Submitted to Photochemistry and Photobiology

UCRL-19336
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September 1969

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UCRL-19336

# ACTIVATION KINETICS OF PHOTOSYNTHETIC OXYGEN EVOLUTION UNDER 20-40 NANOSECOND LASER FLASHES

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Running Title: Laser Activation of Photosynthetic Oxygen

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Abstract—*Chlorella* samples, incubated for varying periods in darkness, were exposed to a series of 20-40 nanosecond flashes, spaced 15 seconds apart, from a Q-switched ruby laser. A stationary platinum electrode measured the microjet of oxygen produced by each flash. After a dark preincubation exceeding 3 minutes, at 23°C, little or no oxygen is evolved until the third flash in a sequence. The yields from subsequent flashes increase monotonically until a constant value is reached. If low levels of background light are supplied, or if the interval between series of flashes is decreased, oxygen is evolved on the second or even the first flash. Very similar results were obtained from analogous experiments with 28-microsecond flashes from a xenon flashtube. This means that there are no systematic differences between the effects of saturating 20-nanosecond and 28-microsecond light flashes on the activation processes during the first few flashes following a long dark period. No oscillations of flash yield with successive flashes were observed because of the long interval between flashes. These results are consistent with the idea that the reaction center of Photosystem II must undergo a dark process lasting considerably longer than 28 microseconds before it can absorb a second photon.

If green plants or algae are incubated in the dark for several minutes and then subjected to a single brief flash of light, they evolve no oxygen, even though the flash would ordinarily have been enough to saturate the photosynthetic apparatus. This arresting observation was first made by Franck and Allen[1], and has since been amply confirmed by Whittingham[2] and by Joliot[3].

Joliot's extensive and careful studies showed that the first flash following prolonged darkness acts as if it "activates" the reaction center so that it can evolve oxygen the next time it receives a photon. The action spectrum of this "activating" flash is that of photosystem II. This fact prompted Kok[4] to suggest that the oxidizing end of photosystem II must be "primed" with one oxidizing equivalent per reaction center. Later work by Joliot[5] indicated that the second in a series of flashes following prolonged darkness produces only a small quantity of oxygen, whereas the third flash gives rise to a much larger oxygen yield than does either the second or the fourth. The yields from subsequent flashes[6] show a damped oscillation with a period of four. The 3rd, 7th, 11th, and 15th flash yields are above average, the 5th, 9th, 13th, 17th, etc. below. Kok[7] has reported similar observations. These dramatic results led Joliot[6] to propose a complex cycle by which four oxidizing equivalents were combined to form a mole of oxygen.

In order to interpret these results, it is important to determine whether each reaction center is turning over only once during each flash. The fact that the fluorescence yield of chlorophyll a *in vivo* varies with the oxidation state of the electron transport chain[8]

indicates that the primary photochemical event of photosystem II must take about 0.6 nanoseconds, the experimental fluorescence lifetime of *in vivo* chlorophyll *a*[9]. This is 1/16,000 the duration of the ten-microsecond flash used by Joliot. It might be that such flashes give the reaction center time to absorb one photon, to store its energy in a dark reaction, and to absorb a second photon before the flash is over.

We have therefore studied the induction of photosynthetic oxygen evolution under 20-40 nanosecond flashes from a Q-switched ruby laser. These flashes are 1/250-1/500 as short as those used by Joliot, and also lack the long tail characteristic of xenon flashes. We therefore hoped to come closer to the kinetics expected from a single turnover of the reaction center per flash.

Available equipment restricted us to one laser flash every 15 seconds. We have therefore supplemented this work with parallel experiments with 28-microsecond xenon flashes shaped to avoid "tailing". These flashes could be repeated at closer intervals and enabled us to bridge the gap between our results and those of Joliot and of Kok. Our data show no major differences between the yields of successive 40-nanosecond and successive 28-microsecond flashes spaced 15 seconds apart following long dark periods. This result excludes most mechanisms that would require two "hits" on a single reaction center during a 28-microsecond flash.

## EXPERIMENTAL

Most of the experiments reported here were performed with a water-cooled Korad ruby laser with a Pockels cell active Q-switch, model K1Q,

graciously made available to us by Professor W. M. McClain of the Department of Chemistry, University of California, Berkeley. A timing circuit automatically flashed the laser every 15 seconds.

Earlier experiments were performed with a high-repetition rate, passively Q-switched ruby laser (Korad model K15QP) graciously made available by Professor H. Pantell and Dr. H. Puthoff of the Stanford University Department of Electrical Engineering. We are grateful to them and to Mr. Jerry Gelbwachs and Dr. Giovanni Soncini for their patient training in its use. Water cooling of the laser head and a larger power supply made it possible to fire this laser as often as every three seconds. In these experiments, the interflash interval was timed with a metronome and the laser fired by pressing a button.

The output pulse of the Berkeley laser was measured at the Korad plant and found to be 20 nanoseconds in duration. In some experiments, slight misalignments of the electronics of the Pockels cell produced double laser pulses. For this reason, we have spoken of 20-40 nanosecond pulses in this report. The Stanford laser pulse lasted less than 20 nanoseconds at half-height as measured with a Hewlitt-Packard Associates HPA 4203 photodiode and a Tektronix 555 oscilloscope.

