

# The dependence of the shapes of fluorescence induction curves in chloroplasts on the duration of illumination pulses

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**ABSTRACT** The shapes of fluorescence induction curves in spinach chloroplasts, measured using double-flash pump-probe techniques, are shown to depend on the duration of the actinic flashes. For flash durations  $\tau_0 \leq 2 \mu\text{s}$ , the variable fluorescence  $F_v$  grows exponentially (or nearly so) with increasing fluence  $J$  of the actinic pulses and the fluorescence induction ratio  $R = F_{\text{max}}/F_0$  is  $\leq 2.6$ . When  $\tau_0 \geq 50 \mu\text{s}$ , the shapes of the  $F_v$  vs.  $J$  curves are sigmoidal, and  $R > 3.2$ . Overall, the experimentally observed trends suggest that, as the duration  $\tau_0$  of the actinic pulses is increased, the degree of sigmoidicity, the deduced values of the interunit excitation transfer parameter  $p$ , and the fluorescence induction ratios  $R$ , also tend to increase. These results can be accounted for in terms of a simple double-photon hit model in which a dark lag time  $\tau_1 = 0.4\text{--}10 \mu\text{s}$  between the two hits is necessary for the observance of sigmoidal fluorescence induction curves and relatively high  $R$  ratios. It is shown that, in principle, such a model can account for the exponential and sigmoidal shapes of the fluorescence induction curves either within the context of a lake model of the photosynthetic antenna bed (free transfer of excitation between photosynthetic units) or the isolated (puddle) model of photosystem II reaction centers. However, from the known values of the  $R$  ratio measured with actinic pulses of different durations, or under continuous illumination, the lake model offers a better description of the experimental phenomena than the puddle model.

## INTRODUCTION

The fluorescence yield in green plants depends on the state of the Photosystem II (PS II) reaction centers. As the PS II reaction centers (RC's) are progressively closed during continuous steady-state illumination, the fluorescence yield increases in a sigmoidal manner on time scales of milliseconds to seconds (for a review, see Lavorel and Etienne, 1977). The sigmoidal shapes of these fluorescence induction curves have been interpreted in terms of a cooperativity between photosynthetic units. In this model, an excitation arriving at a closed reaction center can migrate to an adjacent open one and undergo capture (Joliot and Joliot, 1964; Lavorel and Joliot, 1972; Paillotin, 1976). The progressive closing of the RC's results therefore in increases in both the fluorescence yields and mean excitation lifetime (Keuper and Sauer, 1989; Hodges and Moya, 1986; Holzwarth, 1987). A complicating feature in interpreting the shapes of experimental fluorescence induction curves in terms of this model, is the presence of the two independent photosystems PS II<sub>a</sub> and PS II<sub>b</sub> (Melis and Homann, 1976; Melis and Duysens, 1979).

The sigmoidicity of fluorescence induction curves is one of the major arguments in favor of the cooperativity,

multi-central, or lake model of energy transfer in photosynthetic units of green plants. In this model, the apparent cross-section of PS II is predicted to increase as the PS II reaction centers become progressively closed (Mauzerall and Greenbaum, 1989). However, utilizing single-turnover flashes, Ley and Mauzerall (1986) showed that the apparent cross-section of PS II in *Chlorella* is nearly independent of the fraction of PS II reaction centers closed; this result implies that either the probability of escape from closed PS II reaction centers is small (which would be consistent with exponentially shaped fluorescence induction curves) or that the escape probabilities from open and closed traps are nearly equal.

The shapes of fluorescence induction curves measured by the pump-probe flash technique (Mauzerall, 1972) are exponential in shape and are well approximated by cumulative one-hit Poisson distributions (exponential growth) when short pump flashes ( $< 1 \mu\text{s}$ ) are employed (Mauzerall, 1976; Ley and Mauzerall, 1982a,b, 1986; Deprez et al., 1983; Geacintov et al., 1984; Falkowski et al., 1986; Mauzerall and Greenbaum, 1989). Nonsigmoidal fluorescence induction curves using  $\sim 2 \mu\text{s}$  xenon flashes and weak probe flashes  $20 \mu\text{s}$  after the actinic flash, have also been reported by Joliot and Joliot (1977).

According to presently available theories, the shapes

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of the fluorescence induction curves should not depend on the mode of delivery of photons involving either flash or continuous illumination. The fraction of closed RC's, and thus the fluorescence yield probed either during the continuous illumination or with a probe flash after the actinic pulse, should depend on the number of photons absorbed (Geacintov et al., 1987). In this paper, a simple two-photon hit mechanism is proposed and developed which can account for these variations in the shapes of the fluorescence induction curves and the ratios  $R$  as a function of the duration of the actinic flashes, either within a context of the lake model or the puddle model. While the focus of this work is not on a differentiation between these two models, the experimental results are shown to be more consistent with a lake model than with the puddle model, as suggested by other results as well (see for example Paillotin et al., 1979; Van Grondelle, 1985; Geacintov and Breton, 1987).

## SUMMARY OF EXPERIMENTAL RESULTS

Recent measurements (France, 1989; France et al., 1990; Jursinic, unpublished observations) have shown that the shape of the fluorescence induction curves, when measured by the pump-probe method (Mauzerall, 1972), depends on the duration of the pump flash; if the actinic flash has a duration of  $< \tau_0 \approx 2 \mu\text{s}$ , the shapes of the fluorescence induction curves (determined by weak probe flashes 30  $\mu\text{s}$  or longer after the actinic pump flashes of variable fluences) are approximately exponential in shape. If, on the other hand,  $\tau_0 \geq 50 \mu\text{s}$ , the induction curves assume the familiar sigmoidal shape. The differently shaped fluorescence induction curves are also characterized by differences in the fluorescence ratios  $R = F_{\text{max}}/F_0$  ( $F_{\text{max}}$  and  $F_0$  are the fluorescence yields when all of the PS II reaction centers are closed and

