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FACTORS INFLUENCING HYDROXYLAMINE INACTIVATION OF PHOTOSYNTHETIC WATER OXIDATION

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Summary

The kinetics of Mn release during NH_2OH inactivation of the water oxidizing reaction is largely insensitive to the S-state present during addition of NH_2OH. This appears to reflect reduction by NH_2OH of higher S-states to a common more reduced state (S_0 or S_{-1}) which alone is susceptible to NH_2OH inactivation. Sequences of saturating flashes with dark intervals in the range 0.2—5 s^{-1} effectively prevent NH_2OH inactivation and the associated liberation of manganese. This light-induced protection disappears rapidly when the dark interval is longer than about 5 s. Under continuous illumination, protection against NH_2OH inactivation is maximally effective at intensities in the range 10^3—10^4 erg · cm^{-2} · s^{-1}. This behavior differs from that of NH_2OH-induced Mn release, which is strongly inhibited at all intensities greater than 10^3 erg · cm^{-2} · s^{-1}. This indicates that two distinct processes are responsible for inactivation of water oxidation at high and low intensities. Higher S-states appear to be immune to the reaction by which NH_2OH liberates manganese, although the overall process of water oxidation is inactivated by NH_2OH in the presence of intense light. The light-induced protection phenomenon is abolished by 50 μM DCMU, but not by high concentrations of carbonyl cyanide *m*-chlorophenylhydrazone, which accelerates inactivation reactions of the water-splitting enzyme, Y (an ADRY reagent). The latter compound accelerates both inactivation of water oxidation and manganese extraction in the dark.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $DCIPH_2$, reduced dichlorophenolindophenol; ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; Chl, chlorophyll; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tricine, tris(hydroxymethyl)methylglycine.

Introduction

Of the numerous treatments which may be used to inactivate the oxygen evolving reaction system associated with PS II, exposure of isolated thylakoid membranes to Tris [1—3] or to NH_2OH [4—7] represent relatively mild procedures; inactivation by these agents produces membranes in which oxygen evolution can be partially or wholly restored by added Mn(II) [8,9], and in which coupled electron transport activity can be restored by appropriate donors to the oxidizing side of PS II [10]. In a series of elegant studies, Cheniae, Martin and Frasch [2,3] established that low light intensities accelerated Tris inactivation of oxygen evolution; the locus of this attack by unprotonated Tris was shown to be the S_2 state as defined by the model derived from studies by Kok et al. [11]. Factors other than light also influence the rate at which Tris inactivates water oxidation. For example, the presence of ascorbate plus $DCIPH_2$, ADRY agents, ammonia, NH_2OH or prior fixation of thylakoid membranes with glutaraldehyde serve to protect against Tris attack [2,3]; ferricyanide, on the other hand, facilitates Tris inactivation of oxygen evolution.

Studies by Cheniae and Martin [5] on the mechanism of NH_2OH inactivation of water oxidation produced results which differ from those found for Tris. The former agent produces a rapid inactivation of activity at low concentrations in the dark, in a reaction whose rate is unaffected by the presence either of ascorbate plus $DCIPH_2$ or by ferricyanide. When incubation with NH_2OH was carried out in strong light, an acceleration of the inactivation process was observed. Thylakoids so treated were found to be defective in their ability to photo-oxidize an added donor (NH_2OH) to PS II. Later studies by Horton and Croze [7] showed that exposure of thylakoids to NH_2OH plus EDTA in the dark produced a lowering of the midpoint potential of cytochrome *b*-559 to +0.24 V. When inactivation was carried out in the light, the midpoint potential of cytochrome *b*-559 was further decreased to +0.080 V, and the inhibition of donor photo-oxidation seen earlier by Cheniae and Martin [5] was also observed.

The use of measurements of proton spin-lattice nuclear magnetic relaxation rates (R_1) of thylakoid suspensions can provide a useful technique for monitoring the inactivation of water oxidation by NH_2OH; we [12] have shown that the kinetics of the increase in R_1, produced by addition of NH_2OH to thylakoid suspensions from which non-functional contaminating Mn(II) has been removed [13], correlate closely with the inactivation of water oxidation as monitored by assays of oxygen evolution. The species producing enhanced relaxivity in these experiments has a dispersion profile (R_1 vs. Larmor frequency) with a maximum near 20 MHz, characteristic of Mn(II) bound to sites of reorientationally restricted mobility [13,14]. Our kinetic analyses of the NH_2OH inactivation process, by both NMR measurements and assays of oxygen evolution activity, suggested that this reaction is complex; although the net effect of exposure of thylakoid membranes to NH_2OH is inactivation of water oxidation, the inhibition process appeared to be countered by a repair or protection reaction. In this communication we present the results of further investigations on the action of NH_2OH on the oxygen evolution reaction in which we show that low intensity light or short intense flashes can arrest or

prevent entirely the NH_2OH inactivation process. DCMU, but not the ADRY reagent CCCP, suppresses light-induced protection against inactivation. On the other hand, CCCP accelerates the process of dark inactivation.

