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LIGHT INTENSITY SATURATION PROPERTIES OF O_2 YIELDS IN A SEQUENCE OF FLASHES IN *CHLORELLA*

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Summary

As a function of the light intensity of flash n in a sequence, the O_2 yields Y_n, Y_{n+1} and Y_{n+2} have been measured: $n = 1, 2, 3$ and 6 in the examples given. It is shown that:

(1) No double hit exists in the first saturating flash in *Chlorella.*

(2) The flash saturation curve of the O_2 yield Y_{n+1} as a function of the intensity of flash n exhibits a small sigmoidal shape at weak light.

(3) If Y_{n+1} is detected at different times after the flash n of variable intensity, a well developed lag distinguishes the saturation curve of the O_2 yield measured a long time after flash n (200 ms) with respect to that measured at shorter time (300 μs).

Nevertheless, a large amount of double hits with the transitions $S_1 \rightarrow S_3$ cannot occur in each flash, because it would lead to a periodicity of three rather than four in the O_2 yield pattern.

The saturation curve of the transition $S_2^* \rightarrow S_3$ is different from the other S-state saturation curves which are close to an exponential function; even with a short flash (0.3 μs), this curve shows a small lag at low light intensity, and its saturation intensity is higher than that of the other transitions.

The low quantum yield of the transition $S_2^* \rightarrow S_3$ at low flash light intensity is explained by a product, T, partially inhibiting the formation of S_3; at higher intensity, the quantity of formed S_2^* being larger than that of available T, only a part of S_2^* is inhibited and the quantum yield is higher than at low intensity.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll.

Introduction

The oxygen flash yield pattern has been extensively studied under various conditions [1—3]. Up to now, many experimental results seem to confirm the model of Kok et al. [2] which supposes that four consecutive photochemical reactions per center are necessary for an oxygen molecule to evolve.

However, recently, some anomalies in Photosystem II were mentioned in the literature. On the one hand, Joliot and Joliot [4] showed that, in dark-adapted chloroplasts poisoned by DCMU, the saturation curves of fluorescence rise after the first flash change, according to the time interval between the flash of variable intensity and the following analysing flash: in the saturation curve of the slow fluorescence rise a lag exists, which is not observed for the fast rise. On the other hand, in chloroplasts treated with ferricyanide, Velthuys and Kok [5] found a sigmoidal saturation curve of the O_2 yield following the first flash of variable intenisty in a sequence; they concluded to the presence of double hits in the first flash correlated to oxidizing conditions.

These two results were the origin of our study in *Chlorella.* This kind of experiment was extended by varying the light intensity of any flash of a series and also by varying the time interval between the flash of variable intensity and the following flash. The interpretation of the authors cited above cannot be applied to our data. A detailed analysis shows that our results are not consistent with a double-hit model. The low quantum yield of the transition $S_2^* \rightarrow S_3$ at low flash light intensity can be explained by a product, T, inhibiting some part of the S_2^* state.

Materials and Methods

Chlorella pyrenoidosa was grown and harvested as previously described [6]. Before use, it was centrifuged and resuspended in 0.1 M phosphate buffer, pH 7.5. The concentration of the chlorophyll used was 450 $\mu g \cdot ml^{-1}$ for O_2 measurements.

O_2 yields were measured with a polarographic device described previously [3]. The thickness of the *Chlorella* cells on the platinum electrode was 0.1 mm. With a Cary 14 spectrophotometer, the absorbance of these layers (concentration 450 $\mu g \cdot ml^{-1}$) was found equal to 0.17 in the red (680 nm) and 0.15 in the blue (460 nm).

Saturating flashes (3-μs duration at one-third peak intensity) were provided with two xenon flash lamps (General Radio 'Stroboslave') driven by a pulse generator. The intensity of one of the flashes was attenuated by calibrated stainless screens.

We have used another type of Xenon flash, with the same electrical energy, of which the duration at half-height is about 0.3 μs (see Fig. 1).

Saturation curves in Figs. 3 and 5 indicate that some flashes are completely saturating, because the O_2 yield is independent of intensity around the maximum light intensity $I = 100\%$, but that other flashes are not completely saturating (in Fig. 4). After investigation of the published O_2 yield saturation curves, we concluded that the intensity of our flash might be of the same order as that used by Velthuys and Kok [5]. This incomplete saturation, in particular conditions, is related to the different components in the saturation curves as is explained below.

Results and Interpretation

1. Variation of the light intensity of any flash of a series

Our first step was to try to repeat the experiment of Velthuys and Kok [5], in the same conditions. As a function of time, we observed that our experimental conditions were not reproducible, i.e. more precisely, in a saturating sequence after dark adaptation the value of Y_2 continuously increased with time. Thus, *Chlorella* was chosen because the experimental conditions seem more reproducible with respect to time, and 7 mM NH_4Cl (pH 7.8) was added in order to increase the value of Y_2 after dark adaptation. The origin of this high value of Y_2 is unknown: it may be due to the presence of some S_2 in the dark, induced by the exposure to NH_4Cl (active S_2 issuing from inactive S_2 as in Ref. 7, or slowed deactivation); or it may come from double hits in the first flash. In chloroplasts treated with ferricyanide, Velthuys and Kok measured the turnover time of centers, and because the corresponding turnover kinetics were relatively fast after the first flash (between 50 μs and 100 μs), they concluded to double hit due to overlap of the flash tail and a rapid relaxation of the center.

Our Fig. 2 represents the saturation curves Y_2, Y_3, in *Chlorella* exposed to 7 mM NH_4Cl (pH 7.8); our result is qualitatively similar to that of Velthuys and Kok; when the light intensity of the first flash, I_1, increases, the slope of Y_2 versus I_1 is smaller at low light intensity than at intermediate intensity and is not yet saturated at high light intensity ($I = 100\%$).

Contrary to Velthuys and Kok's conditions, the 7 mM NH_4Cl, added to *Chlorella*, does not fundamentally change the turnover times of the S-states following the first flash: the half-times remain longer than 400 μs (see Fig. 2B). As explained in Appendix I, if the turnover kinetics of the S-states, and the intensity versus time of the flash (i.e. the duration of the flash) are known, one can calculate quantitatively the proportion of double hits related to the flash tail overlap and the turnover kinetics. The 'effective times' of the 'stroboslave' flash (Fig. 1) was found to be around 10 μs; and if we extrapolate linearly the turnover kinetics obtained with 7 mM NH_4Cl in the μs range, the calculated proportion of double hits is of the order of 2%. Therefore, we have examined the possibility of intrinsic double hits.

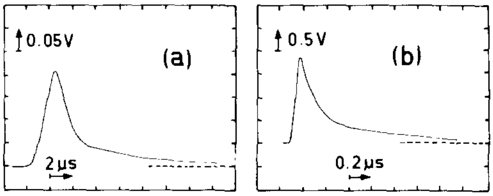

Fig. 1. Light intensity vs. time of the stroboslave flash (a), and of the short flash (b). The measurement was made by means of a photodiode Monsanto MD1 (on an impedance of 50 Ω) and recorded on an oscilloscope of bandwidth 100 MHz.