Xenon flashes were produced from flashlamps model L-391, manufactured by ILC Co., 164 Commercial St., Sunnyvale, Calif. Flashes were slightly underdamped by appropriate pulse forming networks[10], and current reversals (a prime cause of long tails) were prevented by silicon controlled rectifier switches in the pulse circuitry. The flash used in this experiment lasted 28 microseconds measured at one-third height, and has a shape shown in Fig. 1. The system, which

was built by ILC, affords a choice of 15 flash durations from 20 microseconds to 7.5 milliseconds, as well as timing circuits and switches allowing the discharge of four different capacitors through the same flashlamp at preselected intervals. As presently constructed, the system is limited by a maximum repetition rate of four flashes every 8 seconds. Successive flashes must be separated by at least ten times their duration.

In the laser experiments, the sample was protected from stray light by a light-tight box, a lucite tube wrapped with black tape to keep out stray light, and a Corning 2-64 red filter to keep out the light from the xenon pump flash. The laser flash was attenuated with negative lenses, frosted glass diffusers, and/or neutral density filters. Light from the xenon flashlamp was filtered through Corning 1-69 infrared and 3-75 ultraviolet filters, as well as appropriate screens. In both cases, saturation curves of reasonable shape were obtained for the long term average flash yield. Continuous weak "background" light was provided by a 300-watt Bell and Howell projector. It was attenuated by varying the voltage with a Variac, and filtered with 2 cm of water, a Corning 1-69 infrared filter, a 645 nm interference filter, and appropriate neutral density filters.

The algae used in these experiments were Chlorella pyrenoidosa, grown under a variety of conditions in Myers' nutrient medium[11]. Aerated Myers' medium (sometimes enriched with 4% $CO_2$) was circulated by a gravity feed past the dialysis membrane that forms the upper boundary of the sample chamber of the electrode.

Measurement of the oxygen yield of individual flashes was carried out with the teflon-covered stationary rate electrode described by Pickett[12]. The detection circuit is adapted from that of Myers and Graham[13]. We thank Professors J. Pickett and U. Heber, then of the Carnegie Institution of Washington at Stanford, Calif., for help in getting the oxygen electrode to work.

Each flash produced a spike of oxygen evolution with a rise time limited by the electronics. The spike decayed to half-height in 1.5 seconds, presumably because of the slow diffusion to the electrode of oxygen originally evolved in the opposite direction. The oxygen content of the spike was taken to be proportional to its height.

## RESULTS

### 1. Experiments with laser flashes

In a typical laser experiment, a dark period of 0.5 to 5 minutes precedes a series of 5 to 15 flashes spaced at least 15 seconds apart, a spacing dictated by the repetition rate of the laser. This burst of flashes is followed by another dark period and still another burst of flashes, and so on.

In most experiments the algae sample was changed every hour or two. In one experiment, algae exposed to the most intense flashes showed a decline of about 15% in flash saturation yield over a period of 8 hours.

After 3-1/2 minutes of darkness, we found no oxygen evolved from the first Q-switched ruby laser flash, in agreement with the results of Franck[1], of Whittingham[2], and of Joliot[3]. What is more,

following such a dark interval, we found little or no oxygen evolved on a second flash 15 seconds after the first. The third such flash did produce oxygen.

Fig. 2 shows the most general trace observed after dark periods greater than 3 minutes. In this early experiment, laser flashes were produced by pushing a button every 7-9 seconds. Spikes corresponding to the oxygen evolved in response to each flash are superimposed on a slow rise in the baseline presumably due to photoinduced respiratory transients. This slow rise, which does not appear in every experiment, is evident after the first flash before any photosynthetic oxygen is evolved.

Following a long dark period ($\geq$ 3 minutes) the first flash gives rise to a small negative signal (presumably a small uptake due to oxygen-mediated cyclic electron flow in photosystem I). The second produces nothing at all, or at most a very small output spike. We were not able to determine why oxygen was sometimes evolved on the second flash and sometimes not. Successive flashes produce a gradually increasing oxygen yield until the long-term average is reached on about the seventh flash.

As the interval between bursts of flashes decreases, oxygen is evolved on the second flash, and, at still shorter dark intervals, on the first flash as well. A weak 645 nm background of light of 0.75 erg/cm$^2$ sec produces a measurable yield of oxygen on both first and second flashes, while 100 ergs/cm$^2$ sec are sufficient to abolish activation effects completely.

Figs. 3-6 show the yields of the first four saturating flashes as the dark interval before the first laser flash increases. In each case, the interval between flashes in the series is set by the timer at 15 seconds. Flash yields are normalized to the long-term average flash saturation yield for the particular experiment. Each point shown with an error bar is the average of 4-7 experiments. Bare points correspond to individual experiments.

The response to the first flash is most easily treated as the sum of a constant oxygen uptake (a "gasp") and an output that decreases exponentially with increasing dark time with a half-time of 24 seconds, and reaches zero after about 100 seconds of dark preincubation (Fig. 3). This result is in agreement with previous work [1-6]. The second spike falls much more slowly, and reaches zero only at about 3-4 minutes of darkness (Fig. 4). The first-order plots of Figs. 3 and 4 are meant only to be suggestive; second order kinetics fit about as well because of the scatter of the data. The third flash falls off still more slowly at long time intervals, the fourth flash more slowly yet. The data of Fig. 6 hint at a maximum yield of the fourth flash at about a minute of dark preincubation. This conclusion for both third and fourth flashes emerges quite clearly from analogous experiments with 28-microsecond flashes spaced 4 seconds apart. Dark preincubations in excess of 10 minutes produced no further decline in the yield of the third and fourth flashes.

