 2.6% CO<sub>2</sub> in argon. From picture 1 to picture 6 took 20 min in this example; the time required to establish the shape in picture 6 depended on the rate of oxygen removal. Kautsky and U. Franck (17) in 1943 showed that the shape in picture 6 is obtained only when oxygen concentration is below 0.1%. The large photograph of Fig. 3 is from a different anaerobic experiment, chosen to show the *O* level in the anaerobic state.

Fig. 4 (from the same experiment as the sequence in Fig. 3) shows fluorescence levels of the transient during a series of gas composition changes. The effect of

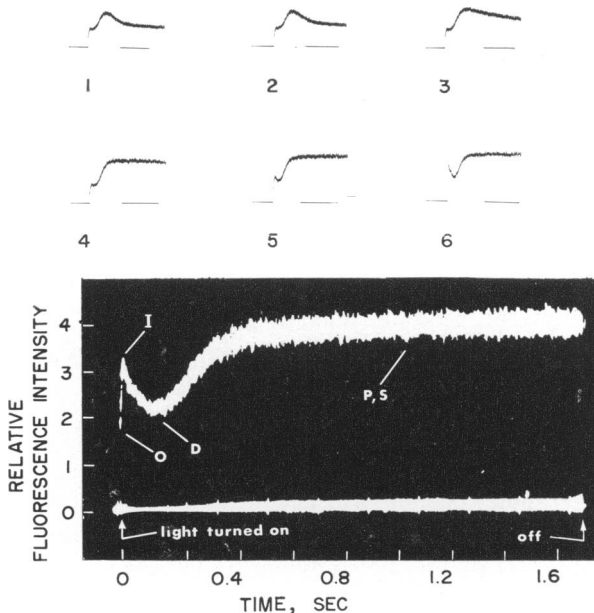


FIGURE 3 Change of the fluorescence transient in *Chlorella* during oxygen removal. The small photographs were taken at 4 min intervals as 2.0% CO<sub>2</sub> in air was replaced by 2.6% CO<sub>2</sub> in argon. Replacement began a few seconds after picture 1. The effect was complete by picture 6, as subsequent transients were identical to that of picture 6. The large photograph, from a different anaerobic experiment, shows the distinction between *O* and *I*.

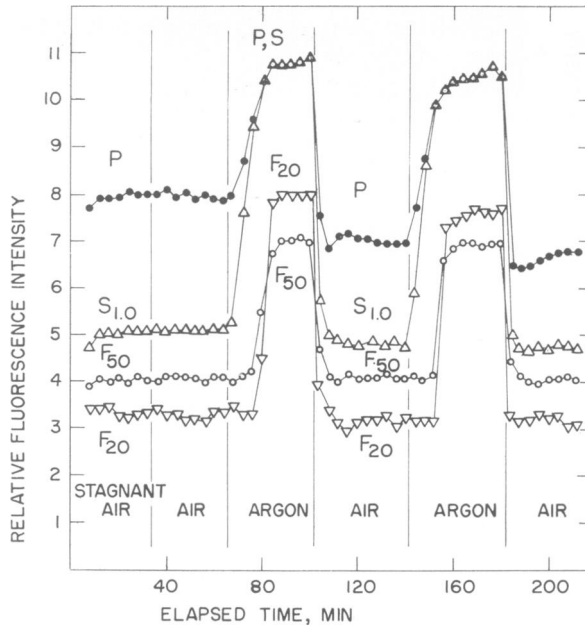


FIGURE 4 The effect of oxygen removal on fluorescence levels during the transient. Levels at 20 and 50 msec after onset of illumination ( $F_{20}$  and  $F_{50}$ ) are plotted because the  $I$  level is near 50 msec in aerobic cells, and near 20 msec in anaerobic cells. Stagnant air was changed to flowing 2%  $\text{CO}_2$  in air, then to flowing 2.6%  $\text{CO}_2$  in argon. Complete replacement of gas required 10–20 min. This experiment produced the small photographs of Fig. 3.

oxygen removal was reversible and repeatable. Data were obtained at 4 min intervals;  $P$  and  $S$  jumped upward at the first data points after argon flow began and thus were affected within 4 min. When oxygen was restored, the transient returned to normal within 4 min.

The rapid development and reversal of the anaerobic effects signify that the effects are not due to long-term metabolic changes, and that if any disarrangement of the photosynthetic apparatus is involved it is mild and easily repaired. We are confident that short-term anaerobic cells are physiologically healthy.