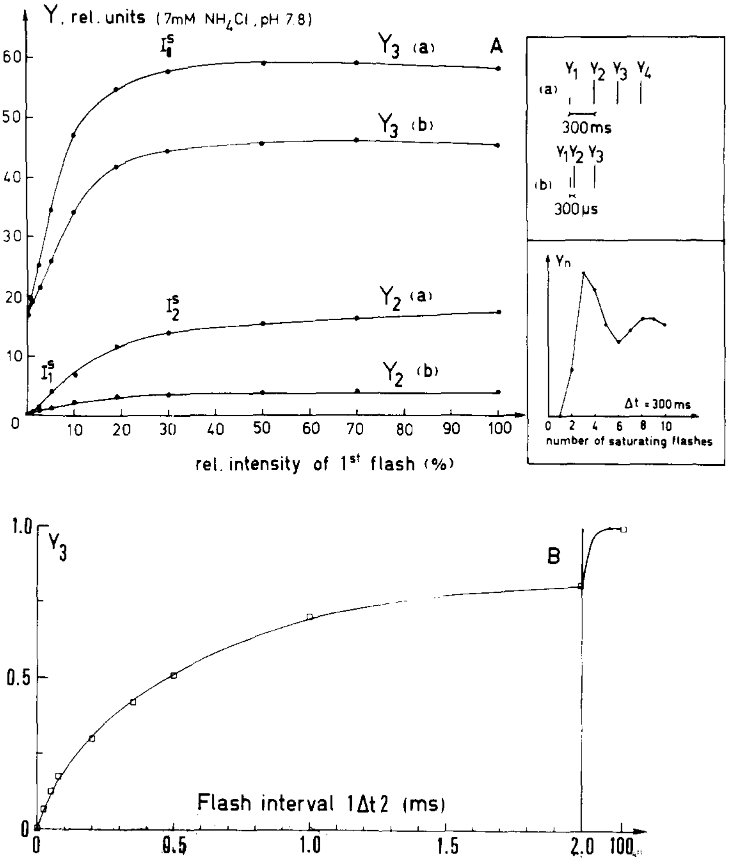

Fig. 2. A. O_2 yields of the second and third saturating flashes, Y_2 and Y_3, in *Chlorella* in the presence of 7 mM NH_4Cl, pH 7.8, as a function of the intensity of the first flash. (a) All flashes were spaced 300 ms apart. (b) The second flash occurred 300 μs after the flash of variable intensity, subsequent flashes were spaced 300 ms apart. 6 min of dark adaptation separated each measurement. B. the $S_1^* \rightarrow S_2$ turnover time in the same conditions of O_2 yield pattern.

The intrinsic double-hit model supposes that, two successive photochemical reactions operate even during a very short saturating flash: $S_i \xrightarrow[(1)]{} S_{i+1} \xrightarrow[(2)]{} S_{i+2}$. The percentage of these double hits is independent of the flash length, in contrast to the double hits due to flash tail overlap; it can be detected only in saturation curve measurements.

At low light intensity, the probability of each step is proportional to intensity I, and therefore the probability of double hits is proportional to I^2. Thus, the observation of a quadratic law such as I^2 can be interpreted by a double-hit model. But in this case, the product of the two saturation curves $f_{12} = f_1(I) \times f_2(I)$ relative to the two successive photochemical reactions, is measured. When both $f_1(I)$ and $f_2(I)$ are linear with I, f_{12} is quadratic. When at least one of the two steps begins to saturate (i.e. as example $f_1(I)$), the curve f_{12} becomes linear with I, and f_{12} is completely saturated only when the other function ($f_2(I)$) is also saturated.

Fig. 2 shows that the quadratic law for Y_2(a) exists only for an intensity of the first flash $I_1^s < 4\%$; thus, it is concluded that one of the two steps is saturated for $I_1^s \sim 5\%$. The second step, as shown by Y_2(a), essentially saturates around $I_2^s \sim 30\%$, as does the simpler curve Y_3(a) which saturates around $I_0^s = 25\%$ (in a double-hit model, Y_3(a) measures the step $S_1 \rightarrow S_2$). With more careful inspection of the Y_2(a) curve, we notice that Y_2(a) is not completely saturated even around $I \sim 100\%$; thus it seems that there is a third step which saturates only at very high intensity $I_3^s > 100\%$. We remark that the saturation intensity of the double-hit first step (I_1^s) and of the single step $S_1^* \rightarrow S_2$ (I_0^s) are different: $I_1^s = 0.2\, I_0^s$ in Fig. 2. One possibility is that the first step of the double hit has not the same properties as the corresponding single hit: the trapping efficiency of the intermediate state S_2^* is different from S_2 in the double hit and would be, in our case, at least 10-times larger than that of S_2 obtained in a single hit. However, this double-hit model would not explain the third step in the saturation curve of Y_2 in Fig. 2. Therefore, the details of our experimental results are not in agreement with a simple double-hit model. It seemed interesting to know if the sigmoidal shape of Y_2 could be observed after a flash other than the first flash of variable intensity.

The following figures show the saturation curves, in untreated *Chlorella*, of the O_2 yields following one particular Flash n in a sequence. As a function of the light intensity of Flash n, the O_2 yields Y_n, Y_{n+1} and Y_{n+2} are plotted; $n = 1$ in Fig. 3, $n = 2$ in Fig. 4, $n = 3$ in Fig. 5, and $n = 6$ in Fig. 6. If these figures are compared, we notice that the different saturation curves of Y_n, Y_{n+1} and Y_{n+2} seem respectively similar; more precisely, the saturation curve of Y_n is illustrated in Fig. 5 by $Y_3(I_3)$ and in Fig. 6 by $Y_6(I_6)$. These curves are close to an exponential function nearly identical to the saturation curves of Wang and Myers [9] though the conditions were not the same (steady state). In contrast, the saturation curve of Y_{n+1} is quite peculiar: in Fig. 4, $Y_3(I_2)$ has a

