

# Evidence for Unequal Misses in Oxygen Flash Yield Sequence in Photosynthesis

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The numerical analysis of the oxygen flash yield  $Y_n$  sequences, alone, does not allow to choose between two models: equal  $S$  state misses with non negligible double hits or unequal misses with nearly no double hits. Nevertheless, the comparison of the sequences in different conditions shows that the equal miss model is unrealistic: in very different experimental conditions (non saturating flash, different batch of *Chlorella* or chloroplasts), a parallel variation of the homogeneous miss and double hit factors is observed. This correlation seems strange within the equal miss model: misses come from incomplete reaction (*i.e.* for example insufficient light) and double hits *i.e.* double advancement come, in principle, from excessive light or too long flash; for these reasons, opposite variation of misses and double hits as a function of light intensity are expected. Within the equal miss model the inverse is exactly observed: at low flash light intensity (11%) which increases the misses, 16% of double hits are needed, which is quite unrealistic. In contrast, the unequal miss model explains such result quite naturally by a mathematical property: any theoretical sequence with only a unique  $S$  state miss and no double hit can be fitted with homogeneous misses and double hits, which increase in parallel as a function of the damping.

Evidence for unequal misses in oxygen flash yield sequence is provided by the heterogeneous properties of the light saturation curves (M. J. Delrieu, *Biochim. Biophys. Acta* **592**, 478–494 (1980)). At high flash intensity, all, excepted the transition  $S_2 \rightarrow S_3$ , are saturated; the transition  $S_2 \rightarrow S_3$  is far from saturation and its very large saturating light intensity is actually not known.

A comparative study, in the same chloroplast batch, of the oxygen yield patterns with attenuated flashes and of the experimental saturation curves of  $S$  states shows that only photochemical misses (due to non saturation) exist. At high intensity, there is only a unique miss for the transition  $S_2 \rightarrow S_3$  *i.e.* the probability for this transition is low. A model involving a second acceptor could explain the slow increase of transition probability of  $S_2 \rightarrow S_3$  at high flash intensity.

## Introduction

As shown by Kok *et al.* [1], liberation of oxygen requires the cooperation of four photochemically formed oxidizing equivalents in the individual reaction centers generated by four successive excitation of system II. They proposed a four step linear mechanism of positive charge accumulation that led to evolution of a molecular oxygen. Forbush *et al.* [2] have explained the damping of the  $O_2$  yield oscillations by assuming in the model, misses  $\alpha$  (centers not converted even in a very short and saturating flash), and double hits  $\beta$  (or double advancement in  $S$  states of oxygen evolution).

A few years ago, a precise analysis on quantitative basis was elaborated by us to study experimental oxygen flash sequences [3]. The double hit

factor  $\beta$  was eliminated because flashes were sufficiently short (3  $\mu$ s) with regard to the  $S$  state turnover ( $t_{1/2} > 500 \mu$ s) in untreated material (*Chlorella* and chloroplasts) [4] ( $\beta \leq 1\%$ ). In these conditions, the least square approximation method allowed us to conclude to a very heterogeneous repartition of misses for  $S$  states; in most cases, only one  $S$  state gives a miss while the other states have no miss [3].

Using a slightly different mathematical method ( $\sigma_i$  calculation method), Lavorel [5] and principally Thibault [6] were led to reconsider these results. Thibault distinguished the first flash from the others and omitting  $Y_1$  found that the damping of the oxygen oscillations can be explained by homogeneous misses and double hits, without proving that unequal misses were not possible.

Supposing the homogeneity of transition probabilities between  $S$  states, Jursinic [7] using the least square fitting procedure noticed that, within this model, a double hit factor is always associated with the homogeneous misses even if the flash duration

**Abbreviation:** DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

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is as short as 5 ns. This was explained by the possibility of "non-photochemical" double hitting *i.e.* a double advancement in  $S$  states associated with one photon in the same center [7].

In the literature, several independent experiments suggest the existence of two primary acceptors  $X_{320}$ ,  $X_a$  [8, 9, 10] or  $Q_1$ ,  $Q_2$  [11, 12] so that possible double hits, a few ns apart [13], can occur at level of photosystem II charge separation. But, photochemical double advancement in  $S$  states in the ns range was never observed. Practically, the only proof for photochemical double  $S$  state advancement would be the observation of an  $I^2$  dependency in the second oxygen yield  $Y_2$  curve as a function of the energy  $I$  of the first flash at low light intensity (in chloroplasts, the small amount of oxygen evolved on the second flash of a sequence was attributed to double hits [14]); the sigmoidal double hit curve has not been observed [15, 16].

Therefore, there is no valuable reasons to exclude *a priori* the heterogeneous miss hypothesis or the homogeneous miss one. We note that the heterogeneous model is more general and includes as a special case the homogeneous one. All the actually known properties of the four  $S$  states are very heterogeneous: turn over or deactivation kinetics [4], saturation curves [18], etc.; for these reasons, it seems natural that the misses can be also heterogeneous. Following this mean idea, in this paper, the two models were considered. The percentage of misses found by the least square fitting procedure used to non saturating flash yield sequences was compared to the misses due to non saturation deduced from experimental  $S$  state saturation curves. Only the model of unequal misses agrees with experiments. A model able to resolve the apparent contradiction between the possibility of double hits at the level of charge separation and a photochemical miss for the  $S_2' \rightarrow S_3$  transition is proposed.

## Materials and Methods

*Chlorella pyrenoidosa* was grown as previously described [17]. Before use, cells were suspended in a 0.1 M sodium phosphate buffer (pH 7.5). Fresh chloroplasts were prepared from market lettuce as in [18], and suspended in a medium containing 0.4 M sucrose, 10 mM NaCl, 3 mM  $MgCl_2$  and 50 mM N-tris (hydroxymethyl) methylglycine (TRICINE) buffered to pH 7.5.

A rate electrode is used for oxygen flash yield measurements as previously described [16]. Flash excitation was provided with two different sources, short flashes with a duration at the half height of 300 ns, are obtained from a Xenon strobe lamp, operating at a voltage of 25 kV. The longest flashes (3  $\mu$ s) come from a "stroboslave" flash lamp. Time profiles of the various flashes have been published previously [18]. The light energy of the different sources has been adjusted to be nearly identical.

