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SUMMARY

Electron abstraction from water by direct photoelectrochemical conversion of visible light is technically perhaps the most challenging performance of green plant photosynthesis. The plant uses the abstracted electrons to produce the strong reductants which power life including technical civilization. Photosynthetic water oxidation occurs in a manganese containing protein complex, and it is driven by a special photochemical reaction center (PSII). The function of the manganese containing protein is threefold: 1.) It receives and accumulates single oxidizing equivalents from the photochemical reaction center; 2.) It levels the different energy demands of the four successive oxidation steps between water and dioxygen. 3.) It traps radical intermediates. Understanding of the catalytic mechanism requires knowledge of the protein structure, in particular the structure of the manganese-water adduct, knowledge of the redox states of manganese and of those of complexed water.

This report is devoted to the protolytic reactions which accompany electron abstraction from water and which were expected to give a clue to the intermediate stages of stepwise catalysis. Broken chloroplasts from higher plants were studied. A spectrophotometric technique to measure proton release into the narrow (20nm) inner phase of thylakoids was perfected for very high sensitivity (10^{-3} pH-units) at high time resolution (1μs) and with regard to spacial resolution. Starting from the lowest oxidation state of the manganese protein the following stoichiometric pattern of proton release per electron abstracted was observed (half-rise-time in parenthesis) 1(200μs) : 0 : 1(250μs) : 2(1.2ms). We furthermore determined the stoichiometry and the kinetics of proton release in the presence of hydroxylamine, which is processed as a water analogue. Based on this information we define restrictions for possible mechanisms of water oxidation. However, the exact mechanism still remains obscure, mainly be-

cause of insufficient knowledge on the state of manganese in the enzyme. Further work will be directed to probe the enzyme with several different water analogues. In comparison with other techniques the approach of this work offers the highest time resolution for such studies.

1. INTRODUCTION

The molecular mechanism of water oxidation by green plants is still a challenging research object. It has been investigated by polarographic techniques for measuring oxygen evolution, by biochemical assay for the polypeptides involved and by spectroscopic techniques designed to follow the path of electrons and protons and to disclose the chemical nature of the intermediate carriers. In the introduction we review the current knowledge on other aspects of water oxidation except the protolytic reactions. These, the main subject of this research, are treated in the following chapters.

In Figure 1 we describe the components which are involved in water oxidation and we speculate about their mutual arrangement. Water oxidation is driven by a photochemical reaction center (photosystem II or PSII) which is built into a polypeptide with 47kD molecular mass (see e.g. Ref.1). A chlorophyll-a molecule (P680) acts as the primary electron donor (2) and a pheophytin-a molecule (PHEO)(3) serves as intermediate electron carrier to a bound plastoquinone (Q_A)(4). The 47kD polypeptide is flanked by two other, probably also transmembrane polypeptides at 41kD and 32kD, which form commonly a binding site for herbicides (79). Light absorption by antennae pigments promotes a very rapid electron transfer from P680 to Q_A (review in (5)), which within less than 200ps crosses the full span of the thylakoid membrane (6) from inside to the outside. The intermediate carrier PHEO seems to be located close to the inner surface of the membrane and close to P680 as judged by fluorescence (7), by triplet-ESR (8) and by capacitive electrodes (9). Oxidized P680 has a midpoint potential of 1.1V (10) which is sufficient to oxidize water. It has been found that the immediate electron donor to $P680^+$, named Z, is embedded in the 47kD reaction center protein. Z was detected via the ESR signals of Z^+ (signals IIf and IIvf, (11)(12)) and by absorption changes in the near UV (13). The reduction of $P680^+$

by Z is complex and rapid. The fastest kinetic component has a half rise time of 30ns (14)(15)(80). The appearence of slower components depends on the redox state of the water-oxidizing enzyme (20ns, 50ns, 300ns, (16)). The spectroscopic properties of Z by ESR (17)(18) and by UV-absorption (13)(19) suggest that Z is a bound quinone molecule. It has been argued that quinones are sufficiently electropositive to oxidize water only if they underwent the transition: $QH_2 \text{--} QH_2^+$ (18). This implied that the redox transients of Z would not be accompanied by protolytic reactions. In modified preparations which lacked the water-oxidizing capacity the pH-dependence of the reaction $Z,P680^+ \longrightarrow Z^+,P680$ was investigated. A very slight dependence of the half rise time over a wide pH-range (20,21,46) was found (factor of 2 in the rise per pH unit). This cannot originate from a univalent deprotonation during oxidation of Z.

Oxidized Z drives the oxidation of water: $2H_2O \longrightarrow 4e^- + 4H^+ + O_2$. From polarographic experiments on oxygen evolution following a series of short saturating flashes it became evident that after a few minutes storage in the dark input of three quanta of light, which caused abstraction of 3 e^-, was needed to produce dioxygen (22). Based on these experiments Joliot and Kok (23) introduced a formalism to describe the cycling of the water-oxidizing enzyme through the redox states: The water-oxidizing enzyme can assume a basic state S_0 and four higher oxidation states (S_1, S_2, S_3, S_4). With state S_1 beeing the most stable one in the dark oxygen evolution is maximum after the third flash. Allowing several cycles of the enzyme one observed a damped oscillation of oxygen evolution with flash number. This was explained by double-hits and misses of the photochemical reaction.

Manganese is an essential component of the water-oxidizing enzyme (24)(28) (reviews in (25)(26)). Treatments which block oxygen evolution are accompanied by differential loss of manganese from the membrane. The functioning of the enzyme appeared to require four manganese atoms (28)(29). Recent work suggests that only two

of them are indispensible, whereas the two others can be replaced by other divalent cations (27)(81)(82). Several models for the catalytic center have been designed. Common to these models is an at least binuclear complex between manganese atoms and oxygens (30)(31)(32). By analogy with cytochrome-c-oxidase which catalyses the reversal of water oxidation it is likely that bound oxygen exists only in the forms: 2 water, peroxide and dioxygen, which differ by two electrons, respectively. Intermediate oxidizing equivalents likely reside on manganese, instead of forming OH radicals. By ESR-measurements it was found that in S_2 the essential manganese exists in a state of mixed valency (Mn^{3+} and Mn^{4+}) (35,36,37). However, the multiline ESR spectrum associated with oxidation state S2 can also be explained by a Mn^{2+}/Mn^{3+} binuclear complex (38)(69)(for model compounts, see Ref.33). XAES measurements confirmed an average oxidation state of functional manganese higher than two (34).

The catalytic site with tightly bound manganese seems to be embedded in a 34kD polypeptide with an isoelectric point at 5.2 (39) (which obviously is identical to that characterized in (40)). Three further polypeptides are also involved, 18kD, 24kD and 33kD. These can be removed by high pH or under high monovalent salt without that the essential manganese is lost (39)(41). Their removal inhibited oxygen evolution. Readdition of either of the larger two partially restored oxygen evolution (33kD: by less than 30% (42) and 24kD: by about 50% (43)(44)). The 18kD polypeptide could be rebound only in the presence of the 24kD one. It appears as if these polypeptides were not directly involved in catalysis but they played a structurally stabilizing role. Another effect of their removal was the slowing of the electron transfer between Z and $P680^+$ by three orders of magnitude (20)(21)(45)(46).

A rigorous understanding of the molecular mechanism of water oxidation requires knowledge of the three-dimensional structure of the manganese-water complex, a record of the oxidation states of manganese and a record of the oxidation and protonation states of bound water. This report is directed to the protonation-deprotonation reactions of the water-oxidizing enzyme. Possible candidates for protolytic reactions are bound water and its oxidation products, the intermediate carrier Z and amino acid side chains of the embedding proteins. A discrimination between these sources for proton release requires high kinetic resolution of pH changes in thylakoids. This has been the objective of this research project.

2. PROTOLYTIC REACTIONS DURING WATER OXIDATION

2.1.1 Proton Release during water oxidation - Survey of the literature

During oxidation of two molecules of water to one molecule of dioxygen four electrons have to be abstracted from water. In green plants this occurs stepwise, driven by the photosensitized charge separation in PSII. The charge separation leads to electron abstraction from the water-oxidizing complex. The redox behaviour of the water-oxidizing enzyme is described by the S state formalism (for a review see Joliot and Kok (23)). Kok et al. (22) excited chloroplasts which had been dark-adapted for about 10 minutes with a series of short saturating flashes and found damped oscillatory yields of oxygen evolution with maxima on the 3rd, 7th, 11th, 15th... flash. They explained these results by the following scheme: The water-oxidizing enzyme cycled through the successive oxidation states S_0, S_1, S_2, S_3, (S_4). When the state S_4 was reached the system decomposed to S_0 and dioxygen in the dark. The oscillation was explained by relaxation of the states S_2 and S_3 to S_1 in the dark, the damping was explained by double hits (abstraction of two electrons upon one flash) and misses (no reaction) (47). Later it was found that the percentage of double hits depended on the flash duration (48). While flashes of e.g. 15µs FWHM caused about 10% double hits flashes of less than 300ns FWHM caused less than 2% double hits (48). Misses were independent of flash duration. Homogeneous as well as inhomogeneous distribution of double hits and misses during the S state transitions has been assumed by different investigators (23,49). Bouges-Bocquet showed that the dark population among the S states could be shifted between the extremes 100%S_0 and 100%S_1 by reductants and oxidants, respectively (50). In the hands of Kok and Velthuys (65), however, the dark equilibrium in the presence of the oxidant hexacyanoferrat(III) was hardly predictable.

The phenomenon of oscillatory oxygen evolution has been confirmed manifold during the last fourteen years . Although its discovery appeared to be a breakthrough at its time it gave us only a key how to plan further experiments by which the water-oxidation mechanism should be unravelled. Dark adaptation opened a way to puzzle out the successive reaction processes via experimental techniques other than polarographic oxygen measurements.
It was interesting to look for proton release which should accompany oxygen evolution. A first attempt to determine the stoichiometry of the water protons was made by Fowler and Kok in 1974 (83) who found simultaneous release of O_2 and $4H^+$ after the third flash in a row. This could not be confirmed. Instead, it became evident that some protons were released during transitions between the lower oxidation levels. This finding is common to all further studies which have been made on the proton release during the oxidation steps. However, the final answer as to the stoichiometric pattern has still not been given. In Table I the proton release stoichiometries are listed as obtained by different research groups. (Note that "experimentally" under the most commonly used redox conditions the water-oxidation cycle starts in S_1, which is most stable in the dark, whereas the stoichiometric pattern of proton release is usually referred to the cycle starting in S_0.)

The proton release stoichiometries under debate are 1:0:1:2, 0:1:1:2 and 1:1:1:1. Even experiments done with the same or similar techniques yielded different results.
In 1977 Fowler (51,84) measured the pH changes of a chloroplast suspension which was submitted to a series of flashes. He discriminated between protolytic reactions at the inner side and those at the outer side of the thylakoid membrane by use of uncoupler; either protolytic reactions at the outer side (-uncoupler) of the net pH changes (+uncoupler) could be indicated. In osmotically shocked chloroplasts, in the presence of 1mM

AUTHORS	EXPERIMENTAL TECHNIQUE	H^+ RELEASE STOICHIOMETRY
FOWLER (1977)	GLASS ELECTRODE	0.75 : 0 : 1.25 : 2
JUNGE ET AL. (1977)	NEUTRAL RED	0 : 1 : 1 : 2
SAPHON & CROFTS (1977)	NEUTRAL RED	1 : 0 : 1 : 2
BOWES & CROFTS (1978)	FLUORESCENCE	1 : 0 : 1 : 2
HOPE & MORLAND (1979)	NEUTRAL RED	(BETWEEN 0:1:1:2 AND 1:1:1:1)
VELTHUYS (1980)	NEUTRAL RED	1 : 0 : 1 : 2
FÖRSTER ET AL. (1981)	NEUTRAL RED	"VARIABLE" BETWEEN 0:1:1:2 AND 1:0:1:2
WILLE & LAVERGNE (1982)	EPR	1 : 0 : 1 : 2
SCHATZ (1983)	pH ELECTRODE	0:1:1:2 OR 1:1:1:1
FÖRSTER & JUNGE (1984)	NEUTRAL RED	1 : 0 : 1 : 2

TABLE I

hexacyanoferrat(III), he observed a four-flash oscillatory behaviour as it was expected for the water-oxidation cycle. This pattern could be explained by a 1:0:1:2 stoichiometry. However, according to the experiences of Junge and coworkers (85) it could not be expected, as assumed by Fowler, that hexacyanoferrat(III) accepted electrons only from PSII in this experiment. The absorption transients of P700 were not monitored at high time resolution which would have been necessary to estimate the amount of proton release which was expected due to PSI reduction upon the respective flashes.