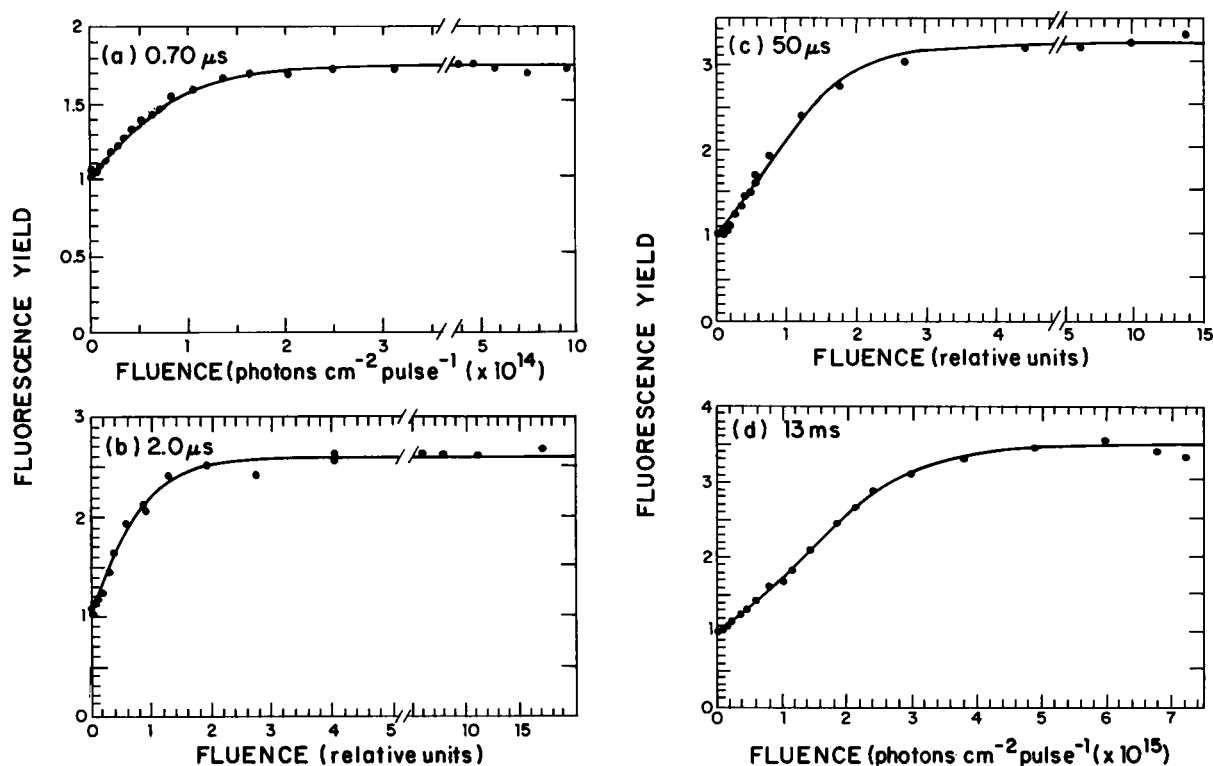


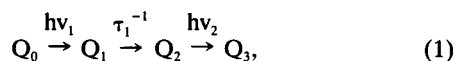
FIGURE 1 Fluorescence induction curves in spinach chloroplasts ( $0^\circ\text{C}$ ) measured with different duration ( $\tau_0$ ) actinic pulses using the pump-probe technique. (a)  $\tau_0 = 0.70 \mu\text{s}$  dye laser flash (650 nm). Weak ( $\sim$ microseconds) xenon probe flash incident on sample 100  $\mu\text{s}$  after the actinic flash. Theoretical curve: plot of Eq. 15 with the connectivity parameter  $p_1 = 0.28$ , which is the value of  $p_1$  which still gives the best fit to the experimental data ( $\chi^2 = 0.0044$ ). (b)  $\tau_0 = 2.0 \mu\text{s}$  (10% of maximum intensity at 6  $\mu\text{s}$ ) followed by a weak (50  $\mu\text{s}$ ) xenon flash 900 ms after the actinic flash; 10  $\mu\text{M}$  DCMU added to prevent reoxidation of the PS II acceptors. Theoretical curve: plot of Eq. 15 with  $p_1 = 0.31$  ( $\chi^2 = 0.0042$ ). (c)  $\tau_0 = 50 \mu\text{s}$  xenon flash followed by a weak (2  $\mu\text{s}$ ) xenon flash 900 ms after actinic flash; 10  $\mu\text{M}$  DCMU. Theoretical curve: plot of Eq. 15 with  $p_1 = 0.53$  ( $\chi^2 = 0.0057$ ). (d)  $\tau_0 = 13 \text{ ms}$  wide, square pulse obtained with He-Ne laser excitation (632.8 nm) and an electronic shutter, with a 2- $\mu\text{s}$  xenon probe flash following 5 ms after the actinic pulse; 10  $\mu\text{M}$  DCMU. Theoretical curve: plot of Eq. 15 with  $p_1 = 0.59$  ( $\chi^2 = 0.0023$ ).

open, respectively); when  $\tau_0 \leq 2 \mu\text{s}$ ,  $R = 1.8\text{--}2.5$ , while  $R = 3.3\text{--}4.0$  when  $\tau_0 \geq 50 \mu\text{s}$  and the induction curves are sigmoidal in shape. It should be noted that in the presence of the inhibitor DCMU, these results do not depend on the time interval (30  $\mu\text{s}$ –900 ms) between the actinic and probe flashes (France, 1989). In the absence of DCMU, similar  $R$  ratios are obtained, but the fluorescence yield measured with the probe flash declines as the time interval is increased beyond a few hundred microseconds because of the reoxidation of the primary acceptor ( $Q_A^-$ ).

Some examples of fluorescence induction curves obtained by the pump-probe technique using different actinic pulse durations are shown in Fig. 1. Each individual datum point was obtained with a fresh, dark-adapted (at least 1 h) sample. In some experiments, DCMU was added to prevent reoxidation of  $Q_A^-$ , which was an important consideration when longer actinic pulse lengths were used. However, the presence of DCMU did not noticeably affect the magnitude of the fluorescence yield probed with the second flash. Independent experiments (data not shown) showed that the fluorescence yield in the presence of DCMU was independent of the pulse separation at least up to 1 s. An extended abstract describing similar experimental results has been published (France et al., 1990), and a fuller account of these and other experimental results is now in preparation (France, L. L., N. E. Geacintov, J. Breton, and L. Valkunas, manuscript in preparation).

## THE MODEL

A simple model which can account for the experimental observations involves a double hit per PS II reaction center in which the dark interval between the two successive excitations is  $\tau_1$ :



where  $Q_0$  denotes open PS II RC's while  $Q_1$ ,  $Q_2$ , and  $Q_3$  denote the PS II RC in different states and  $h\nu_1$  and  $h\nu_2$  denote the first and second absorbed photon hits.

Some important qualitative conclusions which have an immediate bearing on the experimental results can be drawn from this scheme. The state  $Q_1$  acts as a delay for the effective utilization of a second excitation by the RC. If the actinic pulse duration  $\tau_0 \ll \tau_1$ , the transition  $Q_1 \rightarrow Q_2$  cannot occur and the RC can exist only in the states  $Q_0$  and  $Q_1$ . In the case of  $\tau_0 \gg \tau_1$ , i.e., a long actinic pulse or continuous illumination, all states,  $Q_0$ ,  $Q_1$ ,  $Q_2$ , and  $Q_3$  of the RC can exist simultaneously. The relative weights of each of these states at a particular

time after the onset of illumination depends on the kinetic parameters linking these states to one another. The central hypothesis of our model is that the exponentially shaped fluorescence induction curves and low  $R$  ratios occur when  $\tau_0 \ll \tau_1$ , and when  $Q_0$  and  $Q_1$  are present only. On the other hand, sigmoidally shaped curves can occur whenever  $\tau_0 \gg \tau_1$  and there is a sufficient time lag after the onset of illumination so that the states  $Q_2$  and  $Q_3$  can be populated.

