C00-3326-53

PHOTOCHEMISTRY AND ENZYMOLOGY OF PHOTOSYNTHESIS

PROGRESS REPORT

For Period August 1, 1976 through January 30, 1977

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February 1977

Prepared For

The U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION
Under Contract No. E(11-1)-3326

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Abstract

Our studies during the past reporting period were devoted to four topics:

(1) Photooxidation and Photocatalyzed Dismutation of H_2O_2. Most artificial electron donors that can replace water are oxidized monotonously, i.e., they give up one electron after each photoevent in System II. One such donor, H_2O_2, proved to act in a more complicated manner: after an uneven (but not an even) number of flashes, the O_2 system showed a continued catalytic decomposition of peroxide in the dark.

Other studies concerned the long-standing questions of whether O_2 might be (a) evolved in dark during the process of deactivation ($S_3 \longrightarrow S_1$) or (b) able to convert the S_0 to the S_1 state in darkness. Using a new technique, we could definitely exclude both possibilities.

(2) Proton Liberation and Translocation. We have continued our studies of proton release associated with O_2 evolution. Recent results show that 0.75 H^+, 0.0 H^+, 1.30 H^+, and 2.0 H^+ are released during the $S_0 \longrightarrow S_1$, $S_1 \longrightarrow S_2$, $S_2 \longrightarrow S_3$, and $S_3 \longrightarrow S_0$ transitions, respectively. Other studies show that the flash-induced proton uptake and release in chromatophores isolated from photosynthetic bacteria oscillate with a period of two, just as we previously reported for spinach chloroplasts. These results suggest that, in both systems, transport of protons across the membrane is associated with a 2-electron carrier. The most likely candidate is dihydroquinone.

(3) Acid-Base Induced Reduction of System II Acceptor Q. The dependence of the base-induced fluorescence jump on the redox potential of the chloroplast suspension was investigated with the following results: (1) The dependence on the redox potential fit a one-electron titration curve with a midpoint potential of 395 mV at pH 6.9; (2) In dark-adapted chloroplasts, which lack the capacity for an acid-base induced signal, additional dark incubation

with reductants and lipophilic mediators in the potential range +100 to +150 mV restored the full capacity for the signal. These results imply that the acid base-induced "reverse electron flow" towards Q involves intermediates of relatively high midpoint potential.

(4) Mass Spectrometer Studies: Competition for Reducing Power in Photosynthesis. We have determined some of the kinetic parameters related to the O_2 uptake that competes with CO_2 uptake in whole algae. As a first step toward extending this work to include higher plants, studies of O_2 uptake were initiated using whole (Class I) chloroplasts from spinach.

Photooxidation and Photocatalyzed Dismutation of H_2O_2

An intriguingly complicated reactivity of Photosystem II towards H_2O_2 was observed. Preillumination with one flash in the presence of H_2O_2, which was then removed, led to an apparent increase of S_0. An oscillation between two states was observed when a flash series was given in the presence of a high H_2O_2 concentration. After an uneven number of flashes, a continuous oxygen evolution occurred in darkness, presumably due to the alternating 2-electron oxidation of H_2O_2 to O_2 and 2-electron reduction of H_2O_2 to H_2O. This catalase-like activity of Photosystem II was low with dark-adapted chloroplasts or after illumination with an even number of flashes. In addition, rapid oxidation of at least one H_2O_2 molecule was observed after every flash.

Interesting results were also obtained with chloroplasts which had been subjected to Tris-washing, a treatment which leads to the inactivation of the water-splitting system and removes 4 of the 6 Mn atoms of the O_2 enzyme. With this material, each flash still led to the oxidation of some H_2O_2, but no longer to a sustained catalase activity oscillating with flash number. Remarkably, however, charge cooperation effects were still observed with Tris-washed chloroplasts when closely spaced flash pairs were used as illumination.

When a simple 1-electron oxidation mechanism is operating, as is observed with artificial electron donors to Tris-washed chloroplasts, a double flash with a varied flash spacing will reveal the "turnover" kinetics of the system: the amount of product per flash pair will increase from one equivalent to two equivalents when the flash spacing is increased from zero (single flash) to a time which is larger than the turnover half-time. With Tris-washed chloroplasts, however, flash pairs with short spacing produced less oxygen from H_2O_2 oxidation than a single flash. The kinetics of the

phenomenon were dependent on the H_2O_2 concentration. Both phases, the initial decrease and the subsequent increase of product with increased flash spacing, were more rapid at higher concentrations of peroxide. Other peculiarities included the effect of added $MnCl_2$. Manganese ions are known to effectively compete with other artificial electron donors of System II. Mn Cl_2 indeed decreased the yield of oxygen evolved from hydrogen peroxide, but more strongly at short than at long flash spacings.

The interaction of H_2O_2 with Photosystem II which, in its complexity, is quite unique for artificial redox compounds, presumably exposes some of the involved chemistry which is required, eventually, to oxidize water. A further characterization of the described reactions is therefore being attempted.

O_2 Evolution, the "Anomalous" System II Acceptor

As was discussed in the previous report, some questions had arisen which should be resolved by measurements of flash-induced oxygen production under anaerobic conditions. We have obtained these conditions by enclosing the chloroplast samples between gas-permeable membranes. These were stretched on the tip of an oxygen electrode, and the whole assembly was immersed in a solution of hydrosulfite, a strong oxygen scavenger. Because of the low background current in these conditions, the sensitivity of the O_2 measurement was increased to such an extent that a slow oxygen evolution which could accompany deactivation, (particularly that of the S_3 state) would have been detectable. No such evolution was observed, and a long-standing problem was settled.

In another series of experiments using this technique, we found that the anaerobic conditions did not significantly affect the flash-yield pattern obtained with dark-adapted samples. This result excludes that oxygen is involved in poising the S_0-S_1 equilibrium in the dark, if such an equilibrium exists at all.(1)

We are still trying to induce an interconversion of S-states chemically, but cannot yet report positive or even encouraging results. New data have also been obtained concerning the apparent oxidation of S_1 to S_2 by ferricyanide. It was already reported that this effect has been proven to be due to the occurrence of a double hit in the first flash, rather than to a S-state oxidation. We recently found that the phenomenon is probably related to an earlier observed effect of ferricyanide on the fluorescence-induction curve obtained in the presence of DCMU. The two phenomena showed a very similar dependence upon redox-potential and pH. In both cases, also, the effect of flash intensity was similar, indicating that a double-hit phenomenon is involved. We are trying to find a satisfactory explanation for this peculiar System II process so that it "makes sense" in terms of our present knowledge of photosynthetic electron transport.

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(1) "Electron Transport in Photosystem II," B. Kok, R. Radmer and C. Fowler, Proc. 3rd International Congress on Photosynthesis, Elsevier Scientific Publishing Co., Amsterdam, p. 485-496 (1975).

Proton Liberation And Translocation

In a previous report, [2] we presented information about the release of H^+ during oxygen evolution. We reported that $2H^+/O_2$ are liberated in the terminal $S_0 \xrightarrow{hv} S_3$ step and two are released during transitions of precursor states. A careful systematic analysis of the data has shown that the precursor states transition $S_0 \longrightarrow S_1$ releases 0.75 H^+, $S_2 \longrightarrow S_3$ 1.3H^+, and $S_1 \longrightarrow S_2$ zero. A manuscript to be submitted to Biochim. Biophys. Acta. is enclosed. Its summary reads as follows:

1. In a sequence of flashes given to dark-adapted chloroplasts, the flash yield of proton release oscillates with a period of 4, similar but not identical to the oscillation of the O_2 flash yield.
2. Using the proton release associated with ferricyanide reduction as a calibration, we computed that two protons are released in the terminal O_2-liberating reaction; the other two protons are released in precursor conversion steps.
3. Analysis of the effect of preflashes on the oscillation pattern showed that the $S_1 \longrightarrow S_2$ transition did not release a proton, the $S_0 \longrightarrow S_1$ transition somewhat less than one (0.75), and the $S_2 \longrightarrow S_3$ transition more than one (1.25).
4. The precision of the data was sufficient to exclude the possibility that proton uptake in the four one-electron steps of water oxidation follows a simple 1, 0, 1, 2 pattern. In addition, preillumination with two flashes induced an anomalously high proton yield after the second flash in a series.