The results of 8-11 experiments done with laser flashes spaced 15 seconds apart after long ($\geq$ 3 minutes) dark intervals are summarized in the dashed curve of Fig. 7. Flash yields are normalized to the average of many flashes at 15-second intervals taken from another experiment

with the same sample. The first flash produces nothing or a small uptake, the second nothing or a small output ($\lesssim$ 10% of the long-term average) while yields on subsequent flashes rise monotonically until a steady-state is reached.

2. Experiments with 28-microsecond xenon flashes

The experiments described in the previous section were repeated with 28 microsecond xenon flashes in order to determine their dependence on flash duration.

The results of 10 such experiments are shown in the solid curve of Fig. 7. These experiments were done several months after the laser experiments shown in the solid curve, with algae grown under somewhat different conditions. Still, the general features of the two curves are very similar. The first two flashes produce little or no oxygen: the first usually a small uptake, the second a small output ($\lesssim$ 10% of the long-term yield). The third flash is somewhat lower than the corresponding laser flash, and steady state is achieved by the fifth flash.

In order to establish continuity between our experiments and those of Joliot and Kok, we have carried out many preliminary experiments with xenon flashes spaced closer together than 15 seconds following long dark intervals. Our results are consistent with those of Joliot, except that we do not get appreciable oxygen evolution ($\lesssim$ 15%) on the second flash for any interflash interval from 200 microseconds to 30 minutes. We do observe the extra large yield on the third flash spaced 300 milliseconds apart after a long dark period.

DISCUSSION

Joliot[6] has proposed a compact model to explain the unusual oscillations he observed in the oxygen yields from successive short saturating flashes (Fig. 8a). According to this model, there are twice as many electron donors (Z) in photosystem II as there are acceptors (Q). At any given time, one or the other of the Z molecules is specially linked to the reaction center chlorophyll. Such molecules, designated $Z^{/}$ or $Z^{+/}$, depending on their state of oxidation, preferentially donate electrons to the excited reaction center chlorophyll to become $Z^{+/}$ or $Z^{++/}$, respectively. The "preferred" link is now switched by an unspecified flip-flop mechanism to the other Z, which donates an electron the next time that reaction center is excited. An oxygen atom results when either Z has given up two electrons to become $Z^{++}$. The $Z^{++}$ are assumed to last long enough to find a partner and form an $O_2$ molecule. This scheme is represented by the heavy lines of Fig. 8a; the formation of $Z^{++}$ is not shown explicitly.

So far, this model predicts the first appearance of oxygen evolution on the third saturating flash following a long dark period. In Joliot's model, however, the flip-flop controlling the special linkage is only 84% reliable, i.e., the same Z gives up two electrons in a row 16% of the time. This results in the sequence of steps shown in the dashed lines of Fig. 8a. It accounts at the same time for the non-zero yield Joliot observes on the second flash following a long dark period, and for the damping of the oscillations he observes following the third flash.

The kinetic equivalent of the Joliot scheme can be represented by the general 4-state model shown in Fig. 8b. The "majority" (84%)

pathway is shown in heavy lines, the "minority" (16%) in dashed lines. The "linked" $Z^{+/}$ are assumed to back react in the dark faster than the unlinked $Z^{+}$, so that the two equivalent deactivation processes $S_3 \rightsquigarrow S_1$ and $S_2 \rightsquigarrow S_0$ are faster than $S_1 \rightsquigarrow S_0$.

In our experiments, the second flash produces little or no oxygen. This can be accommodated in the Joliot scheme by assuming a much more reliable flip-flop in our algae than in his. This would predict that the oxygen yield from successive brief, saturating flashes should resemble a square wave: high yields on 3rd, 4th, 7th, 8th, etc. flashes, very low yields on 5th, 6th, 9th, 10th, etc. flashes. We have not yet tested this prediction. The strong maximum in the yield of the 3rd and 4th flashes, spaced 4 seconds apart, at about a minute of dark preincubation time does not appear to be consistent with the model proposed by Joliot.

Our data has not revealed any difference between the effects of 40-nanosecond laser flashes and those of 28-microsecond xenon flashes, at least as far as the first few "activation" steps are concerned. Further, no systematic difference was found between the effects of the Stanford laser, whose flash lasted $\leq$ 20 nanoseconds, and the Berkeley laser, whose flash lasted $\leq$ 40 nanoseconds. Each type of flash, applied in succession 15 seconds apart following 3-5 minutes of darkness, produces little or no oxygen until the third flash. Flash yield then rises to the steady state in two or three more flashes.

These results make it likely that only one quantum is used in a given photosynthetic unit during a 28-microsecond light flash. If this were not the case, we should expect that a succession of flashes following a long dark period would produce oxygen on an earlier flash for microsecond than for nanosecond flashes. Experimentally, this is

not the case. By the fourth or fifth 28-microsecond flash, enough reaction centers may have absorbed two quanta on one or another flash for the centers to be out of phase. Such "double hits" could not, however, be essential to the activation kinetics observed on the first few flashes.

We could, of course, suppose that each reaction center is trapping two photons even during a 20-nanosecond laser flash. This would require a dark "reset" process that is substantially complete within 40 nanoseconds. This unlikely possibility could be excluded completely by analogous experiments with a single-pulse, mode-locked laser. These instruments are capable of single pulses of monochromatic light with a flash duration of picoseconds ($10^{-12}$ sec)[14].