Lavorel (1972) has previously proposed a two-hit model, but without the requirement of an intervening dark or lag-time between the two hits. He found that such a model, under certain conditions, could give rise to sigmoidal fluorescence induction curves even without the assumption of energy transfer between photosynthetic units. Thus, the shapes of the fluorescence induction curves cannot, in principle, provide an unequivocal differentiation between the lake (multicentral) or the puddle (monocentral) models of excitation transfer between photosynthetic units in green plants (Geacintov and Breton, 1987; France et al., 1990). Nevertheless, it is shown here that the predictions of our model, coupled with experimentally available parameters, are more consistent with the lake model than with the puddle model. Suitable expressions for evaluating the dependence of the variable fluorescence on the average number of hits per PS II reaction center using the lake and puddle models are described below. We begin with the lake model.

## Dependence of shapes of fluorescence induction curves on duration of pulsed illumination within the context of the lake model

Assuming that excitations can diffuse freely within the photosynthetic membranes, a set of differential equations can be written to describe the time dependence of excitations in photosynthetic systems (Paillotin et al., 1979, 1983; Valkunas, 1986). Defining  $n$  ( $= n(t)$ ) as the mean concentration of excitations per reaction center at any given time  $t$ , the following set of equations is obtained:

$$\frac{dn}{dt} = -[k_0(1 - q_1 - q_2 - q_3) + k_1q_1 + k_2q_2 + k_3q_3]n + I \quad (2)$$

$$\frac{dq_1}{dt} = \mu_0k_0(1 - q_1 - q_2 - q_3)n - q_1/\tau_1 \quad (3)$$

$$\frac{dq_2}{dt} = q_1/\tau_1 - \mu_2k_2nq_2 \quad (4)$$

$$\frac{dq_3}{dt} = \mu_2k_2nq_2, \quad (5)$$

where  $q_1$ ,  $q_2$ , and  $q_3$  are the fractions of the PS II reaction centers in states  $Q_1$ ,  $Q_2$ , and  $Q_3$ , respectively, while  $I$  is

the rate of generation of excitations per reaction center (which is proportional to the fluence and the absorption cross-section). The parameters  $k_0$ ,  $k_1$ ,  $k_2$ , and  $k_3$  denote the overall rate constants of decay of excitations when the RC's are in the states  $Q_0$ ,  $Q_1$ ,  $Q_2$ , and  $Q_3$ , respectively;  $\mu_0$  and  $\mu_2$  denote the respective quantum yields of transformation of the reaction centers from  $Q_0$  to  $Q_1$  and from  $Q_2$  to  $Q_3$ . Eqs. 2–5 describe the generation and the weighted unimolecular decay rates of the excitations.

Denoting the concentration of open RC's by  $q_0$ , the "law of conservation of reaction centers" is equivalent to the following:

$$q_0 + q_1 + q_2 + q_3 = 1. \quad (6)$$

Summing Eqs. 3–5, we obtain the following equation describing the evolution in time of the overall concentration of closed reaction centers:

$$d/dt [q_1 + q_2 + q_3] = \mu_0 k_0 [1 - q_1 - q_2 - q_3] n. \quad (7)$$

Integration of this equation as described previously (Geacintov et al., 1984; Kudzmauskas et al., 1985; Valkunas, 1989) gives:

$$q_1 + q_2 + q_3 = 1 - [1 - (q_1 + q_2 + q_3)_0] \exp [-\mu_0 k_0 \int_0^t n dt], \quad (8)$$

where  $(q_1 + q_2 + q_3)_0$  denotes the initial fraction of closed reaction centers. In the case of very short actinic pulses ( $\tau_0 \ll \tau_1$ ), Eq. 8 determines the fraction of reaction centers transformed into the state  $Q_1$  because  $q_2 = q_3 \approx 0$ .

### Steady-state approximation

When the duration of the actinic pulses,  $\tau_0$ , exceeds several hundreds of nanoseconds, it follows that:

$$\tau_0 \gg k_0^{-1}, k_1^{-1}, k_2^{-1}, k_3^{-1} \quad (9)$$

which justifies the use of the steady-state approximation to solve Eq. 2 as follows:

$$n = \frac{k_0^{-1} I}{1 - p_1 q_1 - p_2 q_2 - p_3 q_3}, \quad (10)$$

where the  $p$  values are defined according to Paillotin (1976):

$$p_i = (k_0 - k_i)/k_0. \quad (11)$$

The parameter  $p$  is defined as the probability of interunit transfer of excitations (Joliot and Joliot, 1964; Lavorel and Joliot, 1972; Paillotin, 1976); more specifically,  $p_1$  is the probability that an excitation which encounters a reaction center in the state  $Q_1$  will be transferred to a neighboring unit. The fluorescence quantum yield  $F$  can

be derived from Eq. 10 as follows:

$$F = \frac{k_F/k_0}{1 - p_1 q_1 - p_2 q_2 - p_3 q_3}, \quad (12)$$

where  $k_F$  is the radiative fluorescence rate constant. The variable fluorescence yield,  $F_v$ , is defined as follows:

$$F_v = \frac{F - F_0}{F_{\max} - F_0} \quad (13)$$

which, normalizing the value of  $F_0$  to unity, becomes:

$$F_v = (F_{\max} - F_0)^{-1} (k_F/k_0) \frac{p_1 q_1 + p_2 q_2 + p_3 q_3}{1 - p_1 q_1 - p_2 q_2 - p_3 q_3}. \quad (14)$$

### Short actinic flashes

In the case of short excitation pulses ( $\tau_0 \ll \tau_1$ ),  $q_2 = q_3 = 0$ , as already noted. Under these conditions  $F_{\max} = (k_F/k_0)(1 - p_1)^{-1}$ , and the following well-known equation is obtained from Eq. 14:

$$F_v = \frac{(1 - p_1) q_1}{1 - p_1 q_1}. \quad (15)$$

This equation, with  $p_1$  replaced by the Joliot and Joliot (1964) parameter  $p$ , describes the variable fluorescence in the classical case when only  $Q_0$  and  $Q_1$  can exist (Paillotin, 1976).

When the pulse duration  $\tau_0$  is of the same order of magnitude as  $\tau_1$ ,  $q_2$ , and  $q_3$  are no longer equal to zero, and the fluorescence yield is now a function of all of the  $Q$  states. Under these conditions, Eqs. 3–5 cannot be solved using the steady-state approximation. Nevertheless, at high fluence rates and for sufficiently long illumination times,  $q_1$  and  $q_2$  approach zero, and  $F_v$  can once again be approximated by Eq. 15, with a replacement of the variables  $p_1$  and  $q_1$ , by  $p_3$  and  $q_3$ :

$$F_v = \frac{(1 - p_3) q_3}{1 - p_3 q_3}. \quad (16)$$

Eq. 15 is similar to the one derived by Ley and Mauzerall (1986) for the case of negligible escape probabilities of excitations from open reaction centers and finite probabilities of escape ( $p_1 = A$  in Ley and Mauzerall's notation) from closed reaction centers. This picture is consistent with the results of picosecond kinetics of fluorescence and absorbance change experiments in photosystem II particles obtained by Schatz et al. (1987); these workers have shown that open PS II reaction centers act as shallow, reversible traps; however, most excitations are ultimately trapped by the open reaction centers, whereas in the presence of closed reaction centers the overall probability of trapping decreases by a factor of 2–3. Keuper and Sauer (1989)

have shown that fluorescence decay parameters obtained under conditions of varying background illumination, can be used to reconstruct sigmoidal fluorescence yield induction curves. The widespread use of Eq. 15 for operationally representing classical, steady-state illumination fluorescence induction curves is thus justified.