We will discuss possible mechanisms to interpret these perturbations.

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(2) Annual Progress Report dated April 1976.

One of the more important problems in bioenergetics is how photosynthetic electron transport is coupled to photophosphorylation. The most currently acceptable model, suggested by Mitchell (Chemiosmotic hypothesis), states that a pH gradient formed by proton-coupled electron transport across a membrane barrier is the primary driving force for phosphorylation. This membrane, which forms a closed "sac" called a thylakoid, separates an inner space (where a proton pool is formed) from an outer space. The electron transport components are arranged in the membrane in a manner which facilitates the transport of proton outside to inside. It has been observed that protons are taken up by spinach chloroplasts during illumination followed by their release in the dark. This observation is essential to the validity of the hypothesis. Many other important questions are implicit in such a model. Some questions which we have addressed ourselves to are: a) the mechanisms by which protons are transported from outside to inside, and b) the number of protons transported inside per electron passing through the chain.

We have previously shown in spinach chloroplasts that $1H^+/e^-$ is released inside the thylakoid during oxygen evolution, 2 H^+ are transported from outside to inside during the passage of one electron from photosystem II to photosystem I, and $1\ H^+/e^-$ is transported directly in photosystem I. In addition, we found that H^+ transport between the photosystems was dependent upon electron pairing at some site within the chain. We postulated that this pairing process was an essential part of the mechanism for transporting H^+ across the thylakoid membrane barrier. The results raised the

following questions: a) how can $2H^+$ be transported in the chain per pass of a single electron? b) how can such a process be linked to electron pairing c) what is the mechanism of energy conservation (proton transport) in photosystem I?

Although we have not answered these questions, some recent results present us some interesting clues. First, we discovered that proton transport (probably $2H^+/e^-$) into the thylakoid of photosynthetic bacteria is related to electron pairing (similar to the pairing observed in electron transport between the photosystems in green plants). We arrived at this conclusion because we observed in both cases, a binary oscillation in pH during flash illumination, which could be correlated with the release of protons inside the thylakoid. In addition, we found that the electron transport inhibitor o-phenanthroline decreased the number of protons transported by half, and, at the same time, changed the phase of the binary pH oscillation.

These results are consistent with a model presented earlier by Mitchell but modified to include an explanation for our observation of electron and proton pairing. This model was designed primarily to give a mechanism for the transport of two protons per passage of an electron across the mitochondrial membrane. It postulates an electron cycle involving high and low potential b-type cytochromes arranged in parallel across the membrane with the photoact, but spatially separated from it. An electron from the cycle and an electron produced by the photoact plus $2H^+$ from outside combine with a mobile quinone. The resulting dihydro-quinone transport its 2 electrons and 2 protons across the membrane carrier

to the "inside" and discharge its electrons and protons. One electron goes to cytochrome c, which is connected to a photoact, and the other goes to a non-protonated intermediate in the cycle, probably cytochrome b. This sequence of events would not lead to oscillating behavior, however, and since we observed that oscillation occurs during the release of protons, it is not sufficient to explain our data. Since the electron pairs must be unpaired on the inside and go to different sites in the electron transport system (cytochrome c and cytochrome b), we make the following additional hypothesis: each of the two sites is accessible only via proton-and electron-paired intermediates, i.e., electrons can enter and exit the carrier membrane only in pairs. We are currently testing this hypothesis.

Other preliminary results may also be related to the mechanism of energy conservation. We have observed that high concentrations of Mg^{++} (~ 50 mM) inhibit all proton transport, including the site in photosystem I, and also inhibits electron transport to some extent. Obviously, Mg^{++} is interfering with the mechanisms for transporting protons to the inside of the thylakoid. One must reconcile this result with the mechanism of proton pumping and the requirement for Mg^{++} in phosphorylation.

Methylamine, a known uncoupler of photophosphorylation in chloroplasts, also has an unexplainable effect on proton transport in the chain and in photosystem I. Normally, it is assumed to rapidly dissipate the proton gradient formed during electron transport, due to its buffering properties and high membrane permeability. We have found, however, that the cationic form of methylamine seems to replace proton uptake

without however inhibiting the pH changes associated with proton transport through the pairing site. Our tentative interpretation is that cations of methylamine are replacing protons at fixed charged sites in the outer surface of the membrane. Methylamine then acts as a direct proton donor to the normal protonation reaction. Normally, in photosystem I, of the $2H^+/e^-$ taken up during a flash one is used in the reduction of O_2 to H_2O_2, and the other is transported to the inside of the thylakoid membrane. Unexpectedly, methylamine doubles the number of protons taken up in peroxide formation. Preliminary results also indicate an increase O_2 uptake; however, this cannot yet be quantitatively determined. Presently, experiments are aimed at unraveling these complicated phenomena.

Acid Base Induced Reduction of System II Acceptor Q

As was described in previous reports, one of our programs concerns the temporary increase of the fluorescence yield that occurs when base is added to a chloroplast suspension with a pH ≤ 6.0. Presumably, the phenomenon reflects a reduction of the trapping center Q caused by a pH gradient across the chloroplast thylakoid membrane. One of our earlier observations was that illumination with far-red light (which excites mainly system I and oxidizes all components in the electron transport chain between the two photoacts) or the addition of ferricyanide (which does more or less the same thing chemically) inhibited the phenomenon. Furthermore, we had found that, in order for the phenomenon to occur, reducing equivalents must be present in the pool of intermediates between the two photoacts. With the aim to pinpoint this site more precisely, we have performed redox titrations of the ferricyanide inhibition. We followed our more or less standard protocol: (a) 10 sec. bright light to reduce the pool, (b) 90 sec. dark (in which Q and intermediate A_2 become reoxidized), (c) switch on the blue, fluorescence exciting beam, (d) add base and view the fluorescence jump. Some two seconds before the base was added, we injected mixtures of ferro-and ferricyanide of known potential. The signal responded promptly to such additions, and we obtained reasonably defined redox titration curves. These curves revealed a single equivalent slope and midpoint potentials around 400 mv. The pH dependence of the midpoint potential is, at the moment, not certain, and we will have to do additional experiments.

The observation is of particular interest because it correlates with other observations which were made recently in our laboratory. These also point to a close interaction between Q (presumably at about 0 Volt) and a "400 mv intermediate." The observations bear directly on long-standing questions concerning the electron transport through the P$\longrightarrow$ Q chain: equilibrium constants, proton translocation, and phosphorylation. Hopefully, we are approaching the point where sufficient information is available for a comprehensive hypothesis.

Mass Spectrometer Studies: Competition for Reducing Power in Photosynthesis

During the past reporting period, our studies centered around the light-induced uptake of O_2 (see Annual Progress Report dated April 1976). We previously observed that molecular O_2 competes directly with CO_2 for light-generated reducing equivalents, and that O_2 is reduced at a high rate under conditions in which CO_2 fixation is suppressed (e.g., during the lag following a dark-light transition). More recent studies have been devoted to (1) determining the kinetic parameters ("K_m's") for CO_2 and O_2 in Scenedesmus D_3, with the aim of obtaining an O_2/CO_2 "forking constant," and (2) extending these studies to other organisms and systems (e.g., chloroplasts).

Recent experiments with Scenedesmus D_3 in the presence of iodoacetamide (a Calvin cycle inhibitor) showed that the net rate of O_2 uptake (and O_2 evolution) is half saturated at about 8% O_2. This relatively low "K_m" for O_2 indicates that O_2 uptake is almost substrate saturated under ambient conditions (~21% O_2) and can thus proceed at a near-maximal rate in the absence of CO_2 fixation. We plan to also do these experiments in the absence of iodoacetamide; in this case, we would determine the O_2 uptake during the brief lag period following a dark-light transition.

We have also studied the O_2 uptake reactions under conditions of low CO_2; in this instance we observed that CO_2 is taken up until it becomes limiting, at which point O_2 uptake again occurs at a high rate. Attempts to determine the CO_2 concentration at which this transition takes place have not been very successful; the experiments require measurements at very low CO_2 concentrations and are thus hampered by the high mass spectrometer background at m/e = 44, the base (and parent) peak of CO_2. We are

currently attempting to overcome this problem by growing ^{13}C-labeled algae.