At the same time Junge et al.(52) published a proton release pattern which was measured with the neutral red technique (87)(88) under conditions when plastohydroquinone oxidation was inhibited by DBMIB. ADRY reagents were used in order to accelerate dark adaptation (86). About equal proton yields were observed from the

very first flash on. The weak oscillation had to be interpreted by a 0:1:1:2 stoichiometry. Probably the proton yield upon the higher-numbered flashes could not be fully visualized in this experiment for the flash distance was too short (10ms) to allow quantitative electron acceptance at the reducing side of PSII within the dark times between the flashes (64).

Also with the neutral red technique Saphon et Crofts (53) obtained a pattern of internal proton release which could be explained by a superposition of oscillations with periods of 2 and of 4. The latter seemed to support the 1:0:1:2 stoichiometry. However, the same argument as to Fowlers work applied as to the contribution of PSI-drawn proton release. No control measurements had been made on P700 oxidation and on oxygen evolution. Thus, the redox state of P700 and of the water-oxidizing enzyme in the dark were not defined. In a review article Saphon and Crofts (78) described the effect of DBMIB on the proton release pattern (documents not shown by these authors). The pattern wich was obtained with 40μM DBMIB was best fitted with the 1:0:1:2 stoichiometry. It remained obscure why the proton yield upon the first flash accounted for more than 60% of the average yield ($1H^+$) while no proton should be released during S_1-S_2, the main transition upon the first flash from the dark.

It had been found by Wraight and Crofts (90) that delayed fluorescence from PSII in the presence of DCMU was stimulated a pH difference across the thylakoid membrane. Bowes and Crofts (54) reversely used this feature in order to study internal proton release. On this rather indirect way they obtained a marked oscillatory pattern which supported the 1:0:1:2 stoichiometry.

A study by Hope and Morland (55) has stirred up the debate about the "water protons" again with an "intermediate" stoichiometry between 0:1:1:2 and 1:1:1:1 but this result has been withdrawn last year.

Ausländer and Junge (75) had discriminated two fractions of internal proton release under repetitive excitation of chloroplasts. Time-resolved measurements of the pH_{in}-indicating absorption changes of neutral red (75)(87)(88) had revealed that roughly half of the protons were released within 1-2ms, the other half was released with about 20ms half rise time. When the plastoquinone antagonist DBMIB was present only the rapid phase was observed. Ausländer and Junge therefore attributed the rapid phase to proton release by PSII and the slow phase to proton release due to plastohydroquinone reoxidation. Based on this work Velthuys (56) found that the rapid fraction behaved according to the 1:0:1:2 stoichiometry.

In Förster et al.(57) we attempted to further discriminate the rapid fraction of internal proton release. It was expected that protons which were produced during the respective S-state transitions could be distinguished kinetically. DBMIB was used to suppress plastohydroquinone oxidation. The results were the following: Surprisingly, different proton yields upon the first flash were obtained when the chloroplasts were dark adapted for 10-30min, the time which is usually required for dark adaptation, or when the measuring suspension was prepared entirely in the dark and filled directly into the cuvette. (In the latter case chloroplasts had been frozen under room light and stored under liquid nitrogen for some weeks "seeing" no light at all before they were flashed four times in the measuring cuvette.) The different proton patterns, which we obtained under these conditions, supported the 0:1:1:2 and the 1:0:1:2 stoichiometry, respectively. High time resolution (20μs/point) under the latter conditions revealed that the half times of proton release widely matched those of electron abstraction from the water-oxidizing enzyme via the intermediate carrier Z (12), i.e. electron transport appeared rate limiting for proton release. However, a biphasic rise upon the second flash appeared contradictory. (We shall discuss this point later in the light of our present results.)

Wille and Lavergne (58) applied the pH-sensitive EPR probe tempamine to solve the problem of PS II proton release. The stoichiometry 1:0:1:2 best explains their results. However, pH transients from the dark have not been time resolved (instrumental time constant 50ms).
In a recent study by Schatz (59), which has been done on PSII preparations from a thermophilic cyanobacterium with a pH electrode, again a stoichiometry between 0:1:1:2 and 1:1:1:1 was found.
In this report we present the continuation of the study of Förster et al.(57). We have clarified the peculiar findings with respect to the proton release stoichiometry and first present both, stoichiometric and kinetic evidence clearly in favour of the 1:0:1:2 stoichiometry.

2.1.2 Literature on the Interference of Hydroxylamine with Water-Oxidation

Hydroxylamine (NH_2OH) in millimolar concentrations is one of the classical inactivators of the water-oxidizing enzyme (66)(67). In micromolar concentrations ($\leq$100μM), however, hydroxylamine interacts reversibly with the water-oxidizing enzyme, apparently as a competitive substrate (68)(50)(70). At hydroxylamine concentrations above 100μM the oxygen evolution capability gets lost, probably with concomitant changes in the Mn catalyst (67). In the dark a Mn(II) which is not hexacoordinated by water becomes NMR visible via enhancement of the spin-lattice relaxation rate of solvent protons (67).

Bouges (68) measured the oxygen evolution pattern in the presence of low concentrations of hydroxylamine. She found that the typical pattern suffered a shift by two flashes, i.e. the maxima of oxygen evolution appeared on the 5th, 9th, 13th,... flash instead on the 3rd, 7th, 11th,... flash in the absence of hydroxylamine. This effect could not be reversed by washing (40min) solely, but the system could return to the normal dark situation when it was washed and then excited only once (68). Bouges explained her results by the following model: NH_2OH reduced S_1 to S_0 in the dark. One molecule of NH_2OH bound to S_0 in the dark which was then oxidized and displaced by water upon the first flash. The water-oxidation cycle then ran normally, starting with S_0-S_1,... The first maximum of oxygen evolution was thus observed upon the 5th flash.

Later, Bouges-Bocquet (50) proposed another model based on an experiment with the following rationales: A reasonable flash distance in a polarographic oxygen pattern experiment is 300ms, which allows a complete turnover within the dark interval. When a distinct dark interval is shortened down to the range of the half time of the rate-limiting step in the electron transport chain this has consequences on the further oxygen yields. Bouges-Bocquet assumed the S-state transitions to be the rate-limiting steps in PSII. From the "deformation" of the oxygen pattern upon varying a distinct dark interval she concluded as to the half time of the respective S-state transition which was expected to occur during that dark interval. Measurements in the absence and in the presence of hydroxylamine lead her to an alternative model of the interaction of hydroxylamine with the water-oxidizing enzyme: Two molecules of NH_2OH bound to S_1 in the dark, which were oxidized upon the first two flashes. However, the rationales of this study have faded for it has turned out that electron transport at the acceptor side of PSII is rate limiting at least when the water-oxidizing enzyme is in S_0, S_1 (and S_2?) (compare (12),(91) with (92),(93)).

Radmer (71) investigated the oxidation products of NH_2OH by mass spectroscopy. When water oxidation was inactivated and when mM hydroxylamine served as electron donor to PSII he found nitrogen (N_2) to be the only stable oxidation product, but no nitrogen oxides, which were common oxidation products of hydroxylamine (71). In a similar experiment Radmer and Ollinger (72) found N_2 to be the oxidation product of hydrazine (NH_2NH_2). The N-N bond was conserved during oxidation (72).

Radmer and Ollinger also searched for nitrogen production from hydroxylamine in dark-adapted, water-oxidizing chloroplasts (70). A flash-series experiment revealed that, proportional to the NH_2OH concentration (in the range of reversible interaction), about equal amounts of N_2 were produced upon each flash plus an extra amount upon the first flash. This surplus nitrogen was assumed to be the product of NH_2OH oxidation at the water-oxidizing enzyme. This interpretation was in agreement with the model of Bouges (68). The concentration-dependent background nitrogen evolution was ascribed to an oxidation cycle acting in centers which were incapable to oxidize water.

The two-flash shift in the oxygen pattern has also been observed with hydrazine (NH_2NH_2) and hydrogen peroxide (H_2O_2), which have similar steric requirements as hydroxylamine (65)(73). Derivatives of hydroxylamine have been much less effective in competition with water, especially those with bulky substituents (74). Radmer and Ollinger (70) emphasized that the N-O bond length in hydroxylamine equals the double of the radius of oxygen in water (1.47×10^{-10}m). They suggested that two H_2O were replaced by one NH_2OH at the binuclear manganese center and that the sterical factor was the reason for the particular effectivity of hydroxylamine (94).

2.2 EXPERIMENTS ON PROTON RELEASE DURING WATER OXIDATION and INTERPRETATION OF THE PROTON RELEASE PATTERN - GENERAL PROBLEMS

Superposition of several protolytic reactions from different sites of the electron transport chain complicates selective detection of the "water protons". In fully functional thylakoids at least four sites of protolytic reactions can be distinguished:

1. proton release at the inner side during water oxidation;
2. proton uptake upon plastoquinone reduction at the outer side;
3. proton release during plastohydroquinone oxidation at the $Cytb_6/f$-complex at the inner side;
4. proton uptake upon reduction of the terminal electron acceptor at the outer side (,which is absent when the physiological acceptor NADP is replaced by an appropriate artificial electron acceptor.)

When cyclic electron transport is running via Photosystem I and the $Cytb_6/f$-Complex a further site of proton uptake was found (63).

The water protons can be worked out from the complexity of protolytic reactions by choosing a proper combination of the following ways:

1. Inhibition of electron transport at the sites of PQ reduction and/or PQH_2 oxidation; (52-55,57,58,60,85)
2. Use of a pH indicator (dye or other pH sensitive probe) which indicates selectively pH changes in the internal phase; (52,53,55-57,58,60)
3. Time resolution of the protolytic reactions and discrimination by kinetic properties; (53,56-58)
4. Separation of internal from external pH changes by use of uncouplers ($\pm$uncoupler); (51),(84)

5. Use of Photosystem II preparations, which have a reduced number of protolytic sites; (59)

The best indication that a signal is related to water oxidation is a pronounced oscillation with a period of four measured in a flash pattern from the dark. For the interpretation of any pattern it is necessary to know the dark state of the system. From the pronounced oscillation in the oxygen pattern one can conclude rather precisely to the values of dark population, double hits and misses. Thus, it would be desirable to have the oxygen pattern as a basis for the interpretation of the proton pattern, especially when only one run through the S states is examined in a four-flash experiment. In none of the studies listed in Table I, except the one by Junge et al.(52) oxygen and protons has been observed in parallel. Thus, the dark population of the S states has been uncertain. Usually a dark distribution of 25-30%S_0 and 70-75%S_1 has been assumed (see also section 2.1.1). However, a principal difficulty exists in a strict correlation of oxygen and proton release with the currently established measuring techniques. Polarographic measurements of oxygen evolution patterns require high chlorophyll concentrations (mM) whereas optical measurements of proton release, which are most powerfull with respect to time resolution, require low chlorophyll concentrations (10-20μM). Thus, it is desirable for a proton measurement to find a set of experimental conditions which 1.) allows the assumption that the majority of the centers are in a distinct S state in the dark and 2.) yields a pattern which allows a conclusive interpretation from the oscillatory behaviour.

2.3 MATERIALS AND METHODS

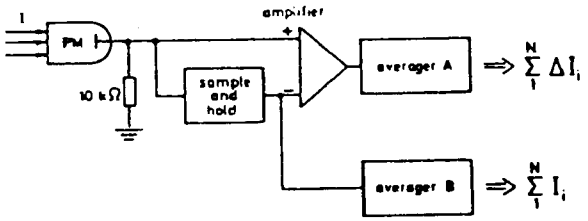
We have studied water oxidation under excitation of isolated chloroplasts by light flashes from a xenon lamp or from a Q-switched ruby laser. Photosynthetic activity was mainly followed via absorption changes in a very sensitive flash spectrophotometer. The preparation procedure for chloroplasts and details of the optical techniques will be descibed in the following.