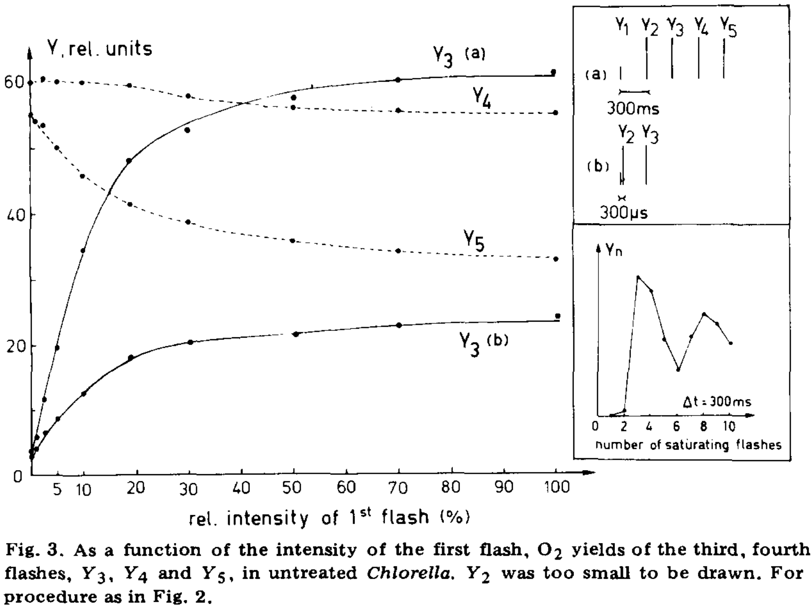

Fig. 3. As a function of the intensity of the first flash, O_2 yields of the third, fourth and fifth saturating flashes, Y_3, Y_4 and Y_5, in untreated *Chlorella*. Y_2 was too small to be drawn. For Y_3(a) and (b): same procedure as in Fig. 2.

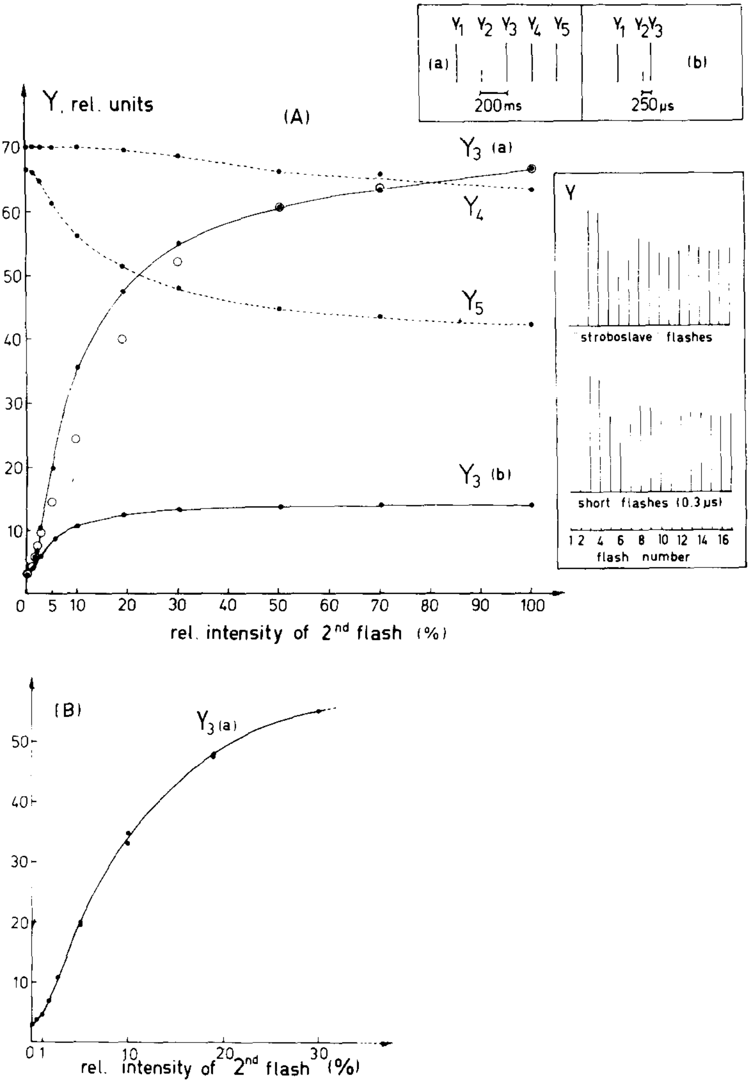

Fig. 4. A. As a function of the second flash, O_2 yields of the third, fourth and fifth flashes Y_3, Y_4 and Y_5, in dark-adapted *Chlorella*, other flashes of the sequence were saturating. ●, 'stroboslave' flash; ○, short flash (0.3 µs). Y_3(a): flashes were spaced 200 ms apart. The 200 ms time spacing has been selected because at larger spacing the deactivation of the states occurs between flash 2 and 4 when the intensity I_3 of the third flash is small. The same remark is valid for the subsequent figures. Y_3(b): the third flash was fired 250 µs after the second flash of variable intensity, all other flashes were spaced 200 ms. On the right of the figures, were drawn the sequences obtained with a 'stroboslave' flash and with the short flash (0.3 µs), for a light intensity of 100% (spacing between flashes: 400 ms). B. The same saturation curve Y_3(a) as in (A) with an extended scale for the relative light intensity.

sigmoidal shape at very low Flash 2 intensity. Otherwise, the variations of Y_{n+2} are well illustrated in Fig. 3 ($n = 1$) in which $Y_3(I_1)$ is close to an exponential.

In order to discuss these curves theoretically, we call E_i the proportion of centers in the S_i state which are converted into S_{i+1} just after the flash n of variable intensity; we also introduced the double hit factor θ_i, the proportion of S_i centers which are converted into S_{i+2} after the flash n of variable intensity of flash n is saturating, E_i is related to the miss α_i and the double hit β_i: $E_i = 1 - \alpha_i - \beta_i$. In Appendix II, Table I gives Y_n, Y_{n+1}, Y_{n+2}, at given concentrations of S_0, S_1, S_2, S_3 just before the flash of variable intensity. Double hits due to overlap of the flash tail and S-state turnover kinetics were neglected. This simplification does not introduce errors: when short flashes (<0.3 μs) with no tails were used rather than 'stroboslave' flashes, no important changes in the saturation curves were observed; in Fig. 4, when the light intensity of the short second flash increases, the variations of Y_3 present the same characteristic feature, especially a slow rise at high light intensity of the flash and a change in the slope at low light intensity. We do not know if the small difference in the shape of the two curves in Fig. 4, obtained with a short and a 'stroboslave' flash is related to differences in the *Chlorella* samples or is specific to the shorter flashes. The relative intensity, 100%, of the short flash gives O_2 yield sequences identical to those obtained with the 'stroboslave' saturating flash (see Fig. 4).