It is also possible that the oscillations observed by Joliot owe their origin to phenomena quite different from those that give rise to the first two nonproductive activation flashes, and that these oscillations are somehow related to the absorption of two quanta per flash per reaction center. Our experiments did not exclude this possibility, since the deactivation processes had progressed far enough in the 15 seconds between our flashes to damp out the Joliot oscillations. This hypothesis can be tested with a Q-switched laser with a higher repetition rate.

In general, then, our results are consistent with the prevailing idea that the reaction center of photosystem II must undergo a dark process which lasts considerably longer than 28 microseconds before it can absorb a second quantum.

Acknowledgments.—This research was supported by the U. S. Atomic Energy Commission. We thank Mr. Gerald T. Babcock for running the flashtube experiments.

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## Figure Captions

Fig. 1. Intensity of xenon flash as a function of time. Ordinate is proportional to number of visible photons (400-700 nm) impinging on phototube per unit time. Time scale is 10 microseconds per division.

Fig. 2. Response of algae to successive laser flashes following 5-minute dark period.

Figs. 3-6. Oxygen yield from successive laser flashes, spaced 15 seconds apart, as a function of the dark interval preceding the first of the series. Points with error bars are the average of 4-7 experiments; bare points are individual experiments. Point at 15 seconds is common to all curves.

Fig. 7. Oxygen yield of successive flashes, spaced every 15 seconds, after dark period exceeding 3 minutes. — — o — — 20-40 nanosecond laser flash; — x — x — 28-microsecond xenon flash.

Fig. 8. (a) Joliot scheme for "charge cooperation" in photosynthetic oxygen production. The reaction center chlorophyll and its electron acceptor are not shown. Electron transfer to the acceptor is assumed to happen very fast. Bar indicates "special bonding" to a particular electron donor Z that favors electron withdrawal from that Z. "Specially bonded" $Z^+$ is especially fast both in losing electrons and in gaining them back in wasteful dark processes. Solid line indicates light steps in the favored ("majority") pathway, dashed line the "minority" pathway due to failures in the "flip-flop".

(b) General kinetic scheme abstracted from Joliot model.

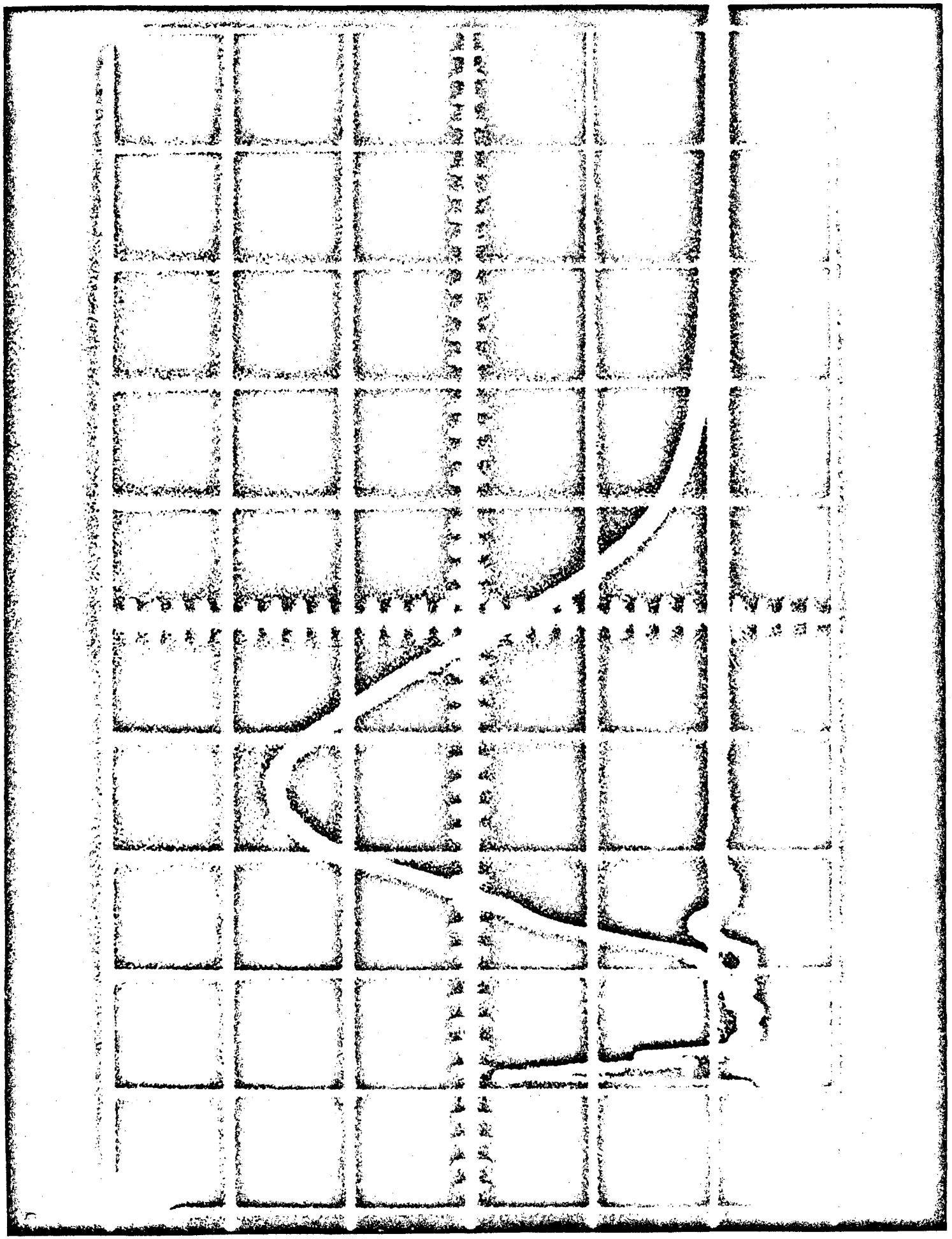
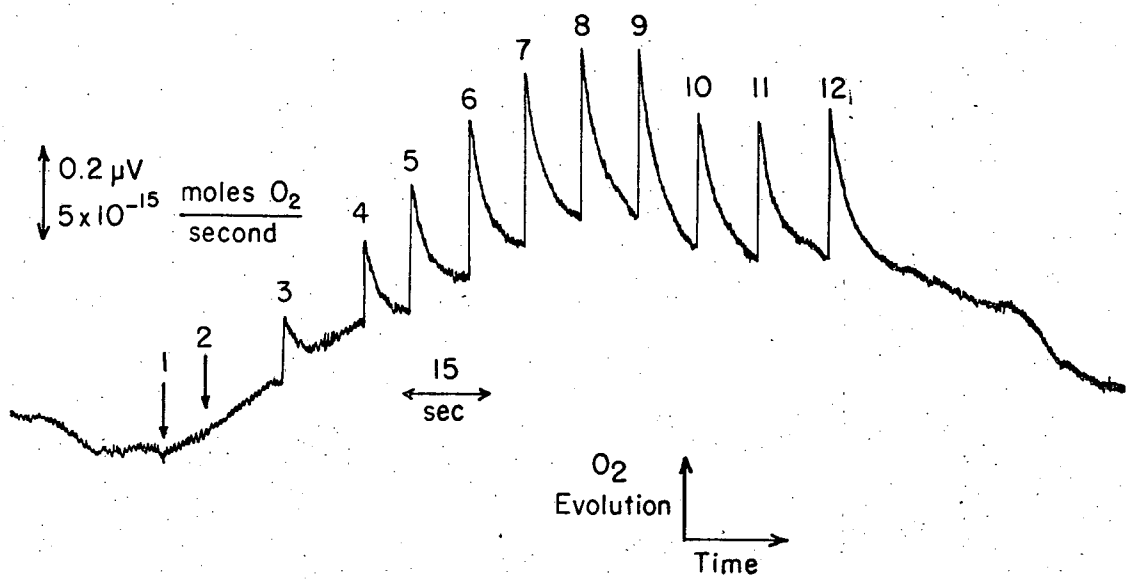