Materials and Methods

Broken chloroplasts were prepared by the procedure of Robinson and Yocum [15] with the modifications that Hepes (pH 7.7) replaced Tricine, and the homogenizing buffer contained 1 mM EDTA, and the isolated thylakoids were stored frozen in 20 mM Hepes-NaOH (pH 7.7)/0.4 M sucrose/15 mM NaCl at −70°C until use. Inactivation of oxygen evolution by NH_2OH was assayed polarographically in a stirred, thermostattically controlled (25°C) 1.6 ml cuvette fitted with a YSI Clark electrode. The electron transport reaction mixture contained 50 mM Tricine (pH 8.0), 50 mM NaCl, 2.5 mM ferricyanide and 3 μg/ml gramicidin D. Saturating illumination was provided by two microscope illuminators; light from these sources was passed through round-bottomed flasks containing 0.2% $CuSO_4$, and red filters (cut-on at 600 nm). The incubation mixture for NH_2OH inactivation studies was the viologen assay mixture of Cheniae and Martin [5] containing chloroplasts equivalent to 291—300 μg Chl. Incubation at 25°C to produce inactivation was initiated by addition of neutralized NH_2OH from a 100 mM stock solution in 10 mM HCl. The incubation mixture (200 μl) was placed in a vial (1.25 cm inner diameter) positioned above a yellow filter (Corning CS2-63) and was illuminated, where indicated, by an Ealing fiber optics source. The intensity from this source was varied by means of calibrated neutral density filters. Alternatively, samples were subjected to short saturating flashes from a General Radio Stroboslave lamp. Light intensities were measured with a YSI model 65 Radiometer.

The apparatus for NMR measurements has been described [14]. Measurements were conducted at 25°C and 20.7 MHz. Effects of flash sequences on the time course of NH_2OH extraction were studied using the NMR cell shown in

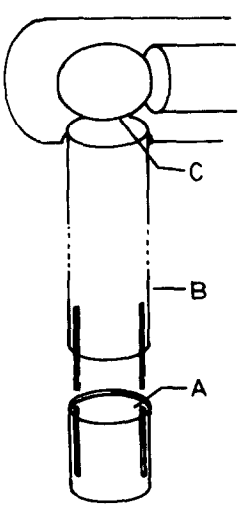


Fig. 1. NMR sample cell used in flash experiments: A, sample compartment; B, light pipe; C, xenon lamp.

Fig. 1. The cell is constructed of 10 mm lucite tubing, which serves as a light pipe. The chloroplast suspension is contained in a disk-shaped recess, 0.5 mm thick (40 μl total volume), machined into the lower segment of the cell. Upper and lower segments of the cell are positioned by two copper prongs that fit snugly into guiding holes in the lower segment. A special collar on the flash lamp allows reproducible positioning of the xenon bulb with respect to the light pipe. In the course of experiments, NH_2OH is added to the chloroplast suspension and mixed outside the NMR cell. Then 70 μl of suspension are transferred to the cell in very weak light, the lower segment is gently pushed into position, excess liquid is removed with tissue, and the cell is placed into the NMR probe. This process requires about 25 seconds, after which the computer-generated timing sequence initiates both T_1 measurements and the desired flash sequence. Flash intensities obtainable using this cell and the Stroboslave flash lamp were studied by monitoring photochemical NADPH production by 2400 flashes in the presence of $NADP^+$ and ferredoxin. Relative flash intensity was measured with a photodiode, and NADPH was measured optically by the difference in absorbance changes at 340 nm before and after addition of phenazine methosulfate [16]. $NADP^+$ reduction saturated at flash intensities equal to approximately half the maximum value, indicating that at maximum intensity our illumination system delivers saturating flashes in dense chloroplast suspensions.

Experiments involving production of specific S-states through preillumination of chloroplast suspensions utilized a coil of clear-walled catheter tubing (0.59 mm internal diameter) positioned between a pair of synchronously triggered flash lamps. The coil contains up to 300 μl of suspension and attaches directly to a No. 26 hypodermic needle, which permits facile loading and unloading of the suspension. Photochemical experiments similar to those described above indicated that flashes obtained with this system were saturating. Illuminated chloroplast suspensions contained 50 μM methyl viologen, 10 μg/ml gramicidin D, and catalase (20 μg/ml) in addition to the standard resuspending buffer.

Results

Effect of S-state on the kinetics of Mn release by NH_2OH

Frasch and Cheniae [3] have shown that the attack of Tris on the oxygen

TABLE I

EFFECT OF INITIAL S-STATE ON THE HALF-TIME FOR NH_2OH EXTRACTION

S-states were prepared by preilluminating with 15 flashes (0.2 s dark interval), a 3 min delay, 1 flash, a 5 min delay (at this point S_1 is most abundant), followed by 0, 1, 2 or 3 flashes to produce $S_{1,2,3}$ respectively. Following this sequence chloroplasts were immediately mixed with NH_2OH (1 mM final concentration, 25°C) and the kinetics of extraction followed by NMR. n = number of experiments; $t_{1/2}$ in seconds.