During the past six months, we also observed that NO_3^- reduction can significantly alter the gas exchange stoichiometry during the lag. We have observed a significant rate of O_2 evolution during the lag that is not compensated by concomitant CO_2 and/or O_2 uptake. In the presence of NH_4^+, which suppresses NO_3^- reduction, a normal stoichiometry is observed. This effect is observed in young (two-day old) cells. In our earlier experiments, done with three-day old cultures, we did not observe this effect, probably because the algae were NO_3^- limited.

We have also extended our mass spectrometer studies to whole (class I) chloroplasts prepared from spinach,* in which we might expect to observe the interplay of O_2 uptake and CO_2 fixation more easily. These chloroplasts are much more permeable than whole cells, and thus one can observe, e.g., the ATP-dependent reduction of phosphoglycerate, the ATP-independent reduction of oxaloacetate, the effect of uncouplers (most of which do not penetrate into whole cells), and the effect of ions (e.g., Mg^{++}). To date these experiments have been reasonably successful, although complicated by the somewhat fractious nature of these whole chloroplast preparations. We have observed that the O_2-uptake kinetics in these preparations differ substantially from those observed in whole cells; at the onset of illumination (after several minutes of dark), the rate of O_2 uptake is not as high as the subsequent rate of CO_2 fixation, and O_2 uptake do not dramatically shut down as it does in Scenedesmus D_3. At the present time we do not know to what extent the gas exchange reactions observed in these preparations reflect those occurring in the whole plant; alterations due to the chloroplast extraction procedure may occur, and some of the O_2-cycle reactions may depend on extra-chloroplastic components.

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*These experiments are being carried out in collaboration with Dr. T. V. Marsho, Univ. of Maryland, Baltimore County.

Status of Publications

"Mechanisms in Photosynthesis," B. Kok and R. Radmer, in: Chemical Mechanisms in Bioenergetics, American Chemical Society Monograph 172, Ed. D. Rao Sanadi, 172-220 (1976).

"Photoreduction of O_2 Primes and Replaces CO_2 Assimilation," R. Radmer and B. Kok, Plant Physiol. 58, 336-340 (1976).

"Photosynthesis: Limited Yields, Unlimited Dreams," B. Kok and R. Radmer, Bio-Science, in press.

"Energy Transfer Between Antenna Components and Reaction Centers," K L. Zankel, Photosynthetic Bacteria, in press.

"Light Conversion Efficiency in Photosynthesis," R. Radmer and B. Kok, in: Encyclopedia of Plant Physiology, in press.

"Photosynthesis: The Path of Energy," B. Kok, Plant Biochemistry, Third Edition, Academic Press, J. Bonner and J. Varner, Eds., in press.

"Proton Translocation in Chloroplasts and its Relationship to Electron Transport Between the Photosystems," C. F. Fowler, Biochim. Biophys. Acta, in press.

"Present Status of the O_2 Evolution Model," B. Kok and B. Velthuys, VII International Congress on Photobiology, Rome, Italy, in press.

"Proton Evolution from Photosystem II:Stoichiometry and Mechanistic Considerations," C. F. Fowler, to be submitted.

Enclosure (1)

(Draft Manuscript to be submitted to Biochim. Biophys. Acta)

PROTON EVOLUTION FROM PHOTOSYSTEM II: STOICHIOMETRY AND MECHANISTIC CONSIDERATIONS

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PROTON EVOLUTION FROM PHOTOSYSTEM II: STOICHIOMETRY AND MECHANISTIC CONSIDERATIONS

INTRODUCTION

In a recent paper, we reported that the flash yield of proton release, in a sequence of flashes given to dark-adapted chloroplasts, oscillates with a period of 4.[1] Because of the similarity to the flash yield pattern of O_2 evolution (and because it was the simplest interpretation of the data), we leaned towards a "concerted" reaction where 4 H^+ were released concurrently with every O_2. We recognized at least two problems with this interpretation: (1) the measurements were made in relative units and the stoichiometry with O_2 evolution undetermined, and (2) several differences of a rather variable nature were encountered between the flash yield patterns of proton and O_2 release.

In more recent papers we presented (1) a procedure to "calibrate" the proton flash yields,[2] and (2) data that showed the occurrence of 2 distinct oscillatory components,[3] during a sequence of flashes. One of these - having a periodicity of two - was described in some detail and was shown to be associated with electron pairing in the transport chain between the photosystems. The other oscillation, which exhibits a dominant periodicity of 4, is associated with O_2 evolution. Its analysis is the subject of this paper.

The methods and materials used in this study have been described before[1,2,3]; additional procedures are included at the appropriate places in the text.

RESULTS

In previous papers we reported three conditions of measurement which lead to a period of 4 oscillation in the pH changes induced by flashes in spinach chloroplasts.[1, 3]

(a) With uncoupled chloroplast and ferricyanide as the electron acceptor, one observes the yield pattern shown in Fig. 1 (curve a). We have shown that this pattern is made up of a linear combination of two oscillatory components, which originate in different sites of the electron transport system.[4] One component has a periodicity of two Fig. 1 curve d). It originates in the electron transport chain between the photosystems and reflects sequential electron pairing and unpairing.[3, 4]

(b) The second component shows an oscillation pattern, characterized by a period of 4 (Fig. 1b). This pattern can be observed independently when the pairing site is bypassed as is shown in Fig. 1b. In this experiment, the chloroplasts were osmotically shocked prior to measurement -- presumably, this facilitates the entry of ferricyanide to a site closer to Q. (See Ref. 3). If the pattern of Fig. 1b is added to the binary pattern (Fig. 1d), we obtain the complete pattern of Fig. 1a. Thus it represents the second component of 1a. In the experiments of Figs. 1a and 1b, there occurs a net release of protons after every flash (except for the first flash one in expt. 1a.) As described in Ref. 2, we may assume that, in the presence of ferricyanide, on the average one H^+/electron/flash is released which allows calibration of our system as used for the ordinate in fig. 1. In this case, the proton flash yields were calibrated by using a parallel sample containing

ferricyanide (Fig. 1a). In expt. Fig. 1c, the oscillations occur about zero as an average because in the steady state every flash-induced proton released is balanced by uptake. Although the measuring conditions are very different, the pattern and amplitude of the oscillation are surprisingly similar to those of Fig. 1b.

We ascribe the absence of the binary component in these two cases to entirely different causes. As already stated above, in case b the pairing site is bypassed because ferricyanide has direct access to Q. In case c, the pairing site in the chain is not expressed in the pH changes. The reasons for this are not clear. However, in a recent paper, it was shown that the observed binary oscillation in pH reflects the release of two electrons at the site of unpairing rather than at the site of uptake. In all likelihood, this release is dependent upon the associated electrons going to non-protonated electron acceptors such as cytochrome f and plastocyanin.[5] If photosystem I is unable to oxidize these non-protonated acceptors efficiently due to the absence of an external acceptor, then the electrons in the pairing site may not be released.

The results shown in Fig. 2 support this hypothesis. Preillumination of a chloroplast suspension containing no external acceptor with far-red light, followed by 5' dark results in a flash-induced proton yield pattern which is very similar to that obtained with ferricyanide for the first 3 flashes. On the other hand, with a chloroplast suspension containing methylviologen, the oscillations are sustained. These results therefore are consistent with the absence of the efficient turnover of some electron transport component essential to the release of protons from the pairing site.

In the following, most of the experiments were done with chloroplast suspensions containing no acceptor because the lifetime of osmotically shocked

chloroplasts is too short for many of the experimental procedures. The presence or absence of the binary oscillation will be critical. It will be dealt with as the question arises. For quantitative analysis of the data, a "real" zero was picked based upon the similarity of the oscillations with and without acceptor. This procedure is demonstrated for Fig. 1c right hand ordinate.

A comparison of the protons yield pattern in fig. 1b to a typical O_2 yield pattern shown in Fig. 1e shows clearly the considerable differences which exist between the two[6]. For example, protons are evolved after flash one, but O_2 is not, the oscillation of the pH yield is much more shallow than the O_2 yield oscillation (e.g., the ratio Y_3/Y_5 is 1.8 for H^+ and 7 for O_2), as if the oscillations were superimposed on a monotonous background flash yield. If only the oscillatory part of the proton yield pattern is considered, we find that Y_2 is larger, Y_3 smaller, and Y_4 larger than the corresponding O_2 yields.