2.3.1 Chloroplasts and Media

We prerared large quantities of chloroplasts according to the following protocol:
Broken chloroplasts were prepared from peas (pisum sativum) grown under artificial illumination by OSRAM 65W/77R Fluora lamps (16h light/8h dark regime). 2-3 weeks old seedlings were grinded in medium A (400mM sorbitol, 10mM NaCl, 4g/l bovine serum albumine and 50mM Mes, pH 6.5). The pulp was squeezed through two layers of nylon tissue (25µm mesh size) and centrifuged for 10min at 10,000xg. The pellet was resuspended in medium B (like medium A, but without sorbitol, in order to break the envelope) and centrifuged for 2min at 200xg. The supernatant was discarded and centrifuged for 10min at 10,000xg. For freezing in liquid nitrogen the pellet was homogenized in 100mM sorbitol, 25mM KCl and 20mM Tricine, pH 7.5. 30% ethylene glycol (v/v) served as cryoprotective.
Chloroplasts were thawed in absolute darkness, and transferred into an optically sealed reservoir which contained the standard medium: 25mM KCl, 3mM $MgCl_2$, 2.6g/l bovine serum albumin, ±13µM neutral red, pH 7.5, 21°C; further additions as indicated.

2.3.2 Sampling and averaging

We measured time-resolved absorption changes at 20, 200 or 2000μs per channel of the averaging computer (Tracor TN 1500). Signals of 50-300 samples were averaged. The same number of signals were taken with and without the indicator dye. The latter one was subtracted from the former one to yield the "absorption change of neutral red" as described previously (87). To accomplish averaging of such high numbers of dark-adapted samples, 0.5l of chloroplast suspension (20μM Chl) was prepared and pumped through an automatic flow system upon triggering. A peristaltic pump (Perifill) was used to deliver a new sample into the absorption cell before each flash group. After sampling, the flow-through cell (1x1x3cm, optical path- length 1cm) required a pulse of 7.5ml to be refilled again at 97 purity with dark-adapted material (tested optically). The whole arrangement was kept in total darkness until the measuring light was applied. The energy of the measuring light pulse (λ=548 nm for neutral red) was kept below 5μJ/cm^2 which excited less than 10% of the reaction centers. (This was calculated as well as experimentally verified by optical detection of the reduction of hexacyanoferrate(III) by chloroplasts exposed to different light intensities.) The detection system is outlined in the following scheme:



The photomultiplier (EMI 9558 QB with 10kΩ load and supplied via a Nucletron NU 1250 B) plus amplifier (Tektronix AM 502) required less than 20 ms in order to reach a sufficiently stable plateau. About 1ms before triggering the averaging computer, a sample-and-hold amplifier was activated which had two functions. The hold voltage was fed into the negative input of the differen-

tial amplifier to compensate for the d.c. voltage and fed via an A/D converter into a single-channel averager. Averaging of the d.c. voltages was particularly important, since measurements of pH changes with the neutral red technique required the subtraction of digitized transient voltage changes obtained in the absence from those obtained in the presence of the dye. That is why slow fluctuations of the measuring light and the photomultiplier sensitivy have to be compensated most accurately during the whole experiment. (Difference of the absorption changes are on the order of $\Delta I/I=10^{-4}$!) The absorption change is proportional to the voltage change divided by the d.c. voltage at the photomultiplier load:

$$2.3\Delta A = -\frac{\Delta I}{I} = \frac{\Delta U}{U}$$

If there are only slight fluctuations in the d.c. voltage the absorption change ΔA is given by:

$$2.3\Delta A \simeq \frac{\sum_{i=1}^{N} \Delta U_i}{\sum_{i=1}^{N} U_i}$$

and the pH_{in}-indicating absorption change of neutral red ($\pm$NR) by:

$$2.3\Delta A_{pH_{in}} = \frac{\sum \Delta U_i(+NR)}{\sum U_i(+NR)} - \frac{\sum \Delta U_i(-NR)}{\sum U_i(-NR)}$$

where N is the total number of averaged samples. Hence, we averaged both, the voltage changes ΔU_i and the d.c. voltages U_i.

Further details, especially those concerning the excitation light source, are given in the legends to the figures.

2.3.3 Critical Evaluation of the Method to Measure pH Transients in the Lumen

This research relied on spectrophotometric techniques to follow proton release, proton uptake and electrogenic events at the thylakoid membrane. These techniques have been reviewed elsewhere (98,99). Monitoring of the transmembrane electric field via electrochromism and of pH-changes in the external phase via hydrophilic pH-indicator dyes are generally accepted. However, the method by which we followed proton deposition into the narrow thylakoid lumen may require some further comments.
We used the hydrophobic pH-indicator neutralred (together with the non-permeating buffer bovine serum albumin to quench pH-transients in the external phase) in order to measure pH-transients in the thylakoid lumen as previously (75,87). For this technique we claimed high artefact rejection and a very high sensitivity (better than 0.001 pH-units) at high time resolution (20μs). Since these valuable properties are crucial for the present studies, we summarize the evidence.

Selectivity and Artefact Rejection

The sensitivity of neutral red for pH transients in the lumen of thylakoids is a result of its adsorption to the membrane. pH-indicating dyes like cresol red which are more hydrophilic than neutral red are distributed over the outer volume and the inner volume according to the volume ratio which is some 10^3 in a typical spectrophotometric experiment. In consequence hydrophilic indicators are much more sensitive to pH transients in the external phase than to others of equal magnitude which occur in the internal phase. On the other hand a membrane-bound indicator sees

pH transients at both sides; when pH-transients outside are quenched by a non-permeating buffer it becomes selective for the internal phase. For neutral red this was established earlier (75).

One crux with spectroscopic probes is that they may respond to various events and not only to the one they are designed for. Neutral red, for instance, is a redox agent with midpoint potential of -340mV (100). We checked for a possible non-pH-response of this dye when used with thylakoids. We found that there was no extra absorption change by neutral red in the presence of permeating buffer (87). Its response was artefact free.

Localization of the Probe

We found that neutral red was strongly adsorbed by thylakoid membranes. This has provoked the question whether it indicated pH transients in the aqueous phases adjacent to the membrane or pH transients occurring in some intra-membrane domains. We studied neutral red binding to thylakoids as a function of the medium pH and of the salt concentration and found the following (88): Neutral-red binding was greater at lower pH, and at a given pH it was higher at lower salt. The binding curves followed the theoretical expectation which was formulated under the following assumptions. At high salt, when the surface potential at the thylakoid membrane was screened, the pK of adsorbed neutral red was the same (6.6) as for neutral red in water. The distribution of neutral red between bulk water and the membrane depended in a predictable way on the surface pH at the membrane, which in turn was a function of the surface potential (88). Most interesting was the observation that the apparent pK of adsorbed neutral red was the same as the one of neutral red in water, only if the membrane was electrically neutralized. This was also found for neutral red adsorbed to micelles from neutral detergents (102). The same pK in both environments indicates that the dye resided at

the interface and not within the lipid core of the membrane. In other terms, its protonation does not require its transfer from the interior of the membrane to the interface. We concluded that neutral red was a true indicator of pH changes at the membrane surface (88). Under conditions where protons are rapidly exchanged between bulk water and groups at interfaces such an indicator is also sensitive for pH transients occurring in the bulk. For highly swollen thylakoids this seemed to be the case since we observed that the pH-indicating absorption changes of neutral red were quenched by extremely hydrophilic buffers as phosphate and pyrophosphate. Quenching by these and eight further, chemically and electrochemically very different buffers followed the theoretically expected behaviour and it was dependent on the lumenal volume (87).
In fresh thylakoids the control experiments for the surface location of the dye did not work. Neither were the absorption changes quenched by phosphate nor acted salts on the apparent pK of inside located neutral red (88). However, we found that the time course of the absorption changes of neutral red showed the same complex kinetic phases as it did in the above swollen material. This made us confident, that the dye indicated the same pH-transients in both types of chloroplasts.

On the Quantitative Competence of Neutral Red

Above we mentioned that neutral red binding could be fitted quantitatively with a theory on the distribution of weak acids between aqueous bulk and surface. We proved that the sensitivity of adsorbed dye to flash-induced pH transients behaved in the same way (88). This was used to calibrate the absorption changes of membrane-adsorbed neutral red quantitatively. We found that excitation of both types of reaction centers by one single-turnover flash induced an internal acidification of 0.06 pH-units in the absence of added permeant buffer. The same figure

came out from experiments where the intrinsic buffering capacity of the thylakoid lumen was determined via the influence of hydrophilic buffers on the absorption changes of neutral red (87). Thus, the quantitative competence of this probe was established. However, it was guaranteed only for small pH-transients. Larger changes which are induced if chloroplasts are excited with continuous light cause redistribution of neutral red and very probably also dimerization of the dye at the inner side of the membrane. This makes quantitative studies difficult if not impractical (104).

On the Kinetic Competence of Neutral Red

Model experiments have shown that neutral red when bound to detergent micelles responded at nanosecond time resolution to small pH transients which were photochemically generated in an aqueous phase (102). It was probable that this property is also valid for neutral red at thylakoid membranes. We asked for the most rapid response of neutral red to pH transients in the lumen of thylakoids. Experimentally we were limited by the most rapid way to generate protons. With 2mM hydroxylamine as artificial electron donor to photosystem II we observed half-rise times of the pH_{in}-indicating absorption changes of this dye of 30µs (Förster,V., unpublished). This is the experimentally established time resolution.

2.4 RESULTS AND INTERPRETATION

2.4.1 Proton Release during Water Oxidation

In Fig. 2 we see a pattern of neutral red absorption changes indicating internal pH changes which have been induced by a series of short flashes. Methyl viologen accepts electrons from PSI; electrons flow through both photosystems. Proton release from water oxidation as well as from plastohydroquinone oxidation is expected. The absorption changes are multiphasic. Rapid pH jumps, which are not time resolved at a dwell time of 2ms/point, are followed by slower phases (half rise time about 10ms). The amplitude of the rapid pH jumps oscillates as a function of flash number (insert in Fig.2). Maxima appear on the 3rd and 7th flash as in the typical oxygen evolution pattern. Formerly, the rapid phases have been attributed to the water-oxidation process on the basis of inhibitor studies (75). This is confirmed by the oscillatory behaviour of this proton fraction. Although the signal to noise ratio of the measurement of Fig.2 does not allow a clear separation of the kinetic phases the pattern supports the $1H^+(S_0-S_1) : 0(S_1-S_2) : 1H^+(S_2-S_3) : 2H^+(S_3-S_4)$ stoichiometry when a dark equilibrium of $30\%S_0/70\%S_1$ is assumed.
The slow phases have been attributed to proton release during plastohydroquinone oxidation at the $Cytb_6/f$-Complex. Since plastoquinone is a two-electron carrier binary oscillation might be expected in the pattern of slow proton release. Bouges-Bocquet has observed observed partial pairwise electron transport between the photosystems (61). Partial binary oscillation of slow internal proton release has been found in two studies (53)(56). The measurement in Fig.2 yielded complex results with respect to the slow proton release which are compatible with a weak binary oscillation but are far from conclusive. (Since this experiment is not adequate to investigate proton release during intersystem electron transport we do not discuss this point here.)

