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COOPERATION OF CHARGES IN PHOTOSYNTHETIC O_2 EVOLUTION – I. A LINEAR FOUR STEP MECHANISM

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Abstract—Using isolated chloroplasts and techniques as described by Joliot and Joliot[6] we studied the evolution of O_2 in weak light and light flashes to analyze the interactions between light induced O_2 precursors and their decay in darkness. The following observations and conclusions are reported: 1. Light flashes always produce the same number of oxidizing equivalents either as precursor or as O_2. 2. The number of unstable precursor equivalents present during steady state photosynthesis is $\simeq 1{\cdot}2$ per photochemical trapping center. 3. The cooperation of the four photochemically formed oxidizing equivalents occurs essentially in the individual reaction centers and the final O_2 evolution step is a one quantum process. 4. The data are compatible with a linear four step mechanism in which a trapping center, or an associated catalyst, (S) successively accumulates four + charges. The S^{4+} state produces O_2 and returns to the ground state S_0. 5. Besides S_0 also the first oxidized state S^+ is stable in the dark, the two higher states, S^{2+} and S^{3+} are not. 6. The relaxation times of some of the photooxidation steps were estimated. The fastest reaction, presumably $S_1^* \rightarrow S_2$, has a (first) half time $\leq 200\ \mu$sec. The S_2^* state and probably also the S_0^* state are processed somewhat more slowly ($\sim$ 300–400 μsec).

INTRODUCTION

ALLEN and Franck[1] noticed that in darkness the photosynthetic apparatus looses its ability to produce O_2 in a single flash, an observation which was confirmed e.g. by Whittingham and Bishop[2]. More recently, with the aid of refined polarographic techniques, Joliot and coworkers[3–5] analyzed this 'deactivation' of the O_2 evolving process in greater detail and realized that the phenomena reflect the cooperation in O_2 evolution of photochemically produced intermediates of limited stability. This paper reports additional observations concerning this process and offers a kinetic model for the mechanism of charge cooperation.

METHODS AND MATERIALS

The experiments were performed with chloroplasts isolated from green house grown spinach. Flash yields and rates of O_2 evolution were measured at room temperature (20°–25°C) with the modulated polarograph technique described by Joliot and Joliot[6]. Both flowing media contained $0{\cdot}1\ M$ KCl in phosphate buffer pH 7·5; the flow medium adjacent to the chloroplast suspension in addition contained $10^{-3}\ M$ NADP. Chloroplast samples were diluted to 0·2 mg Chl/ml from a stock suspension in $0{\cdot}4\ M$ sucrose tris buffer pH 7·65 which was stored in a darkened ice bucket during 1–5 hr after its preparation. Enrichment of the chloroplast suspension with ferredoxin was required only if rates were to be measured in intensities higher than the ones used regularly. After being placed on the electrode and allowed to settle in darkness ($\sim$ 10 min) a sample was used during some 30 min, then replaced by a fresh one. With each fresh, dark adapted sample, an activation rate transient was recorded (see below) which served as a standard curve used to normalize subsequent observations with

the sample. Observations made with one sample or with consecutive ones were reproducible within ±5–10 per cent. We noticed, however, a significant seasonal variation of the stability and behavior of the individual chloroplast preparations.

For rate measurements we generally used a modulated detecting beam of long wavelength ($\lambda \geqslant 700$ nm) to insure oxidation of Q and A and optimal performance of system II [3]. The light modulating rotating disc was synchronized with the external address advance of a Fabri-Tek 1052 signal averager. With a modulating frequency of ~ 50 cycles optimal time resolution was ⩾ 10 msec, each 10 msec address stored one half cycle of the rectified signal and the entire time sweep lasted 10 sec. In order to view slower phenomena also, the signal could in addition be fed to a chart recorder (0·5 sec time resolution).

The shutter which admitted the modulated detecting beam was opened electromechanically (within 2 msec) during a preselected dark period of the modulated beam so that the subsequent light period could be measured. In addition to the modulated beam, the discharge of two Xenon flash lamps (E.G. and G. FX 6), mounted in high aperture reflectors, were focussed on the sample. For each lamp a set of capacitors was available, the one selected was charged to 800 V and discharged through the flash lamp at a selected moment. The flash energies, obtained with the various capacitors, were compared by means of a thermopile and the capacitance values were calibrated in terms of relative light emission. This calibration in terms of 'effective capacitance' thus accounted for deviations from the nominal C values as well as from the $E = \frac{1}{2} CV^2$ relation. Initially our optical arrangement required the use of 1–2 μF discharge capacitors to obtain saturation of the flash yields (expts. Figs. 3, 5, 8). Later improvements allowed a more homogeneous illumination of the sample and the use of much smaller C values. Flash yields of O_2 were measured in relative units as described in [6], the flash induced signals being rectified and read on a D.C. recorder. The electronic circuitry, included provisions for programmed triggering of flashes and admission or removal of the measuring beam.

RESULTS

1. *Rate transients after deactivation, concentration of labile* O_2 *precursors*

Figures 1 and 2 illustrate the phenomena we are dealing with. Since most of these were earlier described by Joliot *et al.* [4, 5] a brief enumeration of the more pertinent or novel aspects might suffice. Plotted in these figures is the modulated rate (V) of O_2 evolution induced by a long wave modulated light admitted at zero time. The intensity of the light was within the linear range of the rate vs. intensity curve. After exposure to the weak beam the sample was kept in the dark for 10 min. This darktime sufficed to achieve complete loss of O_2 evolution ability. Before readmitting the modulated beam and recording the rate, the sample was preilluminated with 0, 1 or more light flashes.

Without a preilluminating flash (curve 0) the initial O_2 rate (V_i) is zero, subsequently the rate increases, following an S shaped time course towards the steady state rate V_{ss}. Without deactivation the initial rate V_i would have been identical to V_{ss} and thus the area bounded by the rate transient after 10 min dark (A_0), reflects the total induction loss: the number of absorbed quanta which have not resulted in O_2. Repeating the 0 flash experiment with a higher or lower intensity results in proportionally faster or slower transients which, however, bound the same area in terms of quantum deficit.

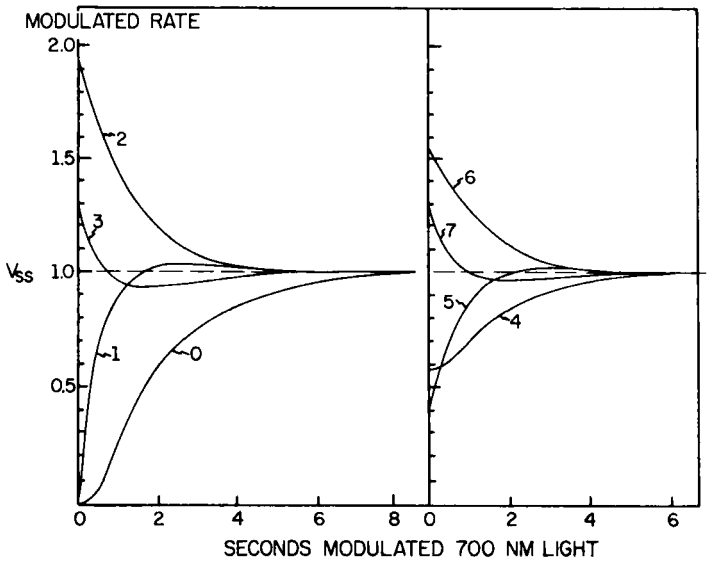

Fig. 1. Time course of the rate of O_2 evolution in 700 nm modulated light as affected by 0–7 preceding light flashes. Each curve was recorded after a 10 min dark period subsequent to the modulated beam. Spacing between the flashes and between the last flash and the opening of the shutter ~ 1 sec.