Contributions of Various S States

Evidently, our earlier speculation viz, that all four protons are released in the final O_2 evolution step in a "concerted reaction," is inadequate since more than one, and possibly all four charge accumulation steps must be involved in proton release. On the other hand, the fact that Y_3 and Y_7 are maximal indicates that a substantial fraction (about half) of the protons are released in the $S_3 \rightsquigarrow S_0 + O_2$ step.

In order to obtain additional information concerning the contribution of the four steps, we performed the preflash experiments shown in Figs. 3a and 3b. Inspection of the data in Figs. 3a and b show that the modification of each individual flash yield induced by preflashes is not influenced by the

presence or absence of the binary oscillation (with and without ferricyanide). Evidently the binary oscillation, which is present in expt. Fig. 3a and absent in Fig. 3b, is unaffected by the preflashes, provided the subsequent darktime is sufficient to allow return to the "equilibrium state" (> 30 sec.).

A cursory examination of Fig. 3 reveals that one preflash causes a decrease of yields 1, 4, and 5, and an increase of yields 2 and 3. The changes induced by three preflashes are more or less the opposite. These modifications are sustained in the subsequent cycle of 4. One preflash increases the initial ratio S_1/S_0 (roughly from 75/25 to 100/0) while 3 preflashes decrease this ratio (to 45/55). Thus, the size of the first proton flashyield Y_1, appears to correlate with the amount of S_0 present before the first flash, i.e., the step $S_0 \longrightarrow S_1$ is associated with proton release, but the transition $S_1 \longrightarrow S_2$ might not be. Similarly, Y_2 is enhanced by 1 preflash and decreased by 3 preflashes, indicating that the transition $S_2 \longrightarrow S_3$ (large after one, small after 3 preflashes) is associated with substantial proton release.

For a more quantitative analysis of the data we calculated the population of each S state during sequences of 8 flashes, using the formulations developed in refs. 6 and 7.

$$Y_{n+1}/1-\alpha = a\,[S_0]_n + b[S_1]_n + c[S_2]_n + d[S_3]_n$$

In which coefficients a, b, c, d denote the number of protons liberated in the four respective transitions (e.g., $S_0 \xrightarrow{h\nu} S_1 + a\,H^+$).

We assumed a homogenous value $\alpha = 0.1$ (representing the misses on each step) and the following three initial distributions of S_1/S_0 : (75/25, 97/3, 45/55) to approximate the distributions after zero, one and three preflashes, respectively.

These initial S state distributions and the first four flashyields provide four equations from which the four coefficients can be calculated. The requirement a+b+c+d=4 is met by the normalization procedure. The values of the four constants, calculated in this way from the three experiments in Fig. 2b, are tablulated in Table 1.

As in our first paper on this subject we recognize that uncertainties exist about the accuracy of the data. Most important of them are: a) the arbitrary positioning of the baseline used for numerical calculation and b) the possibility that the binary oscillations associated with electron transport between the photosystems is contributing to the patterns used in the analysis. This latter possibility is increased because the phase of the oscillation in protons released during transitions between O_2 precursor states is the same as that associated with transport between the photosystems.

The first (a) can be discounted for the most part because of the small variations of the coefficient obtained for the 3 preflash regimes. If baseline were in error, this would imply that the nomalization was wrong. (That is the total contribution of the period of 4 would be different). This is not likely since the amplitude of the period of 4 oscillation in all three measuring conditions are nearly the same. When the baseline was arbitrarily shifted and the same calculation made, it was found that the various coeffients became inconsistent, that is they did not remain the same for the 3 preflashing conditions used in the analysis.

It is also unlikely that the period of two in electron transport make a contribution. This conclusion is based mainly on the similarity of the effect of preflashing on chloroplasts suspension containing no acceptor to that containing ferricyanide. In the latter, the period of two associated with electron transport between the photosystem is maximal. Therefore, a maximal effect should be caused by preflashing. The fact that the modification shows up in the repeat pattern (6, 7, 8, 9 flashes) also makes it unlikely. In the one case where far-red preillumination produced the binary oscillation (fig. 2) it was not sustained for more than one period. Based upon the uncertainties, one can ask now how good are the coefficient. For example if we assume the distribution of a = 1, b = 0, c = 1, d = 2 or a = 0.5, 0_1 1.5, 2 is there a significant deviation from the data? Calculations were made using these coefficients as well as with the (.75, 0, 1.3, 1.9) distribution taken from table 1. The resulting numbers are presented in Table II for the zero pF case only. For the average distribution shown in table I, the calculated yields are in very good agreement for the first 4 flashes, but deviate considerably in 5, 6, and 7. This deviation is however in the same direction for each flash, suggesting that a systematic shift in baseline is occurring. On the other hand, the other distributions cause shifts in yields away from the measured quantities in a predictable and more extreme manner. See, for example, yield one for the (0.5, 0, 1.5, 2 case.) Finally, any set of coefficients which leads to a significant yield during the S_1 to S_2 transition is also not tenable because of the near zero first flash yield for the 1 preflash regime.

Therefore, it is reasonably certain that the value for the coefficient "a" lies between 0.5 and 1 and "c" between 1.0 and 1.5. In order to demonstrate the consistency of the coefficients, flash yields are also calculated for 1 and 3 preflashes and shown in Table I, column designated (Y_n Cal (1)).

The question remains as to why non-integer number occur for the coefficients a and c. One possibility is that these transitions have a different pK. Since the numbers are considerably different from unity, the pH of the suspension medium would not be far from the pK. Variation of the pH over this range (6.5 - 7.5) however does not influence significantly the yield pattern. On the other hand it is difficult to know whether the O_2 system actually equilibrates with the external environment, so this cannot be totally discounted.

Another possibility is that there is more than one pathway leading to oxygen evolution. The only rigid requirements are that $2H^+$ must be released during S_0 to S_3 transition, $2H^+$ in the terminal step (S_3 to S_0) and zero on the S_1 to S_2 transition. Modification of the flash yield pattern by 2 sequential preflashes support this possibility. Figure 4 shows flash yield sequences observed after pretreatment with 1, 2 and 1 plus 2 preflashes. Note that the second flashyield, which reflects mostly the $S_2 \longrightarrow S_3$ transition, is progressively increased, while the other yields are affected much less or not at all. From the first four flashes in Fig. 3, we calculated as before the coefficients: a, b, c and d and find that only c is slightly different. (See Table I). Both pretreatments yield a high concentration of S_1, however, the source of S_1 is different.

Starting from the normal distribution (25, 75, 0, 0) 1 preflash will yield (2, 5, 30, 67.5, 0) which deactivates to (2.5, 97.5, 0, 0) i.e., S_1 is either made in the light or by deactivation from S_2. On the other hand, 2 preflashes will yield the distribution (0.25, 5.25, 33.75, 60.75), which deactivates to (0, 100, 0, 0); but now, a large fraction of the S_1 originates by deactivation from S_3 instead of S_2. This fraction is even larger following a double flash pretreatment (1 plus 2 preflashes).

The data of Fig. 4 then imply that the deactivation of $S_3 \longrightarrow S_1$ leads to a higher yield of protons after the second flash ($S_2 \longrightarrow S_3$) in a subsequent flash sequence than deactivation from $S_2 \longrightarrow S_1$. In terms of proton liberation, there are apparently two forms of S_1, which further suggests that there are a minimum of two pathways leading to O_2 evolution.

There are at least two problems associated with this interpretation. Firstly, measurement of O_2 yields following a double pre-flash treatment also resulted on occasion in an increased second flash yield (unpublished). This result is likely due to slower deactivation of the precursor states than normally occurs,[7] that is 5 minutes is not sufficient time to ensure total deactivation. The maximum amount of O_2 observed on the second flash can be translated into approximately 20% of the O_2 evolving systems being in the S_2 state prior to the measurement (i.e., a distribution of (0, 80, 20, 0)). This distribution, in fact any distribution which has a significant number in the S state should result in a high yield one. This does not occur. In addition any change in yield 2 due to higher S_2 should also be observed in the 6th flash; again this is not observed.