State	$t_{1/2}$, range	$t_{1/2}$, av	n
S_0	0.73—1.21	1.04 ± 0.16	3
S_1	0.63—0.97	0.86 ± 0.11	4
S_2	0.63—0.91	0.74 ± 0.09	4
S_3	0.60—1.07	0.80 ± 0.17	4

evolving reaction is specific for the S_2 state. Since the NMR experiment conveniently monitors Mn(II) exposed during NH_2OH inactivation of the water-oxidizing system [12], we employed this technique to determine whether the kinetics of inactivation can be varied by preillumination with flash regimes that produce various S-states. The results of these experiments, presented in Table I as the $t_{1/2}$ values for the appearance of maximal R_1 enhancements, suggest that there is no significant acceleration of the Mn(II) extraction, as monitored by the NMR experiment, when NH_2OH is added in the presence of higher S-states. A small, but probably not significant retardation of R_1 enhancement, is seen for the S_0 sample.

Effect of flash sequences on Mn release

Cheniae and Martin [5] have previously shown that illumination accelerates Tris inactivation due to production of S_2, and that intense illumination likewise accelerates inactivation by NH_2OH [5]. To test the possibility that these agents might act by analogous mechanisms, we next examined the effect of short, saturating flashes applied during the inactivation process. In these experiments, the dark interval between flashes was varied; the appearance of some dark R_1 enhancement is seen prior to the onset of the flash sequence owing to a delay between the mixing of the sample in the light pipe and the subsequent transfer

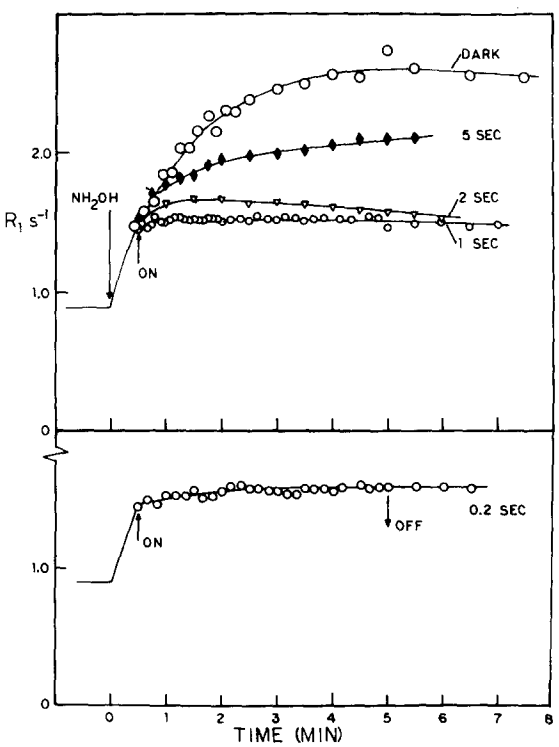


Fig. 2. R_1 as a function of time following NH_2OH addition. Chloroplasts (3.0 mg Chl/ml) were illuminated by a continuous sequence of flashes with the indicated dark interval; 'on' and 'off' denote initiation and termination of the sequence.

of this cell to the NMR probe. Exposure of the sample to very low levels of green light occurred during this procedure. Representative data from these experiments, presented along with a dark control in Fig. 2, show that flash sequences retard or arrest, rather than accelerate, the R_1 enhancement by NH_2OH. At relatively long flash intervals (τ = 2.5—7.5 s), an R_1 enhancement is observed, but the kinetics of its appearance are much slower than in the dark. At flash intervals between 2.5 s and 0.2 s (the shortest flash interval studied), essentially complete inhibition of the NH_2OH-induced R_1 enhancement is observed.

Since the experiments shown in Fig. 2 utilize dense thylakoid suspensions ($\geqslant$3 mg Chl/ml), several control experiments were necessary to verify that the flashes were indeed protecting against NH_2OH-induced Mn extraction. At these chlorophyll concentrations, the trap concentration is approx. 7 μM, and it is therefore possible that the effect seen is due to rapid photooxidative depletion of NH_2OH catalyzed either by PS II or by O_2^- produced by PS I; either of these processes would lower the effective concentration of the inactivating reagent. However, as shown in Fig. 3, the interposition of 30 flashes (upper curve) or 60 flashes (lower curve), spaced 1 s apart, in a dark inactivation experiment produces only a transient cessation of the R_1 enhancement. The subsequent resumption of the inactivation process demonstrates that a substantial portion of the NH_2OH has not been photo-oxidized and that the process leading to inhibition of the R_1 enhancement is reversible. Note, however, that when the R_1 enhancement resumes, the rate is slower than that observed in the dark, probably due to reduction of the NH_2OH concentration. Irreversible inhibition of the R_1 enhancement has been observed in sequences where the number of photochemical equivalents absorbed by PS II exceeds the number of equivalents of NH_2OH present in the suspension (Fig. 2, lower curve).