Fig. 1




XBL 689-6631

Fig. 2

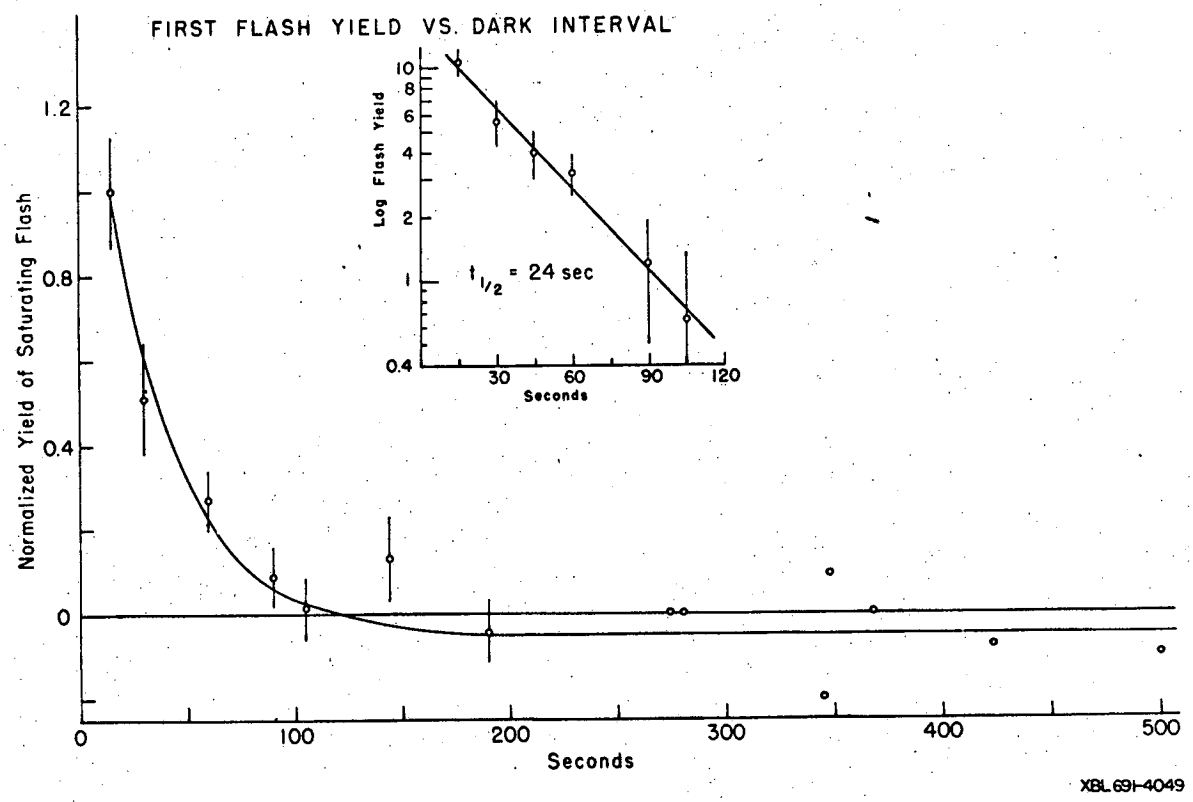

FIRST FLASH YIELD VS. DARK INTERVAL
Normalized Yield of Saturating Flash
1.2
0.8
0.4
0
0
100
200
300
400
500
Seconds
Log Flash Yield
10
8
6
4
2
1
0.4
30
60
90
120
Seconds
$t_{1/2}$ = 24 sec
XBL 691-4049