The second and more serious problem with the two-path interpretation is the possibility that proton transport between the photosystem is contributing to the yield pattern. Intensifying this problem is the similar effect that far red pre-illumination (Fig. 2) and 2 pre-flashes has on proton yield 2 (Fig. 4) without modifying flash 6. The fact that flash yield one and three are not modified by 2 sequential preflashes, whereas far-red preillumination decreases 1 considerably and 3 to some extent argues against this possibility. It is, however, impossible to totally preclude any of these uncertainties. Keeping in mind these objections, but also wishing to pursue the possibilities to its logical conclusion, we carried out the following analysis.

The model presented in Fig. 5 summarizes the results based upon the two pathway model. The initial distributions S_1^+/S_0 were given earlier as: 0 pF = 75/25, 1 pF = 97/3, 2 pF = 100/0, 1+2 pF = 100/0, and 3 pF = 45/55. In the two-pathway model S_0 represents a branch point where 75% release a proton during the $S_0 \longrightarrow S_1$ transition (designated S_1') and 25% do not (designated S_1^2). S_1 consists of two states $S_1^T = S_1'$. In order to simplify the calculations, it was assumed that the proton yield on the second flash was due to proton release only during the S_2 to S_3 transition. Since the S_1 to S_2 transition does not release protons, this simplification is exact for the 1 pF, 2 pF and 1+2 pF cases and approximate for the 0 and 3 pF, where a small contribution occurs due to the S_0 to S_1 transition. Therefore $S_3^T = S_3' + S_3^2$ and $Y(2) = 1S_3' + 2S_3^2$. S_3^T can be obtained as before according to the Kok-Forbush model from the initial S_0/S_1 dark distributions followed by two sequential flashes. Y_2 is taken from the data of Fig. 3b. From these equations, S_3^1 and S_3^2 were calculated. The ratio of S_1' to S_1^2 before the flash sequence is the same as S_3^1/S_3^2

(assuming equal misses. Therefore, by knowing S_1^T, S_1^1 and S_1^2 can be calculated. These numbers are presented in Table III column 1, for each of the preflash regimes.

We next proceeded to determine the fraction of S_3^T which deactivates to S_1^1 and S_1^2 following the preflash regimes. We assumed that S_3 (a branch point) can deactivate by the reversal of both pathways, but S_2^1 and S_2^2 are constrained to deactivate to S_1^1 and S_1^2, respectively. From a knowledge of the total S state distribution after the preflash regimes in question the fraction of S_3 going by any pathway can be determined. For all cases, approximately 33% of S_3 deactivated to S_1^1, and 66% deactivated to S_1^2 or $K_3^2/K_3^1 = 2$. Curiously, the fraction deactivating to S_1^1 and S_1^2 correlated with the concentration of S_3^2 and S_3^1, respectively, before deactivation. Whether this is significant or not remains to be determined.

To demonstrate how well this model fits the data we have calculated flash yield as before and these are presented in Table 2 under the heading Y_n (cal) (2). The fit as expected is as good as that obtained with the coefficients (Y_n (cal) (1)). Based upon our assumptions, the results show clearly that there is an obligatory release of 2 protons in the pre-oxygen evolving steps and 2 protons in the terminal step. The constancy of the stoichiometry of proton release ($2H^+/e^-$) associated with the terminal step for all the preflash regimes, even though 3 charge accumulation steps have occurred, might suggest that S_3 exists at the peroxy level. The 3rd charge would combine with that made in the terminal flash to carry out

the final oxidation steps and the ultimate release of O_2 and 2 H^+. However, one must be cautious in interpreting these results on mechanistic grounds because of the inability to distinguish between release of H^+ or uptake of OH^-. The following alternative schemes demonstrate this complexity. For example, as an alternative to the peroxy compound, S_3 might be a superoxide. It would be oxidized on the final step to O_2 followed by the uptake of 2 hydroxyls, i.e., two OH^- would always be associated with S_0. Another possible sequence of events would include the active uptake of OH^- on the S_0 to S_1 and S_2 to S_3 transitions followed by the release of 2 H^+ on the terminal step. No water or OH^- would be associated with S_0. On the other hand, S_0 might contain 2 water molecules and protons would be released during the S_0 to S_1, S_2 to S_3 transitions and the terminal step. These water molecules could either be freely exchangeable or be taken up only during the S_3 to S_0 transitions.

The evolution of 2 H^+ in 3 steps leaves one charge unstabilized. Since O_2 evolution shows a strict requirement for one of the anions chloride, bromide or iodide, [8] but none for cations, this might require the uptake of an anion or the release of a cation, other than a H^+. According to the model, this would be likely to occur on the S_1 to S_2 transition.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Energy Research and Development Administration Contract E(11-1)-3326. This material is also based upon research supported by the National Science Foundation under Grant No. PCM74-20736. Appreciation is also expressed to Dr. Bessel Kok for helpful discussions during the preparation of this manuscript.

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Table I

Values of the four coefficients, reflecting the number of protons released in the four S state conversions calculated from the 3 experiments in Fig. 3b (0, 1, 3 pF) and Fig. 4 (2 pF, 1+2 pF).

Coefficient	0 pF	3 pF	1 pF	Ave	2 pF	1+2 pF
a	.70	0.78	0.78	0.75	0.76	0.80
b	.10	0.00	0.00	0.03	0.00	0.00
c	1.40	1.27	1.28	1.32	1.50	1.70
d	2.00	2.00	1.80	1.93	1.90	1.90
Σ	4.2	4.05	3.86	4.04	4.16	4.40

Table II. A comparison of flashyields of proton liberation measured experimentally (see also Fig. 3b) with values obtained by calculation. Data obtained for a variety of models and initial conditions are included. Y_n (obs) = observed flash yields (Fig. 3b and Fig. 4), Y_n (1, 01, 2)≡ flash yields obtained according to the Kok/Forbush model[6, 7] and for coefficients a=1, b =0, c=1 and d=2, Y_n (.5, 0, 1.5, 2) ≡ same except different coefficients, (1) Y_n (cal) ≡ flash yields calculated using a=0.75, b=0, c=1.33 and d=1.93, (2) Y_n (cal) ≡ flash yields calculated according to two pathway models. Yields are 0.9 that shown in all figures due to inclusion of 10% miss factor in calculations. Other details can be found in text.

Flash No.	0 pF Y_n(obs)	Y_n(1, 0, 1, 2)	Y_n(.5, 0, 1.5, 2)	(1) Y_n(cal)	(2) Y_n (cal)
1	0.19	0.23	.11	0.19	0.17
2	0.84	0.63	.92	0.83	0.86
3	1.45	1.4	1.56	1.43	1.49
4	1.05	1.23	1.04	1.09	1.11
5	0.64	.64	.40	0.47	0.46
6	0.76	.56	.70	0.63	0.63
7	1.25	1.11	1.27	1.16	1.16

	3 pF			1 pF		
	Y_n(obs)	(1) Y_n(cal)	(2) Y_n(cal)	Y_n(obs)	(1) Y_n(cal	(2) Y_n(cal)
1	.35	.35	.37	0.00	0.02	0.02
2	.55	.57	.61	1.04	1.06	1.03
3	1.32	1.29	1.27	1.55	1.60	1.64
4	1.28	1.24	1.34	0.94	0.94	0.98
5	0.68	0.64	0.68	0.48	0.30	0.31
6	0.56	0.57	0.57	0.86	0.76	0.75
7	1.20	1.04	1.05	1.33	1.34	1.34

	2 pF			1 + 2 pF	
	Y_n(obs)	(2) Y_n(cal)		Y_n(obs)	(2) Y_n(cal)
1	0.0	0.0		0.03	0.0
2	1.20	1.23		1.40	1.30
3	1.60	1.70		1.60	1.72
4	0.95	0.95		1.0	.99
5	0.47	0.27		.45	.26
6	0.85	0.75		.83	.75
7	1.30	1.34		1.35	1.34

Table III

Values obtained for S_1^1 and S_1^2 and S_0 in the dark following described preflash regimes. Model shown in Fig. 5 was used for calculations. * Deactivation distribution was obtained by assuming the ratio of $K_3^{(2)} / K_3^{(1)} = 2$. CFD = calculated from data. See text for details.