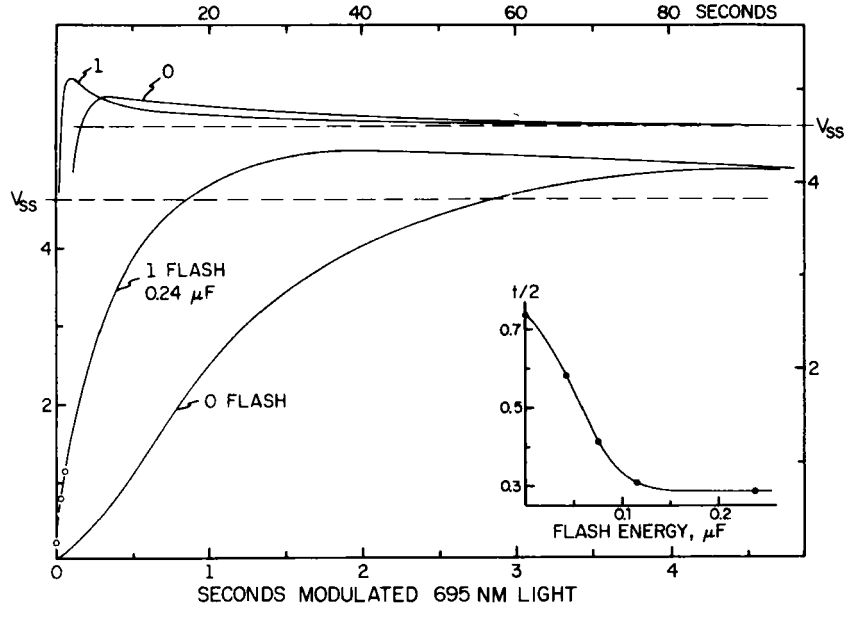

Fig. 2. Time courses of the rate of O_2 evolution in 695 nm modulated light observed 10 min (in dark) after modulated light. Simultaneous recording with two instruments, time resolution respectively 20 msec (left hand ordinate, bottom abcissa) and 0·5 sec (right hand ordinate, top abcissa). Curves 0 without, curves one with, one preceding flash. Open circles: the first three 20 msec points of the integrator. The slow recorder traced the two signals during 150 sec, their final phases were superimposed to account for a small (~3 per cent) difference of V_{ss}, ascribed to settling or ageing of the sample. Insert: halftimes of initial rises observed in similar experiments with various flash capacitors.

This $I \times t$ relation and the assumption of constant quantum efficiency imply that quanta are not really lost, but used to fill a finite pool of photochemical intermediates. These intermediates are presumably precursor(s) of molecular O_2 which are unstable and disappear in darkness. The area (A_0) bounded between V_{ss} and curve 0 thus reflects the number of these unstable procursor molecules which are present during steady state photosynthesis.

The second transient shown in Figs. 1 and 2 was a repeat of the previous one, except that the modulated light was preceded by a single light flash. In this case the rate, initially quite low, rises rapidly and shows a small overshoot before slowly attaining the steady state. A similar but slower overshoot occurs in the transient after 0 flashes (Fig. 2) and such secondary oscillations can be noticed in nearly all rate transients. As far as we can judge, from transients after 1 flash, where the effect is analyzed most easily, also these secondary oscillations occur more rapidly in stronger light, and the rate transients follow the $I \times t$ relation throughout. The amplitude of these oscillations varies (5–15 per cent of V_{ss}), their long duration complicates the measurements (demanding an additional rate recording) and introduces large uncertainties in the area computations, because in this time range the rate is affected by unrelated phenomena such as readjustment of the system II acceptor pool.

Our evaluation of the 0 and 1 flash transients (see also section 7), indicated that one flash removed most ($\geq$ 80 per cent) of the deficit area (A_0), i.e. filled most of the steady state precursor pool. If one assumes that one flash hits all trapping centers in the system once, one estimates that the deficit area (A_0) amounts to $\sim$ 1 equivalent/trap.

2. O_2 *evolution, a four step process*

The third rate transient, in Fig. 1 (left, curve 2) was seen if two flashes preceded the modulated beam. Now the rate initially exceeds the steady state $\sim$ two-fold and the transient bounds an excess rather than a deficit area. Evidently two flashes produce an excess of precursor equivalents which is correlated with an enhanced rate.

After three preceding flashes (left, curve 3) the rate again initially exceeds the steady state rate ($V_i/V_{ss} = 1{\cdot}04$–$1{\cdot}3$), but now drops rapidly below this level and then slowly returns to it. The transient bounds a small net deficit area. Four preilluminating flashes (right, curve 4) yield a curve which resembles the 'zero' transient, except for the fact that the initial rate is about half V_{ss}. The initial slope is zero, the rise curve is S shaped, approaches the steady state rate slowly, and bounds a deficit area $\sim 0{\cdot}5\,A_0$. Similarly, the transient after five flashes resembles the one after flash (1): a rapid rise towards the steady state, which starts however, at $V_i \simeq 0{\cdot}5\ V_{ss}$ and bounds a small area. Six flashes induce a transient similar to the one induced by 2, 7 a pattern like the one induced by 3, etc. After additional flashes these four types of rate transients are repeated with decreasing amplitude until after three to four cycles steady state conditions are approached ($V_i = V_{ss}, A = 0$).

Figure 3, computed from transients as shown in Fig. 1 shows the variation of the initial rate (V_i) and of the activated area (A) with the number of preceding flashes. Although generally a high initial rate correlates with a high level of the precursor pool, the two quantities, which both reveal a cycle of four, are not proportional and their oscillations are out of phase.

If one plots the relation between rate and area one finds four patterns, one induced by 0, 4, 8, 12 flashes, another by 1, 5, 9, 13 flashes, etc. We assume that these four

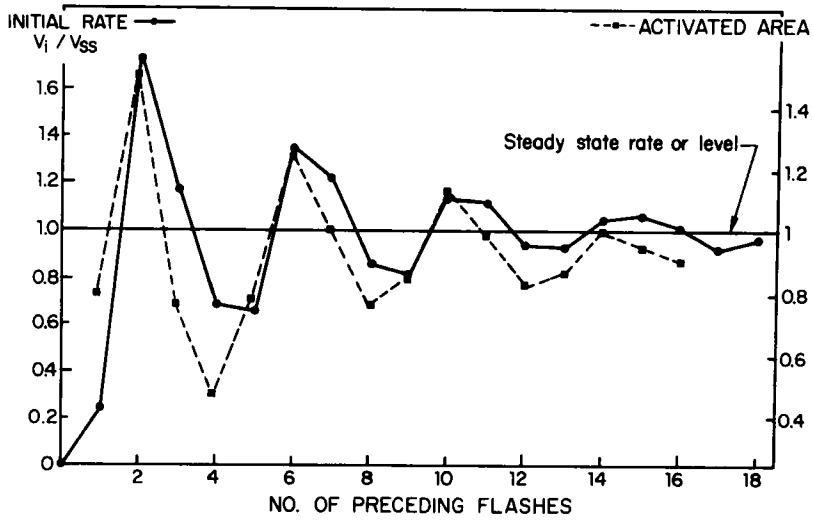

Fig. 3. Dots (left ordinate): initial rate V_i as a function of the number of preceding flashes, spaced 1 sec apart. Pretreatment 10 min dark after weak light. Squares (right ordinate): relative areas bounded by the same rate transients as used for computing V_i, normalized to the area bounded by the 0 flash transient. In this experiment and that of Fig. 5 the illumination system required the use of a 2 μF discharge capacitor to obtain saturation (see Methods).

different patterns reflect the fact that liberation of an O_2 molecule requires the cooperation of four oxidizing equivalents, generated by four excitations of system II.

Apparently, in a sequence of light flashes there occurs a large fluctuation of the relative concentration of the intermediate oxidation states.