Signals were recorded by a Physical Data Incorporation 523-A transient wave-form recorder (10 bit amplitude resolution and 4096 words of memory), connected to a microcomputer Apple II plus. The recording of an oxygen flash yield pattern (Fig. 2) shows amperometric signals characterized by a first maximum followed by a small minimum; the amplitude of the positive signal is taken as the experimental value of the oxygen yield. A program on the Apple II plus, computes automatically these values, draws on the screen the flash yield sequence and prints directly on a printer the screen figure and the numerical values of the flash yields. These values are used directly for the theoretical analysis on the same computer.

We selected, for this work, oxygen flash yield patterns with no artefacts. Oxygen consuming reactions of photosystem I [19] and photoelectric artefacts [15], where not negligible in particular conditions, are also observed at the first flash in a sequence after dark adaptation without the oxygen yield signal.

## Calculation method for obtaining theoretical parameters for oxygen flash-yield patterns

The transition of the  $S$  states on the  $n$ -th flash is give by the following:

$$S^{(n)} = \mathcal{H} S^{(n-1)} \quad (1)$$

where  $S^{(n)}$  is a column vector the elements of which are  $S_0^{(n)}$ ,  $S_1^{(n)}$ ,  $S_2^{(n)}$ ,  $S_3^{(n)}$ , the  $S$  state distribution after flash  $n$ .  $\mathcal{H}$  is a matrix; its elements are the transition probabilities between states: no advancement  $\alpha_i$  ( $i = 0$  to 3), single advancement  $\gamma_i$  and double advancement  $\beta_i$  in  $S$  states.

$$\mathcal{H} = \begin{bmatrix} \alpha_0 & 0 & \beta_2 & \gamma_3 \\ \gamma_0 & \alpha_1 & 0 & \beta_3 \\ \beta_0 & \gamma_1 & \alpha_2 & 0 \\ 0 & \beta_1 & \gamma_2 & \alpha_3 \end{bmatrix}$$

( $\alpha_i + \gamma_i + \beta_i = 1$ ).

The information provided by the oxygen yield experiment is limited to

$$Y_n = \gamma_3 S_3^{(n-1)} + \beta_2 S_2^{(n-1)}.$$

For this reason, as long as we do not need to know the actually not measurable of  $S_0, S_1, S_2, S_3$ , it is better to eliminate these quantities from the recurrence equation (1) obtaining a recurrence equation giving  $Y_{n+4}$  as a function of the four preceding  $Y_{n+i}$  ( $i=0$  to 3) [5], simply by noting that the matrice  $\mathcal{H}$  verifies its characteristic equation giving the eigenvalues  $\lambda$ :  $|\mathcal{H} - \lambda| = 0$ .

$$Y_{n+4} - \sigma_1 Y_{n+3} + \sigma_2 Y_{n+2} - \sigma_3 Y_{n+1} + \sigma_4 Y_n = 0. \quad (2)$$

(This equation corresponds to Eqn. (1) for the four values of  $n$  (1, 2, 3, 4).)

The  $\sigma_i$  ( $i=1, 2, 3, 4$ ) are functions of the elements of  $\mathcal{H}$ :

$$\sigma_1 = \alpha_0 + \alpha_1 + \alpha_2 + \alpha_3$$

$$\sigma_2 = \alpha_0 \alpha_1 + \alpha_0 \alpha_2 + \alpha_0 \alpha_3 + \alpha_1 \alpha_2 + \alpha_1 \alpha_3 + \alpha_2 \alpha_3 - \beta_0 \beta_2 - \beta_1 \beta_3$$

$$\sigma_3 = \alpha_0 \alpha_1 \alpha_2 + \alpha_0 \alpha_1 \alpha_3 + \alpha_0 \alpha_2 \alpha_3 + \alpha_1 \alpha_2 \alpha_3 - \beta_0 \beta_2 (\alpha_1 + \alpha_3) - \beta_0 \beta_3 (\alpha_0 + \alpha_2) + \gamma_0 \gamma_1 \beta_2 + \gamma_1 \gamma_2 \beta_3 + \gamma_2 \gamma_3 \beta_0 + \gamma_3 \gamma_0 \beta_1$$

$$\sigma_4 = \sigma_1 - \sigma_2 + \sigma_3 - 1.$$

(The notation of Kok et al. [1] were kept;  $\beta$ : double hit.)

If there is no errors on the value of experimental  $Y_n$ , the functions  $\sigma_i$  ( $i=1, 2, 3, 4$ ) can be directly calculated from the first eight flash yields in a  $Y_n$  sequence; they are the solution of a linear system of the four equations, in which eight consecutive  $Y_n$  values enter as coefficients [5, 6].

The calculation method used in this paper is nearly the same as described previously [3]. The values of  $\bar{\alpha}$ ,  $\bar{\beta}$  (if misses and double hits are homogeneous four states), or those of  $\alpha_0, \alpha_1, \alpha_2, \alpha_3$ ,  $\bar{\beta}$  (unequal repartition of misses) are varied by a computer program to minimize the sum of the quadratic deviations.

$$E_c = \sqrt{\sum_n (Y_n^{\text{th}} - Y_n^{\text{exp}})^2} \quad (3)$$

( $Y_n^{\text{th}}$  oxygen yield calculated from Eqn. (2),

$Y_n^{\text{exp}}$  experimental oxygen yield under the number  $n$ .)

In Tables I, II, III, the minimized sum of the quadratic deviations has been normalized by dividing  $E_c$  by  $n Y_{st}$  for *Chlorella* ( $n$ , number of oxygen

yields,  $Y_{st}$  steady state oxygen yield) and by  $n Y_9$  for chloroplasts ( $Y_9$ , ninth oxygen yield), because  $Y_{st}$  in chloroplasts is not perfectly stationary and thus,  $Y_9$  is a valuable approximation of  $Y_{st}$ .

A slightly different program from that we used [3] was elaborated.

The recurrence law derived from Forbush *et al.* [2] model being linear,  $Y_n^{\text{th}}$  is a linear function of the four first members of  $Y_n$  sequence (in [3], we used the values of the four  $S_i$  states before the first flash).

$$Y_n^{\text{th}} = a(n) Y_1 + b(n) Y_2 + c(n) Y_3 + d(n) Y_4. \quad (4)$$

The computer calculated these coefficients  $a(n)$ ,  $b(n)$ ,  $c(n)$ ,  $d(n)$ , by permuting the value 1, 0, 0, 0 between the  $Y_1, Y_2, Y_3, Y_4$ . Therefore the quadratic deviation takes the form:

$$E_1 = \sum_n \left( \sum_{j=1}^{j=4} a(n) Y_j - Y_n^{\text{exp}} \right)^2$$

$j$  varying from 1 to 4.