### II. The Light-Requiring Dip

Kautsky et al. (19) found that the  $I$ - $D$  decline in anaerobic cells requires light, i.e., the decline progresses only during illumination. We tested which pigment system is responsible for the  $I$ - $D$  decline by studying the hastening of  $D$  by different background lights. Anaerobic cells were used for the test because  $D$  is more pronounced in anaerobic than in aerobic cells. 705 nm was chosen to excite system I and 650 nm for system II (some 650 nm light reaches system I); the half-maximum bandwidth was 10 nm; the 705 nm intensity was 1500 ergs/sec-cm<sup>2</sup> incident; the 650 nm intensity was adjusted to give absorbed intensity equal to that of 705 nm. Effects were tested

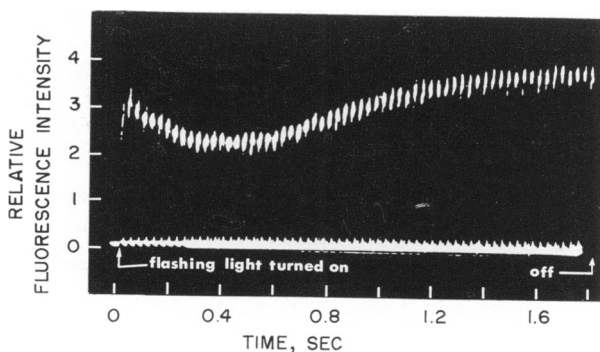


FIGURE 5 The anaerobic transient excited by flashing light (40 msec on and off).  $D$  is delayed in flashing light (compared to continuous light) because the  $I-D$  decline progresses only during the light periods. Compare  $D$  in Fig. 3.

in several experiments, each involving at least five trials at each wavelength, alternated to eliminate influence of long-term fluorescence changes. Shutters for the background and exciting lights were opened simultaneously.

Because of low absorbed intensities and the short time until  $D$ , few background light quanta could be absorbed before  $D$ . Consequently, we used a flashing rather than continuous exciting light to delay  $D$  (see Kautsky et al. (19)) and to allow longer than normal duration of the (continuous) background light. Fig. 5 shows the flash-excited transient. (The transient in the presence of background light is similar, having a slightly raised baseline due to leak of the background light.) Fluorescence is higher at the end of each flash than at the beginning of the next.  $QH$  is apparently being oxidized between flashes.<sup>2</sup> This oxidation is independent of the light-requiring character of  $I-D$  discovered by Kautsky et al (19). Confirming their discovery, we find the time of  $D$  nearly doubles when flashes and dark periods are equally long.

705 nm was slightly more effective than 650 nm in hastening  $D$ . Data from one experiment are shown in Table I. The effects, expressed as fractional changes in the time of  $D$ , were averaged and divided by absorbed intensity to give relative quantum yield.

The relative quantum yields for the background light effects were, for 650 nm, 7.0, and for 705 nm, 8.2. These values rule out the possibility of a long-wavelength "red drop" in quantum yield, as found in *Chlorella* for quantum yields of oxygen evolution (36) and fluorescence excitation (37). (In aerobic cells under steady-state conditions about half of 650 nm light is absorbed by system I (32, 33). We had expected, presuming our anaerobic and transient conditions would not change this fraction, that the 705 nm yield would be higher compared to the 650 nm yield; see below.)

The simplest interpretation is that system I oxidizes  $AH$  and  $QH$ , thereby causing

<sup>2</sup> (Lavorel (35) found two phases in the dark-oxidation of  $QH$  after a 33 msec flash; the faster phase was sensitive to photosynthetic inhibitors.)

TABLE I  
EFFECT ON TIME OF  $D$  ( $t_D$ ) CAUSED BY 650 nm  
OR 705 nm BACKGROUND LIGHT IN  
ANAEROBIC *CHLORELLA*

Exciting light exposure	No back- ground $t_D$	650 nm—background		705 nm—background	
		$t_D$	$\Delta t_D/t_D$	$t_D$	$\Delta t_D/t_D$
	<i>sec</i>				
1*	0.20				
2	0.22				
3	0.21				
4	0.20				
5‡	0.38				
6	0.38				
7	0.38				
8	0.38				
9	—	0.31	0.184		
10	—	—	—	0.31	0.184
11	0.37	—	—	—	—
12	—	0.33	0.108	—	—
13	—	—	—	0.32	0.135
14	0.36	—	—	—	—
15	—	0.31	0.161	—	—
16	—	—	—	0.30	0.167
17	0.38	—	—	—	—
18	—	0.32	0.158	—	—
19	—	—	—	0.30	0.210
20	0.37	—	—	—	—
21	—	0.34	0.081	—	—
22	—	—	—	0.34	0.081
23	0.37	—	—	—	—
24	—	0.34	0.081	—	—
25	—	—	—	0.33	0.108
Average $\Delta t_D/t_D$		0.129		0.147	
Standard deviation		0.0407		0.0443	
Absorbed quanta/sec-cm <sup>2</sup>		$1.83 \times 10^{13}$		$1.78 \times 10^{13}$	
Relative quantum yield		7.05		8.25	

\* The exciting light was continuous in exposures 1–4.

‡ The exciting light was flashing in exposures 5–25.

the  $I$ - $D$  decline. This interpretation implies that oxidation of  $AH$  by system I during the decline is faster than reduction by system II. A measure of the reduction rate is the ratio of  $QH$  to  $Q_o$  (total  $Q$ ) since  $QH$  donates electrons to  $A$ . We presume that at  $O$  of aerobic cells, all  $Q$  is oxidized, and at  $P$  of anaerobic cells, all  $Q$  is reduced.<sup>3</sup> In the experiment of Fig. 4,  $O$  (aerobic, now shown) had a value of 20 (relative units), while  $P$  (anaerobic) was 108. The difference, 88, is proportional to  $Q_o$ . The

<sup>3</sup> Anaerobic  $P$  is higher in our experience than fluorescence under poisoning with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU is thought to block oxidation of  $QH$ ; therefore,  $P$  must signify completely reduced  $Q$ .