### Shapes of fluorescence induction curves in relation to the values of the parameters $p$ and $R$

The shape of the steady-state illumination fluorescence induction curves depends on the parameter  $p$ . If  $p_i$  in Eqs. 15 and 16 is zero, then  $F_v \approx q_i$ , and the fluorescence curves are exponential in shape. The same result is obtained by Ley and Mauzerall (1986) when either the escape probabilities from open and closed reaction centers are very small, or when these two probabilities are equal to one another. Paillotin (1976) showed that sigmoidicity appears only when the parameter  $R = F_{\max}/F_0 \geq 1.5$ ; since, in Paillotin's model,

$$R = (1 - p)^{-1} \quad (17)$$

this means that fluorescence induction curves are sigmoidal for  $p$  values above one-third only. In practice, it is difficult to distinguish sigmoidal from exponential induction curves as long as  $p_i \leq 0.5$  (Eq. 15), while sigmoidicity is clearly apparent when  $p_i \geq 0.60$ . Induction curves plotted according to Eq. 15 for different values of  $p$  ( $= 0.3, 0.5$ , and  $0.75$ ) are shown in Fig. 2; these curves were calculated according to the methods outlined below for the case  $\tau_0 \ll \tau_i$ ; this corresponds to the classical fluorescence induction model (Joliot and Joliot, 1964; Paillotin, 1976) when only one fluorescence quencher ( $Q_i$ ) is formed via a single hit, and  $F_v$  is described by Eq. 15. When  $p = 0.3$ ,  $F_v$  is almost proportional to  $Q_i$ , as predicted by the puddle model (Lavorel and Etienne, 1977) and the induction curve is almost exponential. The  $p_i = 0.5$  curve (Fig. 2 b) is just barely sigmoidal, while the  $p_i = 0.75$  curve (Fig. 2 c) shows clearly sigmoidal behavior.

Utilizing explicit equations relating the fraction of reaction centers to the fluence (Joliot and Joliot, 1964), best fits of Eq. 15 to the experimental data using  $p_i$  as an adjustable parameter were determined using a nonlinear, regression least-squares technique based on the Marquardt algorithm (Bevington, 1969). The values of  $p$  ( $= p_i$ ) and the values of  $\chi^2$  (sum of squares of the residuals divided by the degrees of freedom) are listed in the legends to Fig. 1. For actinic pulses with durations of  $2 \mu\text{s}$  or less, these curves are almost exponentially shaped and the  $p_i$  values do not exceed values of  $\approx 0.3$ . It is interesting to note that the  $\tau_0 = 50 \mu\text{s}$   $F_v$  data cannot be adequately fit by Eq. 15 at low values of the fluence,

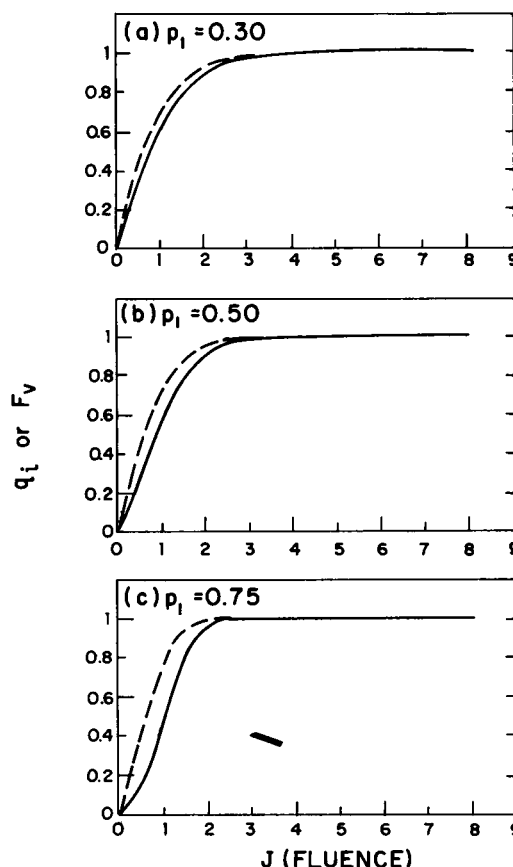


FIGURE 2 Theoretical values of  $q_i$  (-----) and the variable fluorescence  $F_v$  (—) calculated for short actinic flashes ( $\tau_0 \rightarrow 0$ ) according to the lake model. (a)  $p_i = 0.30$ ; (b)  $p_i = 0.5$ ; and (c)  $p_i = 0.75$ .

whereas the  $\tau_0 = 13 \text{ ms}$  data (Fig. 1 d) is well approximated by this equation with  $p_i = 0.59$ .

The foregoing considerations, especially Eq. 17, suggest that exponential fluorescence induction curves (low  $p$  values) should be associated with lower values of  $R$ . For example, if  $p_i = 0.5$ , the induction curves are expected to be almost exponential in shape and, according to Eq. 17,  $R$  is predicted to have a value of 2. On the other hand, if  $p_i = 0.7$ , the induction curves are clearly sigmoidal and  $R = 3.3$ .

In the examples shown in Fig. 1 and in other observations (France, 1989), a trend in which low values of  $R$  are linked with nonsigmoidal fluorescence induction curves is clearly observed. The fluorescence induction curves are exponential (or nearly exponential) in shape when the excitation flashes are  $< \tau_0 \approx 2 \mu\text{s}$  in duration, and the  $R$  values lie in the range of 1.8–2.6. Similar low values of  $R$  were also obtained by other workers who used the pump-probe method for measuring fluorescence induction with short ( $\leq 2 \mu\text{s}$ , or shorter) actinic

pulses (Mauzerall, 1972; Falkowski et al., 1986; Kolber et al., 1988).

When  $\tau_0 \geq 50 \mu\text{s}$ ,  $R \geq 3.2$  and the induction curves are sigmoidal. These trends have been found to be consistent over several years of experimentation in our laboratory; however, more quantitative comparisons between the shapes of the fluorescence induction curves and the  $R$  ratios are difficult because of the known variabilities of the  $R$  ratios for different samples and even for the same sample over periods of time. Furthermore, because of the simplicity of the model on which Eq. 15 is based, we believe that it is unrealistic to expect a close correlation between values of  $R$  and  $p$  deduced from shapes of the fluorescence induction curves. Also, the PS II<sub>a</sub> – PS II<sub>b</sub> heterogeneity of the PS II antenna (Melis and Homann, 1976; Melis and Duysens, 1979; Holzwarth, 1987), would tend to give values of  $R$  higher than those predicted from the values of  $p$ ; however, in this work, to a first approximation, we have neglected the contributions of the  $\beta$  centers to the fluorescence induction curves when we estimated values of  $p$  from experimental  $R$  values.