With respect to  $Y_j$ , the derivative of this expression lead to the resolution of a linear system of four equations  $\frac{\partial E_1}{\partial Y_j} = 0$ .

Then, knowing the optimum four first members of the  $Y_n$  sequence, the theoretical sequence and the corresponding quadratic deviation are calculated. Therefore, we used the trial and error method to find the least quadratic deviations with respect to the parameters; values of  $\bar{\alpha}$  or  $\alpha_0, \alpha_1, \alpha_2, \alpha_3$ , and  $\bar{\beta}$  are proposed, from which are calculated the  $\sigma_i$ 's, the theoretical  $Y_n$  sequence (Eqn. (2)) and the quadratic deviation (Eqn. (3)). By successive approximations, the least quadratic deviation may be found.

## Results

### Least square approximation method

Our results in Table I are not different from those of Thibault [6]: a very good fit between experimental and theoretical  $Y_n$  sequences is found for a model of homogeneous misses for the  $S_i$  states, provided that an important double hit factor  $\bar{\beta}$  is added on each flash. As noted by Thibault, when the first flash yield  $Y_1$  is omitted, the fit is often slightly better than if unequal misses (without  $\bar{\beta}$ ) are searched for. We also remark in this Table I that the quadratic deviation, in the case of homogeneous

Table I. Least square fitting procedure used to our experimental  $Y_n$  sequences (as defined in Materials and Methods). In this Table, every sample was preilluminated by 20 flashes before dark adaptation (5 min);  $E_c$  is the mean square deviation as defined in Eqn. (3); in parenthesis, the successive oxygen flash yields  $Y_n$ ; 1) with all the  $Y_n$ ; 2) the first flash yield  $Y_1$  omitted; for the equal miss model, two quadratic deviations are given: the least square deviation with optimum double hit  $\bar{\beta}$ , and without  $\bar{\beta}$  ( $\bar{\beta} = 0$ ). (Permutations between the misses  $\alpha_{0 \rightarrow 3}$  give the same  $E_c$ ).

	Equal misses			Unequal misses					
	$\bar{\alpha}$	$\bar{\beta}$	$E_c$ [%]	$\alpha_0$	$\alpha_1$	$\alpha_2$	$\alpha_3$	$\bar{\beta}$	$E_c$ [%]
<i>Chlorella</i> (stroboslave)									
(0, 4, 618, 607, 399, 256, 371 478, 464, 384, 383, 425)	1)	0.223	0.058	0.630	0.018	0.018	0.018	0	0.584
		0.223	0						
	2)	0.204	0.041						
		0.204	0						
<i>Chlorella</i> (short flashes)									
(0, 20, 487, 452, 287, 182, 295, 374, 348, 287, 287, 331 345, 322, 307)	1)	0.208	0.052	0.610	0.01	0.01	0.01	0	0.76
		0.208	0						
	2)	0.190	0.034						
		0.190	0						
<i>Chlorella</i> (stroboslave)									
(0, 26, 377, 303, 168, 101, 218, 266, 223, 163, 189, 232, 227, 198)	1)	0.178	0.033	0.542	0	0	0	0	0.51
		0.178	0						
	2)	0.169	0.024						
		0.169	0						
Chloroplasts (stroboslave)									
(20, 84, 672, 390, 202, 162, 417, 409, 293, 216)	1)	0.147	0.034	0.485	0	0	0	0.01	0.708
		0.147	0						
	2)	0.139	0.024						
		0.139	0						
Chloroplasts (short flashes)									
(17, 63, 433, 216, 113, 89, 270, 244, 163, 116, 194, 209, 175)	1)	0.140	0.028	0.466	0	0	0	0	1.21
		0.140	0						
	2)	0.137	0.022						
		0.137	0						

misses and no double hit, is always large, as observed by us previously [3].

If Table I is carefully examined, a fact is apparent. The double hit  $\bar{\beta}$  that must be added to the equal miss  $\bar{\alpha}$  to obtain the best fit, is a function of the miss factor  $\bar{\alpha}$ . In *Chlorella*, when  $\bar{\alpha}$  decreases from 0.223 to 0.177,  $\bar{\beta}$  decreases from 5.8% to 3.3%; similarly, in chloroplast examples,  $\bar{\beta}$  is varied from 3% to 2% as function of the damping. These particularities are also found in the literature results; in Table VI of Thibault's paper [6] and in Table I of that of Jursinic [7], the highest values of double hits  $\bar{\beta}$  are found in *Chlorella* (6% to 4%) whereas in fresh chloroplasts,  $\bar{\beta}$  never exceed 4%. In our Table I, no differences distinguish the  $Y_n$  sequences investigated with saturating flashes of 3  $\mu$ s or 300 ns duration.

Jursinic [7] who analysed  $Y_n$  sequences with the a priori hypothesis of homogeneous misses concluded to non photochemical double hit of about 3% (found even with 5 ns flashes) sensitive to the physiological condition of the sample. In Table I,  $\bar{\beta} = 2-3\%$  are the percentage of double hits found in the less damped  $Y_n$  sequences; thus, within the homogeneous miss hypothesis, we find the same result as Jursinic. However, the decrease of  $\bar{\beta}$  correlated to the decrease of the miss factor, as shown in Table I seems suspicious; we expect no relation between  $\bar{\alpha}$  and  $\bar{\beta}$  which are quite different. In order to see if this correlation is an artefact, it was interesting to test by the same best fit procedure, exact synthetic sequences. As explained previously, for obtaining a theoretical  $Y_n$  sequence, the values of the first four  $Y_n$  and those of the param-



Table II. Least square fitting procedure used to synthetic  $Y_n$  sequences simulating a unique miss on one  $S$  state. The synthetic  $Y_n$  sequences are calculated with Eqn. (2) and the four first flash yields of an experimental oxygen yield pattern;  $E_c$ : mean square deviation as defined in Eqn. (3); 1) with all the  $Y_n$ ; 2) the first flash yield  $Y_1$  omitted.