We shall discuss our results within two hypotheses:

(a) If intrinsic double hits are taken to be negligible, the saturation curve of the transition $S_3^* \rightarrow O_2$, (E_3), is measured by the variation of Y_n as a function of the light intensity of Flash n (see $Y_3(I_3)$ in Fig. 5 or $Y_6(I_6)$ in Fig. 6). The saturation curve of the transition $S_2^* \rightarrow S_3$, (E_2), is illustrated by the curve $Y_3(I_2)$ in Fig. 4 because just before the second flash the concentration of S_3 is nearly

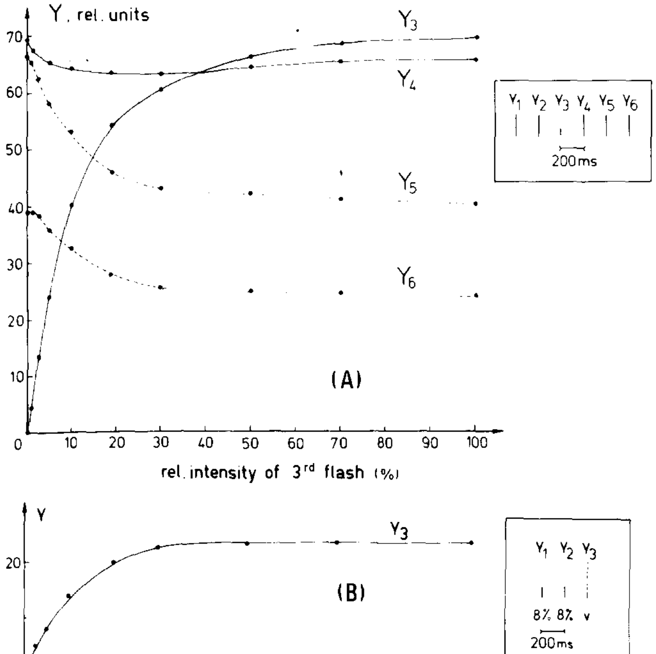

Fig. 5. A As a function of the intensity of the third flash, O_2 yields of the third, fourth and fifth flashes, Y_3, Y_4, and Y_5, in dark-adapted *Chlorella*, other flashes of the sequence were saturating; flashes were spaced 200 ms apart. B. O_2 yield of the third flash, $Y_3(I_3)$, the two first flashes of the sequence being weak (8% I).

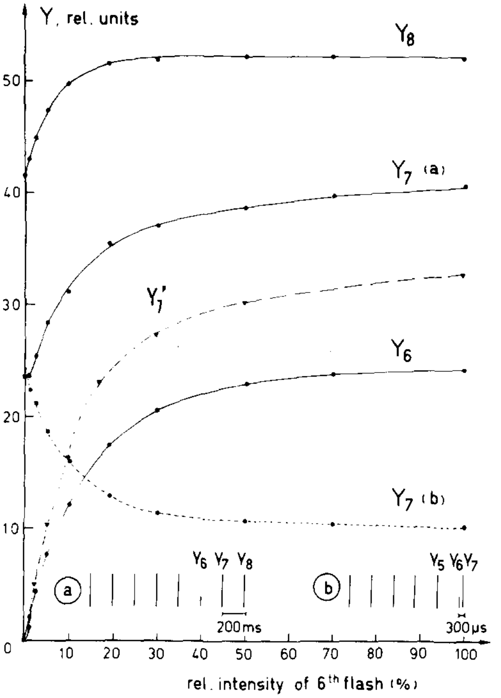

Fig. 6. As a function of the intensity of the sixth flash, O_2 yields of the sixth, seventh and eighth flashes, Y_6, Y_7 and Y_8, in dark-adapted *Chlorella*; other flashes of the sequence were saturating. Y_7(a): flashes were spaced 200 ms apart. Y'_7: curve obtained by substracting from Y_7(a), the complementary part of the saturation curve $Y_6(I_6)$ (see text). Y_7(b): the seventh flash was fired 300 µs after the sixth flash of variable intensity, all other flashes were spaced 200 ms apart. (Experimentally, Y_6 and Y_7 (300 µs) are superimposed. The value of Y_7 (300 µs) is obtained by substracting the values of $Y_6(I_6)$ from the experimental values).

zero; the saturation curve of the transitions $S_1^* \rightarrow S_2$, (E_1), is exhibited by $Y_3(I_1)$ in Fig. 3, the concentration of S_2 and S_3 being very small before the first flash.

(b) If there are double hits, the saturation curves must agree with the superimposition of an exponential (E_i) and a sigmoidal curve: the sigmoid must be apparent, or if the exponential curve is the preponderant one, a slow increase must be observed at high light intensity, which corresponds to double hits only, when the single-hit reaction is completely saturated.

The shape of the saturation curve may indicate the presence or not of double hits. For example the shape of the saturation curve Y_n produced by Flash n is close to an exponential ($Y_3(I_3)$) in Fig. 5 and $Y_6(I_6)$ in Fig. 6); thus, we conclude that Y_n, represents the saturation curve of the transition $S_3^* \rightarrow S_0 + O_2$ and that no double hits as $S_2 \rightarrow S_0 + O_2$ exists in a saturating flash sequence. Similarly, the saturation curve of the transition $S_1^* \rightarrow S_2$ given by the curve $Y_3(I_1)$ versus the intensity of flash 1 in Fig. 3 is close to an exponential. This result seems inconsistent with the hypothesis of Thibault [10]. He proposed a model to explain published O_2 yield patterns. The model of equal misses for all states S_0, S_1, S_2, S_3 [18], never rigorously explains the experimental O_2 yield patterns [3]. Therefore, he proposed to keep the homogenous hypothesis of Kok et al. except for the first flash, and explained the anomaly on the first flash by the occurrence of a high double hit of around 50—60% at this flash. This implies that only the S_0 state would be stable in the dark. Thibault's proposed that 50% of double hits on the first flash would generate a strongly sygmoidal Y_3 saturation curve, because a large part of the O_2 yield, Y_3, would come from double hits on the first flash. However *, if there are abnormal double hits after a first saturating flash following dark adaptation and if these double hits are due to an extra acceptor [8], then, when the first flash is weak, the abnormal double hits would occur on the second flash (which is the first saturating flash) and no lag would be observed in the saturation curve of Y_3. In order to test this possibility the following experiment has been performed: after dark adaptation, *Chlorella* were illuminated with two weak flashes ($I_1 = I_2 = 8\%$ $I_{\max}$) spaced 200 ms apart, followed by a third flash of variable intensity I_3. The shape of the saturation curve of the O_2 yield produced by this flash, $Y_3(I_3)$, should clearly determine whether double hits occur on the third flash which is the first saturating flash in this experiment. Just after the third flash, two types of center can give O_2: those which were in the S_1 state in the dark, and those in the S_0 state in the dark generating double hits in the third flash; these latter centers would induce a sigmoidal $Y_3(I_3)$ saturation curve. Experimentally the saturation curve for $Y_3(I_3)$ is found to be in Fig. 5B, strictly linear at low light intensity I_3 of Flash 3. Thus O_2 yield produced by this flash comes from the S_1 state in the dark. Therefore, the first saturating flash in a sequence does not induce double hits in dark-adapted *Chlorella*.