The shapes of the fluorescence induction curves change rather abruptly when the actinic flash duration is increased from 2 to 50  $\mu\text{s}$ . Therefore, within the context of the model in scheme 1, the dark-reaction time  $\tau_1$  which transforms  $Q_1$  into  $Q_2$ , is therefore likely to be  $\geq 2 \mu\text{s}$ , but  $< 50 \mu\text{s}$ . Furthermore, it follows that for the short pulses,  $p_1 \leq 0.5$ , while for the longer pulses  $p_1 \geq 0.6$ .

### Choice of parameters for calculations of theoretical fluorescence curves

In this section, the rationale for selecting certain values of the parameter  $p$  in our calculations (see below) is summarized. These values are consistent with known experimental data. The double-flash fluorescence induction experiments with  $\tau_0 \leq 2 \mu\text{s}$ , are usually performed with a pump-probe pulse separation of at least 30  $\mu\text{s}$ . Thus, given the above estimate of  $\tau_1$ , the probe flash always arrives when the system is in the state  $Q_2$ . Since, in these short actinic pulse experiments,  $R \approx 2$ , it is reasonable to assume that  $p_2 \approx 0.5$ . In the double-pulse experiments of Mauzerall (1972) and Deprez et al. (1983), the pulse separation was varied from the nanosecond to the microsecond range. With a pulse separation of 100 ns, the observed  $R$  ratio was also  $\sim 2$ . Based on these considerations, we conclude that  $p_1$  is probably also equal to  $\sim 0.5$ , although the role of carotenoid triplet quenchers on these short time scales (Breton et al., 1979; Itoh et al., 1984) cannot be entirely excluded. The nonexponential growth of the fluorescence induction curves when  $\tau_0 \geq 50 \mu\text{s}$ , clearly justifies a value of  $p_3$

larger than 0.5; we have selected a value of  $p_3 \approx 0.75$  (Joliot and Joliot, 1977) in our calculations, because the sigmoidicity is clearly pronounced in the calculated  $F_v$  curves.

For the sake of simplicity, we assumed that the lifetime of the state  $Q_3$  is infinite. Therefore, the results are not strictly applicable to actinic flashes of long duration or to continuous illumination. However, this approximation allows for a rather simple solution of the set of coupled differential Eqs. 2–5, which permits us to evaluate the qualitative aspects and ramifications of the model expressed in scheme 1.

### Solution of differential equations and analysis of results

While the steady-state approximation in Eq. 10 is a valid approximation for  $n$ , the mean number of excitations per RC, this quantity is really time dependent because the quantities  $q_i$  depend on time. Therefore, the evaluation of the integral in Eq. 8 is not simple. As already noted, for short actinic illumination flashes,  $q_2 = q_3 = 0$ ; under these conditions, with  $\tau_1 \rightarrow \infty$ , substituting Eq. 10 into Eq. 8, and integrating, leads to the expression:

$$(1 - p) \ln[(1 - q_1)/(1 - (q_1)_0)] - p[q_1 - (q_1)_0] = -\mu_0 I \tau_0. \quad (18)$$

This equation can be represented in a form which is similar to that of Eq. 8:

$$q_1 = 1 - [1 - (q_1)_0] \exp \left\{ -\frac{\mu_0 I \tau_0}{(1 - p)} + \frac{p[q_1 - (q_1)_0]}{(1 - p)} \right\}. \quad (19)$$

In the case of long pulses, the states  $Q_2$  and  $Q_3$  become significantly populated, and the approximations leading to Eqs. 18 and 19 are no longer applicable. To solve Eq. 8 and to integrate Eqs. 4 and 5, we have employed an iterative procedure using a spreadsheet program (Quattro, Borland International Inc., Scotts Valley, CA). In this procedure we have assumed that the fluence rate  $I$  is constant, and the actinic pulse length  $\tau_0$  was broken up into  $j$  equivalent time intervals. We assumed that in each particular time interval  $j$  the values of  $q_i(j)$  are constant, and that the initial values are given by the value  $q_i(j - 1)$  calculated in the preceding time interval  $j - 1$ . The following iterative equations are then obtained:

$$\begin{aligned} q_1(j + 1) + q_2(j + 1) + q_3(j + 1) \\ = 1 - [1 - q_1(j) - q_2(j) - q_3(j)] \\ * \exp \left\{ -\frac{\mu_0 I \Delta t_j}{1 - p_1 q_1(j) - p_2 q_2(j) - p_3 q_3(j)} \right\} \end{aligned} \quad (20)$$

$$q_2(j+1) = q_2(j) \left[ 1 - \frac{\mu_2(1-p_2)I\Delta t_j}{1-p_1q_1(j)-p_2q_2(j)-p_3q_3(j)} \right] + \frac{\tau_0}{\tau_1} q_1(j) \frac{\Delta t_j}{\tau_0} \quad (21)$$

$$q_3(j+1) = q_3(j) + \frac{\mu_2(1-p_2)I\Delta t_j}{1-p_1q_1(j)-p_2q_2(j)-p_3q_3(j)} q_2(j). \quad (22)$$

In these equations,  $q_i(j)$  is the value of  $q_i$  in the  $j$ th interval, and  $\Delta t_j$  is the duration of each time interval  $j$ . In our calculations, the pulse  $\tau_0$  was divided into 80 equivalent time intervals so that  $\Delta t_j/\tau_0 = 0.0125$ . For simplicity, the quantum efficiencies  $\mu_i$  were set equal to unity. The basic variable in these calculations was the quantity  $\tau_0/\tau_1$  (pulse duration/lifetime of the  $Q_1$  state). The  $p_i$  values were treated as parameters within the constraints obtained from experiments as discussed earlier. In the iterative calculations, values of  $J = I\Delta t_j$  were selected incrementally so that the fluence rate  $I$  remained constant; the values of  $q_i$  and  $F_v$  were calculated for each successive value of  $\Delta t_j$  until the end of the iteration, corresponding to  $\sum_j \Delta t_j = \tau_0$  (i.e., the end of the actinic pulse). This procedure is also applicable to the results of pump-probe experiments in which the calculations are carried out for different fluences  $I$ , and only the values of  $q_i$  and  $F_v$  at the end of the pump pulse are considered.

The effects of the length of the actinic pulse (expressed as the ratio  $\tau_0/\tau_1$ ) on the concentrations of the states  $Q_1$ ,  $Q_2$ , and  $Q_3$ , and on the shapes of the fluorescence induction curves  $F_v$  are shown in Fig. 3.