Synthetic $Y_n$ sequences ( $n = 14$ ), $E_c = 0$ with the corresponding miss $\alpha_i$ and double hit $\bar{\beta}$	Best fit for the synthetic sequences if equal misses for $S$ states are considered		
	$\bar{\alpha}$	$\bar{\beta}$	$E_c$ [%]
a) $Y_1 = 0, Y_2 = 15, Y_3 = 480, Y_4 = 451$			
$\alpha_i = 0.7, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.252	0.088	0.60
	2) 0.234	0.063	0.32
$\alpha_i = 0.65, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.225	0.068	0.51
	2) 0.212	0.054	0.28
$\alpha_i = 0.6, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.198	0.053	0.43
	2) 0.190	0.045	0.27
$\alpha_i = 0.55, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.177	0.041	0.36
	2) 0.170	0.036	0.24
$\alpha_i = 0.5, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.156	0.032	0.30
	2) 0.151	0.030	0.22
b) $Y_1 = 0, Y_2 = 54, Y_3 = 721, Y_4 = 369$			
$\alpha_i = 0.5, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.156	0.031	1.21
	2) 0.150	0.021	0.71
$\alpha_i = 0.45, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.136	0.023	1.03
	2) 0.132	0.016	0.64
$\alpha_i = 0.40, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.118	0.017	0.85
	2) 0.115	0.0125	0.55
$\alpha_i = 0.35, 0, 0, 0 \quad \bar{\beta} = 0$	1) 0.101	0.0117	0.69
	2) 0.098	0.0095	0.47

eters  $\bar{\alpha}$ ,  $\bar{\beta}$  or  $\alpha_0, \alpha_1, \alpha_2, \alpha_3, \bar{\beta}$  (according respectively to the homogeneous or heterogeneous model) are sufficient. Using this method, synthetic sequences were built, simulating exactly a photosynthetic system in which only one  $S$  state should have only one miss  $\alpha_i$  with no double hit. Two kinds of synthetic sequences were investigated by the least square method:

1) five synthetic sequences in which the first four  $Y_n$  are equal to an experimental *Chlorella* flash yield sequence; characterized by a very unequal miss distribution: only a unique miss on one  $S$  state which successively takes the values 0.7, 0.65, 0.6; 0.55, 0.5 ( $\bar{\beta} = 0$ ).

2) Four synthetic sequences in which the first four  $Y$  are those of an experimental chloroplast flash yield sequence; the unique miss being successively 0.5, 0.45, 0.4, 0.35 ( $\bar{\beta} = 0$ ).

Table II shows that the minimum quadratic deviation,  $E_c$ , between the synthetic  $Y_n$  sequence and

a theoretical sequence simulating equal misses  $\bar{\alpha}$  and double hits  $\bar{\beta}$ , can be very small, smaller or of same order of magnitude as that found in experimental  $Y_n$  sequences (compared Tables I and II). At first sight, it seems unfortunate that a mathematical coincidence prevents the possibility to discriminate clearly from experiments between the homogeneous or heterogeneous miss model. Thus, we need to study the effect of other parameters in order to obtain more informations to discriminate between the models.

The analysis of these synthetic sequences (very unequal misses and no double hit), gives the same results as if they were experimental  $Y_n$  sequences. First, we note a similar correlation between  $\bar{\beta}$  and the miss  $\alpha_i$  or  $\bar{\alpha}$ ;  $\bar{\beta}$  decreases progressively from 8% to 1% for a variation of the miss factor  $\alpha_i$  from 0.7 to 0.35 in Table II. Secondly, the mean quadratic deviation resulting of the fitting of these synthetic sequences with an homogeneous miss model, is always smaller if the first flash yield  $Y_1$  is omitted in the  $Y_n$  sequence. This result, also observed in experimental  $Y_n$  sequences (Table I), was a Thiabault's argument to distinguish the first flash from the others. We show here that his finding can have no real significance. In contrast, with unequal misses and negligible double hits, the mean square deviation is of the same order in both cases, with the whole  $Y_n$  sequence or without the first flash-yield  $Y_1$  (not shown in Table I).

Results of Tables I and II have been summarized in Fig. 1. Only qualitative relations can be made between the value of the unique miss of the synthetic sequences,  $\alpha_i$ , and  $\bar{\beta}$  found if the equal miss model is chosen, because the parameter values change slightly with the value of the first four  $Y$  of the  $Y_n$  sequence; (in Table II, the two synthetic sequences for  $\alpha_i = 0.5$  do not give identical parameter values of  $\bar{\alpha}$  and  $\bar{\beta}$ ).

Therefore, we show that a theoretical analysis within the model of equal misses applied to an hypothetical experimental system of unequal misses and no double hit, gives the same results as that observed on real experimental sequences. This is not sufficient to prove the reality of unequal misses, but the correlation between  $\bar{\alpha}$  and  $\bar{\beta}$  is very disturbing, because these parameters are expected to be strictly independent. Therefore, we studied the effect of non saturating flashes which also give large misses at low light intensity.

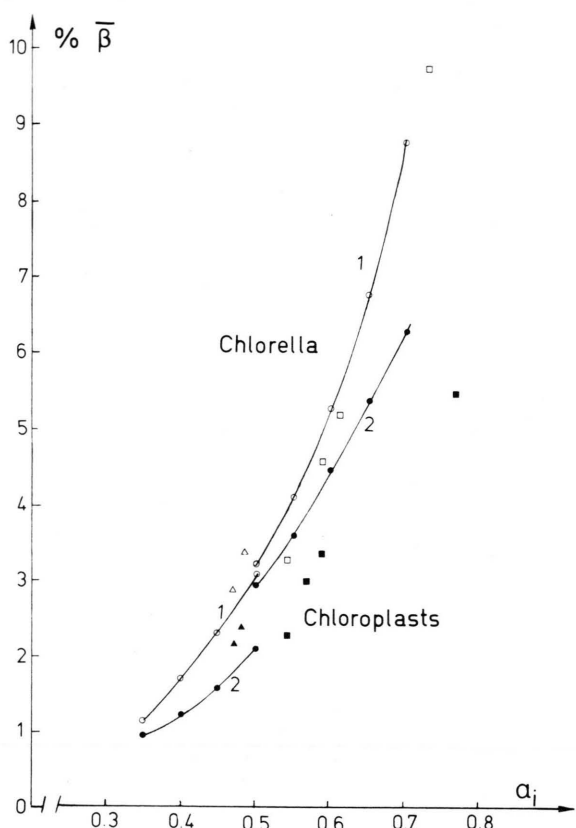


Fig. 1. ●—● or ○—○ joined by full lines: relationship between the value of the unique miss,  $\alpha_i$ , in theoretical synthetic  $Y_n$  sequences, and the percentage of double hits found from these synthetic sequences if equal misses are investigated.  $\bar{\beta}$  is obtained by the least square fitting method described in material and methods; see Table II. ■□:  $\bar{\beta}$  as a function of  $\alpha_i$  for experimental *Chlorella*  $Y_n$  sequences of Table I. ▲△: same relation, for chloroplast  $Y_n$  sequences of Table I; 1, blank symbols: with all  $Y_n$ ; 2, black symbols:  $Y_1$  omitted (see Tables I and II).