3. O_2 *liberated in a one quantum step, independent reaction chains*

The preceding data show that, depending upon its pretreatment, the system can attain different states in which a greater or lesser fraction of quanta arriving in the trapping centers are able to evolve O_2. An important question is whether or not these specific states are recognized in the same fashion by weak and by strong light: In a bright flash all trapping centers are hit simultaneously, in weak light quanta arrive randomly and infrequently in the different centers. If, for instance, a collaboration between unstable photoproducts (such as $T^* + T^* \rightarrow O_2$, $T^* \xrightarrow{k_d} T$) were involved, the relative yield of O_2 produced by a flash (denoted Y) could differ greatly from the relative efficiency with which O_2 is evolved by the first quanta of a weak beam admitted to a comparable state of the system. On the other hand, if in the final O_2 evolving step each quantum acted by itself, the flash yield (Y) and the initial rate in weak light (V_i) would reflect the same number of 'ready' centers and thus should be proportional.

For the plot of Fig. 4 we used experiments as shown in Fig. 1, and computed for each transient the initial rate, expressed as the ratio V_i/V_{ss}. In parallel experiments we gave a sequence of flashes (spacing 1 sec), 10 min after an exposure to the modulated beam. The steady state flash yield Y_{ss} was determined either as the average value of e.g. the 10–20th flash, or the flash yield observed on a background of the modulated light – which yielded the same results as long as $\lambda > 700$ nm was used to eliminate interference through acceptor (A) pool adjustments. Otherwise Y_{ss} and V_{ss} were normalized.

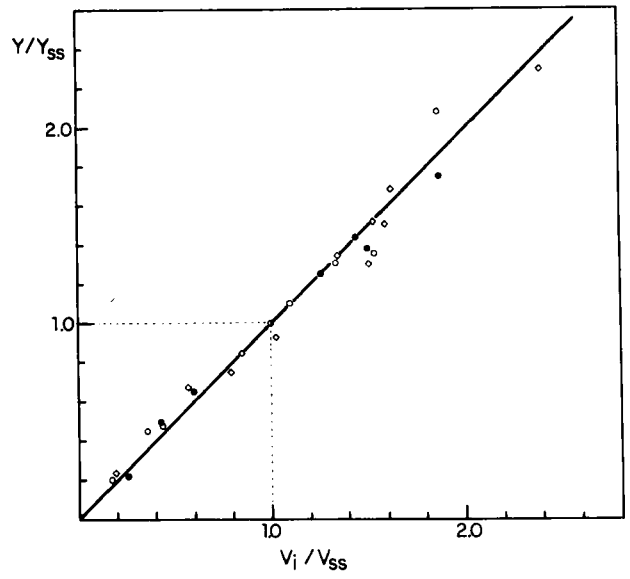

Fig. 4. Plot of the initial rate V_i observed after flash (n) vs. the yield of flash ($n+1$). All observations preceded by 10 min darkness following weak light. Spacing between flashes and between flash n and admission of the ~700 nm beam 1 sec. Different symbols represent different experiments.

In Fig. 4 we plotted the value of V_i after the flash (n): (V_{i_n}) vs. the yield of flash ($n+1$): (Y_{n+1}).

The plot indicates a linear relation between flash yield and initial quantum efficiency, apparently both quantities reflect the number of trapping centers which, upon excitation yield molecular O_2. Although, as will be mentioned in a later paper[7] under some conditions this linearity may not quite hold, we conclude that the final O_2 evolution step is essentially a one quantum process.

The data of Table 1 suggest that not only in the final step but also in the intermediate ones the trapping centers operate independently of each other.

Table 1. Effect of inhibition of system II upon oscillation of flashyield

	Control	10 min u.v.	20 min u.v.	$3\cdot10^{-7}M$ DCMU	Manganese extracted
Y_{ss}	100	47	11	12	40
Y_2/Y_{ss}	26	23	25	13	26
Y_3/Y_{ss}	195	219	220	210	240
Y_4/Y_{ss}	121	131	130	140	110
Y_5/Y_{ss}	47	58	60	–	37

Aliquots from a chloroplast preparation were irradiated with a germicidal lamp, provided with DCMU or extracted so as to remove part of the bound Mn[8]. Samples were placed on the electrode and after 10 min darkness exposed to a series of twenty flashes given at 1 sec intervals. The top line shows for each sample the value of Y_{ss}, the constant flash yield observed in the latter half of the sequence, expressed as a percentage of the control value. Lower lines show the relative yields of the first five flashes, Y_1 was negligible in all cases.

In these experiments we used three different methods to specifically inhibit the activity of system II: u.v. radiation, DCMU and Mn deficiency[8]. Inactivation of up till 90 per cent of the traps did not significantly alter the relative yields of the first five flashes, given after a 10 min dark period. This indicates a negligible role of dark reactions between charges generated in different trapping centers.

4. *Conservation of excitations*

Interpretation of the observations would be quite difficult unless one could assume that (except in long dark times, which allow detectable deactivation) no oxidizing equivalents are lost because they escape detection. For instance, a slow generation of O_2 in darkness following illumination would contribute neither to the observed flash yield nor to the modulated rate measurement.

One thus would like to assume that excitation of a trapping center produces either an equivalent of O_2 or an equivalent of precursor. Our conclusion in the previous section, that O_2 occurs in a one quantum process, implies that in this event three equivalents are being consumed from the precursor pool.

To check this point, we computed plots as illustrated in Fig. 5 (open circles). The abcissa shows the initial rate V_i observed in modulated light admitted 1 sec after flash $(n-1)$. As discussed in the previous section, these values reflect the amounts of O_2 evolved by each flash (Y_n). The ordinate shows the difference between the transient areas before and after each flash, i.e. the number of equivalents which flash (n) added or subtracted from the pool: ΔA_n. After long dark, the first flash yields no oxygen,

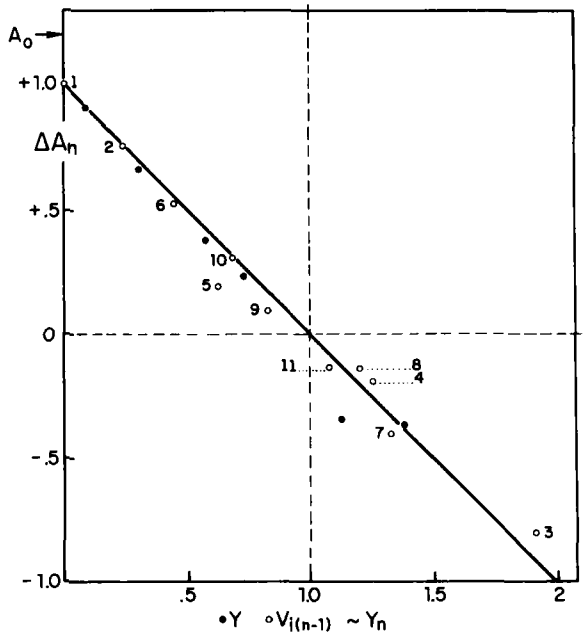

Fig. 5. Open circles: change of activated area (ΔA) caused by flash (n) is plotted vs. V_i after flash $(n-1)$ which, according to Fig. 4, is proportional to Y_n. Dots: abcissa, the O_2 yield of flashes given at various moments during the recording of rate transients (see curves 0 and 2 in Fig. 1); ordinate, flash induced change of the area (ΔA) bounded by the subsequent part of the transient.

it adds one unit of charge to the pool, i.e. for $n = 1$ $V_{i(n-1)} = 0$ and $\Delta A_n = 1$. On the other hand, the 20th flash, or a flash given during steady state illumination yields one unit of oxygen (Y_{ss}) but should not alter the filling of the pool so that for $n = \infty$ $V_{i(n-1)} = 1$ and $\Delta A = 0$. Flashes 2, 3 etc. produce variable quantities of O_2 and either add or extract equivalents from the precursor pool.

Within the limits of experimental accuracy – the main uncertainty being in the area computations (see above) the data support a linear relation between V_i and area, i.e. the constancy of $Y_n + \Delta A_n$.