In contrast to the saturation curves of the transitions $S_3^* \rightarrow S_0$ and $S_1^* \rightarrow S_2$, when the intensity of the second flash is variable, $Y_3(I_2)$ is sigmoidal at very low intensity (Fig. 4). The sigmoidal shape of this saturation curve $Y_3(I_2)$, at first sight, might come from the occurrence of double hits with the transitions $S_1 \rightarrow S_3$ ($\theta_1 \neq 0$) on the second flash. However, the second flash is not different to the others: as previously shown in Fig. 2, the saturation curve of $Y_2(I_1)$ is sigmoidal at low light intensity of the first flash; the saturation curve of $Y_7(I_6)$ as a function of the sixth flash of variable intensity also exhibits a similar sigmoidal curve (Fig. 6). These curves (Figs. 2, 4, 6) may be tentatively taken as a proof of a double hit θ_1 (only $S_1 \rightarrow S_3$) on each flash of a sequence. This first assumption raises some inconsistencies:

(1) Comparing the curves $Y_7(I_6)$ and $Y_3(I_2)$, we remark that the concentration of S_3 is very low before flash 2, whereas, before flash 6, it is higher because it is not far from steady-state conditions. Thus, when the light intensity of flash 6 increases, the curve $Y_7(I_6)$ reflects two superimposed effects: firstly, the formation of S_3 either with a single hit from S_2 or double hit from S_1 ($E_2S_2 + \theta_1S_1$) and, secondly, the disappearance of S_3 present before the flash, through the reaction $S_3 \overset{h\nu}{\rightarrow} O_2$ $(1 - E_3S_3)$. At flash 2, in Fig. 4, this second reaction is negligible, because S_3 is negligible before flash 2. In contrast, just before flash 6, the concentration of S_3 is important. By subtracting the complementary part of the saturation curve $Y_6(I_6)$ from $Y_7(I_6)$, we obtain a resulting curve Y'_7 which only represents the formation of S_3 by reaction $S_2^* \rightarrow S_3$ and by a double hit from S_1 if it exists. The curve Y'_7 is sigmoidal and looks like that of $Y_3(I_2)$ in

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* We are indebted to one of the reviewers for pointing out this possibility.

Fig. 4. This similarity is not in favor of double hit θ_1 ($S_1 \rightarrow S_3$). Because the concentration of S_1 before flash 6 (nearly steady-state conditions) is larger than that before flash 2, the sigmoidal shape would be much more marked for $Y'_7(I_6)$ in Fig. 6 than for $Y_3(I_2)$ in Fig. 4. Therefore our experimental results are against the hypothesis of a large proportion of double hits θ_1 ($S_1 \rightarrow S_3$).

(2) The discrepancy between the assumption of double hits θ_1 and the experiment increases, if the percentage of double hits θ_1 in each flash is evaluated from the sigmoidal shape of our saturation curve. At very weak intensity, the slope or the saturation curve Y_3 in Fig. 4 is equal to about half the maximum slope at intermediate light intensity; assuming that this finite slope comes from single hit and the sigmoidal shape from double hit, a percentage of around 50% is estimated. It is shown in Fig. 7 how theoretically double hit θ_1 on only the transitions $S_1 \rightarrow S_3$ in each flash affects the O_2 yield pattern, leading to a periodicity of three rather than four in the oscillations of the O_2 yield pattern. Consequently, it is likely that the sigmoidal shape of our experimental curves is no a proof of double hit θ_1 in each flash but is more fundamentally a characteristic of the transition law E_2: $S_2^* \rightarrow S_3$ as a function of the light intensity. The possible origins of this behavior will be discuss below.

We notice that the transition $S_2^* \rightarrow S_3$ is also sigmoidal as a function of the time interval Δt after a saturating flash [11], and this property is true for any flash of a series: in steady-state relaxation experiments, a sigmoidal kinetics is still observed for the sum of the four kinetics of S_0, S_1, S_2, S_3 states (Ref. 12, and Lavorel, J., unpublished results); we have verified directly that the kinetics of Y_7 as a function of the time interval between Y_6 and Y_7 is sigmoidal. Therefore, the properties of the S_i state (turn-over kinetics and saturation curves), within our experimental precision, do not change with the number of flashes after dark adaptation.

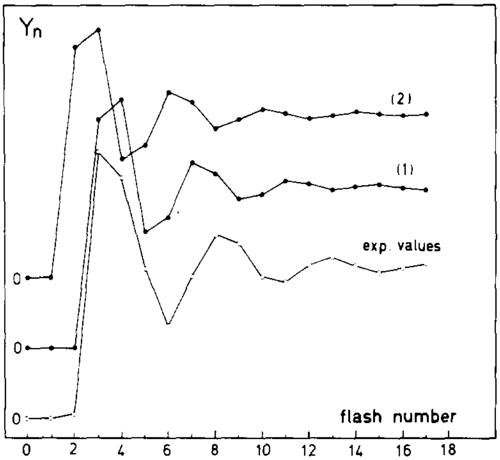


Fig. 7. Experimental flash O_2 yields pattern in dark-adapted *Chlorella* (spacing: 200 ms) compared to theoretical ones: (1) initial conditions $S_0 = 1$, $S_1 = 0$, $S_2 = 0$, $S_3 = 0$; equal miss $\alpha = 0.25$; double hit, only for the transition $S_1 \rightarrow S_3$: $\beta_1 = 0.6$ ($\beta_2 = \beta_3 = \beta_0 = 0$); (2) the conditions are identical to those of (1) except for the initial conditions: $S_0 = 0.25$, $S_1 = 0.75$, $S_2 = 0$, $S_3 = 0$. For other values of α and β_1 in the same range, the theoretical pattern has always an intermediate period between 3 and 4, in contradiction with the experiments.

2. *Flash yield saturation curves as a function of time after the variable intensity flash*

In a way similar to Joliot and Joliot's fluorescence studies [4], we detected the O_2 yield a short time after the variable intensity flash ($t = 250$ μs or $t = 300$ μs), and compared it to the one measured at a longer time (200 ms or 300 ms). The saturation curves of these O_2 yields are not proportional (see in Fig. 2, Y_2 (a) and (b)(I_1) and in Fig. 4, Y_3(a) and (b)(I_2)). For example in Fig. 4, the slopes of Y_3(a) and Y_3(b) are identical near zero intensity. In contrast, at saturating light, Y_3 (200 ms) is much larger than Y_3 (250 μs). A well developed lag distinguishes the long time flash yield with respect to that detected at shorter time. The ratio $Y_3(a)/Y_3(b)$ at saturating light, approx. 4 (Fig. 4), leads one to suppose that there are very large double hits θ_1. As explained above (Fig. 7), preponderant double hits by transitions $S_1 \rightarrow S_3$ cannot describe the experimental O_2 yield pattern. The phenomenon described must concern the transition $S_2^* \rightarrow S_3$.