Besides the kinetical separation of the internal protolytic reactions we have concentrated our efforts in the suppression of PQH_2 oxidation and the selective measurement of the fast proton release from PSII. Formerly, this had been done by DBMIB (53,57,58), which inhibits electron transport at two sites, at the PQH_2-oxidation (pI_{50}=1μM) and at the PQ-reduction site (pI_{50}=10μM) (76). Saphon and Crofts have described the effects of DBMIB on the proton release pattern (78). Qualitatively, we have made similar observations. Depending on the mode of dark adaptation we had found more or less proton release upon the first flash in Förster et al.(57). We have repeated these experiments with concentrations of DBMIB up to 40μM (as Saphon and Crofts (53)), where both DBMIB-specific sites are expected to be blocked. Usually we found considerable proton release upon the first flash which we previously interpreted to indicate that one proton was released during transition S_1-S_2. The variability of the proton release upon the first flash seems to be a function of DBMIB concentration and dark time. Since DBMIB is a quinone-analog redox reagent a possible explanation for this phenomenon is that DBMIB is involved in a so far unexpected redox reaction, possibly driven by PS I. With high concentrations of quinoic PSII acceptors ($\geq$100μM phenyl-p-quinone), which seemed not to impair internal proton release under repetitive excitation, we have found peculiar distortions of the proton release pattern in the first flashes (Förster, unpublished). Therefore, we have chosen an inhibitor of PQH_2 oxidation which was not expected to act as a redox reagent. In Fig.3 we show a pattern of internal proton release measured in the presence of DNP-INT, 0.1μM of which inhibits 50% of the PQH_2 oxidation at the $Cytb_6/f$-complex at 200μM Chl (89). Since PQH_2 is not reoxidized otherwise within 80ms between the flashes the plastoquinone pool gets exhausted after a few flashes, resulting in a decline of the higher-flash proton yields. In order to "enlarge" the plastoquinone pool we added 5μM dimethylquinone which corresponds to about

20 times the pool size but is below the quinone concentrations in former experiments. We observe multiphasic rises: rapid, unresolved pH_{in} jumps (half rise time<2ms) and slower phases (half rise time 5-10ms), which become negligible with the second period of four flashes. The amplitudes of the rapid rises are plotted as a function of flash number in Fig.4. The pattern shows a pronounced oscillation with a period of four which clearly indicates the relation to the water-oxidation process. Four protons are expected to be released during one period of oxygen formation, i.e. one proton per flash on the average. A normalization of the pattern to this value, which is roughly in agreement with the absolute calibration of the absorption changes, shows that protons are released in packages of 1 or 2, respectively. Open triangles in Fig.4 show a fit with appropriate values of dark population, double hits and misses. Since 2mM hexacyanoferrat(III) was present in the chloroplast suspension and no rapid phase was observed upon the first flash we assumed a dark population of $100\%S_1$, which was in line with (50). 6% misses and 5% double hits yielded the best fit. Besides, 5% double hits is a reasonable value at the flash duration in this experiment (1µs FWHM) (48). (The 5% double hits contain double hits caused by flash excitation as well as those caused by continuous measuring light between the flashes, the latter of which contributed by less than 1%.) Thus, from this proton release pattern it follows the stoichiometry of $1H^+(S_0-S_1) : 0(S_1-S_2) : 1H^+(S_2-S_3) : 2H^+(S_3-S_0)$. Fig.5 shows a four-flash pattern measured at higher time resolution. The rapid phases are partially time resolved at the dwell time of 200µs; a phase of about 1ms after the third flash becomes apparent. We should like to mention at this point that in our preceeding study on this subject (57) we followed the absorption changes only for 10ms after each flash. Beyond 10ms no further changes were observed under repetitive flashing. As we now know slower phases ($\tau_{1/2}$=5-10ms) are always observed upon the first few flashes when one starts flashing from the dark (see

Fig.3 and Fig.5) but these slow phases do not exhibit the oscillatory behaviour characteristic of water oxidation. So far we do not know their origin. At the low signal-to-noise ratio of the results in (57) we did not visualize that the slower phase which was observed upon the second flash had not developed to its full amplitude within 10ms. This led us falsely to the attribution of the slower phase to the water-oxidation process. Fully time-resolved measurements of the PSII protons are shown in Fig.6 (each trace expanded on the right-hand side). The following phases are distinguishable:

	experimental half rise time	*mainly expected transition* S_i-S_{i+1}		
		6% misses	*10% misses*	
2nd flash	200μs 2ms	*88%*	*81%*	S_2-S_3
3rd flash	200μs (40%) 1.2ms (60%) (between 60 and 80% slow phase have been observed)	*83%*	*74%*	S_3-S_0
4th flash	250μs 2ms	*78%*	*70%*	S_0-S_1

TABLE II

The stoichiometric interpretation of the multi-flash pattern of Fig.3 suggests that only the phases of half-rise time $\leq$2ms are indicative of the water-oxidation process, i.e. we can disregard the slower phases, which are observed upon the second and fourth flash, when we try to interprete the kinetics in terms of proton release during the S state transitions.

Unfortunately, we cannot transfer the fit parameters (dark population, double hits and misses) of the above multiflash measurement to the time-resolved measurement of Fig.6 since different light sources have been used which give rise to a different percentage of double hits. In the time-resolved measurement only nanosecond flashes have been applied (Q-switched Ruby- and Nd-YAG lasers, 50ns FWHM) which are expected to cause less than 2% double hits per flash (48). The measuring light (200µW at 548nm) was applied only 20ms before the respective measuring flash allowing excitation of less than 10% of the reaction centers before the "measuring" flash. Since the signal-to-noise ratio does not allow a distinction between more than two phases we neglect the double-hit parameter here.
The best fit of the multiflash measurement was obtained with a percentage of misses of 6%, which is a rather low value. From oxygen measurements usually 8-10% have been obtained (23). In the last columns in Table II the main transitions are listed which are expected to occur upon the respective flashes when a dark population of 100%S_1 and 6 or 10% misses, respectively, are assumed (no double hits). The experimental results suggest the following interpretation:

amount of H^+release	half rise time	transition
1 H^+	250µs	S_0-S_1
1 H^+	200µs	S_2-S_3
2 H^+	1.2ms	S_3-S_0

TABLE III

The rapid phase upon the third flash thus would be due to proton release during S_2-S_3 (misses) and, at a negligible amount, to S_0-S_1 (double hits).

2.3.2 Internal proton release from hydroxylamine-loaded chloroplasts

The H^+_{in} flash pattern measured in the presence of hydroxylamine should reflect the two-flash shift in the water-oxidation cycle, which is effected by this compound. Moreover, proton release upon the first two flashes should reveal information about the mechanism of hydroxylamine oxidation.

Fig.7 shows a pattern of internal proton release which has been measured in the presence of 30μM hydroxylamine. The following features are evident: From the third flash on we observe a proton pattern which is very similar to the "normal" pattern (compare upper and lower trace in Fig.7). From the amplitudes of absorption changes it is obvious that the whole population of water-oxidizing centers is active, i.e. the interaction of hydroxylamine with the water-oxidizing enzyme appears complete and is widely reversible. About two protons are observed upon the first flash, one proton upon the second flash.

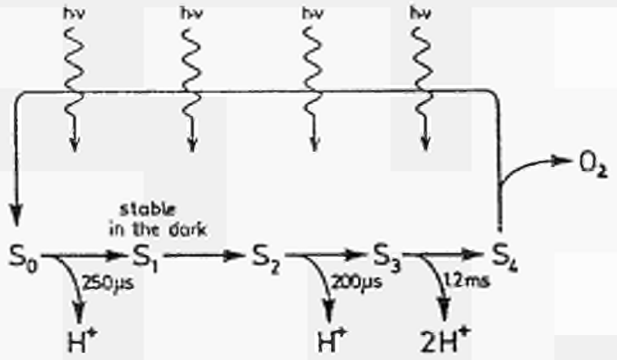
Slight differences in the appearence of the water-oxidation period are expected with and without hydroxylamine because of foregoing damping during the first two flashes in the presence of hydroxylamine. Fig.8 shows the rapid proton yields as a function of flash number (as in Fig.4). Open triangles represent a calculation which we have made under the assumptions of complete homogeneity of the water-oxidizing centers before the first flash, $2H^+$ upon the first, $1H^+$ upon the second flash, followed by the sequence normally observed from the dark, and equal occurrence of 5% double hits and 6% misses upon each flash.

The proton release pattern nicely confirms the two-flash shift of the water-oxidation period by hydroxylamine. The question remains as to the protolytic reactions upon the first and second flash. We have tried to answer this question by higher time resolution. Fig.9 shows the absorption changes of neutral red upon the first

two flashes. The first flash induces an apparently monophasic rise of 1.7ms half rise time (three measurements; 3ms was obtained with another chloroplast preparation). This rise is slower than any of the protolytic reactions which we have ascribed to the water-oxidation process. It is probably due to the oxidation of hydroxylamine at the water-oxidizing site. Upon the second flash a rapid rise of 250µs half rise time is observed, followed by a slow phase of 5-10ms half rise time. Bouges (68) and Radmer and Ollinger (70) could detect only the final products of hydroxylamine and water oxidation, N_2 and O_2, respectively. O_2 and N_2 patterns led them to the assumption that the system undergoes the transition S_0-S_1 upon the second flash. With the pH_{in}-indicating absorption changes we have a probe for the intermediate reactions. In Fig.10 we have compared the signal which is observed upon the second flash in the presence of hydroxylamine with the signal of the S_0-S_1 transition, which is expected mainly upon the fourth flash in the absence of hydroxylamine (about 60% of the signal). From the lower trace it can be seen that the most rapid rises of both signals match each other. Therefore, most probably transition S_0-S_1 occurs upon the second flash given to hydroxylamine-loaded chloroplasts. (Measurements shown in Fig.9 and 10 have been made in the presence of 4µM DBMIB. Experimental conditions were chosen such that DBMIB was not expected to cause an "extra" proton yield upon the first flash. Moreover, measurements in the presence of DNP-INT yielded similar results.)

2.5 Discussion

The aim of this work has been to investigate the four-step water oxidation with respect to proton production which is necessarily involved in this process. Since water oxidation is located at the inner side of the thylakoid membrane we have used the neutral red technique which allows to measure selectively internal proton release. Moreover, we have minimized proton release due to plastohydroquinone oxidation, which is driven by PS I. When the water-oxidizing centers are forced into a distinct redox state (S_1) by dark adaptation a series of flashes induces a striking proton release pattern. A rapid fraction of half rise time below 2ms exhibits a pronounced oscillatory pattern with a period of four flashes which is characteristic of the action of the water-oxidizing enzyme. Slower phases (5-10ms) are superimposed declining in amplitude with flash number. Their origin is so far unknown (Fig.3). The oscillatory fraction is explained by proton release during the S-state transitions with the following stoichiometry: $1H^+(S_0-S_1)$: 0 $H^+(S_1-S_2)$: $1H^+(S_2-S_3)$: $2H^+(S_3-S_4)$. The fully time-resolved rises of the oscillatory rapid pH jumps can be interpreted in terms of this stoichiometry as follows:

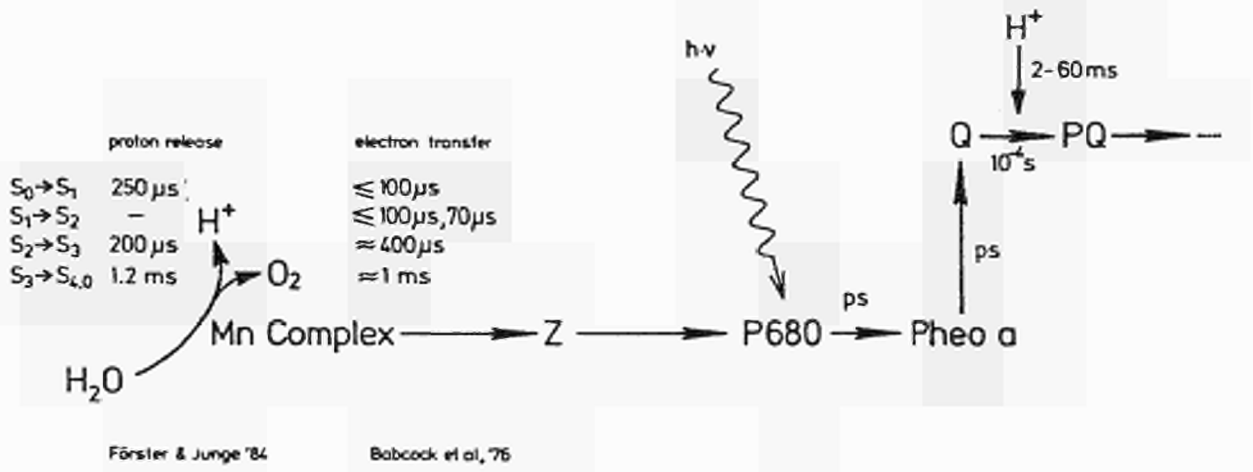


The relation of the oscillatory fraction to water oxidation is further confirmed by the two-flash shift in the presence of low concentrations of hydroxylamine which has formerly been observed in the oxygen pattern (Fig.7). We should like to emphasize that the pronounced oscillation only becomes obvious at high time

resolution. Among the current techniques to monitor the action of the water-oxidizing enzyme the neutral red technique is the one with the highest time resolution.

The question has to be discussed as to the molecular origin of these protons. Although they are doubtless related to the cycling of the water-oxidizing enzyme we do not have an indication of the nature of the molecular group where they are released from. Two possibilities are conceivable: 1.) A proton can be released upon formation of a water-oxidation intermediate which is bound to the catalytic Mn center. 2.) Electron abstraction may lead to a higher oxidation state of the Mn atoms and protons are released by nearby acidic amino acid side chains in order to stabilize the higher oxidation states of Mn. The remaining proton "holes" may be filled up "behind the curtain" when water is decomposed upon the third flash. Compensation of a positive charge by release of a proton may already occur at the level of the intermediate electron carrier Z. In Tris-treated chloroplasts Renger and Völker (62)(95) as well as ourselves (95) have observed proton release from the donor side of PSII which is obviously coupled to the redox reactions of Z. This proton release ($\tau_{1/2}$=100μs) is significantly slower than the oxidation of Z by which it is triggered ($\tau_{1/2}$=5-10μs in Tris- washed chloroplasts). Therefore, a proteinaceous diffusion barrier has been assumed (62)(95). The proton is observed with a half rise time of 100μs which is on the order of the fastest protolytic step which is observed in water-oxidizing chloroplasts. However, in Tris-washed chloroplasts the microenvironment of Z is altered compared to the unmodified chloroplasts. Three proteins which are located near the water-oxidizing enzyme are released from the membrane (see for example (96)) and Z becomes accessible to lipophilic donors (60). It is therefore impossible to conclude from the protolytic reaction occurring in Tris-treated chloroplasts to its occurrence in unmodified chloroplasts.