*Analysis of non saturating flash yield sequences and comparative study of the misses found from this analysis and those deduced from experimental saturation curves*

The percentage of double hits  $\bar{\beta}$  associated with homogeneous misses  $\bar{\alpha}$ , if they exist, would be of two kinds of origin: either a photochemical one due to some reaction centers recovering rapidly enough from an initial quantum absorption to be able to absorb another quantum, or a "non-photochemical" one as defined by Jursinic [7, 20]: in his model, a side carrier "C" [21] is able to exchange charges with the  $S$  states so that a double transition would occur with the absorption of only one photon

(provided that there is a finite concentration of  $C^+$  in darkness, before the flash).

According to these definitions, analysis of non saturating flash yield sequences can give information about the real existence of these double hits. The photochemical double hits should diminish at low light intensity like the square of the light intensity (if for  $I = 100\%$ ,  $\bar{\beta} = 6\%$ , for  $I = 10\%$ ,  $\bar{\beta} = 0.06\%$ ).

The proportion of "non photochemical" double hits should also diminish with decreasing flash intensity, because the positively charge side carrier  $C^+$  is formed by light in the Jursinic's model [7]. (We note that if  $C^+$  is present in the dark in this model, oxygen can be evolved with only three or two photons.)

Fig. 2 represents the successive oxygen yield patterns obtained with progressively uniformly attenuated flashes in freshly prepared chloroplasts. In Table III are shown the results of the numerical analysis corresponding to these patterns. The best fitting for homogeneous transition parameters leads to a non sense: the double hit factor  $\bar{\beta}$  associated with the homogeneous factor  $\bar{\alpha}$ , increases strongly with decreasing flash intensity; in Table III,  $\bar{\beta}$  increases from 3% to 15% as the relative intensity of the sequence flashes decreases from 100% to 11%. The remarks made about Table I are also true for Table III: in very different experimental flash yield sequences the homogeneous transition parameters  $\bar{\alpha}$ ,  $\bar{\beta}$  increase in parallel as a function of the damping showing clearly that these analysis results are artefacts. It seems difficult to explain the observation of the same correlation between  $\bar{\alpha}$  and  $\bar{\beta}$  as a function of as well the variation of light intensity as the change of properties of *Chlorella* or chloroplasts with the mode of batch preparation, the season, the temperature. Excepted the mathematical coincidence that we point on here, no model can give with certitude this parallel variation of  $\alpha$  and  $\beta$  for the influence of very different external parameters on the *Chlorella* or chloroplast properties. The miss  $\alpha$  related to the absence of reaction of the  $S$  state and the double hit  $\beta$  related to a double advancement of  $S$  states cannot be always in parallel in all very different experimental conditions. For example, in the model of "non photochemical double hit" of Jursinic [7], there is a lot of parameters (concentration of  $C^+$ ,  $k_1$ ,  $k_2$  etc.) which will be different in different batch or experimental conditions, so that