The same is borne out by the other set of data shown in Fig. 5 (dots). In this experiment we recorded a number of rate transients and gave a single flash at various moments (t_m) during each transient (see Fig. 7, section 5). The flash caused a sudden change of the rate and the time course subsequent to t_m bounded an area which differed from the one bounded by the unperturbed transient after t_m. In this case the O_2 yield of the flash was plotted vs. the area change induced by it. We conclude that, at least in first approximation, all flashes produce the same total number of oxidized equivalents, i.e. that excitations are conserved.

5. A linear, four step model for charge cooperation

To facilitate the discussion of further data we will at this moment introduce a model for charge accumulation which seems to generally satisfy the observations.

The cycle of four, seen in flash yields and rate transients probably reflects that O_2 liberation requires four electron transfers. About the simplest conceivable mechanism, would be a linear four quantum process in which four consecutive flashes induce four increasingly oxidized states of a trapping center ($S_{0\to4}$), each excitation adding one + charge:

$$S_0 \xrightarrow{h\nu} S_1^{+} \xrightarrow{h\nu} S_2^{2+} \xrightarrow{h\nu} S_3^{3+} \xrightarrow{h\nu} S_4^{4+} \longrightarrow S_0 + O_2.$$

We need an additional assumption to explain why the yield of the third flash is high and exceeds that of the fourth flash. The additional assumption is that in dark the system does not completely revert to the ground state (S_0) but that charges remain in the pool. In section 8 and a subsequent paper, we will present evidence that the S_1 state is stable and that, even after a long dark time, the system contains a mixture of S_1 and S_0 states their ratio being dependent upon pretreatment. In first approximation, since three excitations suffice to make O_2 from S_1 traps, Y_3 reflects the fraction of the traps in the S_1 state, while Y_4 reflects the fraction which is in the S_0 state.

In this model an O_2 molecule is evolved when a trapping center in its S_3 state receives a quantum, so that the rate of O_2 evolution equals $V = I(S_3)$. We assume that one of every four absorbed quanta evolves an O_2 molecule so that in the steady state, a quarter of the trapping centers are in the S_3 state. A flash given during the steady state would yield 25 O_2/100 traps or 0·25 O_2/trap. Since in the steady state all steps have the same rate, the concentration of the four intermediates is equal: $[S_0] = [S_1] = [S_2] = [S_3] = 0{\cdot}25$, if the sum of all states is 1.

Trapping centers in the S_3 state contain three oxidizing equivalents, S_2 centers 2, S_1 centers 1, so that in the steady state, the precursor pool should contain $3+2+1+0 = 6$ equivalents per four traps or 1·5 eq./trap.

In section 2 (see also section 7) we observed that 1 flash, which presumably adds

one charge to each trapping center, just about filled the pool deficit seen after 10 min darkness. A deficit area $A_0 = \sim 1$ eq./trap traps now implies that after 10 min dark the pool still contains $\sim 0{\cdot}5$ eq./trap – presumably half the traps are in the S_1 state, the other half in the S_0 state. One predicts the first four flash yields to be: 0, 0, 0·5, 0·5 (if $Y_{ss} = 0{\cdot}25\ O_2$/trap).

The behavior of such a cyclic set of events in weak light can be evaluated with relative ease since the four differential equations:

$$\frac{dS_3}{dt} = I[S_2] - I[S_3], \frac{dS_2}{dt} = I[S_1] - I[S_2] \text{ etc.}$$

which describe the time course in continuous weak light (I) can be solved explicitly.

Figure 6 illustrates that so computed time courses simulate the observed transients (Fig. 1) rather well, if one selects the right initial conditions (S_1/S_0 ratio). The model predicts that upon illumination the concentrations of the various intermediate states,

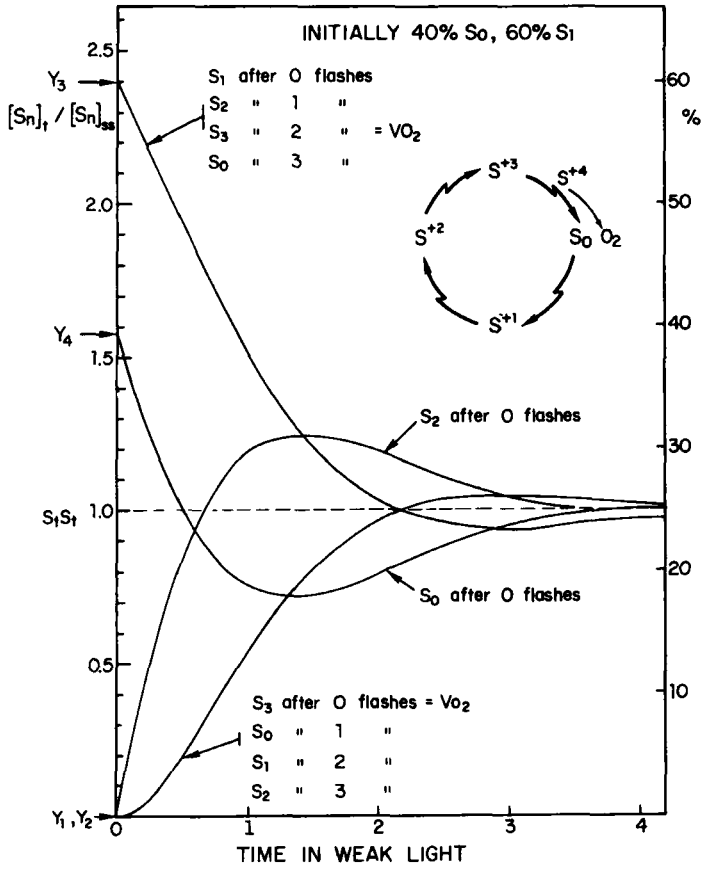

Fig. 6. Prediction of the time course of the concentration of the four intermediate states S_0, S_1, S_2, S_3 upon admission of weak light. It is assumed that initially 40 per cent of the traps are in the S_0, 60 per cent in the S_1 state. The predicted yields of the first four flashes are indicated on the ordinate. A flash moves all states to the next one, and the four curves predict the time course of the O_2 rate ($[S_3]$) after 0–3 preceding flashes.

including S_3—and thus the O_2 rate—show a damped oscillation before attaining the steady state value. The four states participate in a cycle, a flash moving all states to the next one. Thus, the time course of $[S_3]$ after zero flashes is identical to the time course of $[S_0]$ after 1 flash, etc. and the four curves simulate the first four rate transients in Fig. 1.

Assumption of this model thus offers a simple way to analyze the momentary composition of the precursor pool *viz.* by measuring the yields of four consecutive flashes: The first flash yields an amount of O_2 proportional to the number of S_3 states ($Y_1 = \alpha[S_3]_0$) and moves all other states one step up. Flash (2) liberates O_2 from S_3 states which were S_2 states at zero time: $Y_2 = \alpha[s_2]_0$. Similarly the yield of flash (3) reflects the traps which are initially in the S_1 state $Y_3 = \alpha[S_1]_0$ and $Y_4 = \alpha[S_0]_0$.