Time-resolved measurements can give restrictions as to the possible mechanisms of water oxidation and proton release. If water-oxidation intermediates are formed at the Mn center during the S-state transitions proton release involved in the respective reactions is expected to occur slower than the foregoing electron transfer, which is the rereduction of Z by the water-oxidizing center. The pattern of Z^+ reduction from the dark has been measured via transients of the EPR signal IIvf (12). Unfortunately, only rough estimations of the half times of electron transfer could been made from the EPR traces because of instrumentally limited time resolution (100µs) and because of low signal-to-noise ratio. The kinetic data of electron transfer and proton release (half rise times) are compared in the following scheme:



At the accuracy of these data we have to conclude that protons are released as fast as electrons are transferred between the water-oxidizing enzyme and Z only during the transitions S_2-S_3 and S_3-S_4,S_0. Thus, the rereduction of Z seems to be the rate-limiting reaction at the donor side of PSII. During transition S_0-S_1 proton release is significantly slower than electron abstraction. Since S_0 is the lowest oxidation level of the water-oxidizing enzyme this could be an indication of the

following reaction in the Mn ligands:

$H_2O \longrightarrow e^- + \cdot OH + H^+$

During transition S_2-S_3 protons appear faster than reported for the transfer of one electron to Z^+. If this will be confirmed in a correlated measurement, which is so far missing, it would suggest that during oxidation of state (S_2,Z^+,P680) a proton is released as a counter ion in order to stabilize Z^+. The proton "hole" remaining in the protein may be filled up again by the protolytic reaction which follows oxidation of water. We have already discussed this mechanism in (95). On the basis of the presently available data we do not need to assume a compensatory "transient proton".

The proton release pattern which we have found in hydroxylamine-loaded chloroplasts confirms the delay in water oxidation which has been found by other authors (see section 2.1.2). Furthermore, our data suggest that the release of two protons, which are observed upon the first flash from the dark, is due to the oxidation of hydroxylamine. From the absolute values of proton release it is evident that PSII reacts about quantitatively with hydroxylamine in the dark. A side reaction in which hydroxylamine is oxidized upon each flash in a row, as proposed by Radmer and Ollinger (70), is unprobable in the light of our results. If it occurred we would have to assume that it is not driven by PS II and that it occurred via a different mechanism not involving millisecond internal proton release.
Since hydroxylamine and water appear to be competitive donors it is reasonable to speculate about a mechanism of hydroxylamine oxidation at the catalytic manganese center. The current understanding of the catalytic center involves two Mn atoms with water or water-oxidation intermediates as ligands. The oxidation states of the Mn atoms have been investigated by EPR in the state S_2 which, according to current debate, is either a Mn(II)/(Mn(III) pair or a Mn(III)/Mn(IV) pair (32,35-38,77) and

possibly part of a tetramer cluster (77,69). Since no proton is released during the S_1-S_2 transition (see above) and the formation the Mn-EPR signal of state S_2 is pH independent (69) the S_1-S_2 transition is supposedly a one-electron oxidation in the Mn atoms. This means that the S_1 state is a Mn(II)/Mn(II) pair or a Mn(III)/Mn(III) pair, respectively; (principally, Mn(II)/Mn(IV) would also be possible in the latter case). Thus, in the former case hydroxylamine would interact with the Mn(II)/Mn(II) state, in the latter one to Mn(III)/Mn(III). The Mn(III)/Mn(III) state could be reduced by hydroxylamine to Mn(II)/Mn(III) or even Mn(II)/Mn(II). Reduction of Mn from a higher oxidation state to (non-water-coordinated) Mn(II) by hydroxylamine in the dark has been observed by Robinson et al.(67) only at hydroxylamine concentrations above 100μM where the water-oxidizing center is irreversibly distroyed.

Unfortunately, we cannot suggest a plausible mechanism for a one-electron oxidation of hydroxylamin which is compatible with all conceptions about the manganese oxidation states and lead to stable oxidation products, especially molecular nitrogen and $2H^+$/PS II. In the following, we assume for sure that $2H^+$ are released upon hydroxylamine oxidation since this is a quantitative result and more reliable than the interpretation of nitrogen patterns (70) and of the manganese multiline EPR signal in state S_2 (see above). In Fig.11 and Fig.12 we present a hypothetical mechanism which is compatible with the model for water oxidation proposed by Andreasson et al.(32) and which leads to the formation of one N_2 molecule per center. Two molecules of NH_2OH bind to a Mn(III)/Mn(III) complex in the dark. Nitrogen is produced in analogy to water oxidation. When one electron is abstracted photochemically from hydroxylamine a second electron flows into manganese by which the S_0 state is formed, from which the water oxidation cycle runs normally.

However, if manganese has to be assumed in the oxidation level Mn(II)/Mn(II) in the dark (S_1) the S_0 state most probably also consists in a Mn(II)/Mn(II) center. If we account for the fact that the system obviously returns to S_0 upon the first flash there is no net variability in the manganese oxidation states during hydroxylamine oxidation and we do not need to consider the manganese atoms to be involved in the overall reaction. Dismukes and coworkers have obtained results by EPR which might be explained by the formation of one (or two?) bound ·NHOH radicals to "S_1" in the dark (69). It is unknown, however, how the radical is formed. We can imagine only one oxidation mechanism under these preassumptions which accounts for the production of $2H^+$ and thereby leads to the formation of dinitrogen oxide, N_2O:

$$2\ \cdot NHOH - e^- \longrightarrow 2\ H^+ + H_2O + N_2O$$

This mechnism is of course not compatible with the interpretation of the measurements on nitrogen release by Radmer and Ollinger (70). Nevertheless, we consider this reaction to be possible for two reasons: 1.) N_2 has been proved to be the only stable volatile product when hydroxylamine served as the donor to $P680^+$ in Tris-washed chloroplasts (71). However, it has not been mentioned in this work whether the same hold true if the water oxidizing enzyme was kept intact (no tris treatment). Possibly, hydroxylamine is oxidized in the two cases via completely different mechanisms which yield different reaction products and N_2 is not the only stable oxidation product with the active photosystems. 2.)The N_2 flash pattern (70) exhibits nearly equal yields of nitrogen upon each flash, increasing with hydroxylamine concentration (see 2.1.2), only a slight surplus is observed upon the first flash. Above we have argued that a side reaction driven by one of the photosystems, which should account for the high background yield, is very unprobable. At worst, nitrogen is produced in a reaction which is completely unrelated to photosynthetic electron transport.

3. CONCLUSIONS

We studied the functioning of photosynthetic water oxidation by green plants. Thereby we closely followed the outlines given in the original proposal. Our approach was directed to thylakoid membranes with photosystem II and with the water oxidizing enzyme intact and with the natural sidedness of the membrane as well as the proton pumping properties conserved.

We studied three items: (A) Charge separation in photosystem II was measured at unpreceeded time resolution (100ps) by novel electric measuring techniques (6 and unpublished work). The aim was to infer the distances between the very primary electron carriers. (B) We measured proton liberation in the thylakoid interior which is associated with water oxidation. Owing to the also unpreceedingly high time resolution we could attribute proton release to distinct partial reactions in the water oxidation cycle. (C) In side studies we became involved in questions related to localized pathways for protons from water oxidation into the ATP synthase (97,101) and in studies on the protolytic reaction of the intermediate electron carrier Z under conditions where water oxidation was impaired (95,103). While the aspects (A) and (C) were treated in some of the periodical reports we concentrated here on aspect (B) since this seems to be most relevant in the context of this RD program.

Understanding of the catalyst of photosynthetic water oxidation requires knowledge of the construction and of the redox states of the metal center (manganese center) and of bound water and its derivatives. Proton liberation during redox transitions is one indicator for the redox state of bound water. We showed that the kinetics of proton release closely matched those of electron abstraction and we could resolve a standing controversy about the stoichiometric pattern of proton release during the partial reactions of water oxidation. Even more, the particular spectrophotometric technique to monitor proton release allowed

higher sensitivity and higher time resolution for these partial reactions than other methods.

Despite of this progress a unified concept for the redox situation of the manganese center and its bound water could not be constructed. This was due to the existing ambiguities concerning the state of manganese. Several groups are investigating the state of manganese in situ by spectroscopic means (ESR, IR). We plan to continue our line of research and to investigate proton release during the oxidation of water analogues. The rationale is to infer the properties of the enzyme from the kinetic effects of isotopic or chemical substitutions to these analogues.

5. FIGURES

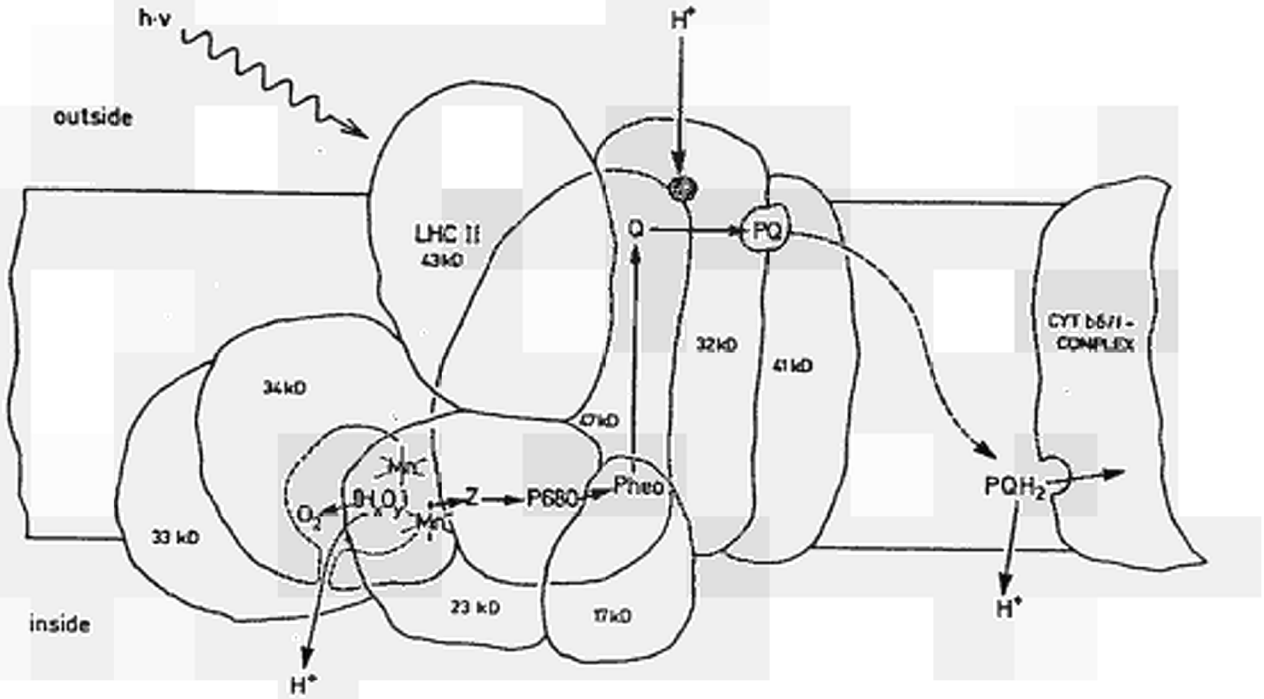


FIG.1:
Model for the structural organisation of the main components of Photosystem II based on present understanding of the chemistry and the localisation of electron carriers