	0 pF		-3 pF		1 pF		2 pF		1+2 pF	
Dark State	CFD	*Deact	CFD	*Deact	CFD	*Deact	CFD	*Deact	CFD	*Deact
S_1^1	47	45	48	48	67	64	48	48	41	41
S_1^2	29	30	52	52	30	33	52	52	59	59
S_0	25	25	0	0	3	3	0	0	0	0

Legends

Fig. 1. Flash induced pH changes in spinach chloroplast plotted as a function of flash number. Chloroplasts were dark adapted 5' prior to the initiation of each flash sequence.

(a) Sample contained gramicidin, 3×10^{-6}M and Ferricyanide (1.0 mM).

(b) Chloroplasts were osmotically shocked prior to measurement. Sample contained only ferricyanide (1.0 mM).

(c) Sample contained only 3×10^{-6}M gramicidin d.

(d) Binary proton exchange associated with electron pairing in the chain connecting the photosystems.

(e) O_2 flash yield pattern obtained after 5' dark adaption.

Fig. 2. Flash-Induced pH changes in spinach chloroplasts following pre-illumination with far-red (720 nM interference filter) and 5' dark. Sample contained no acceptor and 3×10^{-6}M gramicidin. Dashed line is fig. 1C replotted for comparison.

Fig. 3. Flash-induced pH changes obtained in spinach chloroplast following 3, 1 and no pre-flashes. A 5' dark period preceded and succeeded each pre-flash regime. Sample 2A contained 3×10^{-6}M gramicidin d and 1.0 mM ferricyanide and 2B contained gramicidin and no acceptor. ● No preflash ○ 1 preflash, ◆ 3 preflashes. The right hand ordinate was used for numerical computation.

Fig. 4. Flash-induced pH changes obtained in spinach chloroplasts following 1, 2 and 1+ 2 pF regimes. Procedures are identical to that of Fig. 2.

● 1 preflash

▲ 2 preflashes

□ 1 PF + 5' dark + 2 PF

Fig. 5. A model for proton evolution associated with O_2 evolution.

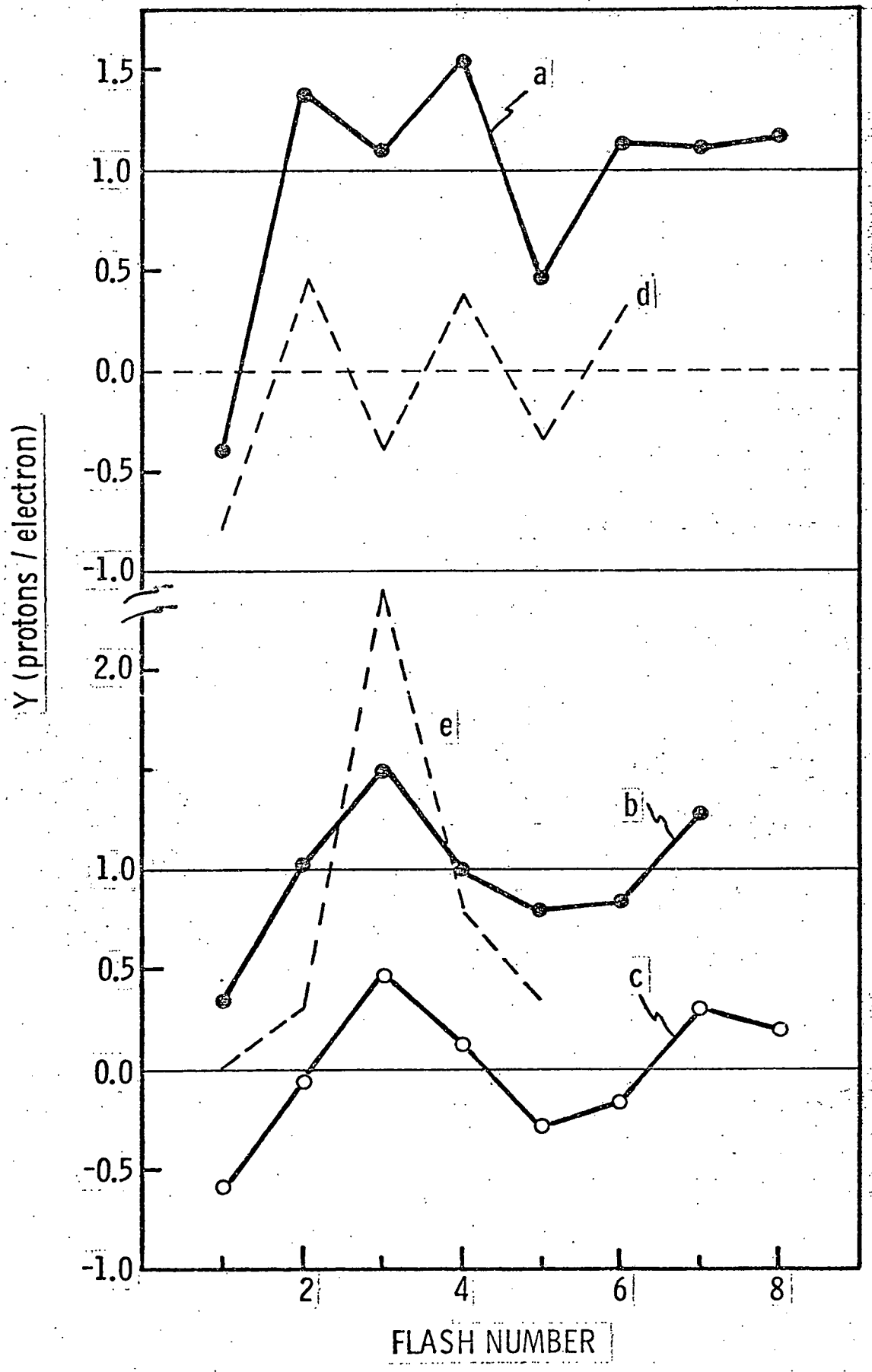
1.5
1.0
0.5
0.0
-0.5
-1.0
a
d
2.0
e
b
1.0
0.5
c
0.0
-0.5
-1.0
2
4
6
8
Y (protons / electron)
FLASH NUMBER