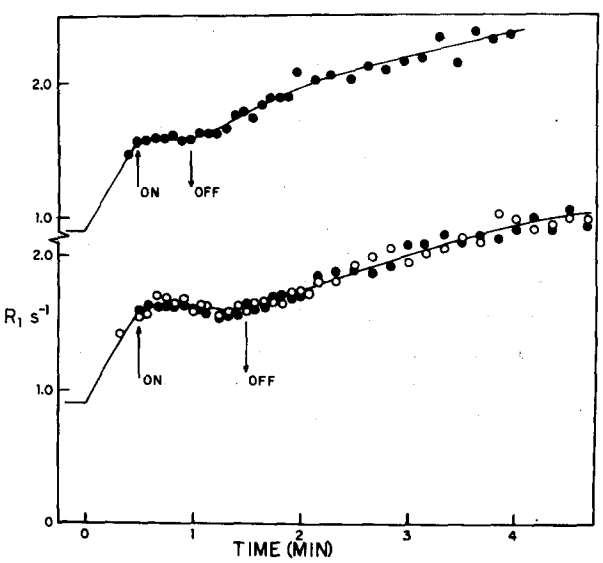
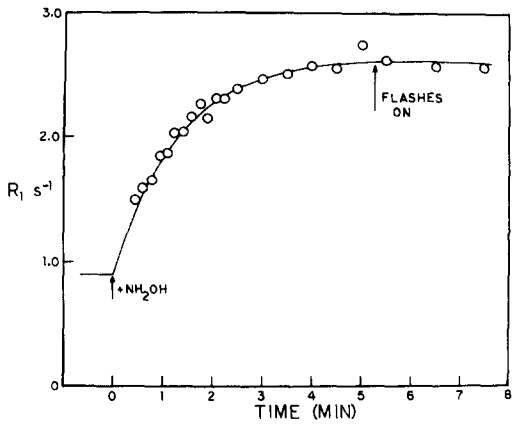


Fig. 3. Effect of short flash sequences (1 s dark interval) on the time course of NH_2OH extraction. Duplicate experiments are shown on the lower curve. Chlorophyll concentration is 3.0 mg Chl/ml.



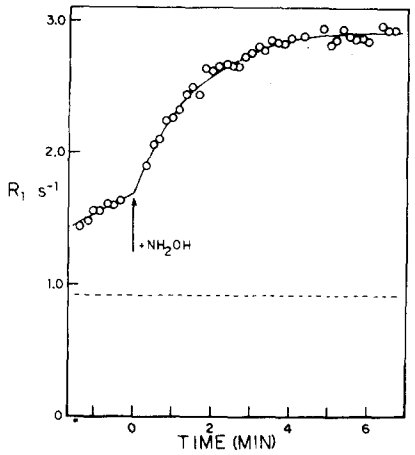


Fig. 4. Effect of a flash sequence (0.2 s flash interval) initiated after full development of the R_1 enhancement (3.0 mg Chl/ml).

Fig. 5. Resumption of NH_2OH extraction by readdition of NH_2OH (1 mM final concentration) to a chloroplast suspension previously depleted of NH_2OH by 1200 flashes (3.0 mg Chl/ml).

Another possible reason for the observed inhibition of R_1 enhancements is that a portion of the Mn exposed by NH_2OH is present in higher oxidation states, which are not monitored by the NMR experiment. This could in principle result from S-state changes that preceed inactivation or it could occur subsequently due to photo-oxidation of Mn(II) by O_2^- [17]. The data of Fig. 4 rule out the latter possibility. Here, the R_1 enhancement was allowed to develop fully in the dark prior to the onset of the flash sequence. No flash-induced change in R_1 is observed, indicating that oxidation of exposed Mn(II) by O_2^- (or by other oxidants produced by photosynthetic electron transport) is not significant in these experiments. An additional experiment investigating the possibility that in flash sequences Mn is exposed in oxidation states higher than Mn(II) is shown in Fig. 5. In this experiment, the thylakoid suspension was flashed for 4 min, arresting the R_1 enhancement induced by NH_2OH. The sample was then removed from the light pipe, transferred to an NMR tube, and NH_2OH (1 mM final concentration) was added. As shown in Fig. 5, the R_1 enhancement process resumes upon NH_2OH addition, and the kinetic profile is similar to that seen in the dark control. We conclude that flash illumination inhibits the inactivation process, rather than liberating Mn in oxidation states above Mn(II). Finally, we have examined the effect of DCMU, which permits only a single charge transfer through PS II, on the ability of short flashes to inhibit NH_2OH-induced R_1 enhancements. DCMU abolishes the flash protection phenomenon, indicating that the NH_2OH-resistant state is produced by PS II photochemistry.