Fig. 3

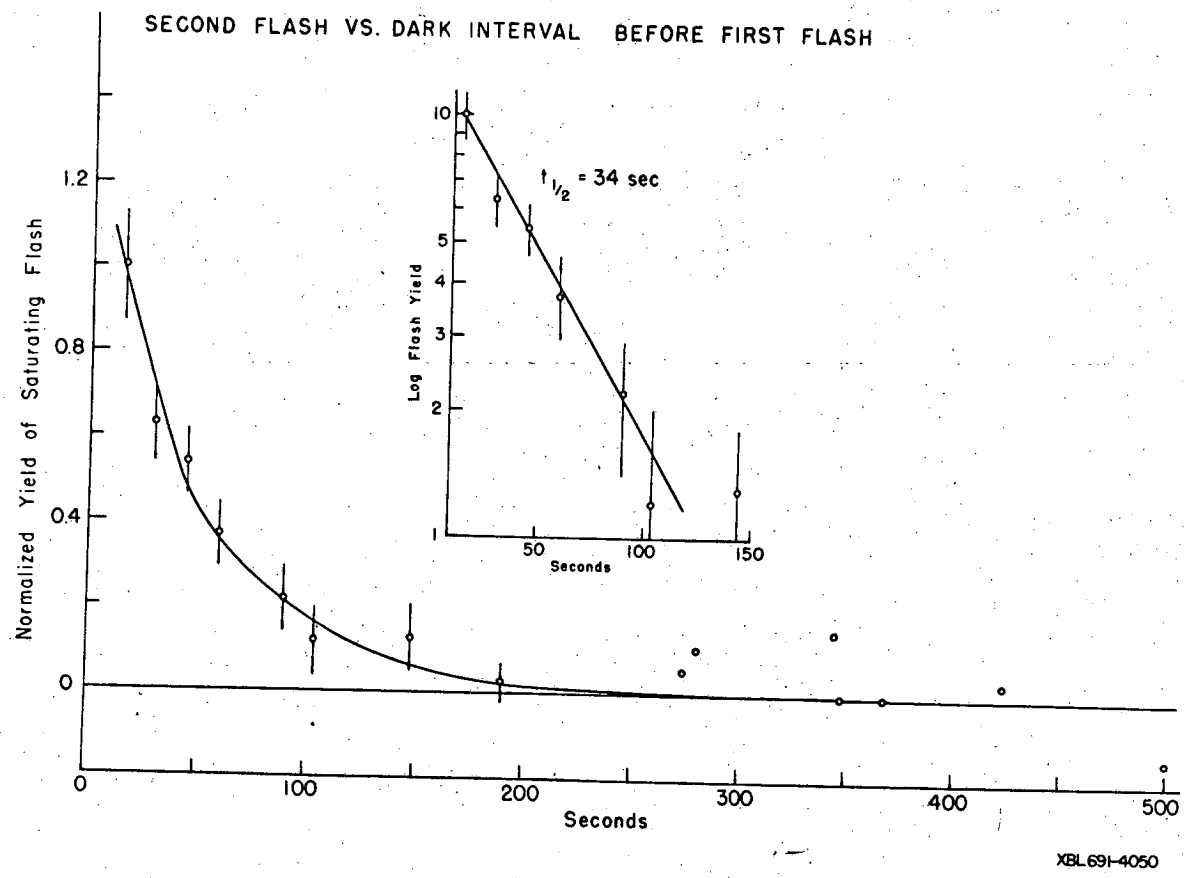

SECOND FLASH VS. DARK INTERVAL BEFORE FIRST FLASH
Normalized Yield of Saturating Flash
1.2
0.8
0.4
0
0
100
200
300
400
500
Seconds
Log Flash Yield
10
5
4
3
2
1
50
100
150
Seconds
$t_{1/2}$ = 34 sec
XBL691-4050