Fig. 1

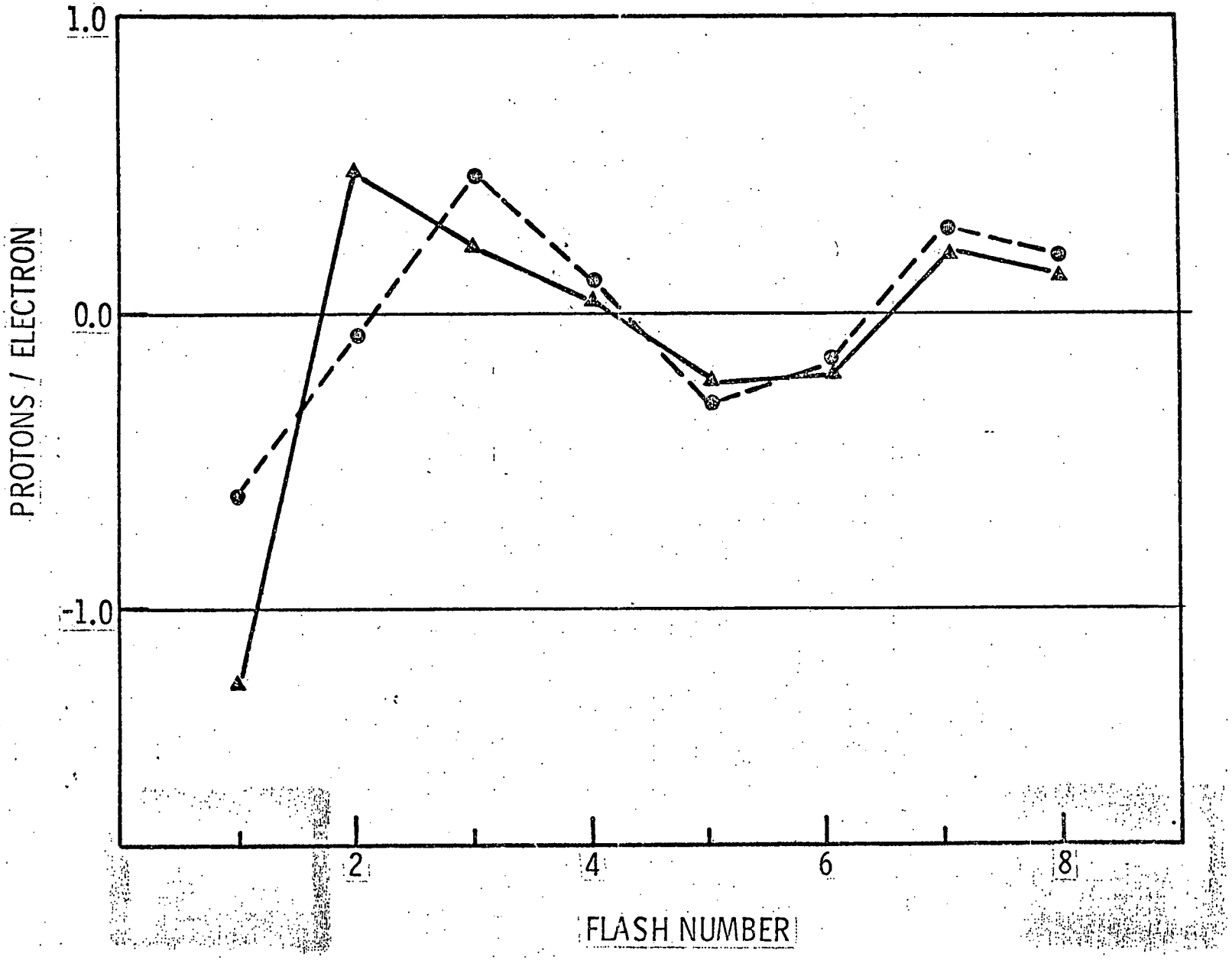

Fig. 2

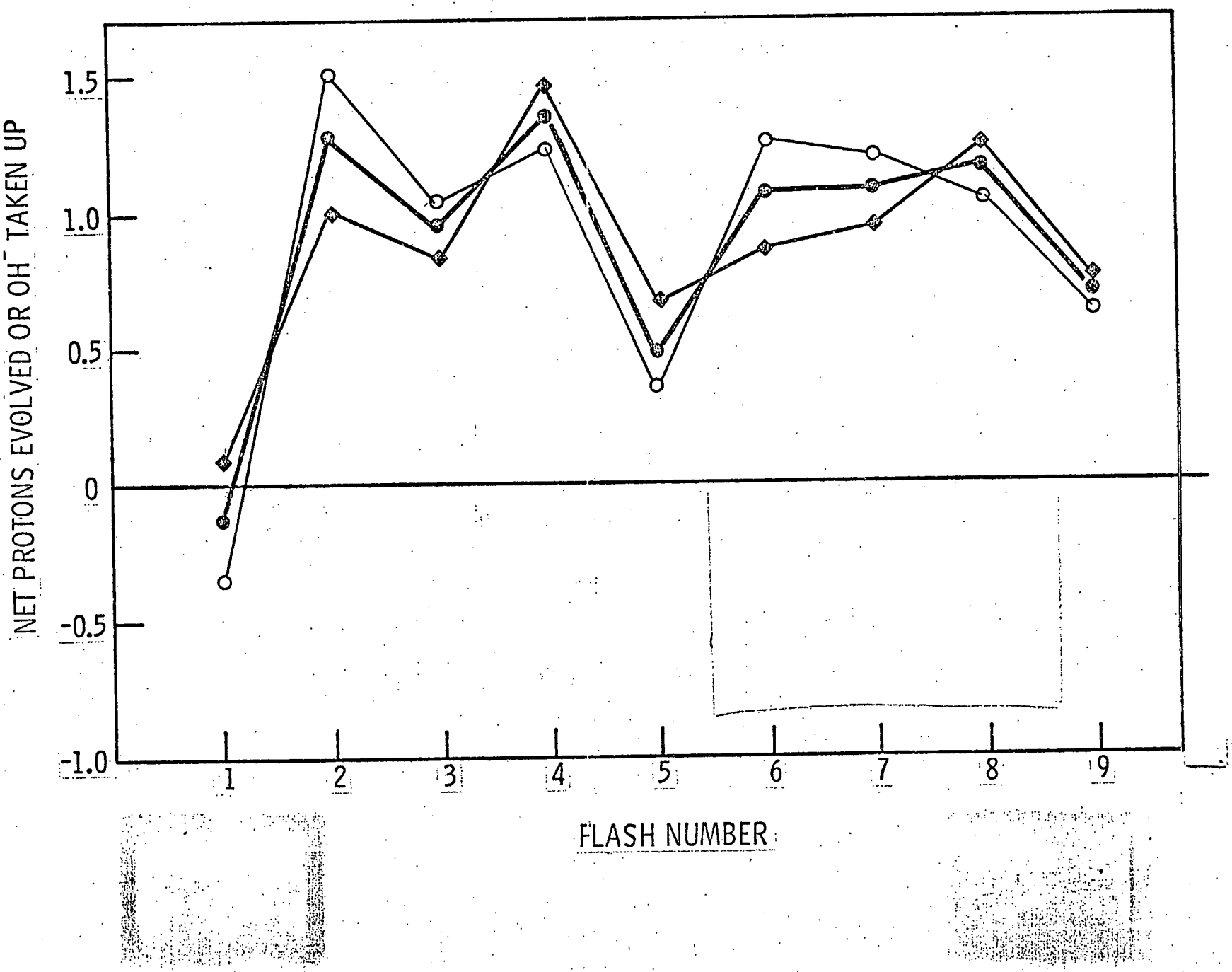
NET PROTONS EVOLVED OR OH⁻ TAKEN UP
1.5
1.0
0.5
0
-0.5
-1.0
1
2
3
4
5
6
7
8
9
FLASH NUMBER