An example of such analysis is shown in Fig. 7. It shows firstly a rate transient seen 10 min after removal of the modulated light (dash–dot). According to the model this curve reflects the rise of $[S_3]$ from 0 to its steady state value. The experiment was repeated, but now a flash was given 1 sec before the modulated light. The rate

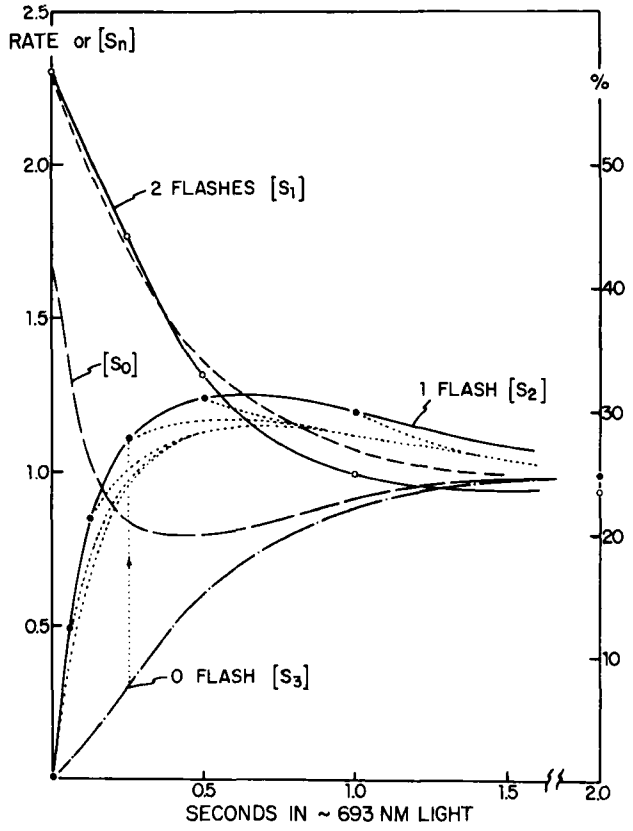

Fig. 7. Rate transients, recorded after 10 min dark subsequent to weak light, were interrupted by a single 0·25 μF flash (dots) or a pair of flashes ($\Delta t \simeq 10$ msec, open circles). The dotted (dashed) curves show the time courses of the modulated rate subsequent to each flash (flash pair). The dots and circles connected by full lines show the rate immediately after the flashes and reflect $[S_2]$ and $[S_1]$ just before each interruption. The uninterrupted curve (dash–dot) shows the time course of $[S_3]$ while the time course of $[S_0]$ (long dash) was calculated from the envelopes assuming a constant sum (4·0) of all states.

followed the dotted trace starting at $V_i = 0$ (dot). In the next experiments flashes were given during the zero flash transient (e.g. as illustrated at $t = 0{\cdot}25$ sec). This caused a sudden increase of the rate followed by an approach to the same (dotted) trace seen when the flash was given at zero time. Presumably the flash depleted all S_3 and converted all S_2 to S_3 so that the rate immediately after the flash reflects $[S_2]$ immediately before it. The full line, connecting the dots in Fig. 7 thus should show the time course of $[S_2]$ during the zero flash transient.

In similar experiments we gave pairs of flashes (spacing 10 msec) instead of a single one. Now, the initial rate after each pair (open circles) should reflect $[S_1]$ at the moment the pair is given and the envelope of the 'spikes' (full line connecting the open circles) should show the time course of $[S_1]$ during the zero flash transient. The dashed line shows the individual time courses of the rate seen subsequent to the flash pairs. Experimental variability prevented a very precise analysis, but these curves tended to deviate from the envelope (full line) in the illustrated fashion.

If we now assume that the sum of all states remains constant, we can compute from the O transient ($[S_3]$) and the $[S_1]$ and $[S_2]$ curves (full lines), the time course of S_0. The so computed time courses of the four states indeed resemble the pattern predicted by the model, and appear to indicate that after 10 min dark 45 per cent of the traps are in the S_0 state, 55 per cent in the S_1 state (in Fig. 6 the assumption was 40 and 60 per cent).

Clearly, however, the correspondence is only approximate, a comparison of Figs. 7 and 8 reveals several discrepancies between prediction and observation. Also, the model is obviously incomplete since it fails to predict the damping of the flash yield oscillation, observed to occur in three to four cycles (Fig. 3). One can conceive several perturbations which 'mix up' the various states and thus damp the flash yield oscillation, distort the rate transients or do both.

Whatever the 'spoiling' perturbations, their effects will compound with increase of flash number or exposure time. Thus, it is not surprising that the computed $[S_0]$ curve in Fig. 7 which is 'preceded' by three flashes and weak light differs notably from the prediction.

Based partly on evidence given in subsequent sections, we assume as major perturbations that the flashes convert a certain percentage of the traps more than once ('double hits') while another fraction of the traps are not converted at all by light ('misses'). Double hits 'advance' while misses 'retard' the stepwise conversion of the states by the flashes. A detailed analysis of this spoil mechanism will be given in a subsequent paper [7].

6. *Minimum time separation between flashes, rate constants*

If in photoact II an electron were transferred from donor Z to acceptor Q:

$$H_2O \xrightarrow{k_s} S_{0\to 4} \xrightarrow{k_0, k_1, k_2, k_3} Z \xrightarrow{h\nu\,II} Q \xrightarrow{k_a} A \qquad (1)$$

this act could be repeated only after the reoxidation of Q^- and the rereduction of Z^+ in dark steps (see equation (1)). In most of our experiments pool A, acting as the final electron sink, was fully oxidized. Under this condition step k_a takes < 1 msec [9]. Joliot [10] found a $\simeq 1$ msec delay between Z and O_2 and also Witt *et al.* [11] report a ~ 1 msec reaction time in system II. As is illustrated in Fig. 8 (left) the relaxa-

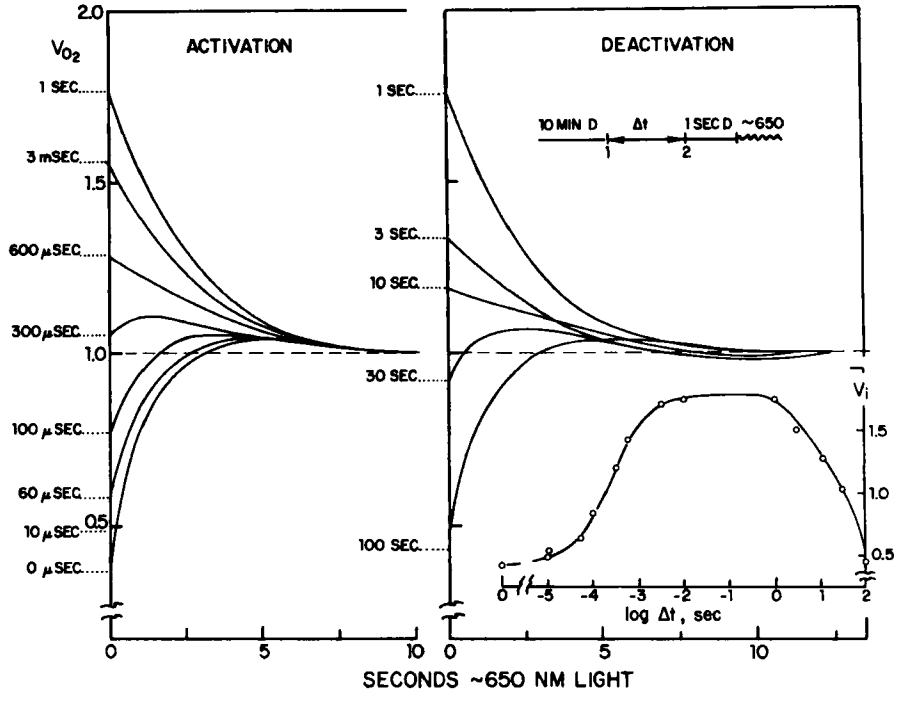

Fig. 8. Effect of the time interval Δt between the first and the second flash, upon the rate transient in modulated 650 nm light, admitted 1 sec after the second flash. Left ('activation') full effect of second flash requires $\Delta t \geq 1$ msec. Right ('deactivation'): the effect decays in ~10 sec. Insert: plot of V_i vs. log Δt shows useful range between 10^{-3} and 10 sec.

tion time of the system—i.e. the minimum spacing (Δt), required between two flashes, to be recognized as a pair rather than a single flash proved to be in this same time range, the effect being half at $\Delta t \simeq 0{\cdot}3$ msec and fully developed at $\Delta t \sim 10$ msec.