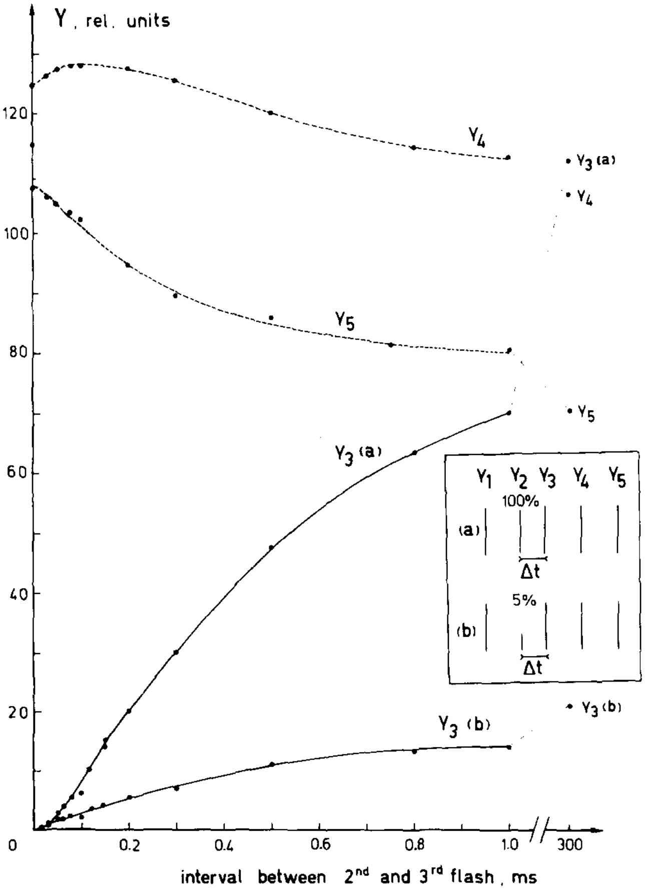


Fig. 8. Turnover time of the transition $S_2^* \rightarrow S_3$ in *Chlorella* in different light intensity conditions. After a first saturating flash followed 200 ms later by a second flash, O_2 was detected at a variable time later by the third saturating flash, Y_3. The relative light intensity of the second flash was either 100% (a) or 5% (b).

In contrast to the transition $S_2^* \rightarrow S_3$, the saturation properties of the transition $S_1^* \rightarrow S_2$ do not change whatever the measurement time. In Fig. 3 and in Fig. 2A with NH_4Cl, Y_3(300 ms) and Y_3(300 μs) are proportional. Fig. 2A shows also that at 300 μs after a saturating flash, 65% of the S_2 centers are active, whilst only 20% of the S_3 centers are. Although this result seems peculiar at first sight, it is qualitatively in agreement with the very different turnover kinetics of the transitions $S_1^* \rightarrow S_2$ and $S_2^* \rightarrow S_3$ measured by Bouges-Bocquet [11].

In Fig. 6, the saturation curve of Y_7(b) detected 300 μs after the sixth variable flash is compared to that of Y_7(a) detected at 200 ms; by increasing the intensity of the sixth flash, Y_7(b) decreases from zero intensity value as opposed to Y_7(a) which increases. This result is also explained by the sigmoidal kinetics of the transition $S_2^* \rightarrow S_3$ [11]; because a lag exists in this kinetics, only a small number of S_2 centers are converted into S_3 at 300 μs after a flash, for any intensity value of this flash, the factor E_2S_2 is small in the Y_7(b) saturation curve, so that this curve principally reflects the factor $1 - E_3S_3$ (see Appendix II) which is negative (E_3 varies from 0 to $1 - \alpha_3$). On the other hand, E_2S_2 is the predominant factor in the Y_7(a) saturation curve. Therefore, at any flash number, the flash saturation curve of $S_2^* \rightarrow S_3$ detected at a long time (200 ms) shows a lag which is not observed at a shorter time (300 μs).

The inter-relation between the effect of the flash intensity and that of the time interval upon O_2 yields is illustrated in Fig. 8. At a short time ($t < 50$ μs) after a flash, the kinetics of the conversion $S_2^* \rightarrow S_3$ is the same whatever the light intensity of the flash: saturating light $I = 100\%$, or weak light $I = 5\%$; in contrast, at a long time $t > 1$ ms, the conversion into S_3 is relatively much larger after a saturating flash than after a weak flash (Y_3 in Fig. 8). This result is in agreement with the non-homothethic saturation curves of the O_2 yield Y_3 measured at different times after the second variable flash in Fig. 4. Fig. 4 and Fig. 8 could be two representations of the same experiments if we had made these measurements in the same batch, and on the same day.

Discussion

We shall first consider the possibility of explaining our results by a photosynthetic double hit on the transitions $S_1 \rightarrow S_3$. We have shown that the sigmoidal saturation curve is not restricted to the O_2 yield following a first flash of variable intensity, $Y_2(I_1)$; the same curves exist following the second and sixth flash for the formation of S_3; $Y_3(I_2)$ in Fig. 4 and $Y_7(I_6)$ in Fig. 6. This would imply a large proportion of double hits, which is in contradiction with the very low value of double hits observed in the flash O_2 yield pattern.

Within the double hit model, it remains the possibility of a single step $S_2^* \rightarrow S_3$ with a double hit: one photon being not used for the photoreaction; this possibility is similar to the models of Glaser et al. [8], Joliot and Joliot [4] and Diner [14]. In their model, at the first saturating flash, oxidized chlorophyll Chl^+ is reduced by a secondary donor Y in a time short compared to the duration of the flash (<1 μs) after the first hit, and after the second hit, Chl^+ is reduced by another donor, D; Y in normal chloroplasts or *Chlorella*, is the photosynthetic donor, and D is an auxiliary donor, not used by the O_2-evolving site. In Fig. 4, the saturation curve $Y_3(I_2)$ at 250 μs is not sigmoidal, so that the difference between the saturation curve at long time, 200 ms, and that at 250 μs shows a very strong lag at low flash intensity. At first sight, this result is similar to that of Joliot and Joliot [4], observing a lag in the fluorescence flash saturation curve of the Δ (2 ms — 20 μs). Nevertheless, our results are different in many respects: the amplitude of the sigmoid is much larger in our case; we observed that the apparent quantum yield of the transition $S_2^* \rightarrow S_3$ is lower by a factor 1.5 to 2 at low intensity (below 4% of the saturating light) than at intermediate intensity (in Fig. 4). This result cannot be explained by assuming that the first hit is used for the reaction $S_2^* \rightarrow S_3$ in *Chlorella* or chloroplasts, and the second hit for an auxiliary donor, D [4,8,14]; in this model, the saturation curve of the reaction $S_2^* \rightarrow S_3$ measuring the first hit must have no lag, because at the second hit, D is not used for this reaction: $S_2^* \rightarrow S_3$; the double hit of this model cannot be seen in the O_2 evolving reactions. At weak flash intensity, the low quantum yield of the one-hit reaction $S_2^* \rightarrow S_3$ may be explained if, in the double hit, the first hit is used by an auxiliary donor, D′, for non-photosynthetic reactions and, the second hit, for the reaction $S_2^* \rightarrow S_3$; but this assumption is in desagreement with the conclusions of Glaser et al. [8], Joliot and Joliot [4] and Diner [14]. We have, thus, two possibilities: to keep this double-hit hypothesis as a starting point, or to consider other models explaining the low quantum yield at low flash intensity.