*I* hump (anaerobic) was 80; therefore at *I* (anaerobic),  $QH/Q_0$  is (80–20)/88 or about  $\frac{2}{3}$ , and the reduction rate should be high. Still, the dip occurs.

A. Joliot and P. Joliot (38) and Delosme (18) suggested that the S-shaped *O–I* rise of the transient, excited by very intense light, signifies energy migration from system II units where *Q* is reduced to neighboring system II units. Perhaps migration also occurs from system II to system I (“spill-over”) when *Q* is reduced. At *I* (anaerobic) when  $\frac{2}{3} Q_0$  is reduced, the transfer of 650 nm light might be large enough to account for the similar quantum yields of Table I. However, P. Joliot et al. (33) have concluded that spill-over does not occur in aerobic algae and isolated chloroplasts.

### III. The Peak

A. *Effect on P of System I Illumination During the Transient.* One test whether *P* involves *XH* is the effect of a short system I exposure supplementing the exciting light during the phase *D–P*. If *P* does not involve *XH*, and involves only a temporary maximum in *AH* and *QH*, then the system I exposure should increase oxidation of *AH* and decrease and/or delay *P*.

700 nm light was chosen to excite system I. According to P. Joliot et al. (33), system I is preferentially excited by all wavelengths beyond 678 nm, and at 700 nm, system I is excited six-fold more than system II.

A 150 msec flash of 700 nm light was timed to start after *D*, and terminate 100 msec before *P* usually occurs. The 100 msec delay was included to allow intersystem balancing of the flash effect. If our view of *D* is correct, then balancing is indicated by the interval between *O* and *I–D*, which under our conditions is less than 100 msec. The 700 nm intensity was 1500 ergs/sec-cm<sup>2</sup> incident, and the half-maximum bandwidth 10 nm.

In many trials in several experiments, the flash slightly hastened and raised *P*. Fig. 6 from one trial shows superimposed drawings of a transient excited normally, and the succeeding transient modified by the flash. *P* was hastened by 7%.

We must consider the possibility that 700 nm light excited system II enough to hasten and raise *P*. We rule out this possibility by the following reasoning: Quanta which excite system I begin to affect fluorescence earlier than 100 msec, because the *I–D* decline, sensitized by system I absorption, begins within 50 to 75 msec. Also, the system II effect, being direct, must precede the indirect system I effect. Finally, the system II effect is smaller than the system I effect because the majority of the 700 nm quanta excite system I (32, 33). We may conclude that if there is a system II effect 100 msec after the flash, it is opposed by a larger system I effect.

Despite the above arguments, we decided to test further, using 710 nm flashes, which excite system I ten-fold more than system II (33). Neither the flashes at *O* nor between *I* and *P* produced any effect, presumably because the cells absorb about 3.5 times fewer quanta at 710 than at 700 nm. However, administering the 710 nm

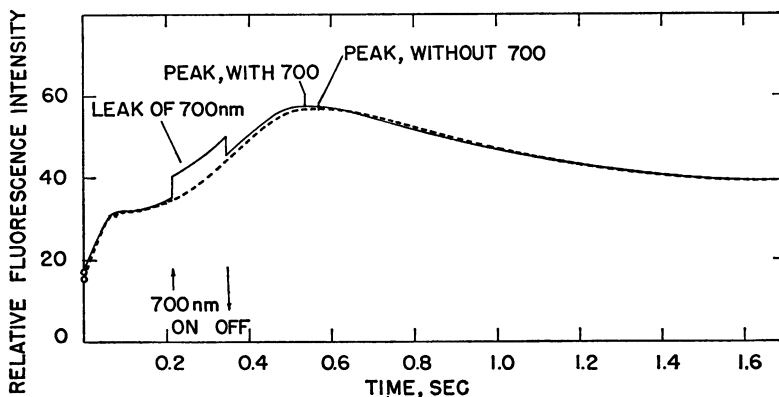


FIGURE 6 The effect on  $P$  of a 700 nm flash after  $I$ . Transient without flash, dotted line, followed by transient with flash, solid line. Some of the flash intensity leaked into the detection system as shown.

illumination throughout  $O$  to  $S$  of the transient, simultaneously with the exciting light (with the half-maximum bandwidth 13 nm for sufficient intensity) hastened  $P$ , and raised  $P$  by 3% (standard deviation 1.9%, 20 trials). To make certain the 710 nm was exciting mainly system I—again despite the above arguments—we tested its effect on steady-state fluorescence. During 5 min exposures to the exciting light (separated by 10 min dark periods), the 710 nm illumination was administered at various times for 15 sec. For cells in buffer (see Methods), the result during the period 1–5 min after onset of the exciting light was a consistently reproducible quenching of fluorescence. For example, the fluorescence amplitude at 685 nm for exciting light alone was 70.3 (relative units), for the 710 nm light alone, 2.0 (including scattering leak), and for both lights together, 67.5. Under the given conditions, effects of the same magnitude were observed in more than 35 trials without exception. The quenching of steady-state fluorescence by long wavelength illumination is well known (8, 9, 39), and proves that the 710 nm illumination which raised  $P$  is exciting primarily system I.