In the linear sequence of equation (1), primary photooxidant Z is connected to (or identical with) a charge accumulating and O_2 producing catalyst $S_{0=4}$. The various steps k_0, k_2 etc. such as $Z^+ + S_0 \xrightarrow{k_0} Z + S_1$ (which we can write more simply as $S_0{}^* \xrightarrow{k_0} S_1$ and $S_4 \xrightarrow{k_1} S_0 + O_2$) probably have different rates. If all of these were rapid compared to the rate of the $Q \rightarrow A$ transfer, one would expect to find a single time constant (k_a) regardless which flash pair is studied. Actually we do find different relaxation times and one cannot *a priori* decide whether the fastest of these should be ascribed to one of the Z^+ discharges or to the k_a step.

In experiment Fig. 9 we attempted to determine k_1 and possibly also k_0. We varied the time (Δt) which separated flashes (1) and (2), given after 5 min dark, and recorded yields of flashes (3) and (4) given 2 and 4 sec later. 'Y_3', which for $\Delta t = 0$ actually corresponds to Y_2 in a normal sequence, reflects the number of S_3 states left by the preceding flash pair. These were initially S_2 states for $\Delta t = 0$ and S_1 states for $\Delta t \simeq$ 1 sec. Since, as we will describe in[7], after 5 min dark $[S_2]_0$ is small but $[S_1]_0$ considerable, Y_3 will rise with Δt and reflect mainly k_1 in: $Z^+ + S_1 \xrightarrow{k_1} Z + S_2$. If this were a first order reaction, $Y_3 = [S_1]_0\,(1\text{-exp} - k_1 t)$ in which $[S_1]_0$ is the concentration of S_1 before the flash sequence is given.

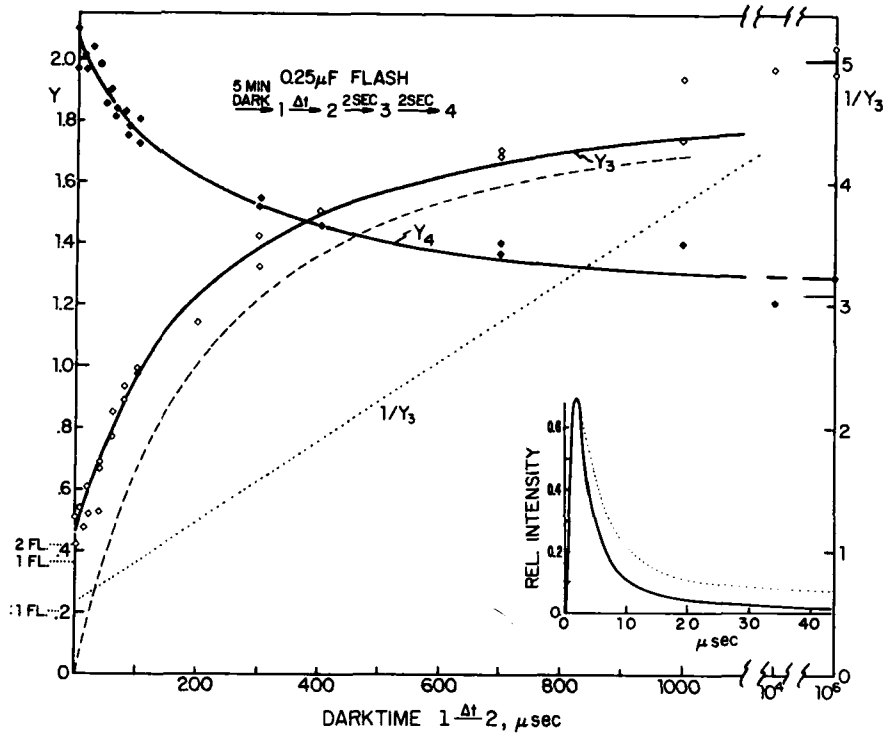

Fig. 9. Dependence of Y_3 and Y_4 upon the time spacing (Δt) between the first and second flash. Pretreatment for each point: fifteen flashes followed by 5 min dark. Full lines, observed curves; dashed line, Y_3 curve corrected for spoiling and incomplete deactivation according to[7]. Dotted line: reciprocal plot of the dashed Y_3 curve. Indicated on the ordinate are the yields of a single flash after 5 min and 10 min dark and the yield of a double flash ($\Delta t = 0$) after 5 min dark. Insert: the time course of the flash intensity using a 0·25 μF (full line), or a 1 μF capacitor (dotted). The maxima of the curves were normalized, the larger C value yielded nearly twice the peak intensity.

Similarly, Y_4 will reflect $[S_1]_0$ for $\Delta t = 0$ and $[S_0]_0$ for $\Delta t \simeq 1$ sec, so that, again assuming first order kinetics:

$Y_4 = [S_0]_0 (1\text{-exp } -k_0 t) + [S_1]_0 (\exp -k_1 t)$ and the sum $Y_3 + Y_4$ thus should reflect k_0.

In Fig. 9 the open symbols connected by a solid line show the actual observations of Y_3. A relatively short (5 min) darktime was used and deactivation was incomplete. In addition the Y_3 curve does not truly reflect the time course of S_1 because of spoiling. Applying our analysis of these two interferences descussed in[7], we computed a corrected time course for Y_3 which is shown as a dashed curve. The dotted slope is a reciprocal plot of the dashed one, $1/(Y_{3\max} - Y_{3t})$, and shows that the time course is approximately second order. The first halftime is ~ 200 μsec.

Determination of k_0 from the plots of Y_4 and Y_3 vs. Δt proved beyond the precision of our experiments and spoiling analysis. There is some indication that k_0 is rather similar to k_2, i.e. slower than k_1.

In experiment Fig. 10 we tried to determine k_2. Again in each observation four flashes were given after 5 min dark and 'Y_3' and 'Y_4' recorded. Now the spacing Δt between flashes (2) and (3) was varied, the other spacings being 2 sec.

The flash yield signal is a damped oscillation, lasting $\simeq$ 0·1 sec (c.f. Fig. 3 in [6])

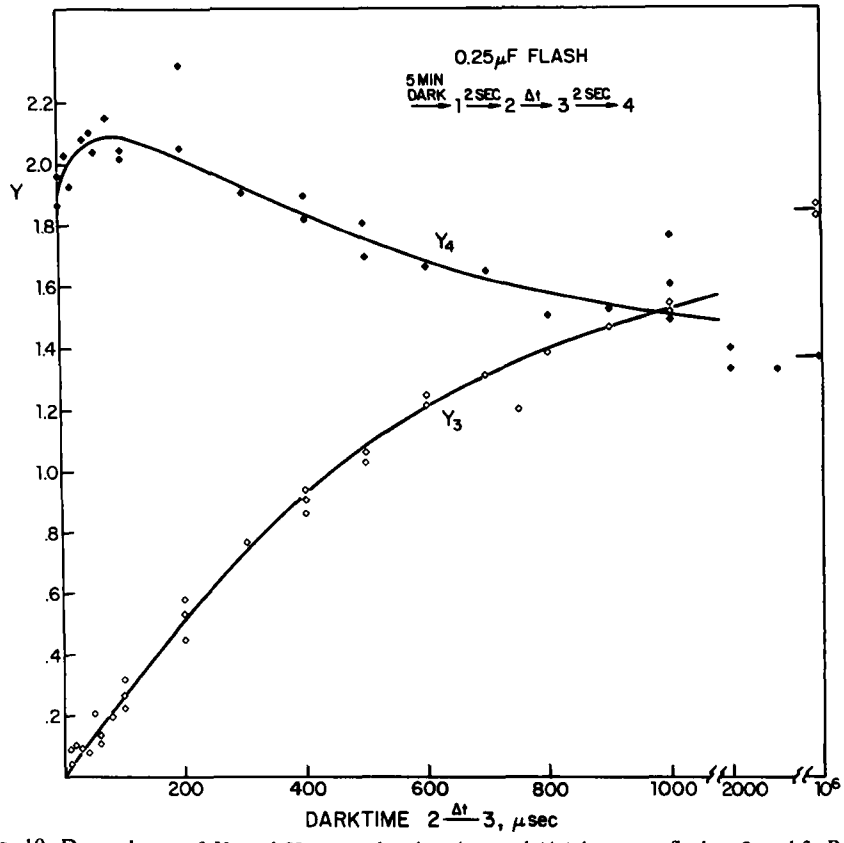

Fig. 10. Dependence of Y_3 and Y_4 upon the time interval (Δt) between flashes 2 and 3. Pretreatment for each set of points: fifteen flashes followed by 5 min dark. For all but the highest Δt values ($\geq$ 1 sec) the 'Y_3' signal included that of Y_2, therefore the yield observed for $\Delta t = 0$ (0·38) was subtracted from these data.