Effect of an ADRY reagent, CCCP, on Mn release

The simplest explanation for the data presented above is that NH_2OH can inactivate oxygen evolution only by attack on lower S-states; higher S-states, produced by single turnover flashes, are immune from attack. If this hypothesis is

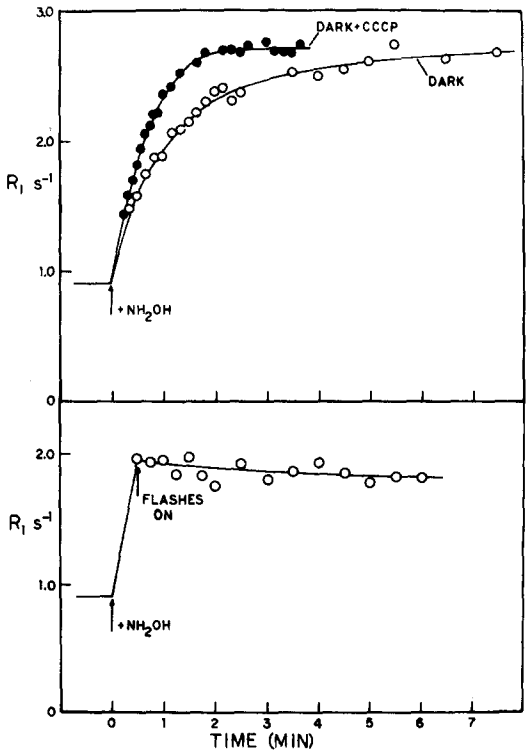


Fig. 6. Effect of CCCP on NH_2OH extraction in the dark (upper, 50 μM CCCP) and during flash illumination (lower, 67 μM CCCP, 1 s dark interval).

correct, then agents which modify higher S-states should be capable of countering the flash protection effect. The effect of CCCP, and ADRY reagent [18—20], on flash-induced protection against R_1 enhancement is shown in Fig. 6. A sequence of flashes separated by 1 s effectively arrests the normal R_1 enhancement, even at a relatively high concentration of CCCP (50 μM) which is easily saturating with respect to the properties of CCCP as an ADRY reagent [20] or as an uncoupler of photophosphorylation [21]. Surprisingly, CCCP is seen to accelerate the NH_2OH-induced R_1 enhancement in the dark (Fig. 6). This result is in strong contrast to the effects of FCCP noted by Cheniae and Martin [2], where the ADRY reagent served to retard light-stimulated Tris inactivation of O_2 evolving centers.

NH_2OH inactivation of O_2 evolution; effects of light and CCCP

In order to examine the correlation between the effects of NH_2OH on R_1 enhancements and on inactivation of oxygen evolution, a series of experiments were conducted using polarographic measurements of O_2 evolution activity (Figs. 8—11). For these studies, the viologen reaction mixture of Cheniae and Martin [5] was used for exposure of thylakoids to NH_2OH (1 mM throughout). Fig. 7 shows the results obtained for inactivation of water oxidation in darkness (plus and minus CCCP) and in intense light (400 kerg · cm^{-2} · s^{-1}). In agreement with the NMR experiment (Fig. 6), CCCP (50 μM) accelerates inacti-

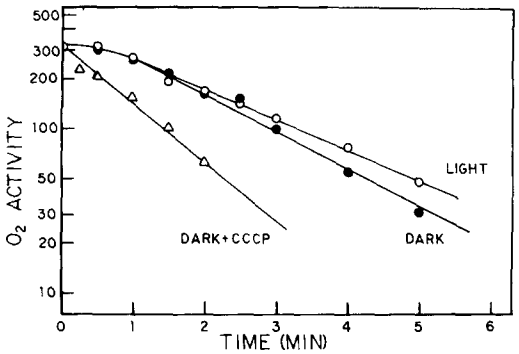


Fig. 7. Effect of steady-state illumination (400 kerg · cm^{-2} · s^{-1}) and of CCCP (50 μM) on the kinetics of NH_2OH-inactivation of O_2 evolution activity.

vation of water oxidation in the dark; no lag in the onset of inactivation is seen. Intense light, on the other hand, does not produce the accelerated inactivation of water oxidation reported by Cheniae and Martin [5]. It should be noted, however, that our assay procedure (direct transfer of the thylakoids to the assay system with a 16-fold dilution of the NH_2OH) differs from the procedure used by Cheniae and Martin (centrifugation and resuspension of the membranes to remove NH_2OH), suggesting that the assay itself may have an influence on residual O_2 evolution activity after NH_2OH extraction. One observation that may be relevant to this point is that the mild shear forces that occur during resuspension produce temporary lesions in the thylakoid membrane, permitting release of internally-liberated Mn(II) (unpublished data).

The effects of light on NH_2OH inactivation of water oxidation were further explored using saturating flashes at different repetition rates, as shown in Fig. 8. Good correlation is observed between the NMR experiment, which directly monitors Mn(II) exposed during NH_2OH treatment, and similar experiments monitoring inactivation of oxygen evolution. At repetition rates between 1 and 5 Hz, protection against NH_2OH inactivation is essentially total. Some of this