The increase in  $P$  caused by system I illumination during the transient supports our view that  $P$  involves a block in oxidation of  $XH$ , as well as  $AH$  and  $QH$ .

*B. Effect of Methyl Viologen.* According to our view,  $P$  should be absent if most  $X$  can be kept oxidized. Kautsky et al. (19) tested on anaerobic cells the effect of  $K_3Fe(C_2O_4)_3$ , but this substance can oxidize both  $XH$  and  $QH$ . For conclusive results, the acceptor should oxidize only  $XH$ . To oxidize  $XH$ , several investigators have used the dye methyl viologen (paraquat dichloride, *i.e.*, 1,1'-dimethyl-4,4'-dipyridilium dichloride).<sup>4</sup> The potentials of methyl viologen (40, 41), the

<sup>4</sup> According to Michaelis and Hill (40), methyl viologen is reduced in two steps by addition of single electrons, and thus its potentials are independent of pH. The polarograms by Black (41) indicate that oxidized methyl viologen begins to accept the first electron at about  $-0.37$  v, and that this first step is complete at about  $-0.52$  v. The midpoint is  $E_0' = -0.426$  v (phosphate buffer; pH 7.8; T =

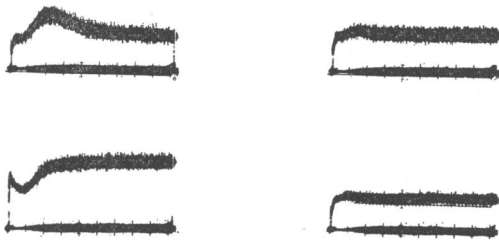


FIGURE 7 The effect of methyl viologen. Top left, before addition. Top right, after addition. Bottom left (new sample), anaerobic cells after addition. Bottom right, after return of the treated anaerobic cells to air.

potentials of the photosynthetic reductants, and the effects of methyl viologen on chloroplasts (33, 42, 45) prove that it interacts only with system I. It apparently has not been shown to penetrate intact cells, but because of its small size it is expected to penetrate.

Methyl viologen at  $10^{-4}$  M after addition caused the progressive disappearance of  $P$  in 15 min (thus proving penetration). The 15 min development was probably caused by diffusion and penetration as the cells were stationary on the bottom of the Dewar flask before addition and the dye was added without swirling at the side of the flask away from the small illuminated area in the center of the flask. There was no noticeable effect on the phase  $O-I$ . Fig. 7 shows transients before and after treatment. The same results have been observed eight times in four different cultures.

Anaerobic cells treated with the dye displayed a normal anaerobic transient. Admission of air led to display (within 4 min, the interval between light exposures) of an aerobic transient without  $P$ , but gradual return to anaerobiosis led again (over several intervals) to a normal anaerobic transient. Fig. 7 shows the results. These results are explained by the fact that methyl viologen after photoreduction is oxidized by oxygen. When oxygen is lacking, photoreduction is unopposed and all of the dye accumulates in the reduced form, whence it can no longer oxidize  $XH$ .

The results confirm the view that a block in oxidation of  $XH$  allows an accumulation of reduced intersystem intermediates and thereby causes the peak  $P$ .

## MATHEMATICAL ANALYSIS

### *I. Basic Assumptions and Equations*

A short analytical review is necessary, to establish basic assumptions and equations.

We assume that (1) the excess of fluorescence above  $O$  is emitted by system II, and that (2) the primary reaction of system II is the photoreduction of  $Q$ . Assumption (2) is common (13, 14, 18) but no electron donor is specified. We accept (2) presuming in effect that the donor concentration is constant.

22°C) (41). Kok et al. (42) determined that the potential of  $XH$ , the reductant produced by system I, has a midpoint below  $-0.6$  v, and that the potential of  $QH$ , produced by system II, has a midpoint at  $+0.18$  v. In chloroplasts, methyl viologen undergoes a one-electron photoreduction (33, 42). It facilitates cyclic photophosphorylation, a function of system I (43, 44). A concentration of  $10^{-4}$  M has a saturating effect on the absorption change of  $P_{700}$ , the trap for system I (45).