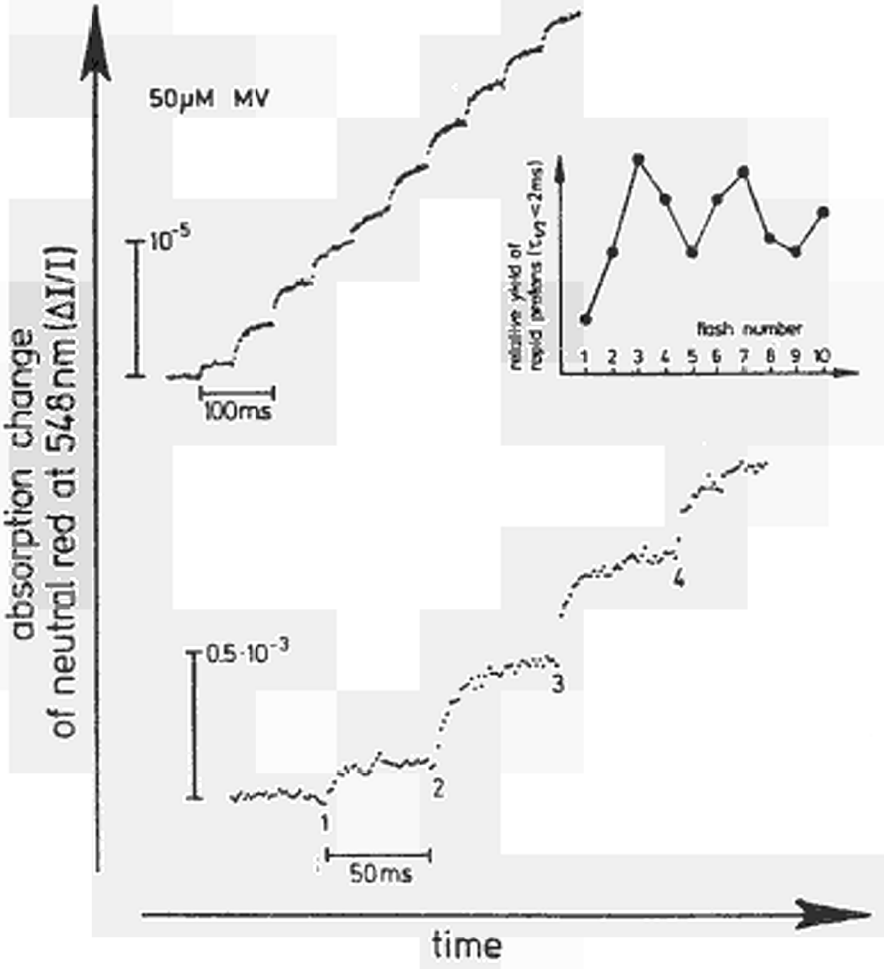


FIG.2:
Pattern of proton release into the thylakoid interior when both photosystems are active.

Pattern of absorption changes of neutral red at 548nm (+NR) evoked by a series of red Xenon flashes ($\lambda \geq 610$nm, 1µs FWHM, $\geq$ 2mJ) spaced 50ms apart; 50µM MV was added to the standard medium, pH 6.8; time resolution 2ms/point; 50 signals were averaged;
Insert: amplitudes of the unresolved rapid phases of half rise time $\leq$ 2ms;

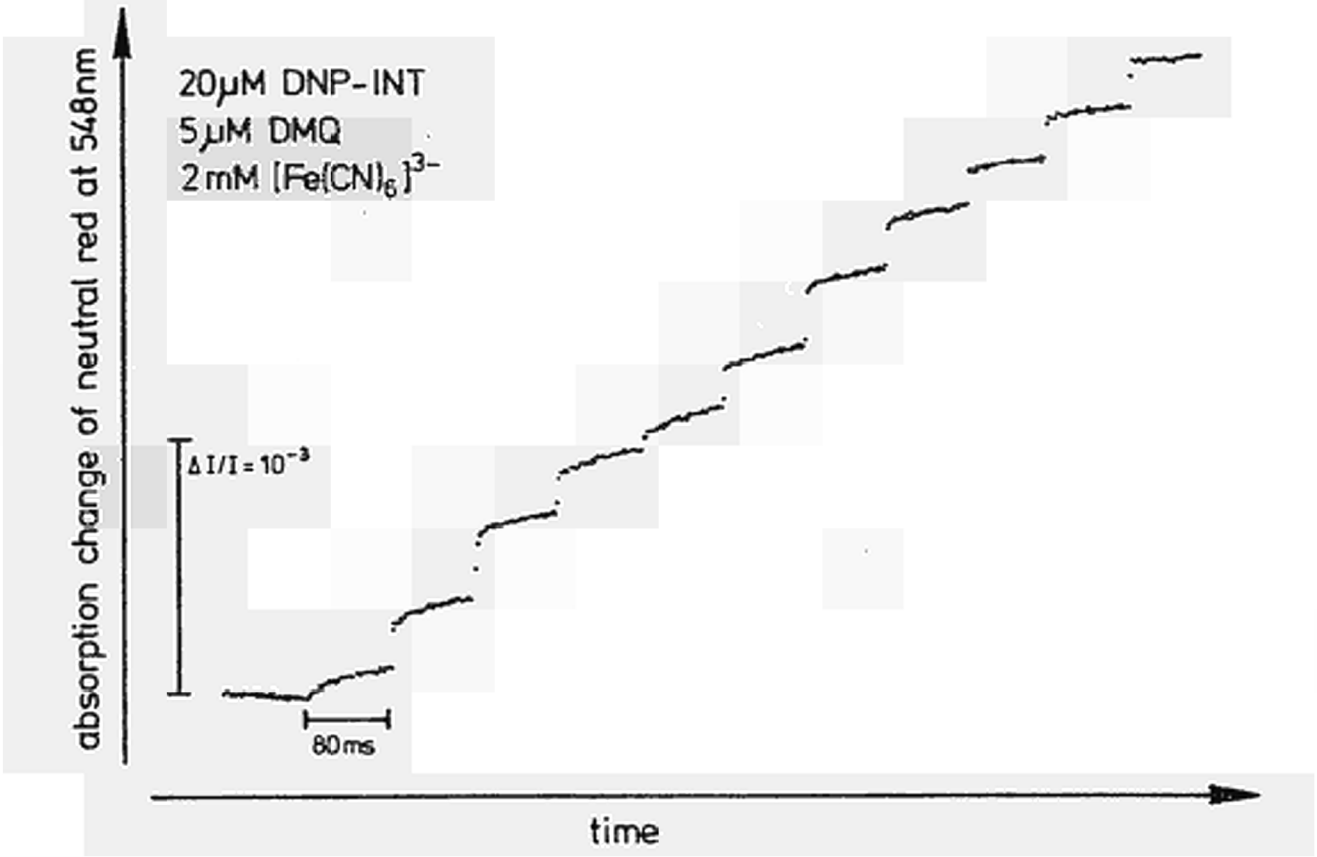


FIG.3:
Pattern of proton release by PSII, only.

Absorption changes of neutral red at 548nm ($\pm_{NR}$) evoked by a series of Xe flashes (as in Fig.2), spaced 80ms apart.
2mM Potassium hexacyanoferrat(III), 20µM DNP-INT and 5µM dimethylquinone were added to the standard medium, pH 7.2; 200 signals were averaged.

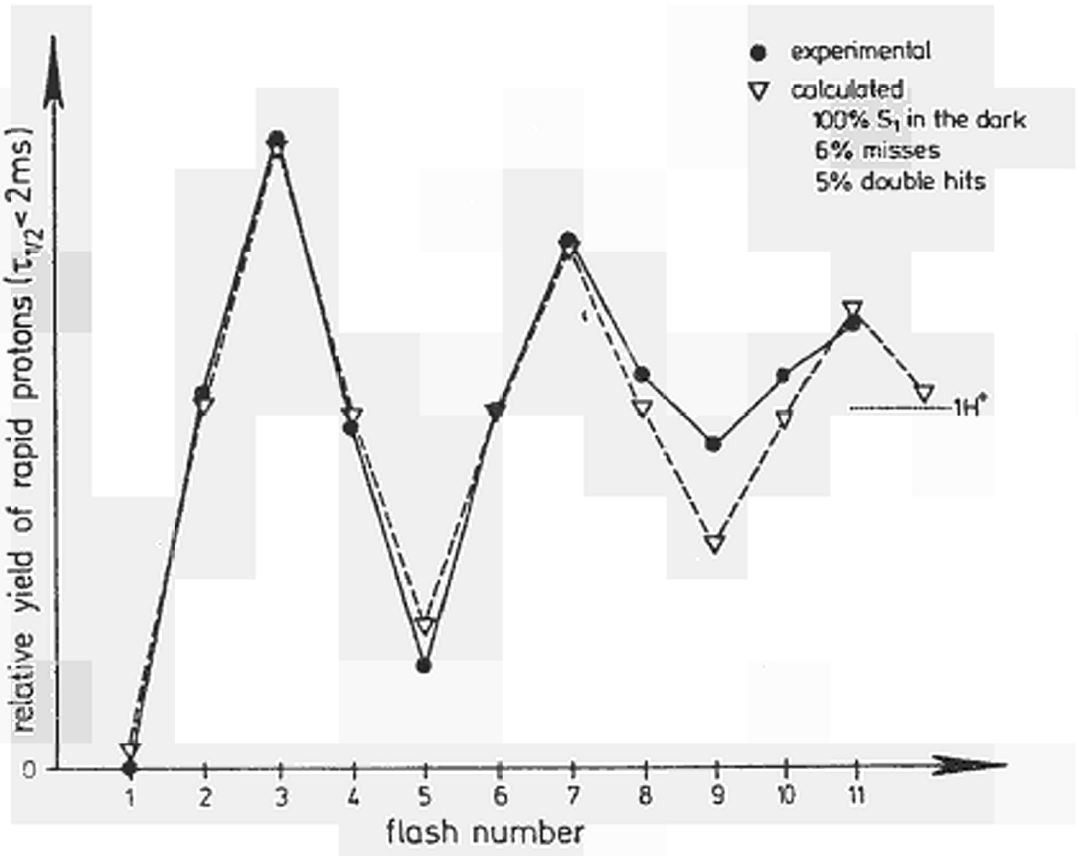


FIG.4:
Amplitudes of the rapid phases of half rise time 2ms of the absorption jumps in Fig.3 as a function of flash number.
Open triangles: Pattern calculated under the assumptions of a proton release stoichiometry
1 H^+ (S_0-S_1) : 0 H^+ (S_1-S_2) : 1 H^+ (S_2-S_3) :
2 H^+ (S_3-S_0), starting with the whole population in S_1 in the dark, 6% misses and 5% double hits per flash.

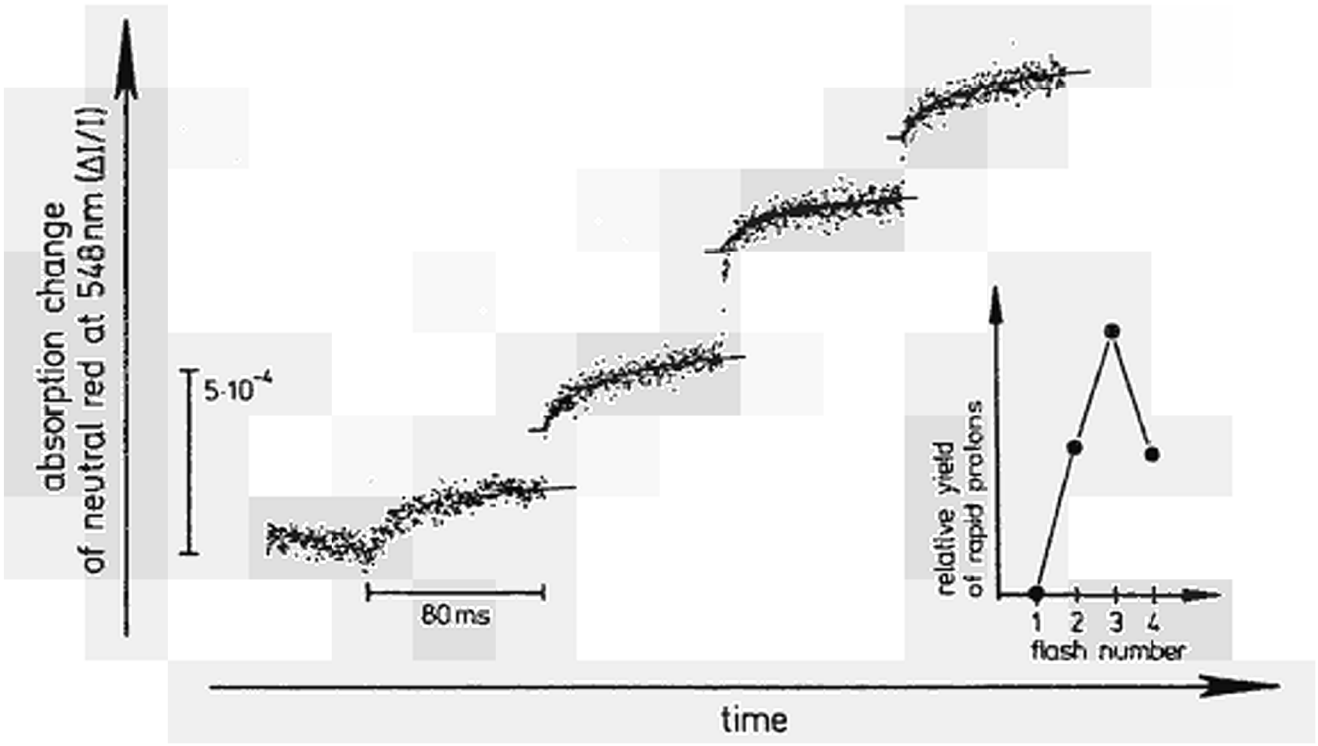


FIG.5:
Pattern of proton release by PSII, only.