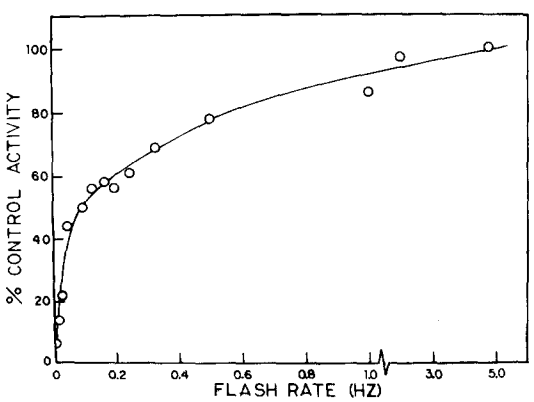
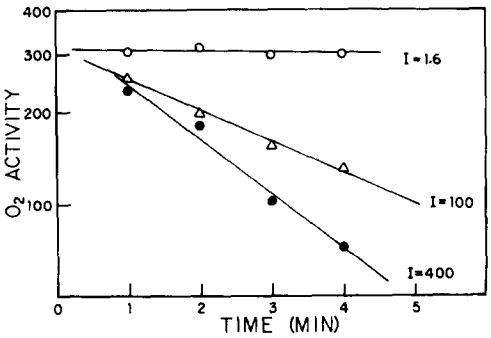


Fig. 8. Effect of flash rate on NH_2OH inactivation of O_2 evolution activity. Residual activity (as a percent of the dark control) after 4 min incubation in 1 mM NH_2OH in the presence of flash illumination.



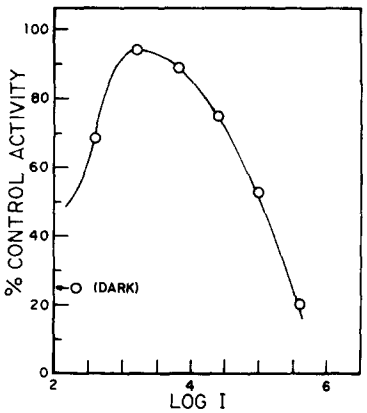


Fig. 9. Effect of various light intensities in erg · cm^{-2} · s^{-1} on the kinetics of NH_2OH extraction.

Fig. 10. Effect of continuous illumination on the kinetics of NH_2OH inactivation of oxygen evolution activity. Residual activity after 4 min incubation in the presence of 1 mM NH_2OH and continuous illumination of intensity I (erg · cm^{-2} · s^{-1}).

protection is lost at higher flash rates, and a slow loss of activity is also observed if the flash repetition rate is lowered from 1 Hz to 0.2 Hz. Interestingly, when the flash repetition falls below 0.1 Hz, a precipitous decline in protection is seen, falling essentially to zero when 1 or 2 flashes per min are presented to the thylakoids.

The observation that flash sequences at moderate repetition rates (approx. 0.2—5 s^{-1}) provide substantial protection against Mn extraction (Fig. 2) and O_2 evolution activity (Fig. 8), is surprising in view of experiments showing that NH_2OH inactivation of O_2 evolution activity proceeds rapidly both in darkness and under intense illumination (Fig. 7 and Ref. 5). We reasoned, therefore, that protection against NH_2OH inactivation might occur only in a range of intermediate intensities of continuous illumination. The results of typical experiments are shown in Fig. 9, where it can clearly be seen that decreasing light intensities are increasingly effective in protecting oxygen evolution activity against attack by NH_2OH. In Fig. 10, data are presented on the protective effects of a wide range of light intensities. Note that a critical intensity (1.6 kerg · cm^{-2} · s^{-1}) exists for maximal protection; at a lower intensity (0.4 kerg · cm^{-2} · s^{-1}) the protective effect of continuous illumination begins to disappear.

NH_2OH extraction of Mn at high light intensity

Surprisingly, the process by which NH_2OH inactivates O_2 evolution at high light intensity is not mirrored in analogous NMR experiments (Fig. 11), which quite directly monitor exposure of Mn. In these experiments, the variation of the NH_2OH-induced R_1 enhancement after 4 min illumination was measured as a function of illuminating intensity. Dense chloroplast samples (approx. 3 mg Chl/ml) were mixed with 1 mM NH_2OH and incubated for 30 s in the dark, then illuminated with white light of the indicated intensity for 4 min. After illumination, the sample cell was transferred to the NMR probe, and R_1 was measured every 30 s for 5 min. The data were extrapolated to a time corre-

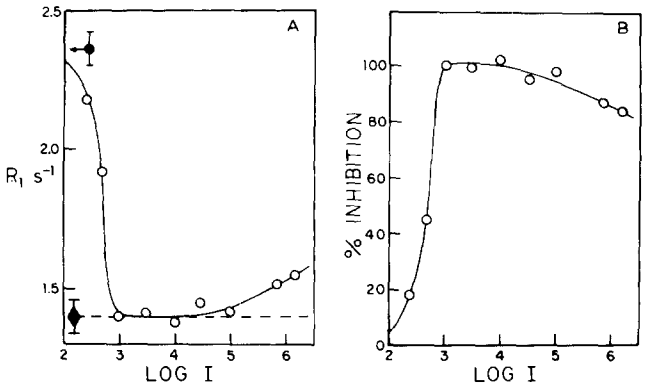


Fig. 11. (A) Effect of continuous illumination on R_1 enhancements produced by 1 mM NH_2OH; R_1 following 30 s dark incubation and 4 min illumination at the indicated intensity. (B) Percent inhibition of NH_2OH-extraction produced by various illuminating intensities.