Let  $F$  be the rate of fluorescence,  $H$  the rate of heat loss, and  $P$  the rate of photochemistry. Duysens (46) assumed  $P$  is equal to  $I$ , absorbed light intensity, times  $K$ , the probability that quanta are trapped for photochemistry, and that  $H$  is proportional to  $F$  (assumed also by Murata et al. (47) and Delosme (18)).

$$P + H + F = I \quad (1a)$$

If

$$F = \frac{k_F}{k_H + k_P + k_F} I \quad (1b)$$

and

$$H = \frac{k_H}{k_H + k_P + k_F} I \quad (1c)$$

then, if  $k_P$  changes but not  $k_F$  and  $k_H$ , one can justify that  $H \propto F$ . Finally, from  $H \propto F$ , and from equation (1a), we obtain

$$c_1 F = I - KI \quad (1)$$

where  $c_1$  accounts for  $H$  proportional to  $F$ .  $F$  is fluorescence from units of system II only and includes bulk but not trap fluorescence.

However, the assumption about  $H$  may be questioned. The series scheme requires 8 quanta per evolved oxygen molecule (48), and Emerson and Chalmers (49) found 8 under optimal conditions. Therefore, the quantum efficiency of each photoreaction under optimal conditions must be above 90%. Fluorescence wastes 3% (50, 51); however, in system II, the fluorescence yield is higher (52) and may reach 6%. In that case, heat loss in system II is 4%. After treatment with DCMU,  $QH$  cannot be oxidized, and oxygen production drops nearly to zero (53). Quantum efficiency of the system II photoreaction must then be lower than 10%. Fluorescence nearly triples (54). Heat loss then must be 72%. Thus, heat loss apparently rises six times as much as fluorescence, whereas the assumption is that the rises are equal.

A way out of this difficulty is to presume that quanta may still be trapped when  $Q$  is reduced.  $K$  for reduced  $Q$  is probably smaller than for oxidized  $Q$ , but is not necessarily zero. This matter is unsettled, but we proceed as if equation 1 is acceptable.

For the probability  $K$ , both Delosme (18) and Duysens (46) have obtained

$$K = 1 - \frac{(1 - a)(1 - Q_o)}{1 - a(1 - Q_o)} \quad (2)$$

where  $a$  is the probability of intermolecular transfer of excitation quanta and  $Q_o$  is

the ratio of total  $Q$  to chlorophyll. Equations 1 and 2 give

$$F = \frac{1}{c_1} I(1 - K) = \frac{1}{c_1} I \frac{(1 - a)(1 - Q_o)}{1 - a(1 - Q_o)}. \quad (3)$$

Equation 3 is correct only if all  $Q$  is oxidized, such as at  $O$  of the transient. The  $O$  fluorescence includes  $F$  and probably a contribution from system I.

If an absorbed quantum has access only to the reaction center of the unit where absorbed, equation 3 applies only to units where  $Q$  is oxidized. For this case  $F$  and  $K$  in equation 3 henceforth take the subscript "o" to stand for "oxidized". Where  $Q$  is reduced,  $K = O$  (the simplest hypothesis), and

$$F_r = \frac{1}{c_1} I \quad (4)$$

where "r" stands for "reduced".

The units contributing  $F_o$  are a fraction  $[Q]/[Q_o]$  of all units, and those contributing  $F_r$  are a fraction  $[QH]/[Q_o]$ , where  $[Q_o] = [Q] + [QH]$ . Total fluorescence from all system II units is then

$$F = \frac{[Q]}{[Q_o]} F_o + \frac{[QH]}{[Q_o]} F_r = F_o + \frac{K_o I [QH]}{c_1 [Q_o]}. \quad (5)$$

Equation 5 is isomorphic with the basic equation used by Malkin and Kok (13). Here, however,  $F_o$  and  $F_r$  have specific meanings, given by equations 3 and 4.

From equations 1, 3, and 4, and the fact that the rate of trapping equals the rate of increase of  $[QH]$  (we acknowledge the independent development of these ideas by Delosme (18)), we obtain

$$d[QH]/dt = K_o I(1 - [QH]/[Q_o]), \quad (6)$$

assuming no oxidation of  $QH$ . If  $[QH] = O$  when  $t = O$ , we solve for  $[QH]$  and substitute into equation 5 obtaining

$$F = F_o + \frac{K_o I}{c_1} (1 - \exp \{-K_o I t/[Q_o]\}). \quad (7)$$

The assumptions are that units are separate, reduction of  $Q$  proceeds unopposed by oxidation of  $QH$ , and the rate of change to heat energy is proportional to fluorescence.

Morin (20) and Delosme (18) recorded the  $O-I$  rise in *Chlorella* in very intense light, when rapid reduction of  $Q$  precludes dark oxidation via  $A$ . Then the  $O-I$  rise is  $S$ -shaped, conflicting with equation 7. A. Joliot and P. Joliot (38) and Delosme (18) suggested that units are not separate, and quanta reaching a trap where  $Q$  is

reduced may transfer to another trap. On this basis, Delosme (18) obtained (in our symbols)

$$F = F_o + \frac{K_o I (1 - p)[QH]/[Q_o]}{c_1 (1 - p[QH]/Q_o)} \quad (8)$$

and (except for a trivial constant we assume equal to unity)

$$K_o I t = p[QH] + (p - 1)[Q_o] \ln ([Q]/[Q_o]) \quad (9)$$

where  $p$  is the probability of transfer between traps. Equations 8 and 9 give an  $S$ -shaped rise of fluorescence with time which matches the  $O$ - $I$  rise observed in intense light. The match is strong circumstantial evidence that quanta may travel among traps.