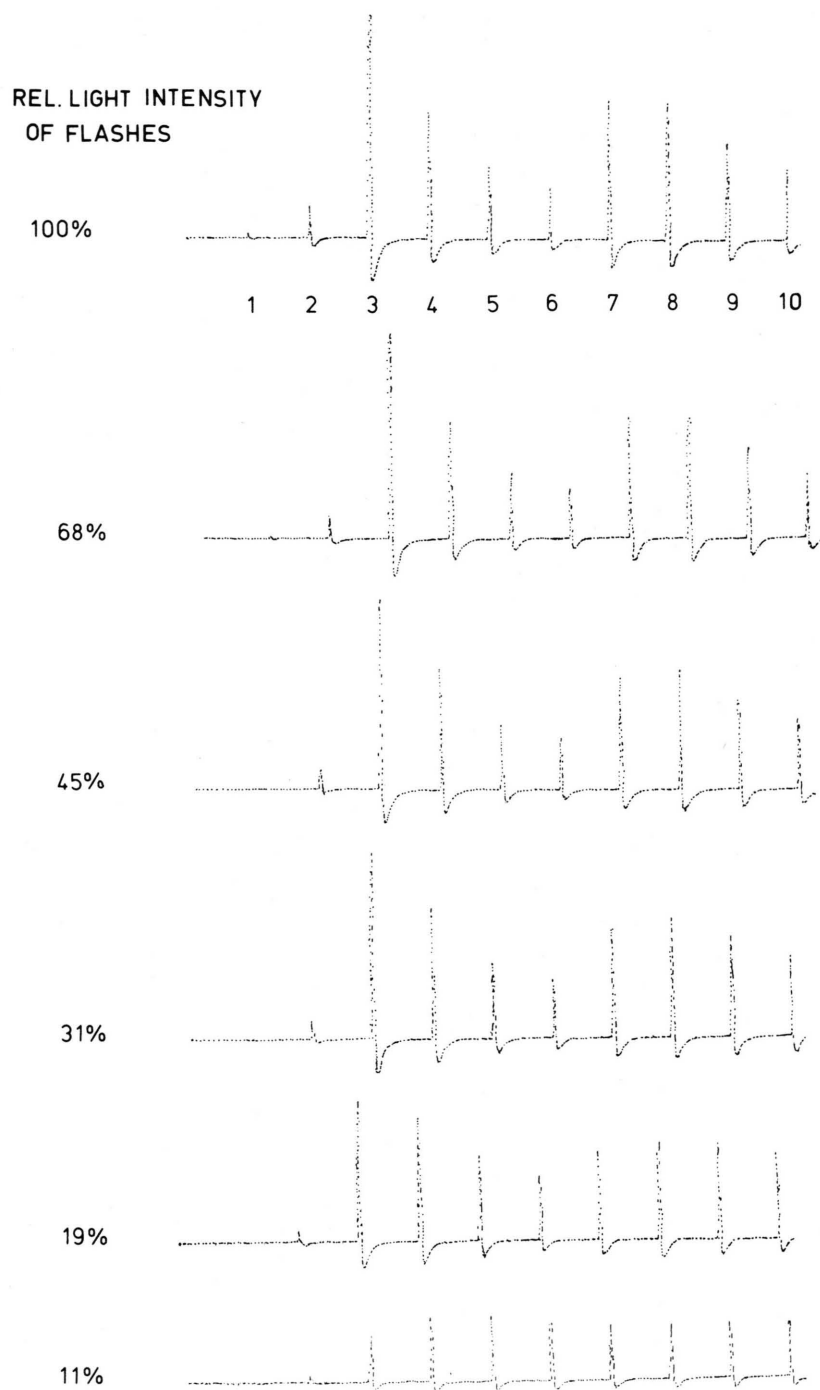


Fig. 2. Oxygen flash yield patterns in dark adapted (5 min) lettuce chloroplasts for different light intensities; the light intensity of the flashes (300 ns) are progressively uniformly attenuated by calibrated Balzers neutral density filters as written on the figure.

Table III. Least square fitting procedure used to the chloroplast  $Y_n$  sequences of Fig. 2 in which the light intensity of all the flashes (300 ns) has been uniformly attenuated; for both models, equal misses and unequal misses. All other information is the same as for Table I.

Relative intensity of flashes (Fig. 2)		Equal misses			Unequal misses					
		$\bar{\alpha}$	$\bar{\beta}$	$E_c$ [%]	$\alpha_0$	$\alpha_1$	$\alpha_2$	$\alpha_3$	$\bar{\beta}$	$E_c$ [%]
100% I; 10 photons/center	1)	0.147	0.031	1.93	0.485	0	0	0	0	1.08
	2)	0.135	0.016	<b>0.77</b>						
68% I	1)	0.160	0.038	1.76	0.517	0	0	0		1.06
	2)	0.151	0.022	<b>0.80</b>						
45% I	1)	0.174	0.047	1.08	0.545	0	0	0		0.72
	2)	0.165	0.034	<b>0.49</b>						
30% I	1)	0.195	0.064	1.17	0.600	0	0	0		0.60
	2)	0.184	0.042	<b>0.37</b>						
19% I	1)	0.254	0.086	0.83	0.685	0.01	0.01	0.01		0.28
	2)	0.233	0.057	<b>0.49</b>						
11% I	1)	0.335	0.147	0.60	0.825	0.14	0.14	0.14		0.62
	2)	0.37	0.16	<b>0.62</b>						

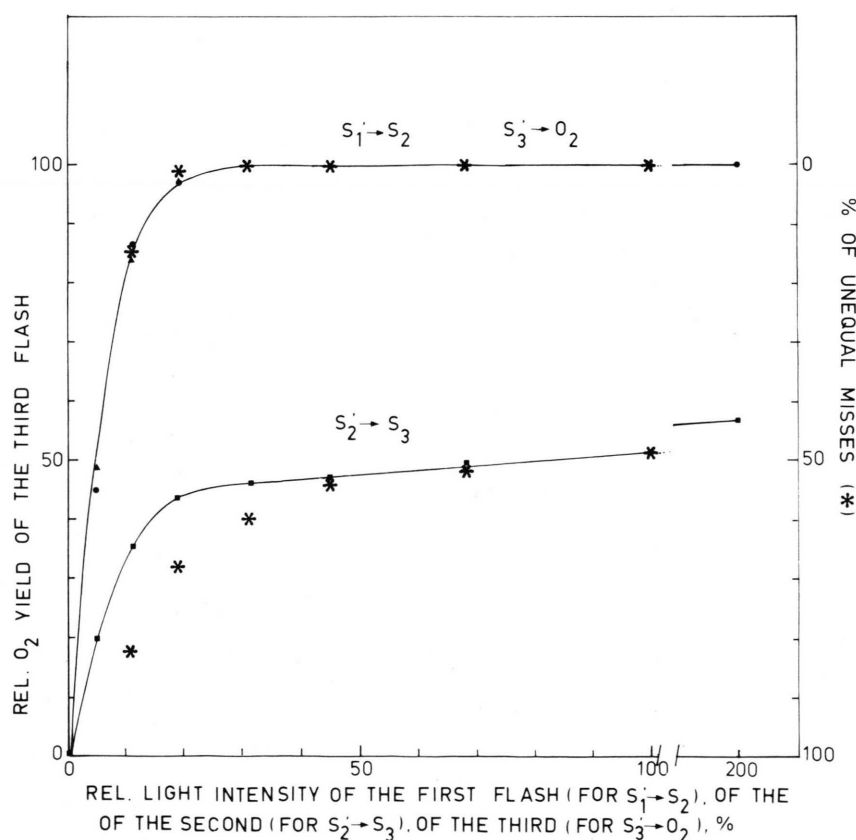


Fig. 3. Left: Oxygen evolved on the third ( $Y_3$ ) flash of a series of non saturating flashes (19%) given 400 ms apart to chloroplasts, as a function of the light intensity of the first flash (for  $S_1' \rightarrow S_2$ ), of the second flash (for  $S_2' \rightarrow S_3$ ), of the third flash (for  $S_3' \rightarrow O_2$ ). For the  $S_2' \rightarrow S_3$  transition, the complementary part of the saturation curve  $Y_2$  ( $I_2$ ) has been subtracted from  $Y_3$  ( $I_2$ ), oxygen evolved on the third flash as a function of the light intensity of the second flash. All these saturation curves were normalized at a 100% flash intensity, as explained in the text. Right: percentage of unequal misses of Table III obtained by the least square fitting procedure used to  $Y_n$  sequences of Fig. 2, which were obtained in the same chloroplast sample as the saturation curves of this figure.



we cannot expect a parallel variation of  $\alpha$  and  $\beta$  identical to that given by the mathematical coincidence pointed out in this paper.

In contrast, in Table III, the unequal miss model leads to no double hits at low intensity. As the flashes are attenuated from 100% to 19%, practically only a unique miss increases, the misses on the other  $S$  states becoming significant for a light intensity of less than 19%.