so that the signals of two flashes given in close succession (Δt in the msec range) are superimposed. The resulting distortion of 'Y_3' is bearable, because at $\Delta t = 0$ 'Y_2' is small ($\sim$ 0·4 in Fig. 10) and can be subtracted. The time course of 'Y_3' thus should provide a relatively faithful reflection of k_2.

The Y_3 curve in Fig. 10 might be first order, it shows a half time of $\sim$ 400 μsec. Apparently reaction k_2 is considerably slower than step k_1—at least initially. This is also seen in the time course of Y_4, which reflects both k_1 and k_2, and shows a distinct initial rise.

We will not at this moment contemplate whether the rapid step k_1 might not, in reality be k_a, or the significance of its second order kinetics. Also, the rate of step k_2 might possibly be underestimated if the Q–A reaction had slowed down after the first two electron transfers driven by flashes (1) and (2).

One important consequence of these rapid relaxations, especially after the first flash, will be discussed in the next section.

7. *Effect of flash intensity and duration*

The rapid relaxation of the system (especially k_1) discussed above, raised doubts

whether the duration of the Xenon flashes could be neglected. The insert of Fig. 9 shows the time courses of the flash intensity observed with a 0·25 μF and 1 μF capacitor (~0·3 and 1·2J), used in many of our experiments. Flashes obtained with various capacitors show a limited variation of peak intensity, especially with high C values the increased output was mainly due to a longer duration of the main discharge, while also the emission in the long 'tail' is enhanced.

Figure 11 (closed circles) shows Y_{ss}, the average yield of a series of flashes spaced ~ 1 sec, as a function of flash energy, expressed in terms of effective capacitance (see Methods). In these, and additional experiments in which wire screens were used to vary intensity, we noticed a small 'lag' in the Y_{ss} vs. energy curve, amounting to ~ 4 per cent of the energy needed for half saturation. Below this limit < 2 per cent of the traps were hit every second and we obviously encountered deactivation.

Beyond this range Y_{ss} rises with increase of flash energy, however, it does not attain a clearly defined saturation level. Correcting for this slow increase at high capacitance values (dashed line in Fig. 11), one computes an adequate first order relation between Y_{ss} and energy in which half of the extrapolated maximum yield is obtained with $C \approx 0{\cdot}04\ \mu$F.

On the basis of data as shown in Figs. 9 (insert) and 11, we ascribe the slow increase of Y_{ss} with (excessive) flash energy to an increase of effective double excitations – in single traps during one flash. While these 'double hits' have a rather secondary effect upon Y_{ss} (10–15 per cent) they cause more severe complications under non-steady state conditions. Figure 12 shows sequences of flash yields such as shown in Fig. 3, observed with different capacitors. The insert of Fig. 12 compares the energy dependence of Y_{ss} with that of Y_2. Whereas the first is half saturated at ~0·05 μF,

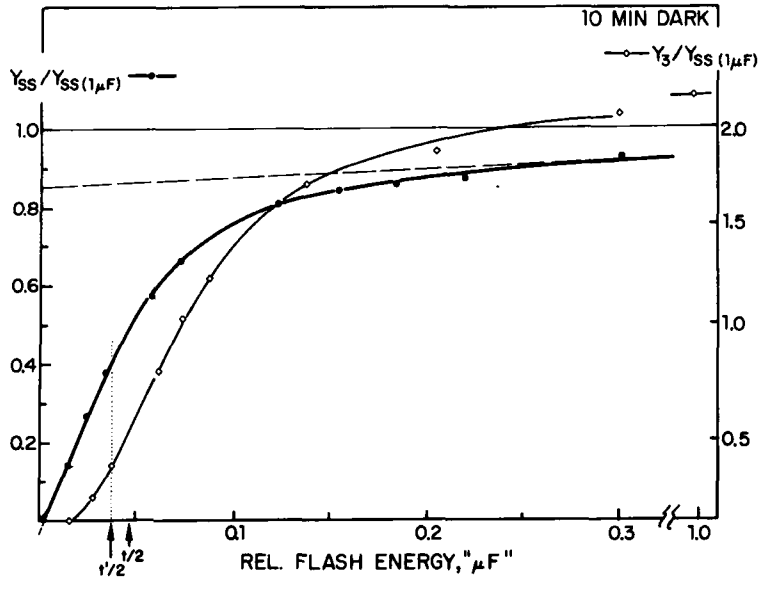

Fig. 11. Effect of relative flash energy, expressed in terms of effective capacitance, upon the steady state flash yield (left ordinate) and the yield of the third flash (right ordinate). Dotted line indicates half saturation of the exponential rise of the Y_{ss} curve, which approaches the level indicated by dashed line.

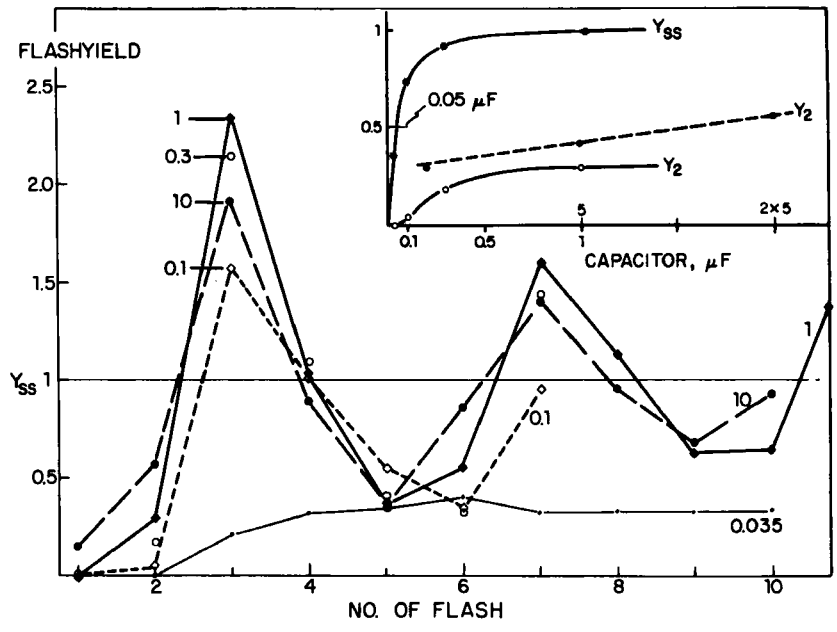

Fig. 12. Flash yield sequences observed with flashes using various discharge capacitors. Before each sequence the sample was in dark during 10 min. Insert shows the variation of Y_{ss} and Y_2 with capacitance.

Y_2 shows an S shaped rise (full line, circles). The dashed line, a plot on a compressed abscissa scale, shows a pronounced secondary rise, so that with $C = 10\,\mu F$ ($1/e$ decay time of the flash ~ 30 μsec) Y_2 approaches 0·6 Y_{ss}. The main plot in Fig. 12 shows that with this capacitance, even flash (1) yields a significant amount of O_2, which indicates the occurrence of triple hits.