**Short actinic pulses** For short pulses ( $\tau_0/\tau_1 = 0.1$ ), the concentrations  $q_2$  and  $q_3$  are negligibly small. Adopting a value of  $p_1 = 0.5$  (see above), the plots of  $q_i$  and  $F_v$  as a function of the fluence  $J$  shown in Fig. 3a are obtained. The variable fluorescence function is practically, but not completely exponential in shape. The double-pulse fluorescence induction experiments are not sufficiently accurate (Fig. 1) to discern the small degree of sigmoidicity predicted for  $p_1 = 0.5$ .

**Intermediate length actinic pulses** For intermediate length pulses corresponding to  $\tau_0/\tau_1 = 5$ , there is sufficient time for the states  $Q_2$  and  $Q_3$  to be populated. For the choice of parameters  $p_1 = p_2 = 0.5$ , and  $p_3 = 0.75$ , the sigmoidicity of the  $F_v$  curve is still weak. The lack of sigmoidicity is due to the rather strong contribution of the state  $Q_1$  to  $F_v$  and the relatively high value of the parameter  $p_1 = 0.5$ . The sigmoidicity becomes more pronounced the greater the difference between the pair of parameters  $p_1, p_2$ , and the parameter  $p_3$ . When  $p_3 \gg$

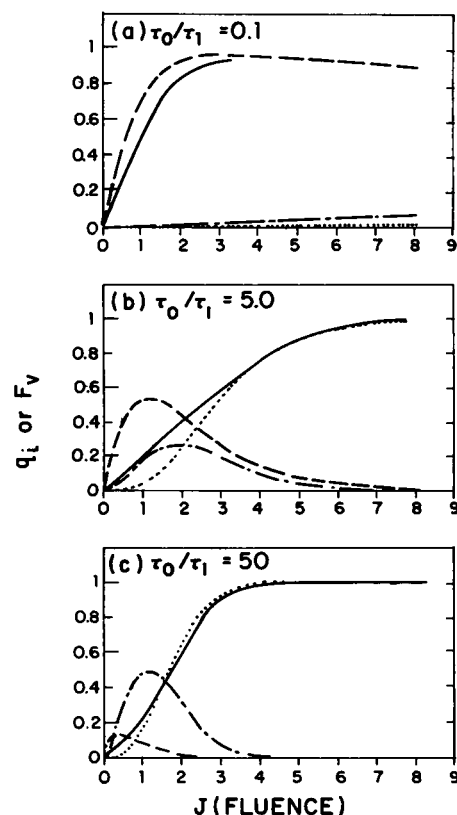


FIGURE 3 Calculations of the concentrations  $q_1$  (----),  $q_2$  (-.-.-),  $q_3$  (.....), and  $F_v$  (—) as a function of  $J$ , the fluence, within the context of the lake model ( $p_1 = p_2 = 0.5$ , and  $p_3 = 0.75$ ) for different durations of actinic flashes expressed in terms of the ratio  $\tau_0/\tau_1$ : (a) 0.1, (b) 5.0, and (c) 50.

$p_1, p_2$ , the function  $F_v$  depends more strongly on  $q_3$  than on  $q_1$  and  $q_2$ , and considerable sigmoidicity is expected. This is evident from the time dependence of the concentrations  $q_1, q_2$ , and  $q_3$  (Fig. 3b) because  $q_3$  rises sigmoidally with increasing  $J$ .

**Long pulses** Using  $p_1 = p_2 = 0.5$ , and a value of  $p_3 = 0.75$ , the fluorescence induction curve is clearly sigmoidal in shape when  $\tau_0/\tau_1 \approx 50$  (Fig. 3c). The reason for the higher sigmoidicity in this case is that the state  $Q_1$  contributes relatively little to  $F_v$  even at low values of  $J$ .

### Comparisons with experimental data

The proposed model can account for the experimental dependence of the shapes of the fluorescence induction curves in chloroplasts on the duration of the actinic pulses. It is evident from the theoretical curves (Fig. 3) that the nearly exponentially shaped induction curves are observed in the case of short actinic pulses, corresponding to the case when the state  $Q_3$  is not yet present.

The calculated  $F_v$  curves suggest that sigmoidicity becomes clearly apparent when the pulse duration  $\tau_0$  is at least five times greater than the lifetime  $\tau_1$  of the  $Q_1$  state. Experimentally, sigmoidicity is observed when  $\tau_0 \approx 50 \mu\text{s}$ , but not for pulse durations  $\leq 2 \mu\text{s}$ ; unfortunately, for technical reasons, intermediate pulse lengths were not available to us which would have permitted a more accurate assessment of the pulse durations associated with the transition from the exponential to the sigmoidal fluorescence induction regime. Nevertheless, from the available data, we can estimate that the value of  $\tau_1$  probably lies in the range of  $\approx 0.4\text{--}10 \mu\text{s}$ ; the lower limit is based on the fact that the  $2 \mu\text{s}$  actinic pulse fluorescence induction pulse was nonsigmoidal, and that the value of  $\tau_0$  must be at least 5 times greater than  $\tau_1$  for sigmoidicity to be weakly manifested (Fig. 3b). The upper limit was arrived at in a similar manner, based on the observation that  $50\text{-}\mu\text{s}$  actinic pulses give rise to sigmoidal curves.

A two-state model (referring to two different states of the reaction centers reached after one or two successive hits per RC, respectively) was previously proposed by Lavorel (1972) to account for the sigmoidal shapes of fluorescence induction curves. However, in Lavorel's model, there was no explicit dark interval time corresponding to the time  $\tau_1$  in scheme (1). In our model, a mean dark interval  $\tau_1$  must elapse before the reaction center can accept a second hit. If multiple hits/RC occur on time scales significantly below  $\tau_1$ , the RC's will not be able to advance to the state  $Q_2$  or  $Q_3$ , regardless of the time interval between the pump and the probe flashes. This dark time lag can account for the changes in the shapes of the fluorescence induction curves when the actinic pulse length is increased beyond the microsecond range.

### Shapes of induction curves predicted from a puddle model

To determine how well a purely photochemical model, i.e., without energy transfer between photosynthetic units, could account for the experimental observations, scheme 1 was viewed as a series of consecutive chemical reactions. This involved solving Eqs. 3–5 with the appropriate modifications (substituting the fluence rates  $I$  for the factors  $k_0 n$  and  $k_2 n$ ). In this model, the overall variable fluorescence yield is simply proportional to the fractions of the reaction centers in the states  $Q_i$  ( $p_i = 0$ ), and the relative quantum yield of fluorescence  $r_i$  of each state. The overall variable fluorescence yield can be shown to equal:

$$F_v = r_1 q_1 + r_2 q_2 + r_3 q_3, \quad (23)$$

where  $F_v$  varies between the limits  $0 \rightarrow 1.0$ , and the relative fluorescence yields  $r_i$  are expressed as follows:

$$r_i = \frac{F_i/F_0 - 1}{F_3/F_0 - 1} = \frac{F_i/F_0 - 1}{R - 1}, \quad (24)$$

where  $F_i$  denotes the fluorescence yield when all RC's are in the state  $Q_i$ ;  $F_3/F_0$  denotes the (maximum) fluorescence yield when all of the reaction centers are in the state  $Q_3$ , and is identified with the experimental parameter  $R$ . Because, experimentally,  $F_v$  increases as the pulse duration is increased (France, 1989), it is assumed that  $r_3 > r_2 \approx r_1$ .