In order to check the validity of these results, the experimental saturation curves of the transition  $S_1 \rightarrow S_2$ ,  $S_2 \rightarrow S_3$ ,  $S_3 \rightarrow O_2$  were studied at the same time, with the same sample in order to eliminate the variations from batch to another (Fig. 3). These saturation curves were measured by the variation of the third oxygen yield  $Y_3$  ( $Y_3$  is the highest  $O_2$  yield in a sequence). Dark adapted chloroplasts were illuminated by a series of flashes 400 ms apart. The first flash (for the  $S_1' \rightarrow S_2$  transition) or the second flash (for  $S_2' \rightarrow S_3$ ) or the third flash (for  $S_3' \rightarrow O_2$ ) was of different energies and was followed or (and) preceded, depending on cases, by non saturating flashes (19%). Weak flashes were used to minimize the probability of double hits they could induce and also the probability of decline occurring at high flash intensity [22]. (For more details on measurements, see the caption of Fig. 3.)

The saturation curves of the transition  $S_1' \rightarrow S_2$  and  $S_3' \rightarrow O_2$  looks like exponential functions in Fig. 3; both normalized to 100% for a relative flash intensity of 100%, they look practically similar; these saturation curves become independent of the flash energy for energies higher than 5 photons/center. In contrast, the saturation curve of the transition  $S_2' \rightarrow S_3$  is not yet saturated at the highest intensity (200%). This non saturation was shown previously by Delrieu [18] and confirmed by Joliot and Joliot after the first flash [15].

A saturation curve  $S_{i \rightarrow i+1}(I)$  of an  $S$  state transition is essentially a measurement of the change of the number of centers in the  $S_i$  state receiving an excitation from the flash as its intensity changes. Thus  $S_{\max} - S_{i+1}(I)$  represents the number of centers which have not reacted. ( $S_{\max}$  is the maximum of  $S_{i+1}$  state observed at high flash energy); and the value  $1 - \frac{S_{i+1}(I)}{S_{\max}}$  gives directly the proportion of misses  $\alpha_i(I)$  related to the non saturating light intensity.

Therefore, we checked the validity of the misses found by the least square calculation as function of flash energy (Table III) with respect to the expected misses  $\alpha_i(I)$  deduced from the saturation curves of the different  $S_i$  states. The fact that the saturation curves are not the same for the different  $S$  states, in particular for the  $S_2$  state, demonstrates that at low or intermediate intensity the misses  $\alpha_i$  are not equal for the different  $S_i$  states. The values of  $\alpha_0$ ,  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  determined in Table III (within the unequal miss model) were plotted in Fig. 3 as a function of flash intensity. Fig. 3 shows that the two strictly independent measurements  $S_i(I)$  and the least square determination of unequal misses are in good agreement. The agreement is precise for the three transitions  $S_0' \rightarrow S_1$ ,  $S_1' \rightarrow S_2$ ,  $S_3' \rightarrow O_2$  which saturates after a definite flash energy easily reached experimentally. (The properties of the transition  $S_0' \rightarrow S_1$  were found to be similar to those of  $S_1' \rightarrow S_2$  [4].) In this check of consistency, we have assumed that even at 100% intensity the main part of the misses are due to the very incomplete saturation curve of the  $S_2' \rightarrow S_3$  transition which is well established [18, 15, 16]; thus, this  $S_2' \rightarrow S_3$  curve in Fig. 3 has been normalized for a flash intensity of 100% to 51.5% corresponding to a miss of 0.485. Below a flash intensity of 45% some discrepancies are observed between the saturation curve of  $S_2' \rightarrow S_3$  and the miss value of Table III: this is due to the more important effect of measurement errors in the mathematical analysis performed on very attenuated flash yield patterns which are very damped (see Fig. 2).

The maximum saturation value of  $S_{\max}$  to infinite intensity of the transition  $S_2' \rightarrow S_3$  needs a more detailed discussion because from our analysis, this value is large:  $\frac{1}{1 - 0.485} = 1.94$ .

The experimental result shown in Fig. 3 and more especially in Fig. 5 of the Joliot and Joliot's paper [15] presents a direct experimental proof of the very large increase of  $Y_2(I_1)$  i.e.  $S_{2 \rightarrow 3}(I_1)$  at very high intensity of short laser flash of 30 ns. By comparison of the saturation curve  $Y_3(I_1)$  of Joliot and Joliot [15] and ours in Fig. 3, it is found that their maximum intensity is 10 to 20 times, larger than in our experiment. For this reason, this result of Joliot and Joliot [15] proves that at 100% flash intensity of Fig. 3, an important part of misses remains on the  $S_2$  state, at least 25 to 40%. This experimental result

of Ref. [15] which is not exclusively observed after the first flash [18, 23] (Fig. 3) shows definitively and directly that misses are unequal on the different states at usual saturating light intensity.

It is quite unfortunate that repetitive laser flashes at very high intensity induce a decline of oxygen yield [22] preventing the possibility of using analysis presented in this paper on flash sequences of very high intensity.

Nevertheless, our analysis shows the complete consistency of all available measurements of oxygen flash yields at very different light intensity with the model of unequal misses only, essentially because the saturation curves of the  $S_2' \rightarrow S_3$  transition is very different from the other  $S$  state saturation curves.

Experimentally, the oxygen flash yield sequences and the  $S$  state saturation curves must be investigated in the same sample because, depending on the physiological condition of the sample (for example chloroplast isolation procedure), the same maximum flash intensity may be largely saturating or not saturating (for  $S$  state transitions other than  $S_2' \rightarrow S_3$ ); the apparent cross sections per photosystem II trap for oxygen production [24] are variable in different chloroplast samples.

## Discussion

The properties of the saturation curves of the  $S_i \rightarrow S_{i+1}$  transitions lead inevitably to an unequal repartition of misses or double hits in oxygen flash sequence. At high flash intensity, all the transitions are saturated except the transition  $S_2' \rightarrow S_3$  which does not seem to saturate, as shown in Fig. 3 [15, 16, 18, 23]. This transition,  $S_2' \rightarrow S_3$ , displays a biphasic increase as a function of the flash intensity; the first phase is nearly exponential up to the absorption of one photon by the other  $S$  states; the second phase is a slow increasing phase at the highest intensity, found after a first flash [15, 16] as well as after every flash in a sequence [18, 23] (Fig. 3).

For explaining this slow increasing phase, Joliot and Joliot [15] have suggested the possibility of a double oxidation of the photoactive chlorophyll (or the oxidation of a second chlorophyll close to the center), the probability of this second photooxidation being extremely low.