Experimental details are the same as in Fig.3 except that the time resolution was 200µs/point and that 100 signals were averaged.

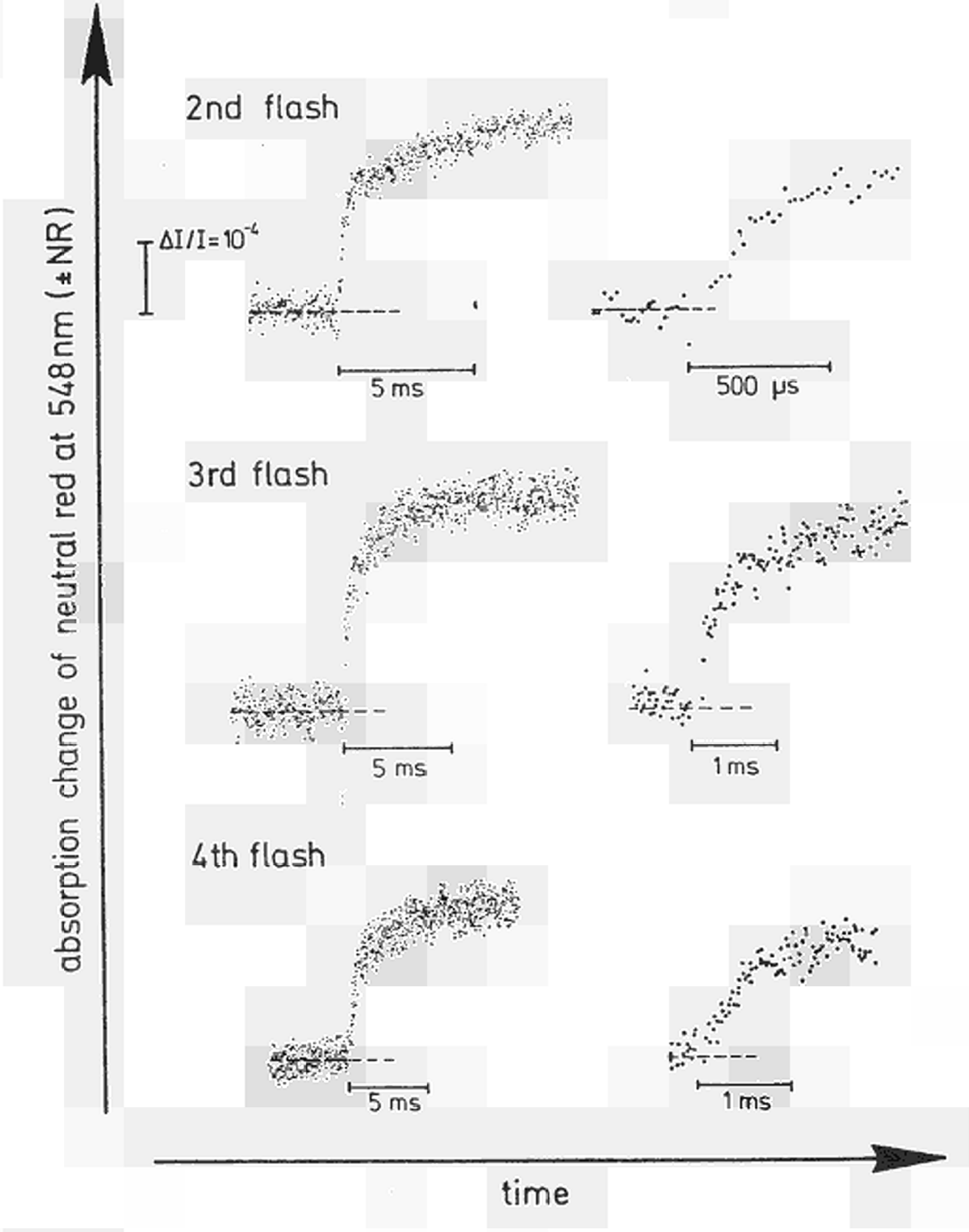

absorption change of neutral red at 548nm (±NR)
2nd flash
ΔI/I=10⁻⁴
5 ms
500 µs
3rd flash
5 ms
1 ms
4th flash
5 ms
1 ms
time

Fig.6

FIG.6:
Time-resolved rise of the internal acidification following the second, third and fourth flash given to dark-adapted chloroplasts.

Preflashes were given by a Q-switched Ruby laser (694nm, 5-7mJ, flash distance 2s), absorption changes of neutral red (+NR) were induced by a Xenon flash (15µs FWHM). 8µM DBMIB and 2mM Potassium hexacyanoferrat(III) were used with the standard medium.
Time resolution was 20µs/point, 150-200 signals were averaged.

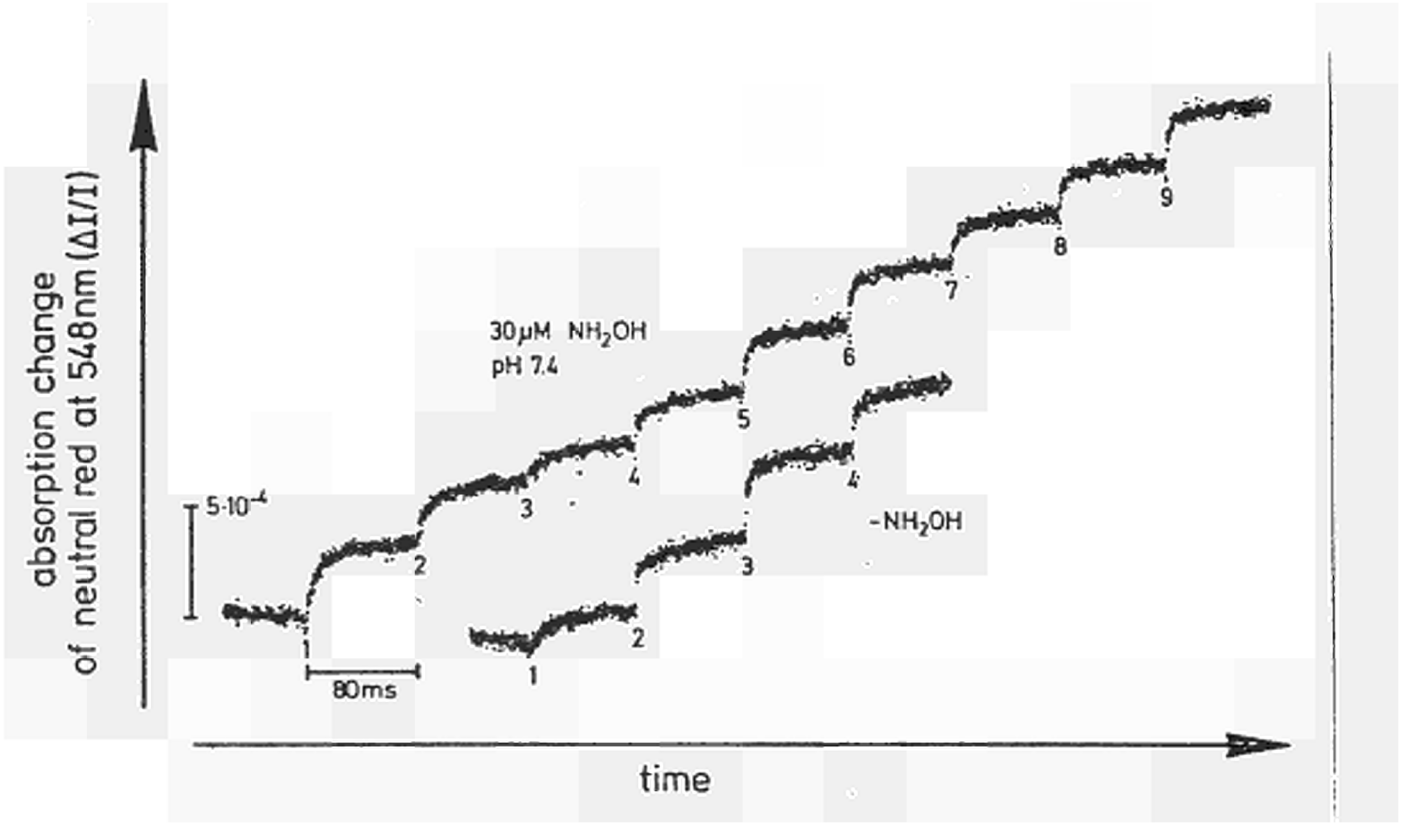


Fig.7:
Interference of hydroxylamine with the water-oxidation process, reflected in the shift of the proton-release pattern.

Pattern of absorption changes of neutral red (±NR) measured in the presence of 30µM hydroxylamine at pH 7.4; 20µM DNP-INT, 2mM potassium hexacyanoferrat and 5µM DMQ were added to the standard medium.
The samples were excited by a series of Xenon flashes ($\lambda \geq 610$nm, 1µs FWHM), spaced 85ms apart;
time resolution 200µs/point; 100 signals were averaged.

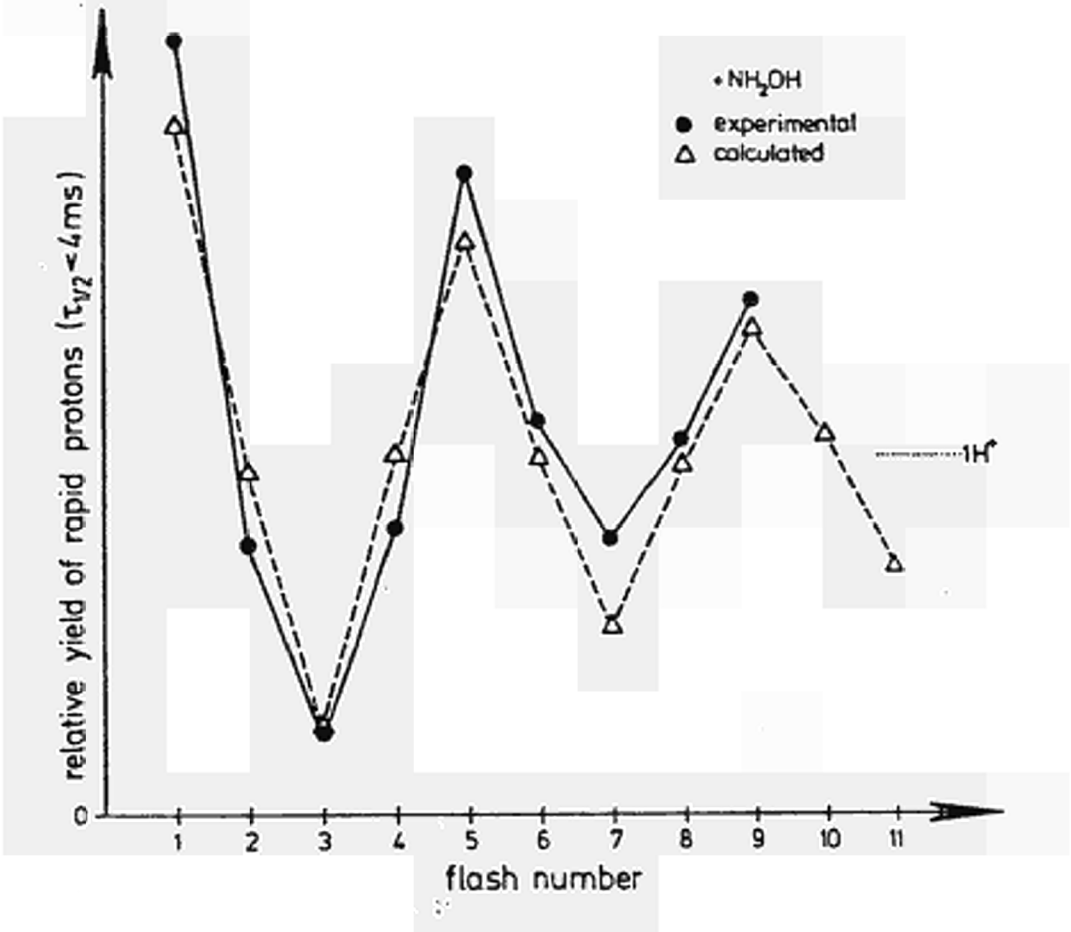


FIG.8:
Amplitudes of the rapid phases (half rise time $\leq$ 4ms) of the proton release pattern measured in the presence of 30µM NH_2OH (Fig.7) as a function of flash number.
Open triangles: Pattern calculated under the assumption of the proton release stoichiometry 2 : (1 : 0 : 1 : 2 :)$_n$, 6% misses and 5% double hits (for details see text).