In solving for the values of  $q_1$  and  $q_3$  as a function of  $J$ , which were then used to calculate  $F_v$  (Eq. 23), we have assumed that the quantum yields of the two photochemical reactions in scheme 1 are the same. As Lavorel (1972) has shown, pronounced sigmoidicity is predicted if the photochemical yield of the second reaction is only 50–85% of the value of the yield of the first reaction. However, there is no experimental evidence indicating that the quantum yields of successive photochemical hits differ from one another systematically. Instead, we have assumed that the source of sigmoidal behavior is due to a time lag in the generation of the state  $Q_3$  and the higher fluorescence yield when PS II is in this state.

To evaluate the validity of this model, it is necessary to examine only the limit of long pulse duration. If the puddle model is valid, it should account properly for the sigmoidicity of the classical fluorescence induction curves ( $\tau_0 \gg \tau_1$ ) with the appropriate values of  $r_i$ . Some typical  $F_v$  curves are shown in Fig. 4 in the case of  $\tau_0/\tau_1 = 100$ .

When  $r_1 = r_2 = 0.15$  and  $r_3 = 1$ ,  $F_v$  is definitely sigmoidal in shape. When the  $r_1$  and  $r_2$  values are increased to 0.33 (with  $r_3 = 1$ ), the fluorescence induction curves are still sigmoidal, but only weakly so.

Because, experimentally,  $R \approx 4$  in the case of steady-state excitation, the  $r_1 = r_2 = 0.33$  values correspond to  $F_1 = F_2 = 2F_0 = 0.5F_3$ . In the case of  $r_1 = r_2 = 0.15$ ,  $F_1 = F_2 = 1.45F_0 = 0.36F_3$ . When short actinic pulses are used,  $R \approx 2$ ; therefore, the experimentally determined relative fluorescence yields are  $F_1 \approx F_2 \approx 0.5F_3$ . Thus, the less sigmoidal  $r_1 = r_2 = 0.33$  case should more closely simulate the experimental conditions, than the  $r_1 = r_2 = 0.15$  case. Pronounced sigmoidicity is predicted when  $F_2 = 1.5F_0$  (or less, see Fig. 4), and when  $F_3 = 4F_0$ ; this value of  $F_2$  is lower than the value of  $F_2 = 2F_0$  inferred from experimental data using short actinic illumination pulses.

### Lake vs. puddle model

Given an appropriate choice of parameters, either the lake or puddle models in conjunction with scheme 1 can



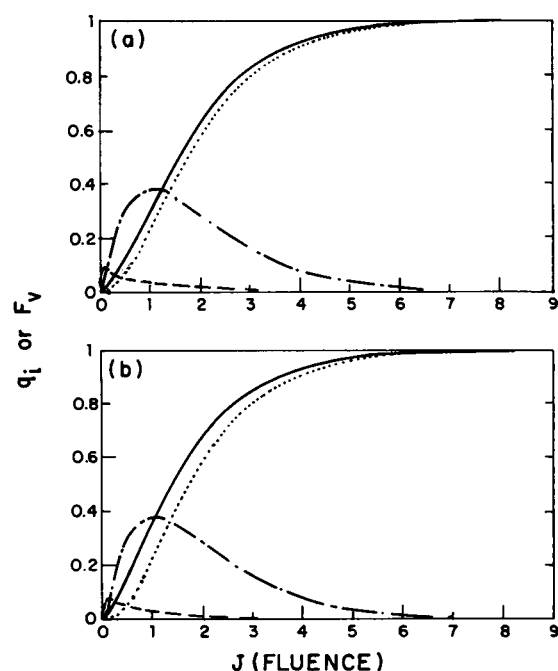


FIGURE 4 Calculations of  $q_1$  (---),  $q_2$  (-·-·-),  $q_3$  (·····), and  $F_v$  (—) as a function of the fluence  $J$  within the context of the puddle model (Eq. 23), for two different sets of relative fluorescence yields  $r_1$ ,  $r_2$ , and  $r_3$  corresponding to the PS II reaction center states  $Q_1$ ,  $Q_2$ , and  $Q_3$ , respectively. (a)  $r_1 = r_2 = 0.15$ ,  $r_3 = 1.0$ . (b)  $r_1 = r_2 = 0.33$ ,  $r_3 = 1.0$ .

account for the shapes of the fluorescence induction curves under conditions of steady-state illumination or when long actinic pulses (relative to  $\tau_1$ ) are employed. However, based on the known values of the relative fluorescence yield values  $R$  after short ( $R \approx 2$ ) and long pulses ( $R \approx 4$ ), it appears that the lake model provides a better fit to the experimental observations than the puddle model. A pronounced sigmoidicity based on the puddle model is predicted only for a choice of parameters somewhat outside those which can be gleaned from the available experimental data.

## DISCUSSION

### Relationships to other fluorescence quenchers

The model of fluorescence induction presented here (scheme 1) is somewhat simpler and is relevant to a different time domain (microseconds) than the coupled differential equation models recently advanced by others (Sorokin, 1985; Renger and Schulze, 1985; Baake and Strasser, 1990). These treatments relate to classical,

steady-state illumination fluorescence induction curves on longer time scales. Specifically, our states  $Q_1$ ,  $Q_2$ , and  $Q_3$ , do not correspond to the two-electron transfer  $Q_A Q_B \rightarrow Q_A Q_B^- \rightarrow Q_A^- Q_B^- \rightarrow Q_A Q_B^-$  because the  $Q_A \rightarrow Q_B$  electron transfer time occurs with a half-time of 100–200  $\mu$ s (Crofts and Wraight, 1983). It is unlikely that  $P680^+$ , a known fluorescence quencher, can account for the changes in the shapes of the fluorescence induction curves. This oxidized state is reduced within  $\sim 100$  ns when single flashes are utilized (see, for example, Mauzerall, 1972, and Deprez et al., 1983). Because in our experiments single flashes were also used and the samples were fully dark adapted, it is unlikely that  $P680^+$ , or any charge recombination processes involving this species, are involved in the phenomena discussed here. However, when multiple flashes are used, the possibility of slow (microseconds) relaxation components of  $P680^+$  must be considered (Schlodder et al., 1985). Other electron acceptors in PS II reaction centers have been identified; for example, Joliot and Joliot (1977, 1979) identified two different fluorescence quenchers in PS II, but their relation to the quenchers  $Q_i$  relevant to this work, if any, is not clear. The presence of a second electron acceptor and fluorescence quencher,  $Q_{400}$ , in PS II has been reported (see for example Dennenberg and Jursinic, 1985); however, this quencher is unlikely to play a role here because it manifests itself in the presence of potassium ferricyanide which was not utilized in our experimental studies (France, 1989).