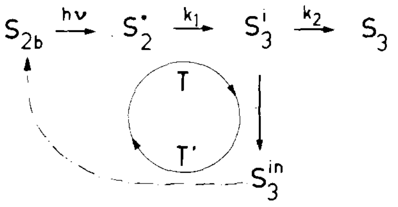
Within the first hypothesis, it seems puzzling that D′ is used before Y on the S_2 state; Chl^+ undergoes a very fast reduction, in less than 0.1 μs [15]. Therefore, the reduction of Chl^+ by Y is very fast, but its reduction by the auxiliary donor, D′, necessary to explain our signoidal saturation curve in Fig. 4, must be much faster than Y. With respect to this property, D′ is thus fundamentally different from D, which explains the slow kinetics of Joliot and Joliot [4].

The quantum yield of photosystem II centers is quite good, around 4 to 5 photons per O_2 [16]; thus, the O_2 yield corresponding to a flash intensity of one photon per center collected by the photosynthetic pigments is equal to $(1 - 1/e)Y_{\max}$, in the exponential saturation curve of transitions $S_i^* \rightarrow S_{i+1}$ (other than $S_2^* \rightarrow S_3$). This intensity is evaluated to $I = 20\%$ in Fig. 5. In Fig. 4, wihin the double-hit hypothesis, the first step becomes saturated around an intensity of 4% i.e. around 1/5 of the intensity corresponding to one photon per center. Our experimental results clearly show (within any model) that, at low intensity some part of the photons are not used for photosynthesis and that the corresponding auxiliary reactions are saturated with one photon per five centers. Within the double-hit hypothesis, our results imply that there are units of five Chl more tightly connected to one D′ than to the five Y; on the donor side, a strong cooperation between a large number of centers must be supposed. For this reason, it is not clear that this cooperation implies a direct transfer of the positive charge from Chl to D′ (Z—Y—Chl—D′) or an indirect transfer through $\mathrm{Y}(\begin{smallmatrix}\mathrm{D'}\\ \mathrm{Z}\end{smallmatrix}\!>\!\mathrm{Y\text{—}Chl})$. The D′ donor is not seen by fluorescence measurements: at low light intensity (Fig. 1 in Ref. 4), there is no lag in the saturation curves of fluorescence yield in DCMU-inhibited chloroplasts. Thus, the quenching property of D′ seems similar to that of Y, although it should give one electron to Chl before any of the five competing Y donors in the double-hit model. Experimentally, the saturation curve with a sigmoidal shape occurs only for the transition $S_2^* \rightarrow S_3$; this shows that D′ is not bound to Chl or to Y, otherwise D′ would take the positive charge for any number of accumulated charges, and thus the sigmoid would be observed in all S-state transitions.

For all these reasons, it seems more likely that D′ does not interact with Chl or Y, but with the water-splitting reactions S_i; in that case, the model becomes fundamentally different from a simple double-hit model. Although we have a lower quantum yield at weak intensity than at higher intensity in Fig. 4, the particularities of our results lead to the conclusion of a cooperation between centers at the level of the water-splitting reactions.

As shown in Fig. 8, the turnover kinetics of $S_2^* \rightarrow S_3$ changes with the light intensity; the one-step kinetics is completely suppressed at high intensity; thus we can distinguish two types of S_2 center: the S_{2a} centers, in smaller number (<10%), observed at low flash intensity, with monophasic kinetics; and the S_{2b} centers with biphasic turnover kinetics. Tentatively, the S_{2a} and S_{2b} centers may be associated to the α and β centers of Melis and Homann [17], having different yields of excitation trapping. Contrary to the conclusions of Melis and Duysens [18], the α and β centers would be embedded in the same pigment bed; at weak intensity, the α centers would strongly trap the excitations (with a low quantum yield), so that a smaller number of excitations would be available to the β centers. At a higher intensity, the α centers disappearing by saturation, the β centers could trap excitations with an increasing efficiency. Unfortunately, this model is not in agreement with the observed fluorescence-induction curve; this model implies a lag in the flash saturation curve of the β center fluorescence yield, which is not observed [18]. It seems more likely that the differences between the S_{2a} and S_{2b} centers comes from differences at the level of the water-splitting reactions and not in the efficiency of excitation trapping.

For these reasons, we propose a model based on the particular properties of the S_2 to S_3 transition. There are two types of S_2 center, as above a and b; after a flash, the S_{2a} centers (10%) are converted into S_3 with monophasic kinetics and saturation curve similar to that of the other S states, whereas the S_{2b} centers exhibit biphasic turnover kinetics and sigmoidal saturation curve. In Ref. 11, the turnover kinetics of $S_2^* \rightarrow S_3$ is related to two successive reactions with an unknown intermediate, S_3^i. Thus, the small lag in the saturation curve of $S_{2b}^* \rightarrow S_3$ can be explained if S_3^i reacts with some product, T, converting this intermediate state into an inactive form: $S_3^i + T \rightarrow S_3^{in}$ (see Scheme I). The concentration of T is relatively small, so that there is a leakage in the reaction $S_2^* \rightarrow$



Scheme I. Proposed model.

S_3 as long as the product T has not completely reacted with S_3^i. After a flash of low intensity, the quantity of S_2^* is smaller than that of T and the apparent quantum yield is low. At sufficiently high intensity, the quantity of S_2^* formed in the flash is larger than that of T, so that the quantum efficiency increases as observed. This kinetics model is sufficient to explain our results; its principal characteristic is that the formation of S_3 is partially inhibited by a small concentration of T. The nature of T is unknown, as the nature of the two step reactions in the turnover kinetics of S_3. T could be an auxiliary donor reducing the S_3^{in} state into S_2; the photosystem II would possess a cyclic oxidation reaction of a compound, T, in parallel to the transition $S_2^* \rightarrow S_3$. This possibility gives a 'miss' on only one transition; this explains quantitatively the damping of the flash O_2 yield pattern oscillations as we have shown in a previous paper [3].