Fig. 4

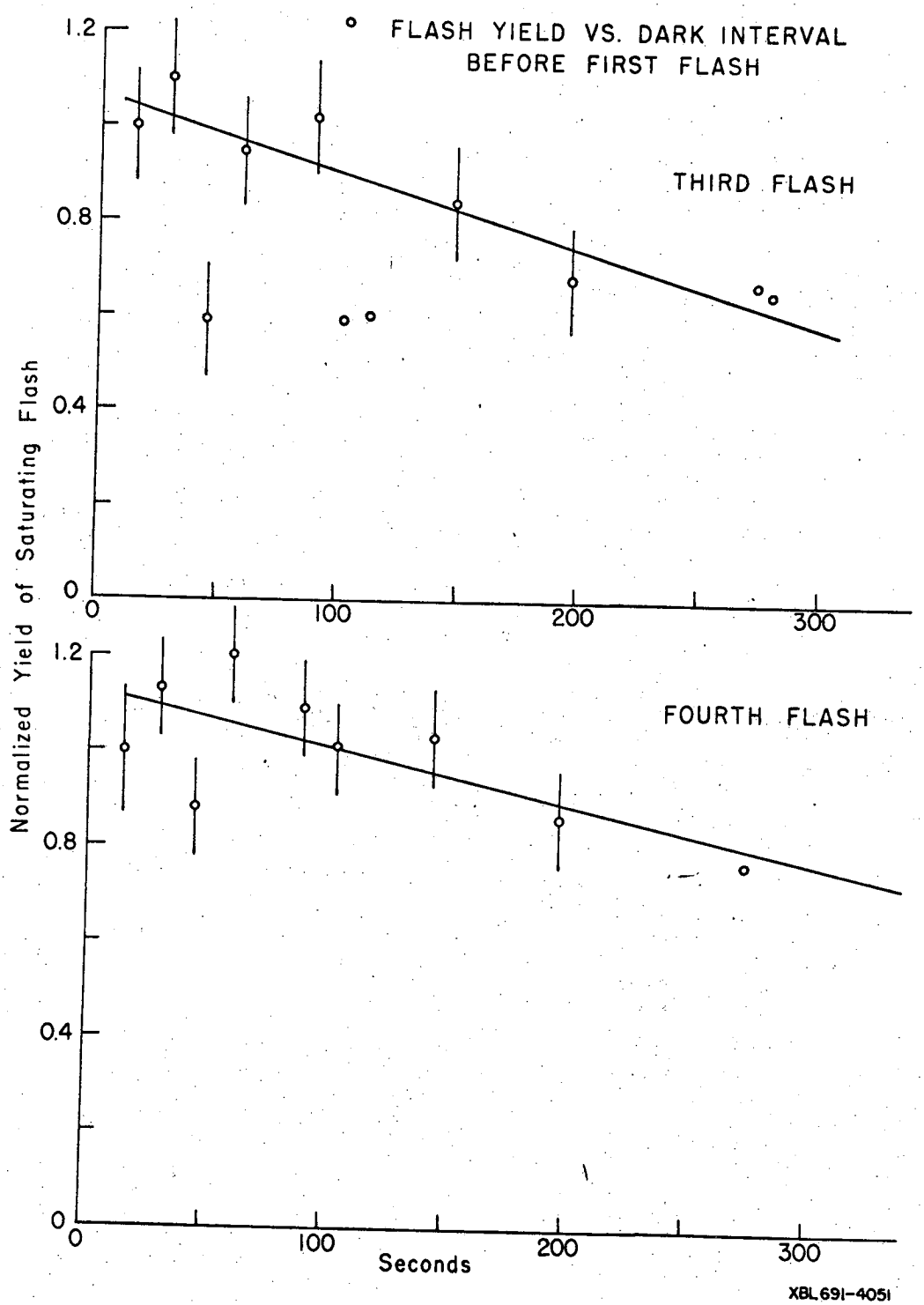
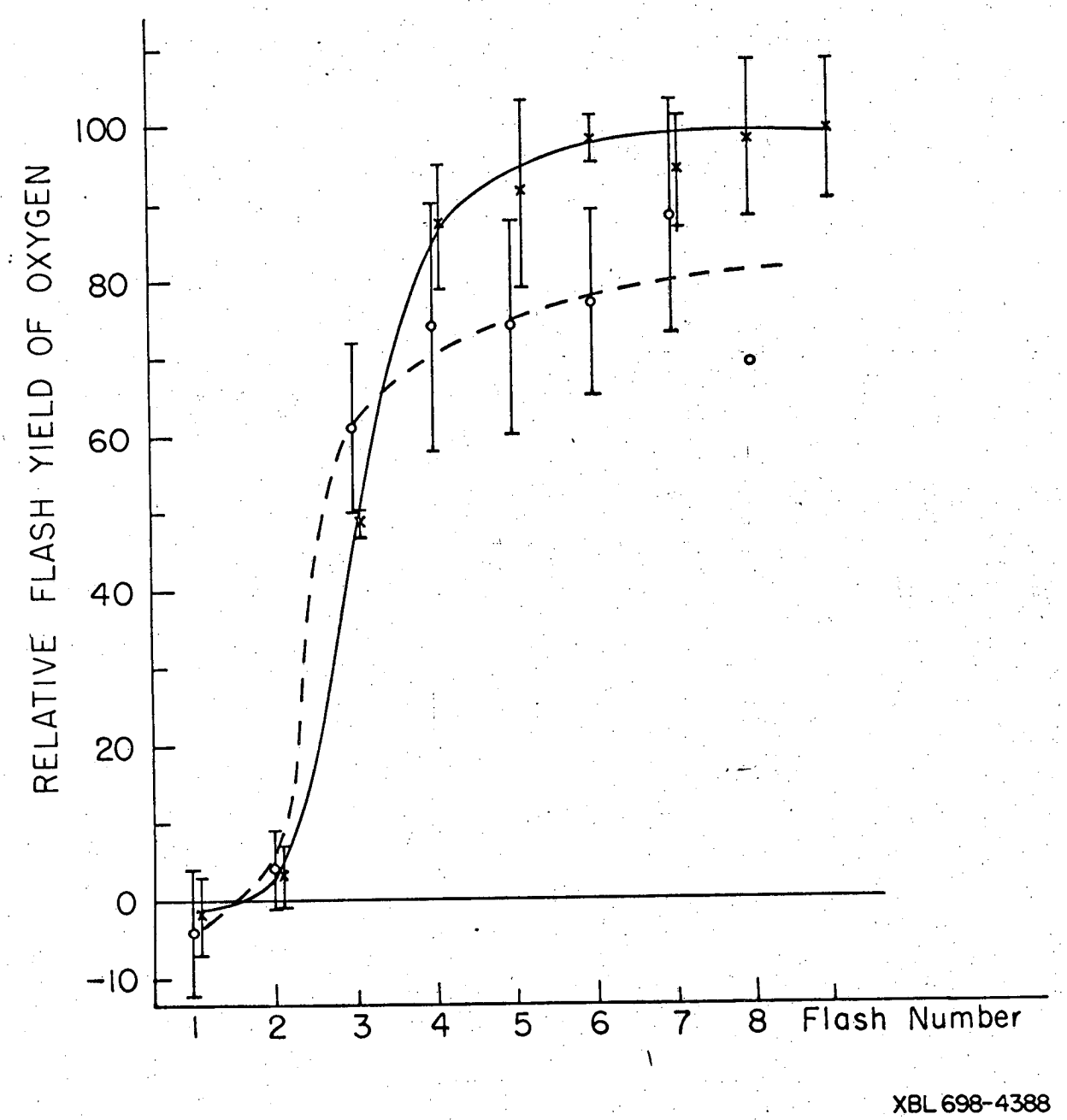