Fig. 3a

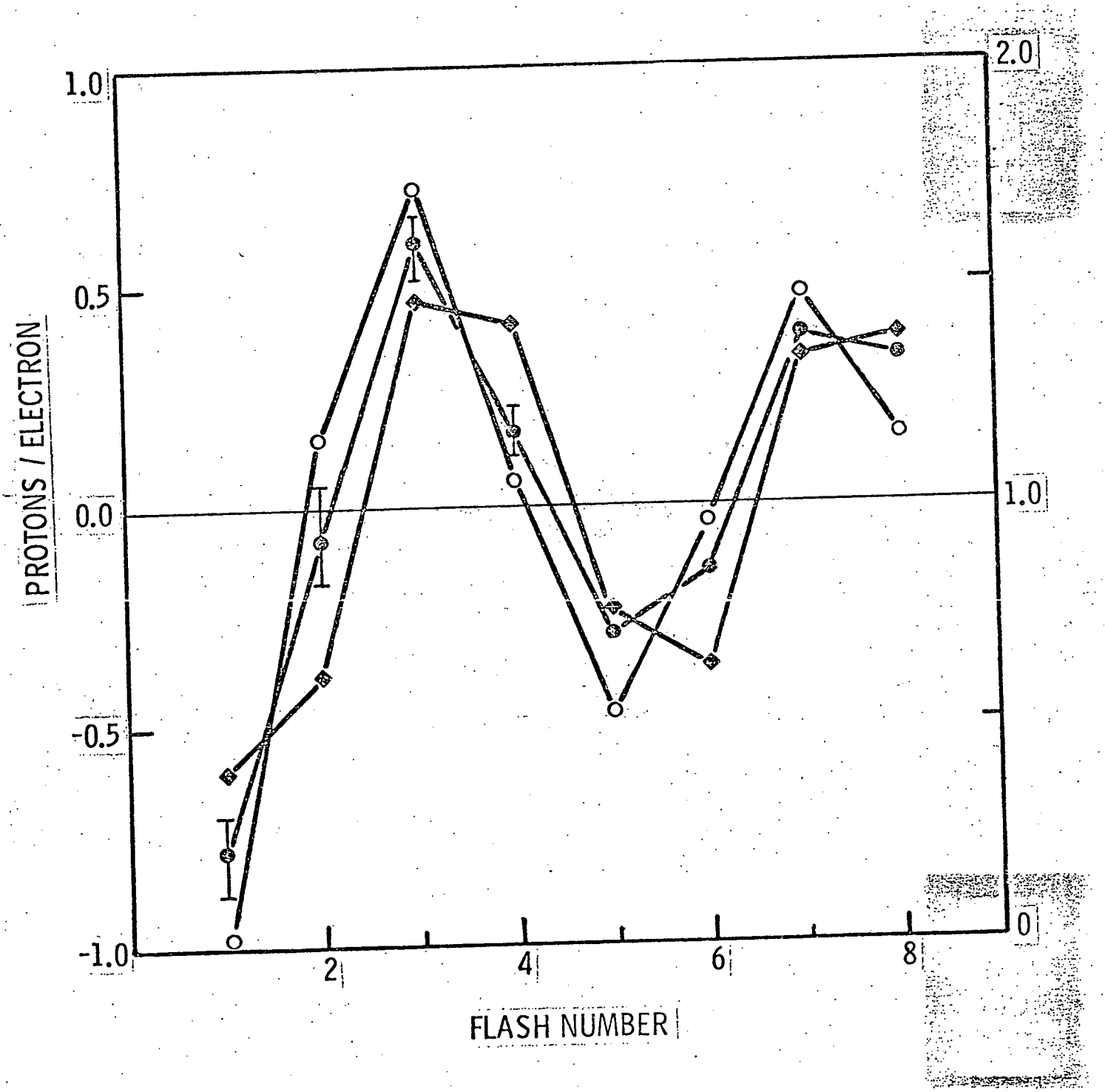

Fig. 3b

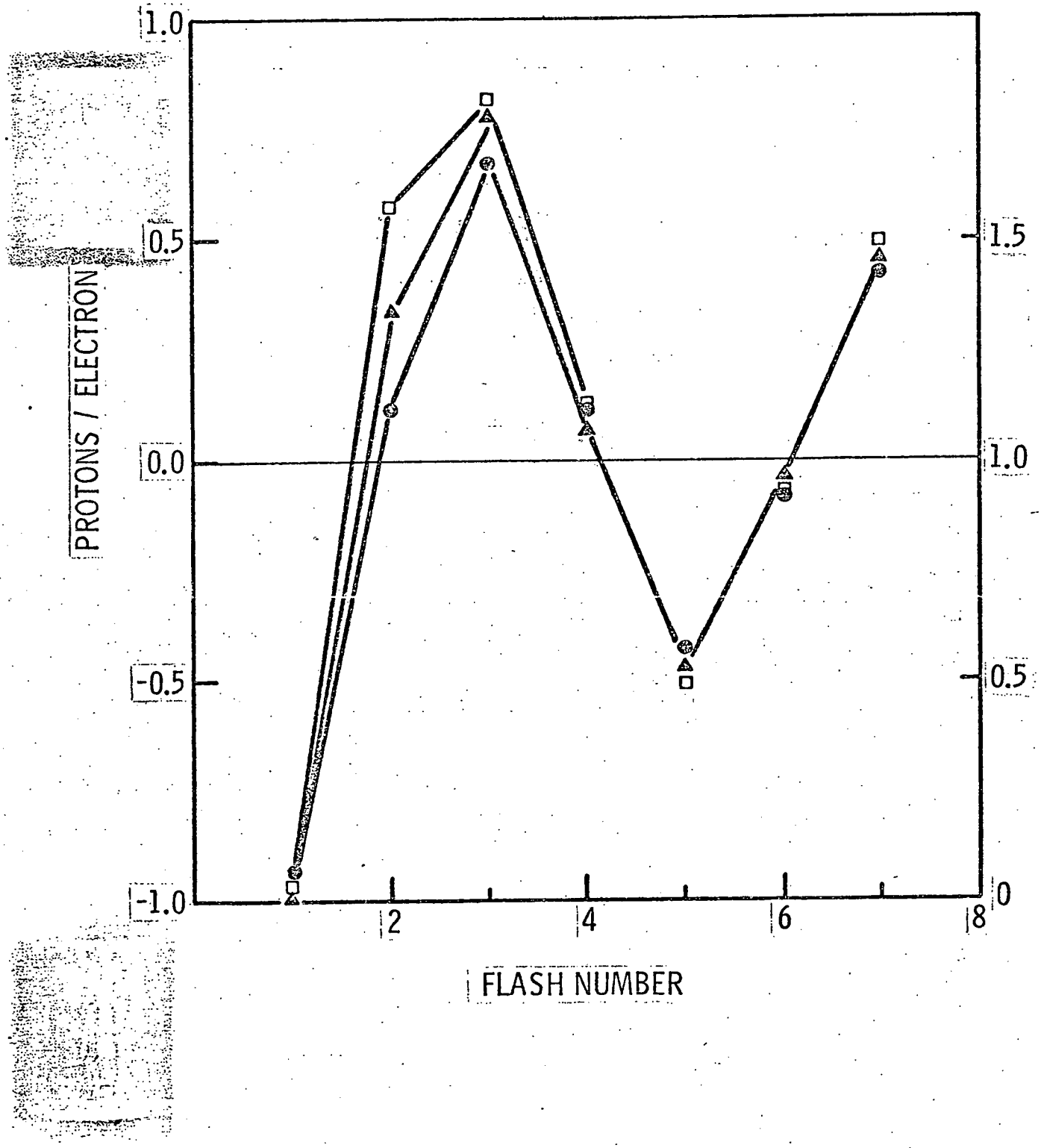

Fig. 4

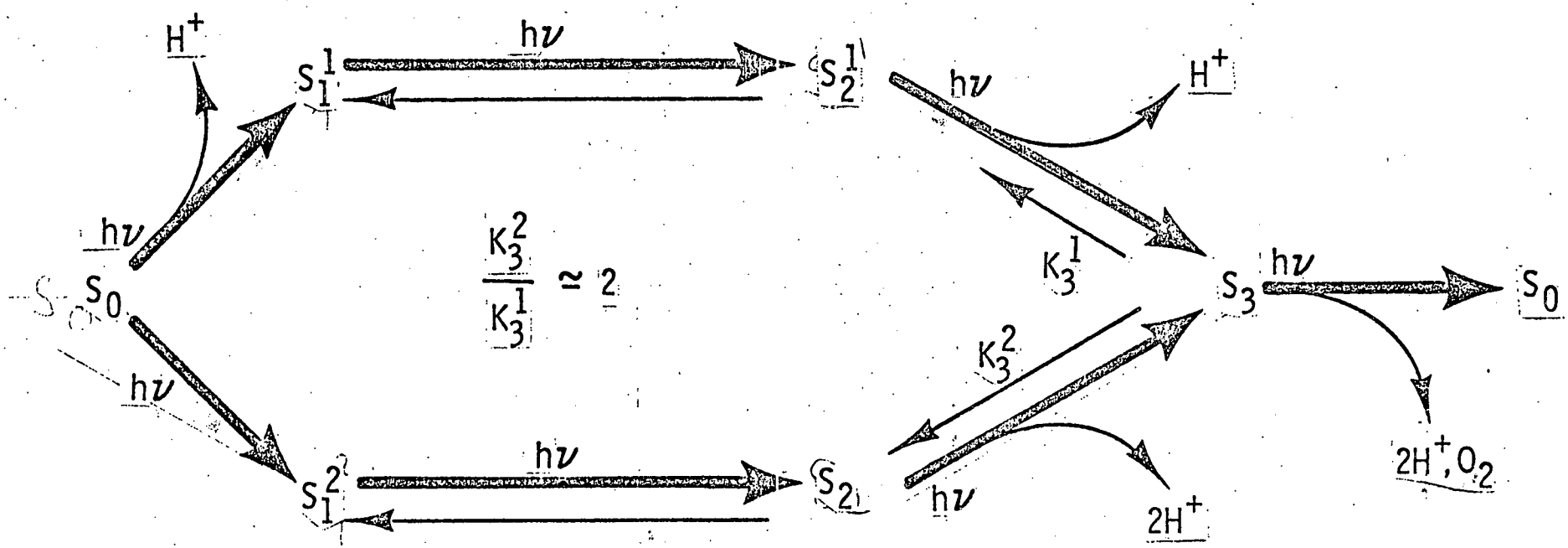
H+
hν
S0
S1 1
hν
S2 1
hν
H+
K3 1
K3 2 / K3 1 ≃ 2
S3
hν
S0
hν
K3 2
S1 2
hν
S2
hν
2H+
2H+, O2

Fig. 5