The above equations imply that fluorescence monotonically follows  $[QH]$ . In equation 5 this property is explicit; in equation 8 it can be seen by dividing both numerator and denominator of the right-hand term by  $[QH]$ .

## II. Mathematics and the Dip

Both nonlinear and linear systems will allow the dip. Analysis requires allowance for the oxidation of  $QH$ , because: (a) preillumination of system I lowers I (55-57), showing that (at our exciting light intensity of  $1.5 \times 10^4$  ergs/sec-cm<sup>2</sup>) the I level involves oxidation of  $QH$  by  $A$ ; and (b) the dip represents a decrease in  $[QH]$  because of the monotonic relation between  $[QH]$  and fluorescence.

*A. A Nonlinear System of  $QH$  and  $A$ .* We assume here that  $Q$  is in only two forms (oxidized and reduced) after I, and obtain a nonlinear system of relations between  $QH$  and  $A$ . We would prefer to use a differential form of equation 9 and include a term representing oxidation of  $QH$  by  $A$ , such as  $K_A[A][QH]$ :

$$d[QH]/dt = K_o I \frac{1 - [QH]/[Q_o]}{1 - p[QH]/[Q_o]} - k_A[A][QH]. \quad (10)$$

However, the algebra becomes unmanageable because of the denominator factor. If equation 6 is used instead and modified, it becomes

$$d[QH]/dt = K_o I (1 - [QH]/[Q_o]) - k_A[A][QH]. \quad (11)$$

Here the  $K_o$  term is linear, a simplification not affecting the argument later, for the following reason: Fig. 8 shows the  $K_o$  and  $k_A$  terms of equations 10 and 11 (curves 1, 2, 3, and 4, 5, 6, respectively) as a function of  $[QH]/[Q_o]$ .  $p$  is 0.45 (18). Different slopes for  $k_A[A][QH]$  results from varying  $[A]$ , and we choose  $[A]_1 > [A]_2 > [A]_3$ . The  $O$  level of the transient, where  $[QH] = O$ , is represented on Fig. 8 by vertical

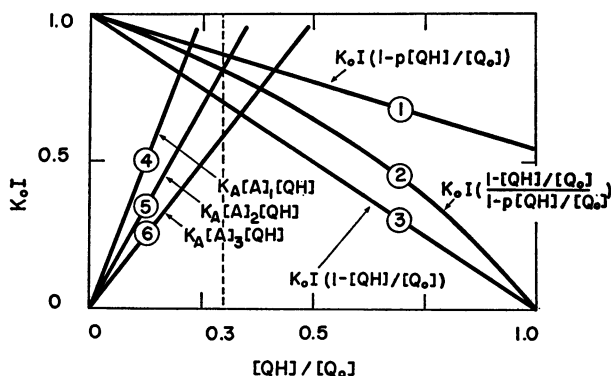


FIGURE 8 Terms of equations 10 and 11 as a function of  $[QH]/[Q_o]$ .  $p$  is taken as  $0.45^{18}$ , and  $[A]_1 > [A]_2 > [A]_3$ .

axis intercepts, whence  $d[QH]/dt = K_o I$ . As the transient progresses,  $[QH]$  rises, and points of interest move to the right along the curves. At  $I$  of the transient,  $[QH]/[Q_o]$  is about 0.3 (calculated according to  $F_{60}$  in Fig. 4 and discussion in part II), and  $d[QH]/dt = 0$ .  $I$  therefore is represented by an intersection of curve 2 or 3 with curve 4, 5, or 6. We chose a slope for curve 5 such that the intersection would occur at  $[QH]/[Q_o] = 0.3$ . Because the difference in intersection points using curve 2 or 3 is small, and we seek only qualitative conclusions, use of equation 11 rather than 10 is permissible.

Because  $[A]$  is an unknown function of time due to coupling with succeeding electron transport intermediates) we will first assume  $[A]$  constant in order to solve equation 11. For  $[A]$  constant, and  $[QH] = 0$  when  $t = 0$ ,

$$[QH] = \frac{[Q_o]IK_o}{K_o I + K_A[A][Q_o]} (1 - \exp[-t\{K_o I/[Q_o] + k_A[A]\}]) \quad (12)$$

By equation 12, if  $[A]$  only decreased (as  $QH$  reduced  $A$ ),  $[QH]$  would never decrease and permit a dip. Also, by Fig. 8, if  $[A]$  only decreased, the intersection point representing  $d[QH]/dt = 0$  at  $I$  would move only to higher values of  $(QH)$ . However, the dip is an experimental fact, and represents a decrease in  $[QH]$ . Therefore,  $[A]$  must increase.

Only if more than two forms of  $Q$  participate beyond  $I$  can this conclusion be avoided.