Fig. 5 (upper)
Fig. 6 (lower)


RELATIVE FLASH YIELD OF OXYGEN
100
80
60
40
20
0
-10
1
2
3
4
5
6
7
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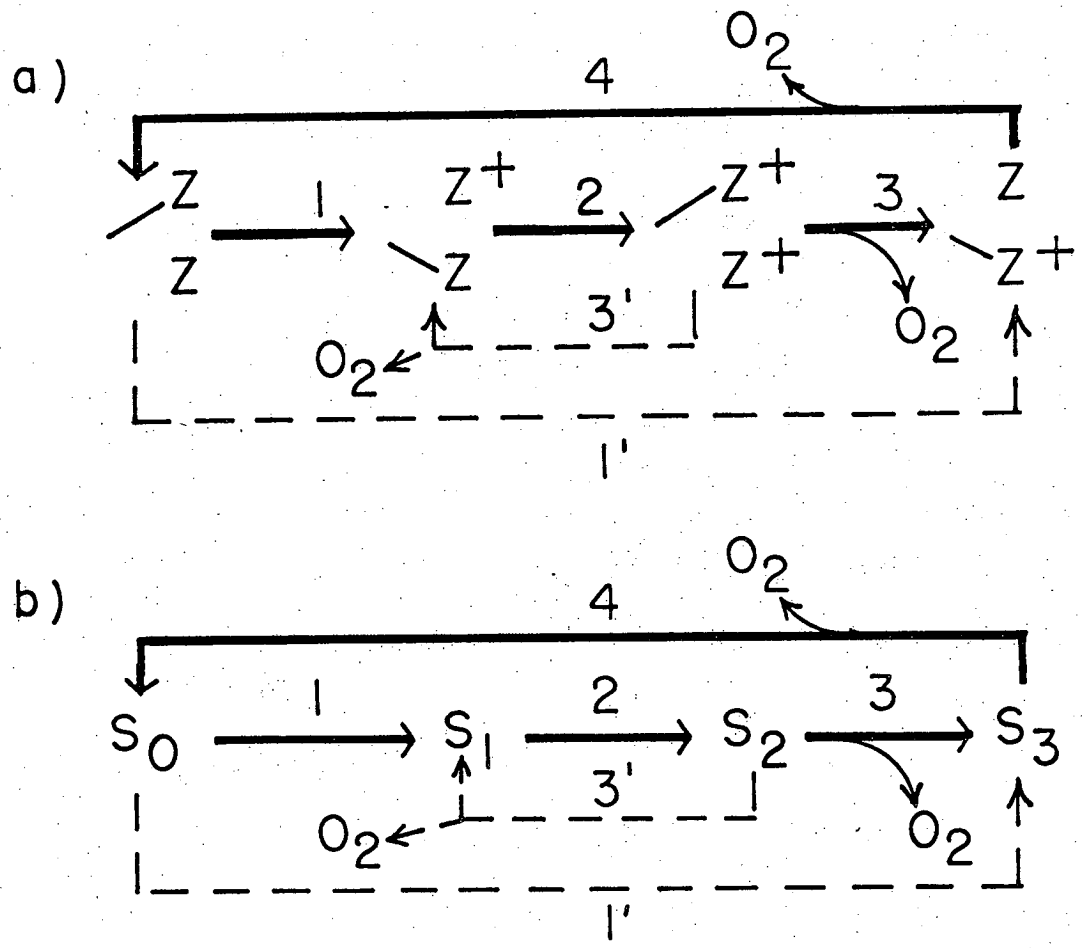
Flash Number
XBL 698-4388

Fig. 7




Deactivation steps: $S_3 \rightsquigarrow S_1$, $S_2 \rightsquigarrow S_0$ $\Big\}$ $k_1$

$S_1 \rightsquigarrow S_0$ $k_0$

$k_1 > k_0$

XBL 698-4389

Fig. 8

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