sponding to the termination of illumination. These extrapolated points, plotted in Fig. 11A, reflect the R_1 enhancement after 30 s dark incubation in addition to enhancements occurring during the illumination period; 30 s dark incubation alone produces an R_1 of 1.41 ± 0.10 s^{-1} (solid diamond), whereas full development of the enhancement in the dark leads to R_1 = 2.35 ± 0.1 (solid circle). The extent of inhibition of NH_2OH extraction conferred by 4 min illumination has been expressed as a percentage of total protection (i.e., R_1 = 1.41 after 4 min) in Fig. 11b. As expected from the O_2 evolution assays, protection is total at $I \approx 10^3$ erg · cm^{-2} · s^{-1}, and falls off rapidly at lower intensities. In contrast to the behavior of O_2 evolution (Fig. 10), however, Mn release is arrested quite effectively between 10^3 and 10^5 erg · cm^{-2} · s^{-1}, and greater than 80% protection remains even after illumination at 1.25 · 10^6 erg · cm^{-2} · s^{-1}. If, after this illumination period, NH_2OH is added to the suspension to restore the concentration to extracting levels, the process of Mn extraction as monitored by R_1 enhancements resumes. Apparently the NH_2OH-induced inhibition of O_2 evolution activity observed at high light intensity is a process distinct from that observed in the dark and does not involve attack on the Mn associated with water oxidation.

NH_2OH inhibition of O_2 evolution; effects of light intensity

Another indication that the reaction leading to inactivation in strong light differs from the dark process is seen in the experiments of Table II. Chloroplasts were incubated in 1 mM NH_2OH for 4 min and illuminated with intense white light for various intervals at the beginning of the incubation period (Table II). Lengthening the illumination period from 0 to 45 s increasingly protects against inactivation, which is consistent with the hypothesis that photooxidation is depleting the extracting concentration of NH_2OH. At longer times (1—4 min) however, this process is reversed, and intense illumination is less effective in inhibiting inactivation. Apparently, a second, slower inactivation process occurs under intense illumination; this process requires NH_2OH (control experiments in the absence of NH_2OH showed little, if any, inactivation of O_2 evolution activity) but the dependence on NH_2OH concentration is

TABLE II

EFFECT OF VARIED ILLUMINATION PERIODS DURING THE ONSET OF NH_2OH INHIBITION OF OXYGEN EVOLUTION

Chloroplasts were incubated in 1 mM NH_2OH for 4 min. Illumination was 400 kergs $\cdot$ cm^{-2} $\cdot$ s^{-1} for the times indicated immediately after addition of NH_2OH. The O_2 evolution is presented in μmol O_2 $\cdot$ mg^{-1} Chl $\cdot$ h^{-1}.

Illumination time (s)	O_2 evolution after 4 min in 1 mM NH_2OH	% Control activity
0	50	17
15	70	22
30	114	36
45	141	44
60	88	18
240	75	15

uncertain because of the probability of substantial photooxidative depletion of NH_2OH during the illumination period.

NH_2OH inhibition of O_2 evolution; effect of CCCP

Lastly, we examined the apparent ability of CCCP to accelerate the inactivation of oxygen evolution by NH_2OH in the dark. For these experiments, a fixed dark incubation period (90 s) was employed with varying concentrations of CCCP. The results of these experiments are shown in Table III. The data show that CCCP exerts a maximal enhancing effect on NH_2OH inactivation at concentrations between 25 and 50 μg. Note also that a small inhibition of oxygen evolution (10%) is seen with 50 μM CCCP in the absence of NH_2OH. These results suggest that CCCP is less efficient in accelerating NH_2OH extraction than it is in its action as an ADRY reagent, where concentrations of CCCP of about 5 μM were effective in producing deactivations of higher S-states [20].

Discussion

The central result of the experiments described here is that light, presented either as continuous illumination or as a sequence of saturating flashes, inhibits NH_2OH extraction of Mn from PS II. This result is in marked contrast with Mn

TABLE III

EFFECT OF CCCP CONCENTRATION ON ACCELERATION OF INACTIVATION OF O_2 EVOLUTION BY 1 mM NH_2OH

Chloroplasts were incubated for 90 s with 1 mM NH_2OH plus indicated concentrations of CCCP. O_2 evolution rates are μmol O_2 $\cdot$ mg^{-1} Chl $\cdot$ h^{-1}.