An increase may occur in the series scheme by two mechanisms: (1) oxidation of  $AH$  by dark reactions with substances at a more positive redox potential, and (2) oxidation of  $AH$  by the action of system I. We believe that (1) is slow (or opposed by a reducing pool) and does not completely oxidize  $AH$  even after 30 min of dark, because weak system I preillumination lowers the dark-adapted I level (55-57). Left with (2), we conclude that the dip signifies an increase in  $[A]$  and  $[Q]$  due to the action of system I.

If system I oxidizes  $AH$  by a first order reaction, whose rate is  $k[AH]$ , then

$$d[A]/dt = -k_A[A][QH] + k[AH] = -k_A[A][QH] + k([A_o] - [A]) \quad (13)$$

where  $[A_o] = [A] + [AH]$ . Simultaneous solution of equations 13 and 11 (or better, 13 and 10) would allow a test of the equations against the  $O-I-D$  phase. Unfortunately, the equations are nonlinear, and require for solution the specific values of all constants and initial concentrations (58). Well-established values are not yet available. In general, such equations lead to a variety of behaviors, only sometimes periodic and/or stable.

### B. Linear Systems.

1. *A linear system with three components.* Among those systems considered, the most promising is:



We solved the rate equations for equation 14 and later found them discussed in the chemical literature. A thorough analysis was published by Lowry and John (59). Our solutions for  $[X_2]$  is

$$[X_2] = X_{2m} \left\{ -\frac{m_2(k_3 - m_1)}{k_3(m_2 - m_1)} e^{-m_1 t} + \frac{m_1(k_3 - m_2)}{k_3(m_2 - m_1)} e^{-m_2 t} + 1 \right\}$$

where

$$X_{2m} = k_1 k_3 / (k_2 k_4 + k_1 k_3 + k_1 k_4)$$

$$m_{1,2} = \frac{1}{2}(p \pm q)$$

$$p = k_1 + k_2 + k_3 + k_4$$

and

$$q = (p^2 - 4\{k_2 k_4 + k_1 k_3 + k_1 k_4\})^{1/2}. \quad (15)$$

The steady-state value of  $[X_2]$  is not zero. The initial transient in  $[X_2]$  depends on the ratio of  $k_1$  to  $k_3$ : if  $k_1 > k_3$ , and  $k_2$  is small, then  $[X_2]$  rises from zero to a temporary maximum which is higher than the steady-state value. This behavior is similar to  $O-I-D$  of the fluorescence transient (except for the  $S$ -shape close to  $O$  at extreme intensity). (Equations 15 do not allow, however, a second maximum as is seen with  $P$  of the *Chlorella* transient.) The question is whether  $X_2$  may be identified with  $QH$ .

A review by Kok and Cheniae (60) has presented an arrangement of the reactions of  $Q$  in the Joliot model (61). (We have assumed that  $Q$  and  $E$  have a one-to-one

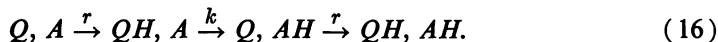


correspondence.  $E$  is Joliot's reaction center complex, either oxidized- $E$ , reduced- $EH$ , or inactive- $EiH$ .) The reactions of  $Q$  are homomorphic with equation 14 by an identification of  $X_1$ ,  $X_2$ , and  $X_3$  as  $Q_iH$ ,  $QH$ , and  $Q$ . The homomorphism is suggestive, but there is an argument against this identification. Joliot (10) measured simultaneously the rate of oxygen evolution and fluorescence during the fluorescence transient and found that preilluminated *Chlorella* show the fluorescence phases  $O$ ,  $I$ , and  $P$ , but not an activation phase in oxygen evolution. The lack of an activation phase indicates that the preillumination has converted all  $Q_iH$  to the active forms  $QH$  and  $Q$ . Since  $O$ ,  $I$ , and  $P$  appear when  $Q_iH$  is apparently not present,  $I$  and  $D$  are probably not due to the interaction of  $Q_iH$  with  $QH$  and  $Q$ .

However, a form  $Q'$  as suggested by Duysens and Sweers (9) may participate in  $D$  according to equation 14. Specifically,  $X_1$ ,  $X_2$ , and  $X_3$  might be  $Q$ ,  $QH$ , and  $Q'$ . If so, then  $k_1$  is proportional to light intensity and a higher light intensity should make  $I$  and  $D$  more prominent. Delosme (18) found an increasing  $I$  and more pronounced  $D$  as intensity rose to high values (10 to 100 times our intensity).

If  $Q$  has three forms connected as in equation 14, then  $[A]$  need not increase to allow  $D$ ; it will occur for any value of  $[A]$  as long as  $k_1 > k_3$  and  $k_2$  is small. Nonetheless, the evidence of section II of Results indicates that  $[A]$  does increase.

2. *The Malkin model.* A linear system has been proposed by Malkin (14) who assumed a chemically first-order reaction between  $QH$  and  $A$ . This assumption allows  $Q$  and  $A$  to be treated mathematically as a single entity,  $Q, A$ . The sequence of reactions is:



The steps  $r$  represent photoreduction of  $Q$ , and the step  $k$  represents dark oxidation of  $QH$  by  $A$ .