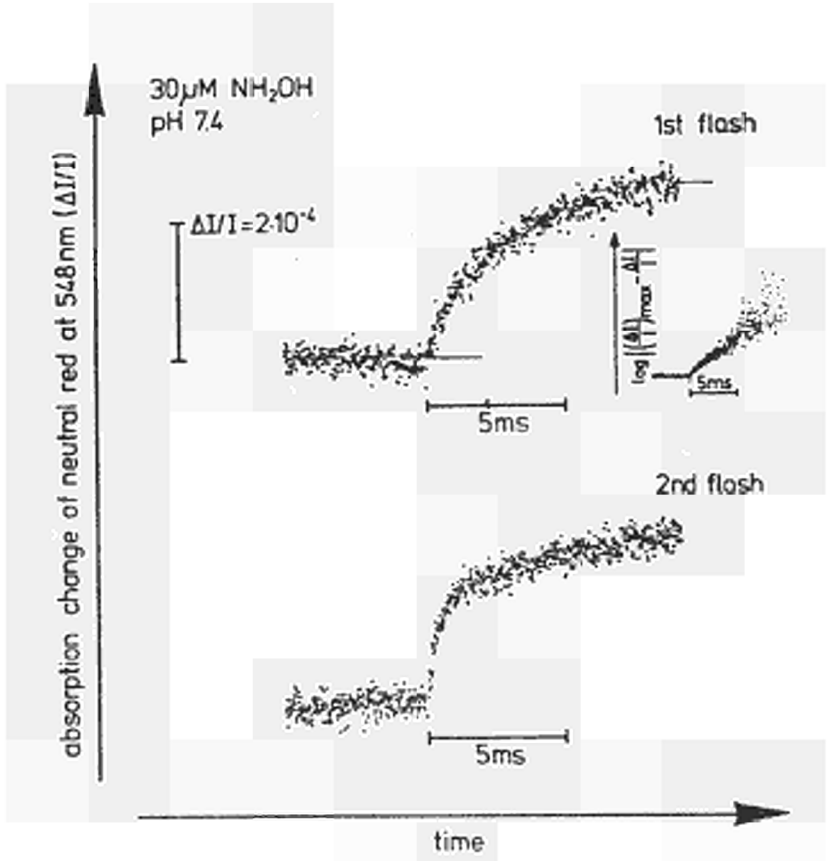


Fig.9:
Time-resolved internal acidification upon the first two flashes given to dark-adapted, hydroxylamine-loaded chloroplasts.

Absorption changes of neutral red were measured in the standard medium plus 2mM potassium hexacyanoferrat(III) and 4µM DBMIB; time resolution 20µs/point;
flashes were given from a Q-switched Ruby laser;
the measuring light was kept low in order not to excite more than 10% of the reaction centers per flash.

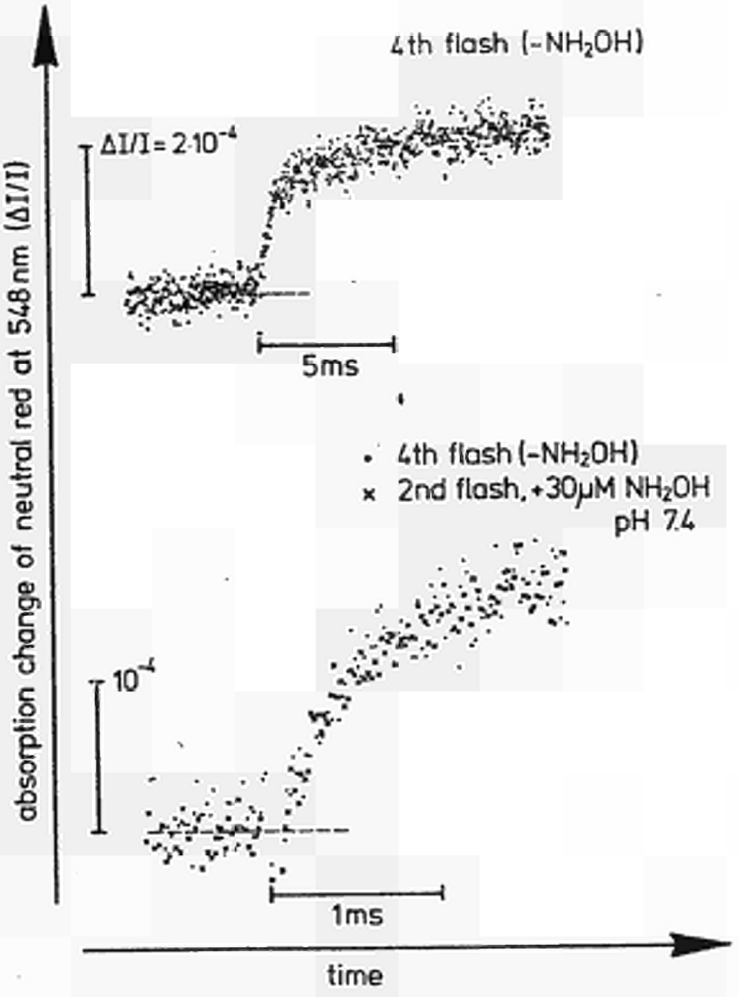


Fig.10:
Comparison of proton release during the S_0-S_1 transition with proton release observed upon the second flash given to dark-adapted, hydroxylamine-incubated chloroplasts.

Absorption changes of neutral red were measured in the standard medium plus 2mM potassium hexacyanoferrat(III) and 4μM DBMIB; time resolution 20μs/point;
200 and 300 signals were averaged, respectively.
Upper trace: Three saturating preflashes were given to dark-adapted chloroplasts from a Q-switched, frequency-doubled Nd-YAG laser (30ns FWHM, 532nm, flash distance 2s) before the measuring flash was fired (Xenon flash, $\lambda \geq 610$nm). - (No hydroxylamine!)
Lower trace: Preflash and measuring flash from a Q-switched Ruby laser (50ns FWHM, 694nm), 30μM hydroxylamine was added, pH 7.4;

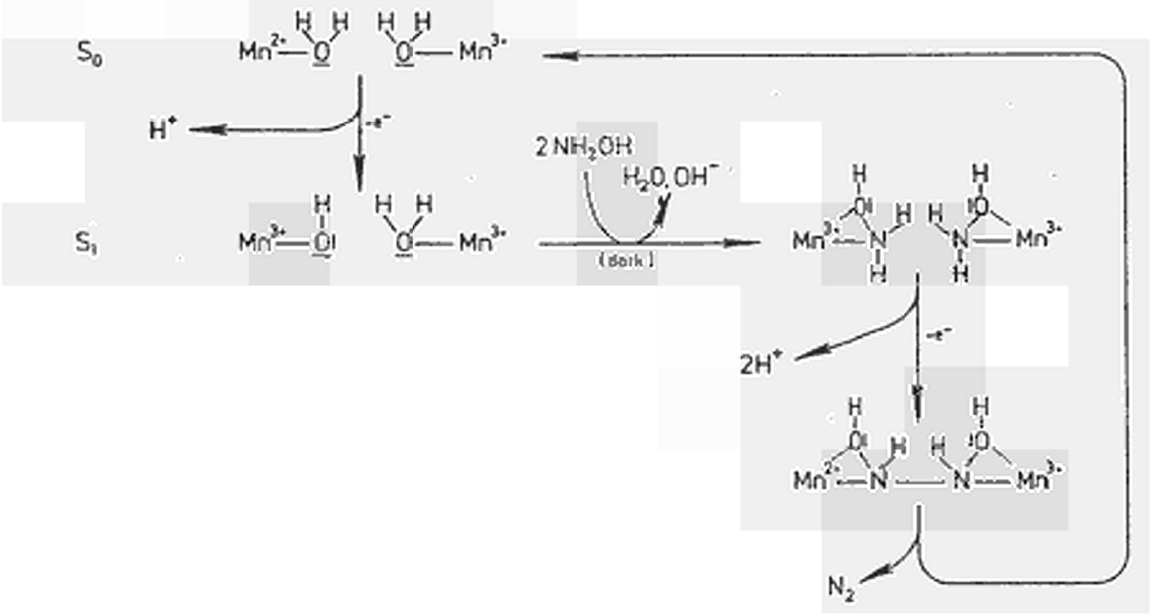


Fig.11:
Hypothetical mechanism of the oxidation of hydroxylamine at the water-oxidizing manganese catalyst.

(The mechanism is compatible with the model for water oxidation by Andreasson et al.(32) and the formation of N_2 as oxidation product (70).)

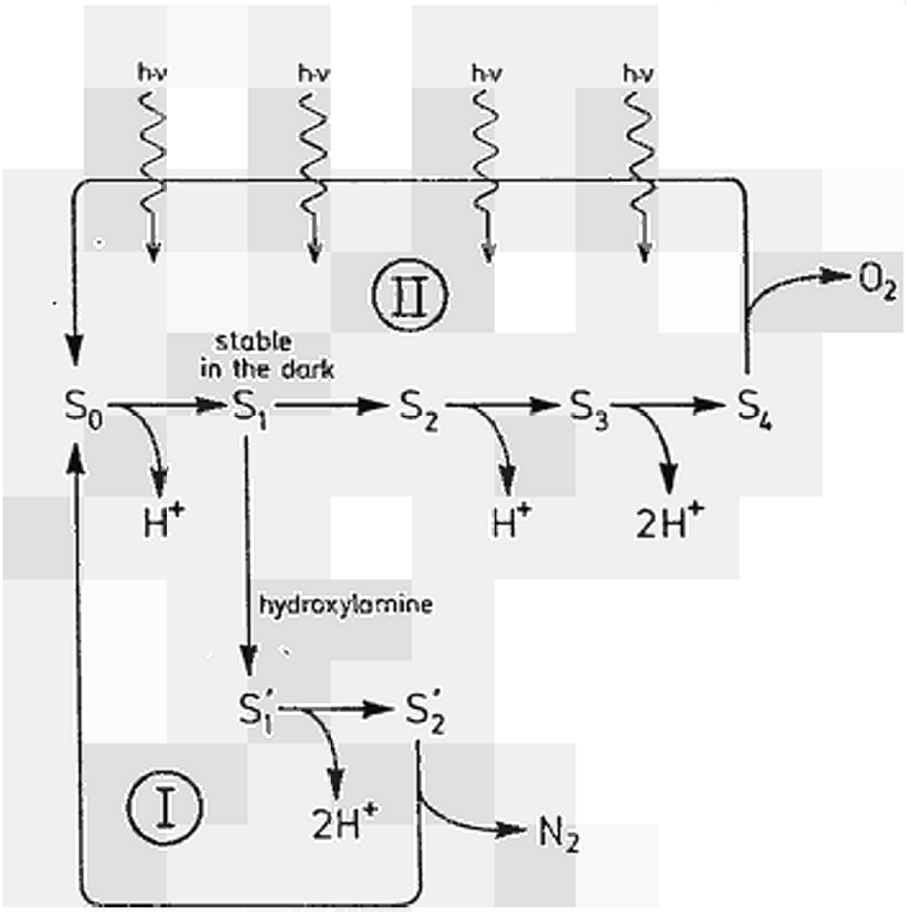


Fig.12:
Hypothetical scheme of the reactions cycles which occur when a dark-adapted, hydroxylamine-loaded chloroplast suspension is excited by a series of flashes.

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