It is important to note that our model is consistent with the known one hit model for the reduction of  $Q_A$ . Our model states that two hits, with an intervening dark time in the tens of microsecond range, is necessary to reach the highest fluorescence state of the system. The different S states on the donor site are known to be characterized by different fluorescence quenching efficiencies (Saygin and Witt, 1987). The shortest dark-transition times between the different S states are 40–50  $\mu$ s. These kinetics are somewhat slower than the lag-time  $\tau_1$  estimated from our fluorescence induction experiments; nevertheless, because the difference is not large, and our estimate of the value of  $\tau_1$  is uncertain, the possibility that the S-state transitions are responsible for the phenomena described here, should be seriously considered.

Fluorescence induction curves are often interpreted as resulting from a major sigmoidal component arising from connected PS II<sub>a</sub> units (multi-central model), and a smaller exponential component arising from mutually unconnected PS II<sub>b</sub> units (Melis and Homann, 1976; Melis and Duysens, 1979; Thielen et al., 1981; Holzwarth, 1987). The smaller contribution of PS II<sub>b</sub> to the overall fluorescence induction curves has been, for simplicity, omitted in our analysis.

## Sigmoidicity and connectivity in different time domains

When PS II reaction centers are closed with brief pulses of actinic light, the fluorescence quenching properties of the system evolve as a function of time (Mauzerall, 1972). We have shown that, when the fluorescence yield of PS II in chloroplasts is probed  $\sim 30$ – $100 \mu\text{s}$  after the onset of the actinic flash, the fluorescence yield also depends on the width of the actinic pulse (France, 1989; France et al., 1990). Ley and Mauzerall (1986) have shown that the apparent cross-section of PS II reaction centers, as measured by oxygen evolution and fluorescence induction, does not vary by more than  $\pm 10\%$  when the traps are progressively closed by background illumination.

The exponential shapes of fluorescence induction curves measured by the pump-probe technique when the actinic pulse duration is less than  $\sim 2 \mu\text{s}$  (Geacintov et al., 1987; France et al., 1990), can be accounted for in terms of the persistence of a fluorescence quencher into the microsecond time range; the fluorescence yield is 2–2.5 times greater than when the traps are in the open state (France et al., 1990; Kolber et al., 1988), and thus the quenching efficiency of this quencher is lower than that of P680 or P680<sup>+</sup> (Deprez et al., 1983). When the increase in the fluorescence yield or lifetime is relatively small upon closure of the traps, the shapes of the fluorescence induction curves are expected to deviate little from exponentiality. Theoretically, pump-probe fluorescence induction curves under these conditions are predicted to be exponential in shape either within the context of the puddle or the lake models of PS II (Geacintov and Breton, 1987), as is observed experimentally using short actinic flashes (Mauzerall, 1976; Ley and Mauzerall, 1986; Geacintov et al., 1987; Falkowski et al., 1988; France et al., 1990). This is consistent with a “low escape probability” (in Ley and Mauzerall’s terminology) from open and closed PS II traps. It follows that connectivity between photosynthetic units cannot be established under these conditions and employing short actinic pulses.

The sigmoidicity manifests itself only when the widths of the actinic flashes are of the order of  $10 \mu\text{s}$  or more. We propose here that this results from the dark evolution on microsecond time scales of the PS II traps after receiving a first hit, and the absorption of a second hit to transform the traps to a highly fluorescent state. The connectivity then manifests itself in classical, steady-state fluorescence induction curves because there is a sufficient difference between the fluorescence yields of the open and the closed traps (Paillotin, 1976). We have shown here that a purely photochemical two-state model can also account for sigmoidicity, even in the absence of

connectivity; however, the values of the parameters  $r$  and  $R$  which must be used in this puddle model are somewhat outside of the bounds of the experimentally observed values. Furthermore, other results, notably those on exciton-exciton annihilation, are consistent with a lake model of PS II (Paillotin et al., 1979; Geacintov and Breton, 1987; Van Grondelle, 1985). Taken together, there seems to be little convincing evidence in favor of the puddle model, and excitations appear to be free to diffuse over hundreds of chlorophyll molecules during their lifetimes (Geacintov and Breton, 1987), the diffusion lengths depending on their lifetimes and thus on the states of the PS II traps (Schatz et al., 1987). The question is whether such an implied connectivity can be revealed by classical, steady-state fluorescence induction experiments. The most serious challenge to the standard interpretations of such fluorescence induction experiments (Joliot and Joliot, 1964; Paillotin, 1976; Melis and Homann, 1976; Lavorel and Etienne, 1977; Melis and Duysens, 1979) remains the observation of Ley and Mauzerall (1986) that the apparent cross-sections of PS II traps are approximately independent of the fraction of closed PS II reaction centers. In this publication and elsewhere (France et al., 1990), we have shown that sigmoidicity can be observed only when actinic flashes of  $50 \mu\text{s}$  or longer are employed. Rigorous conclusions regarding the connectivity of photosynthetic units in PS II from such steady-state illumination experiments should be made only after re-examining the validity of the two-state photo-chemical model and an explanation of Ley and Mauzerall’s (1986) observations.

## CONCLUSIONS

The experimental data coupled with the theoretical analysis presented here suggests that the sigmoidicity of the fluorescence induction curves may arise from double-hit events per PS II reaction center, in which the second hit occurs only after a mean dark lag-time  $\tau_1$  after the first hit. We have deduced that the dark lag-time  $\tau_1$  lies in the range of  $0.4$ – $10 \mu\text{s}$ . These findings suggest that more detailed studies of the shapes of fluorescence induction curves and associated  $F_{\text{max}}/F_0$  ( $= R$ ) ratios, using well-defined actinic illumination with pulse duration in the  $100 \text{ ns}$ – $50 \mu\text{s}$  time range, would be fruitful. Such experiments should focus on measurements of the fluorescence yield during the illumination itself, or using weak probe flashes incident on the sample some  $30 \mu\text{s}$  or more after the actinic pulses. In the former case, interpretation might be complicated by the presence of carotenoid triplet quenchers (Breton et al., 1979; Itoh et al., 1984). The use of the double-pulse method offers two advantages: (a) the effects of carotenoid triplets on

the fluorescence yields can be avoided because these triplets decay within 10  $\mu$ s or less (Breton et al., 1979); (b) the variable fluorescence yield  $F_v$  can be probed while the system is in the state  $Q_2$  (because  $\tau_1$  would be shorter than the pump-probe pulse separation) rather than in a state of mixed  $Q_1$  and  $Q_2$ . Such kinetic fluorescence yield experiments coupled with transient kinetic absorption experiments might provide a deeper insight into the nature of the intermediate states  $Q_1$ ,  $Q_2$ , and  $Q_3$ , and the phenomena which are responsible for the changes in the shapes of fluorescence induction curves on microsecond time scales.

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