A double hit process in charge separation at high flash intensity, as was proposed by Joliot and Joliot

[15] lead to study the possibility of a double advancement in  $S$  states. Thus, the slow phase of the  $S_2' \rightarrow S_3$  saturation curve, at high flash intensity, could correspond to a percentage of double reactions  $S_1 \rightarrow S_3$  increasing slowly with the flash intensity. In Fig. 3, the increase of the experimental  $S_2' \rightarrow S_3$  saturation curve in comparison with the saturation level of an hypothetical exponential curve is equal to 10% at a flash intensity of 100%; this percentage could be due to double hits like  $S_1 \rightarrow S_3$ . We remark that, in Table III, the homogeneous hypothesis predicts a decrease of double hits (from 8% to 3%) as the flash energy increases (from 19% to 100%) *i.e.* the contrary to what is expected.

The possibility of double hits only for the transitions  $S_1 \rightarrow S_3$ ,  $\beta_1$ , was already studied [18]; it was shown that theoretical patterns with  $\beta_1 \geq 10\%$  (with the equal miss model) have an intermediate period between 3 and 4, in contradiction with the experiments. More generally, using the least square fitting method to  $Y_n$  sequences, all our attempts to increase  $\beta_1$  ( $\geq 0.5\%$ ) always increase the quadratic deviations in both models, equal miss and unequal miss models. Therefore, a percentage of  $\beta_1$  does not exist in the  $Y_n$  sequences and the slow increasing phase of the  $S_2' \rightarrow S_3$  saturation curve is not produced by double advancement  $S_1 \rightarrow S_3$ .

As measured recently by Ley and Mauzerall [24], the quantum requirement for photosynthesis in flashing light (like in continuous light [25]) is constant at  $10 \pm 1$  photons absorbed per  $O_2$  evolved. This requirement is 25% greater than the theoretical minimum of 8. Photochemical double advancement in  $S$  states should not change the quantum requirement for photosynthesis. The finding of only one miss for  $S_2' \rightarrow S_3$  in usual saturating light, of order of  $\approx 0.5$  is in agreement with the percentage of inefficiency in photosynthetic photochemistry; the transition  $S_2' \rightarrow S_3$  should require around 2 photons, so that the quantum requirement should be equal to  $\approx 9$  in chloroplasts.

The two components in the saturation curve of the  $S_2' \rightarrow S_3$  transition reveal a double step or possibly hit process for the primary donors and acceptors. But as shown in this paper, the probability that these two photoreactions convert the  $S_2$  state into  $S_3$  is very low. If we suppose that the quantum yield of the transitions other than  $S_2' \rightarrow S_3$  is equal to 1, the quantum yield of the first photoreaction converting  $S_2$  into  $S_3$  would be of order of 0.45

calculated from the slope of the saturation curves in Fig. 3, and that of the second photoreaction increase slowly with increasing light intensity.

In this model, the second photoreaction should increase the probability of transitions  $S_2' \rightarrow S_3$  in each center rather than the probability of double advancement in  $S$  state which are not experimentally observed as explained above. Therefore, the saturation curve of the  $S_2' \rightarrow S_3$  transition is explained if  $Q_2$  a secondary acceptor and  $D$  an auxiliary donor are effective only for the  $S_2$  state; thus, during a flash two sites can accept electrons and two sites can donate electrons only for the  $S_2$  state; if the first photoreaction (with  $Q$ ) able to convert  $S_2$  into  $S_3$  is missing, there is a probability that a second photon has induced a charge separation between  $Q_2$  and  $D$  a donor, so that this latter transfers its positive charge to  $S_2$ , for the transition  $S_2' \rightarrow S_3$ .

The possibility of a second acceptor  $Q_2$  which would be effective only for the transition  $S_2' \rightarrow S_3$  are not in contradiction with numerous reports showing evidence for two acceptors included in the same center [8–12]. There are some indications that our  $Q_2$  should be identical to  $Q_2$  of Joliot and Joliot [11] or  $X_a$  of Ekert and Renger [10]; Joliot and Joliot [11] have shown that the saturation curves of the fluorescence rise induced by a first flash in chloroplasts inhibited by DCMU are different according to the time of measurement: the difference between the slow rise (20 ms) and the fast rise (20  $\mu$ s) shows a lag. The same phenomenon is also observed in oxygen evolution, only for the saturation curve of  $S_2' \rightarrow S_3$ ; the slow increasing phase at high flash intensity is not observed if the concentration of  $S_3$  is measured at short time after the flash (300  $\mu$ s), and progressively becomes apparent as the time between the flash and the measurement increases (200 ms) [18, 23].

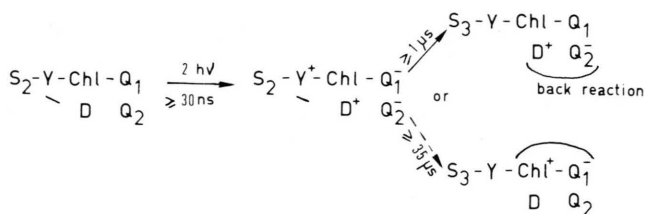


Fig. 4. Scheme I. Proposed model including the rate limiting steps studied in Refs. 13, 8, 11, 18.

Gläser *et al.* [8] have found that the saturation curve of the initial amplitude of the measured 690 nm absorption change,  $\Delta A_0$  (Chl-aII) in Tris-washed chloroplasts can be described by exponential function, whereas  $\Delta A_0$  (Chl-aII) in normal chloroplasts exhibits a more complex biphasic dependency and much higher flash intensities are required for saturation. This proves that the second phase of the saturation curve of  $\Delta A_0$  (Chl-aII) is intimately related to the function of the water-splitting enzyme system  $Y$ .

The model explaining our experimental results incorporates  $Q_1$  and  $Q_2$  [11] or  $X_{320}$  and  $X_a$  [10] (see Scheme I). There are two possibilities in the conversion of the  $S_2$  state into  $S_3$ : either relatively rapid charge stabilization via the primary plastoquinone acceptor  $Q$  (or  $X_{320}$ ) and the secondary donor  $Y$  (the probability for this reaction would be constant and equal to 0.45 in chloroplasts), or a slow one ( $\geq 35 \mu$ s) induced only by a second photon in the center; the probability of stabilization for this possible reaction via the acceptor  $Q_2$  and donor  $D$  occurring only at very high light intensity would be low: 0.01/photon/center.

The low probability for the transition  $S_2' \rightarrow S_3$  in comparison to the other  $S$  state transitions is perhaps related to the necessity of a spacial disposition of enzymes at the level of the  $S_2$  state, which would take some time, decreasing the probability of  $S_3$  formation.

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