CCCP (μM)	Rate of O_2 evolution	% Control
0	216	68
1	198	62
5	150	47
10	132	41
25	101	32
50	88	28
50 (minus NH_2OH)	284	90

extraction by 0.8 M Tris buffer, where light strongly accelerates Mn extraction through production of S_2. The most straightforward interpretation of the data in Figs. 2,8,10 and 11 is that higher S-states are immune to attack by NH_2OH; in the presence of NH_2OH, higher S-states are reduced to the susceptible state (or states) in times of the order of 5—10 s. For this reason sequences with dark intervals longer than about 10 s are ineffective in arresting the inactivation process. Presumably the observed absence of correlation between the kinetics of extraction and the S-state present prior to addition of NH_2OH (Table I) likewise reflects the ability of NH_2OH to reduce higher S-states to the susceptible state on a time-scale of 5—10 s. A consequence of this explanation, however, is that the kinetics at the onset of NH_2OH extraction should be complex, in general exhibiting a lag prior to the appearance of R_1 enhancement or of inactivation of O_2 evolution activity. Lags of this kind have been observed in the O_2 measurements (e.g., Fig. 7) and variable lags are often observed in the NMR experiment. The latter are not easily quantitated however, since it is difficult, using our apparatus, to measure R_1 within a few seconds of NH_2OH addition.

The process of Mn extraction by NH_2OH is arrested quite efficiently at moderate or high turnover rates, i.e., at intensities of continuous illumination above about 10^3 erg $\cdot$ cm^{-2} $\cdot$ s^{-1} or in flash sequences with dark intervals $\lesssim 2$ s^{-1}. The totality of protection under conditions of rapid turnover is at first sight surprising in view of the fact that these conditions should produce equal populations of $S_{0,1,2,3}$, and thus should populate a reduced state, S_0, that appears to be susceptible to NH_2OH attack. On this basis, a limiting rate of Mn release under high light intensity equal to one-quarter the dark rate would be expected. It seems probable, however, that NH_2OH binding to the susceptible state is slow. For example, Bouges [22] found that 50 μM NH_2OH retards the well-known oscillation in oxygen flash yields by two flashes and that this process requires dark incubation times of approx. 60 s. This phenomenon was interpreted in terms of slow NH_2OH binding to the Mn center. Subsequently, an interpretation of the flash retardation based on the slow dark reduction of the S-states by bound NH_2OH was suggested [23]. The latter hypothesis was based on the observation that H_2O_2, like NH_2OH, retards oscillations in the O_2 flash yield by two flashes, but that the first two flashes delivered to dark-adapted thylakoids do not oxidize bound H_2O_2 (since significant O_2 yields are not observed). Apparently H_2O_2 does not remain bound to S_0 or S_1 in the dark, but reduces them to a nonphysiologic level, S_{-1} [24]. A similar interpretation can explain the 2-flash retardation in O_2 yields produced by NH_2OH in flash sequences. In any event, it appears that the interaction of NH_2OH with the water oxidizing center to produce the susceptible state is relatively slow, and that rapid turnover of the S-states decreases the efficiency of this process.

It is somewhat surprising that an ADRY reagent, CCCP, does not noticeably inhibit the protection conferred by a sequence of flashes with a 1-s dark interval (Fig. 6). The concentration of CCCP used should have deactivated S_2 and S_3 in times much shorter than the dark interval in these experiments [20]. It seems likely that the rate-limiting step in the reappearance of the extractable state involves reduction of the lower S-states, S_1 or S_0 to S_0 to S_{-1}, rather than the higher S-states, S_2 or S_3. Alternatively, NH_2OH binding to the S-state may block the ADRY effect of CCCP.

The effect of CCCP in accelerating the dark inactivation of NH_2OH was unexpected. Other uncouplers of photophosphorylation (e.g., gramicidin + 1 mM NH_4Cl) have no effect on the kinetics of extraction, indicating that the properties of CCCP as a protonophore are not involved. An explanation based on the properties of CCCP as an ADRY reagent appears to be ruled out by the high concentrations required to achieve appreciable acceleration (Table II). Furthermore, CCCP has very little influence on the inhibition of NH_2OH extraction of inactivation through the intentional production of higher S-states by means of flash sequences. Although it is possible that CCCP in some way enhances the susceptibility of lower S-states to NH_2OH attack, there is no clear evidence that the dark acceleration by CCCP reflects a direct effect on the S-states at all. CCCP and the closely related ADRY agent, FCCP, affect at least two other sites on the oxidizing side of PS II. Both CCCP and FCCP affect the kinetics and extent of Signal II changes [24,25] and both reagents lower the midpoint potential of cytochrome *b*-559 [26—28]. The action on NH_2OH extraction may therefore be a secondary effect of phenomena at one of these neighboring sites.

The present results appear to conflict with the experiments of Horton and Croze [7], who found that NH_2OH extraction of Mn is more extensive in the light than in the dark. The reason for this discrepancy is not clear at present although we would note that the extraction conditions (incubation in 5 mM NH_2OH plus 1 mM EDTA for 30 min at 25°C) used by Horton and Croze are not identical to ours. Other complexities associated with NH_2OH extraction of Mn(II) also remain to be evaluated. First among these is the quantitation of Mn(II) extraction by this agent. Experiments in the dark [5] and the light [7] suggest that one additional Mn is extracted in the light. It has also been shown that the light extraction procedure (using manipulations avoided in the present work) serves to lower the ability of NH_2OH to act as a donor to the oxidizing side of PS II and enhances Mn(II) extraction. This raises the question of whether a fifth, more tightly bound Mn might be involved in PS II activity.

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