Does this system allow a dip? Analysis shows that it does not. A sketch of the proof follows.

Malkin (14) used the labels  $n_1$ ,  $n_2$ ,  $n_3$ , and  $n_4$  for  $[Q, A]$ ,  $[QH, A]$ ,  $[Q, AH]$ , and  $[QH, AH]$ . The sum of all  $n$  is a constant  $n_0$ . If at  $t = 0$ , only  $n_1$  is present and  $k \neq r$ , the solutions are (after Malkin [14]):

$$\begin{aligned} n_1 &= n_0 e^{-rt} \\ n_2 &= \frac{n_0 r}{k - r} (e^{-rt} - e^{-kt}) \\ n_3 &= \frac{n_0 kr}{k - r} t e^{-rt} - \frac{n_0 kr}{(k - r)^2} (e^{-rt} - e^{-kt}) \end{aligned}$$

and

$$n_4 = n_0 - (n_1 + n_2 + n_3). \quad (17)$$

The case  $k = r$ , a singularity where equations 17 are not valid, will receive special attention later.

Malkin used equation 5 which may be incorrect because it assumes that system II units are separate, an assumption made unlikely by the observations of Morin (20) and Delosme (18). However, fluorescence monotonically follows  $[QH]$ ; since equation 5 incorporates the monotonic feature, it is adequate.  $(F_r - F_o)/[Q_o]$  in equation 5 will be replaced by  $b$ . As  $[QH]$  is equivalent to  $(n_2 + n_4)$  we write

$$F = F_o + b(n_2 + n_4). \quad (18)$$

From equations 17 and 18

$$\begin{aligned} dF/dt = b \left[ rn_o e^{-rt} + \frac{n_o kr^2}{(k-r)} te^{-rt} - \frac{n_o kr}{(k-r)} e^{-rt} \right. \\ \left. - \frac{n_o kr^2}{(k-r)^2} e^{-rt} + \frac{n_o k^2 r}{(k-r)^2} e^{-kt} \right]. \quad (19) \end{aligned}$$

We factor  $n_o r e^{-rt}/(k-r)^2$  and obtain

$$dF/dt = b \frac{n_o r e^{-rt}}{(k-r)^2} [-2rk + r^2 + krt(k-r) + k^2 e^{-(k-r)t}]. \quad (20)$$

The factor  $e^{-rt}$  insures a zero slope when  $t \rightarrow \infty$ , as required. Let  $R$  stand for the terms inside the brackets.  $R$  must be negative at a finite  $t$ , to give negative  $dF/dt$ , because terms other than  $R$  are positive for finite  $t$ . Munday (55) has proven that  $R \geq 0$  for both  $K < r$  and  $K > r$  and  $dF/dt$  is never negative, and  $F$  does not show a dip. The method involves examining derivatives of  $R$  with respect to  $t$ .

For the case  $k = r$ , we obtain new solutions for system 16:

$$\begin{aligned} n_1 &= n_o e^{-rt} \\ n_2 &= rn_o t e^{-rt} \\ n_3 &= (\frac{1}{2})r^2 n_o t^2 e^{-rt} \\ n_4 &= n_o - (n_1 + n_2 + n_3). \end{aligned} \quad (21)$$

As before, we find:

$$dF/dt = bn_o r e^{-rt} [1 + (\frac{1}{2})r^2 t^2 - rt] = bn_o r e^{-rt} R'. \quad (22)$$

The factor  $e^{-rt}$  insures a zero slope as  $t \rightarrow \infty$ , as required. For finite  $t$ ,  $dF/dt$  can be zero only if  $R'$  becomes zero. By analysis of  $R'$  the same as that indicated for  $R$  above, the minimum value of  $R'$  is  $+\frac{1}{2}$ , at  $t = 1/r$ . Thus  $R'$  is never zero. The same conclusion is reached by directly solving  $R'$  for  $t$ , assuming that  $R' = 0$ :

$$1 + (\frac{1}{2})r^2t^2 - rt = 0. \quad (23)$$

By the quadratic formula,

$$t = (1/r) \pm (i/r). \quad (24)$$

The permitted solutions for  $t$  are complex. Therefore, no real finite  $t$  allows  $dF/dt$  to vanish. Consequently, for  $k = r$ ,  $F$  does not show a dip.

Thus, for all cases  $k > r$ ,  $k < r$ , and  $k = r$ ,  $F$  does not show a dip. Consequently, the Malkin (14) model in its present form cannot be used to explain the dip in the fluorescence transient of *Chlorella*.

### CONCLUSION

We believe the dip,  $D$ , in the fluorescence transient of *Chlorella pyrenoidosa* involves oxidation of  $QH$  via system I (see Results And Discussion, II and III). From the mathematics (see Mathematical Analysis), if two forms of  $Q$  participate in  $D$ , then system I action is required for  $D$ . If three forms participate, the mathematics allow that  $D$  does not require system I action. Experimental results suggest, however, that system I action is involved. The  $D$ — $P$  rise is suggested to be due to a rise in  $[QH]$  caused by development of a block in oxidation of  $XH$ .

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