We have measured Y_2 as a function of the effective capacitances (C_1 and C_2) used for flashes (1) and (2). The S shape occurred only when both capacitors were varied ($C_1 = C_2$). With either C fixed and the other varied, the relation between Y_2 and C was similar to that between Y_{ss} and C.

If in the latter type of experiment the invariant capacitor was 'large' (e.g. 2 μF), Y_2 rose to a higher value if C_1 was fixed than if C_2 was. Thus, double excitations are relatively more effective in the first flash than in the second flash, in agreement with the observation that $k_1 > k_2$ as discussed in the previous section.

A quadratic (S shaped) intensity curve of Y_2 is to be expected since the O_2 yield results from two successive flashes. The 'true' value of Y_2, unaffected by double hits, thus can be estimated if we assume that a flash energy which yields 0·5 Y_{ss} will yield 0·25 Y_2. In this way we compute that after 10 min dark, following weak light $Y_2 \leqslant 0{\cdot}1\,Y_{ss}$.

We made a similar evaluation of flash energy in respect to the 'true' effect of flash (1): As shown in the insert of Fig. 2 the halftime of the rise of the subsequent rate transient decreases with increase of flash energy, the bounded area decreasing similarly. With higher capacitance values the 'overshoot' becomes more pronounced, and begins to resemble the transient after two flashes. In Fig. 2 a 0·04 μF flash ($\leqslant$ half saturation) removed 40 per cent of the deficit area A_0 (10 min after continuous light). Twice this

value or ~0·8 A_0 might be the best estimate of the effect of flash (1)—in accord with the data of Fig. 5. As will be discussed in the subsequent paper[7] the so estimated value of A_0 is actually lower than predicted by our hypothesis.

8. *Deactivation, stability of* S_1

In experiment Fig. 8 (right, insert) the effect of the dark interval (Δt) between flashes (1) and (2) upon the subsequent rate transient is maximal and constant between ~10 msec and 1 sec. With longer intervals the high initial rate and excess activated area decline progressively more slowly; after some 15 sec $V_i \simeq V_{ss}$ and after ~100 sec the transient resembles the one seen with a single flash ($\Delta t = 0$), the first flash of the pair having lost its effect.

An essential assumption in our model (Sect. 5) is that during deactivation not all traps return to the ground state (S_0), but charges in the S_1 state are stable and remain in the pool indefinitely. For the computation of Fig. 6 to fit the observations, we had to assume that after deactivation only 40 per cent of the traps had returned to the ground state, (S_0) while 60 per cent had remained in the S_1 state. If S_1 were stable one might expect that under different conditions different amounts of S_1 and S_0 remain after deactivation which would be seen as a variation of the ratio Y_3/Y_4. The data of Fig. 13 show that indeed by manipulating the initial composition of the pool, before deactivation, one can significantly vary the final composition after deactivation.

We assumed in Fig. 6 that deactivation following the steady state leaves $[S_1] > [S_0]$ so that one consequently observes $Y_3 > Y_4$. A single flash which moves all states one step up would bring a pool 0·4 S_0, 0·6 S_1 to 0·4 S_1, 0·6 S_2 and leave no S_0. If S_1 were stable and S_2 converted largely into S_1[7], subsequent deactivation would lead to a quite high concentration of S_1 and a low value of $[S_0]$ so that in a subsequent flash series $Y_3 \gg Y_4$.

Figure 13 indeed shows that if a single flash is given after a 5 min dark period following the steady state the system deactivates to a state which leads to a very high Y_3

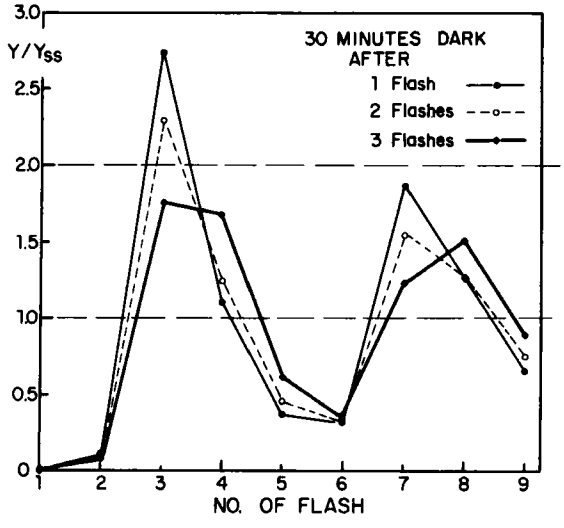

Fig. 13. Sequences of flash yields observed after 30 min darkness following one, two or three flashes. Pretreatment: fifteen flashes spaced 1 sec apart, 5 min dark.

(approaching 3 Y_{ss}) and a low Y_4. An interruption by two flashes induces a similar, be it less dramatic, change. More or less the opposite result is obtained if three flashes are used instead—now Y_3 is quite low while Y_4 is relatively high: three flashes convert S_1 to S_0+O_2 and given to a pool in which $[S_1] > [S_0]$, bring most of the traps into the ground state S_0 so that subsequently Y_4 is high. The less abundant S_0 traps are converted by the three flashes to S_3, these deactivate to S_1 and now Y_3 is low. Subsequent flashes follow suit and the oscillation is 90° out of phase.

The steady state flash yield is not affected by such pretreatments and the darktime unimportant as long as it suffices to allow complete deactivation. To emphasize the stability of the various pool compositions we used, in expt. Fig. 13, long, 30 min dark periods.

DISCUSSION

For didactic reasons we have discussed our proposed model for charge cooperation earlier in this paper. A few additional remarks might summerize the argument: The requirement of four oxidizing equivalents for O_2 liberation, the photosynthetic quantum requirements (~2*hν*/eq. overall or 1 *hν*/eq. in system II), and the cycle of four in the flash yield pattern all suggest a four quantum, four step process. How can this be reconciled with Y_3 being high and exceeding Y_4? If one assumes with Joliot[4, 5] that after long darkness all traps deactivate to the ground state, one is forced to assume a collaboration between + charges, collected in different trapping centers. Joliot assumed the collaboration $(O)+(O) \rightarrow O_2$. This two quantum process would yield oxygen in the second flash, to avoid this a special switching device was incorporated in his model.

We also have considered models which assumed return to the ground state and interactions between photooxidized trapping centers. In a computer program we used a matrix of a statistical number of traps which could be hit all at once (flash) or randomly (weak light). Each trap had four oxidation states ($S_4 \rightarrow O_2+S_0$) and was allowed to interact with a selected number of neighbors in various types of charge dismutations. The most compatible model assumed a restricted number of interacting neighbors (four to six) and $S_1+S_1 \rightarrow S_2+S_0$ as the major dark recombination event.

Later we could consistently observe Y_3 values which exceeded $2Y_{ss}$ and approached 3 Y_{ss}, while Y_4 was still close to Y_{ss}. This was clearly incompatible with models which assume that the ground state is attained in darkness. Furthermore, the data of section 3, which showed that the flash yield pattern is unaffected by system II inhibitors, yielded a forceful argument against interactions between traps and indicated that each trapping center is an independent O_2 generating unit.

A forthcoming paper[7] will present an experimental and theoretical analysis of the deactivation and spoiling events. This analysis supports the straightforward model of Fig. 6 with the assumptions, (a) state S_1 is stable and produced in the dark by deactivation of the S_3 and S_2 states, (b) state S_0 is formed exclusively by way of $S_3 \xrightarrow{h\nu} \longrightarrow S_0+O_2$, and (c) spoiling is due to relatively slight imperfections of the flash excitation: ~5 per cent double hits (see Fig. 11) and ~10 per cent misses, caused by inhomogeneous illumination and non-perfect biological photochemical efficiency.

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