Appendix I

The formula giving the proportion of double hits as a function of flash intensity vs. time $I(t)$ and the turnover kinetics $U(t)$ is a complex multiple integral. At high intensity, the formula is simpler; the first step $S_1^* \rightarrow S_2$ is completely realized at the begining of the flash tail (2 μs with the stroboslave) and only the long flash tail (up to 20 μs) can induce the second transition $S_2^* \rightarrow S_3$, when the turnover reaction $S_2^* \rightarrow S_3$ has begun. This turnover reaction $S_2(t) = U(t)S_3^*(0) \simeq S_3^*Ct$, is linear at short time and is equal to $U(\infty) = 1$ at long time ($C = (\mathrm{d}U/\mathrm{d}t)_{t=0} = (\mathrm{d}S_3/\mathrm{d}t)_{t=0}$ is the relative rate of initial turnover at $t = 0$). Thus, the proportion β of double hits, due to overlap of the flash tail is equal to:

$$\beta = \frac{\int_0^{+\infty} \mathrm{d}t \cdot U(t) \cdot I(t)}{\int_0^{+\infty} I(t)\,\mathrm{d}t} \simeq C\,\frac{\int_0^{+\infty} \mathrm{d}t \cdot t \cdot I(t)}{\int_0^{+\infty} I(t)\,\mathrm{d}t} = Ct_{\mathrm{eff}}$$

with an effective time

$$t_{\mathrm{eff}} = \frac{\int_0^{+\infty} tI(t)\,\mathrm{d}t}{\int_0^{\infty} I(t)\,\mathrm{d}t}$$

t_{eff} measures the importance of the flash tail. The effective time of the 'stroboslave' flash was found to be of order of 10 μs.

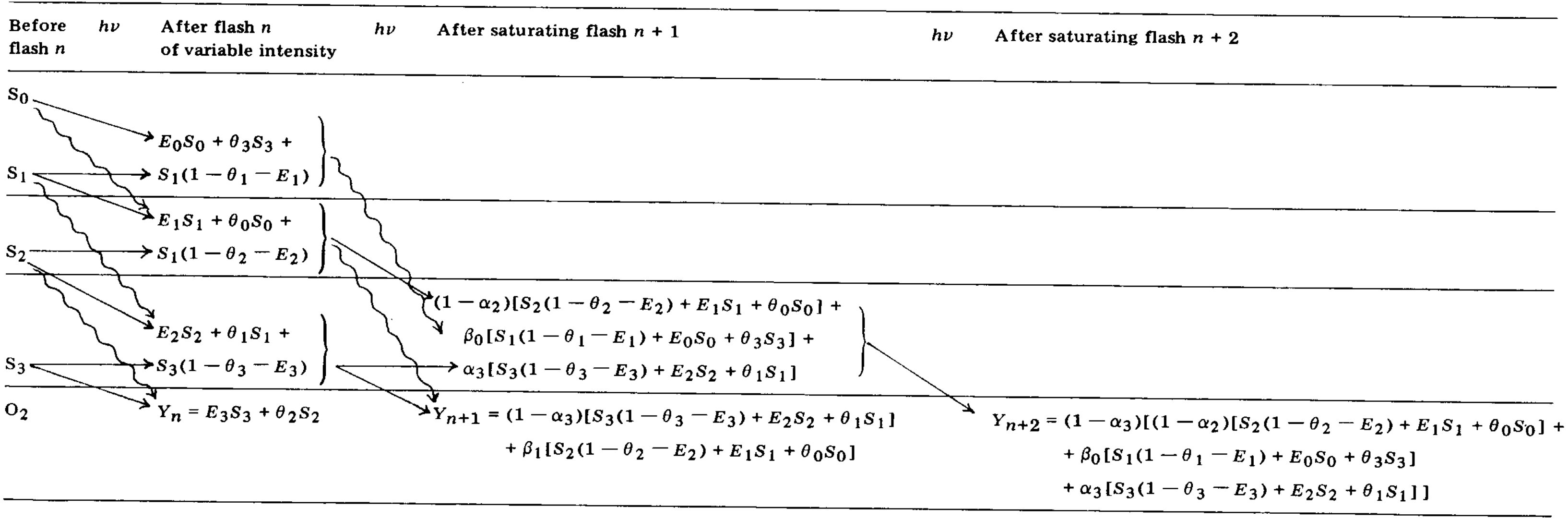
Appendix II

Model for O_2 yield saturation curves

Let us consider a flash O_2 yield sequence spaced 200 ms apart. The light intensity of flash of number n is variable; all other flashes are saturating. We call E_i, the variable proportion of centers in S_i state which are converted in the

TABLE I

EQUATIONS CONTROLLING THE SATURATION CURVES OF Y_n, Y_{n+1} AND Y_{n+2}. DECREASING ARROWS: ↘ ONE HIT, ↘ DOUBLE HIT.



Before flash n	$h\nu$	After flash n of variable intensity	$h\nu$	After saturating flash $n+1$	$h\nu$	After saturating flash $n+2$
S_0		$E_0S_0+\theta_3S_3+$				
S_1		$S_1(1-\theta_1-E_1)$				
		$E_1S_1+\theta_0S_0+$				
S_2		$S_1(1-\theta_2-E_2)$				
				$(1-\alpha_2)[S_2(1-\theta_2-E_2)+E_1S_1+\theta_0S_0]+$		
		$E_2S_2+\theta_1S_1+$		$\beta_0[S_1(1-\theta_1-E_1)+E_0S_0+\theta_3S_3]+$		
S_3		$S_3(1-\theta_3-E_3)$		$\alpha_3[S_3(1-\theta_3-E_3)+E_2S_2+\theta_1S_1]$		
O_2		$Y_n=E_3S_3+\theta_2S_2$		$Y_{n+1}=(1-\alpha_3)[S_3(1-\theta_3-E_3)+E_2S_2+\theta_1S_1]$ $+\beta_1[S_2(1-\theta_2-E_2)+E_1S_1+\theta_0S_0]$		$Y_{n+2}=(1-\alpha_3)[(1-\alpha_2)[S_2(1-\theta_2-E_2)+E_1S_1+\theta_0S_0]+$ $+\beta_0[S_1(1-\theta_1-E_1)+E_0S_0+\theta_3S_3]$ $+\alpha_3[S_3(1-\theta_3-E_3)+E_2S_2+\theta_1S_1]]$

S_{i+1} state after the flash n of variable intensity, θ_i the proportion of centers in S_i state which are converted in the S_{i+2} state (double hit) after the flash n.

$$S_i^n \xrightarrow{E_i} S_{i+1}^{n+1}$$

$$S_i^n \xrightarrow{\theta_i} S_{i+2}^{n+1}$$

The factors E_i and θ_i are functions of the light intensity I_n of flash n.

When I_n is saturating, θ_i and E_i are equal to β_i and $(1 - \alpha_i - \beta_i)$, respectively; α_i is the 'miss' on the S_i state and β_i, the double-hit proportion with saturating flash. We suppose that these values are the same at the following saturating flash. For simplification, we suppose that the total number of centers: $S_0 + S_1 + S_2 + S_3$ is kept constant, i.e. we neglect the possibility of a transitory inactive state, as already proposed [3].

Let us suppose known concentrations of S_0, S_1, S_2, S_3 just before Flash n of variable intensity; Table I gives the equations controlling the saturation curves of Y_n, Y_{n+1} and Y_{n+2}.

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