Effects of Hydroxylamine on Photosystem II

I. FACTORS AFFECTING THE DECAY OF O₂ EVOLUTION

GEORGE M. CHENIAE AND IRIS F. MARTIN
Research Institute for Advanced Studies, 1450 South Rolling Road, Baltimore, Maryland 21229

ABSTRACT

Illumination of chloroplasts in the presence of NH₂OH (2 mM) leads to the destruction of all system II activities without affecting system I activity. The system II primary charge separation remains intact when incubated with this agent in the dark with release of one of the system II Mn pools and simultaneous destruction of O₂ evolving capacity. The size of the Mn pool associated with the O₂ evolving center is calculated to be 4 Mn/O₂-evolving center.

We observed the following properties of the hydroxylamine-induced destruction of O₂ centers in darkness:
1. The rate of destruction proved proportional to the concentration of O₂ centers and hydroxylamine.
2. The temperature dependency showed a Q₁₀ of 2.43.
3. The pH dependency suggested that unprotonated NH₂OH is the effective destructive species.
4. The destructive effectiveness of NH₂OH for inducing loss of O₂ centers and Mn was markedly altered by N- and O-substitution (NH₂OH > NH₄SO₂ > CH₃NH₂OH ≈ NH₄OCH): NH₂OCH showed markedly different behavior than the parent compound.
5. The rate of O₂ center destruction proved independent of redox buffering with either ferricyanide or ascorbate-dichloro-phenolindophenol.
6. Complete loss of O₂ evolution capacity did not affect the photooxidation of NH₂OH, serving as an artificial electron donor to System II; electron transport to methylviologen showed a red-drop and in System II light proceeded with the low quantum requirement of ~2 hr/equivalent.

Chloroplasts from summer greenhouse spinach (4–5 Mn/400 chl., 50 to 60% of the Mn pool of the O₂-evolving center) showed high quantum requirement for O₂ evolution (5–6 hr/equivalent) yet photooxidized NH₂OH with low quantum requirement (~2 hr/equivalent).

Although the inhibitory action of NH₂OH on photosystem II has long been recognized (9, 11, 15, 20), the complex mode of action of this inhibitor has only recently begun to be understood (2–4, 10, 21).

Previously we noted that incubation of chloroplasts in darkness with NH₂OH resulted in a progressive decay of O₂ evolution capacity and a loss of two-thirds of the system II Mn pool (4). The capacity of system II to oxidize NH₂OH, however, remained apparently unaffected.

This paper reports observations on the decay of O₂ evolution induced by NH₂OH and some of its derivatives in darkness and of parameters affecting this decay.

MATERIALS AND METHODS

Rate Measurements of O₂ Evolution and Hydroxylamine Photooxidation. Rate measurements of O₂ exchange were made polarographically as described previously (4). Light from a 750-w projection lamp was focussed through 12 inches of H₂O, 2 inches of 2.5% CuSO₄, and an OG-3 filter onto the polarograph vessel. For assay of spinach chloroplast-Hill activity, the reaction mixture (1 ml) contained in μmoles: Tricine-NaOH, 50: methylamine, 30: FeCN, 1.6; and chloroplasts equivalent to 10 μg of chlorophyll.

Hill activity of Anacystis nidulans and Chlamydomonas reinhardtii was assayed in a mixture containing in μmoles: potassium phosphate buffer, pH 6.75, 50; FeCN, 2; freshly sublimed benzoinone, 1.0; KCN, 1.0; and cells equivalent to 5 and 10 μg of chlorophyll, respectively. Scenedesmus cells (10 μg of chlorophyll) were assayed as described previously (5).

The reaction mixture for DCIPH₂ photooxidation contained in μmoles: Tricine-NaOH, 50; methylamine, 30; methylviologen, 0.1; KCN, 0.1; ascorbate, 5; DCIP, 0.10; DCMU, 2; and chloroplasts equivalent to 10 μg of chl/ml.

Cofactor studies for NH₂OH photooxidation (8) indicated a requirement for viologen for obtaining maximal rates in low as well as high light intensities. However, the addition of methylamine (30 mM) yielded only a 1.1-fold increase in rate of NH₂OH photooxidation in strong light (Vₘₐₓ from 800 to 900 eq/chl-hr). An "uncoupling factor" of 13.5 (Vₘₐₓ from 100 to 1350 eq/chl-hr) was obtained for methylamine with the same chloroplasts in the H₂O-viologen assays (pH 7.5). Though such results suggest that NH₂OH itself is an "uncoupler," methylamine was routinely added in the assays of NH₂OH photooxidation in strong light. The assay medium therefore contained in μmoles: Tricine-NaOH, 50; methylamine 30; hydroxylamine 50 or 100; methylviologen, 0.1; KCN, 0.1; and chloroplasts, extracted or preincubated with NH₂OH, equivalent to 10 μg of chl. In all assays of NH₂OH photooxidation a H₂O/O₂ ratio of 1 was assumed and thus the observed rates of O₂ uptake were multiplied by 2 to express rates as eq/chl-hr.

1 Abbreviations: DCIP and DCIPH₂; 2,6-dichlorophenolindophenol, oxidized and reduced forms, respectively; FeCN: potassium ferricyanide; HQ: hydroquinone; PDA: p-phenylenediamine; STN: 0.4 M sucrose-0.05 M Tricine-NaOH-0.05 M NaCl, pH 7.4.
Absolute quantum yields were determined polarographically as described previously (4, 5). Absorption measurements of chloroplasts were made in a double-beam integrating sphere with correction for scattering, using the same interference filters employed in the rate measurements. These filters (Thin Film Products, Cambridge, Mass.) were of 5 nm half-band width blocked to infinity on both sides of the peak transmission. Extraction of chloroplasts with NH₄OH did not alter the absorption values of chloroplasts at 640, 650, and 710 nm. The absorption values obtained were essentially the same as described previously by Schwartz (17).

Preparation of Chloroplasts, Culture of Algae, and Extraction Procedures. Chloroplasts from spinach were prepared (16) in a medium containing 0.4 M sucrose-0.05 M Tricine-NaOH-0.05 M NaCl, pH 7.4. Chloroplast extractions with hydroxyamine were routinely made at 4°C by suspending chloroplasts (50 or 200 μg of chl/ml) in grinding medium containing neutralized hydroxyamine. After a specified time of extraction, cold STN was added (7 volumes), and chloroplasts were recovered by high speed centrifugation in a Model RC-2B Sorvall centrifuge as described previously (4). The chloroplasts then were washed twice with 8 ml of STN.

Alternately, chloroplasts were "extracted" directly in the polargraph. In this procedure chloroplasts were equilibrated in the FeCN Hill-reaction assay medium after which hydroxyamine was injected through a small hole in the vessel cap. This procedure could not be used with the N-methyl and O-sulfonate derivatives of hydroxyamine, since they reacted rapidly with FeCN. Extraction of algae was made at 23°C by suspending cells (200 μg of chl/ml) in 1 ml of 0.02 M potassium phosphate, pH 6.75, containing neutralized hydroxyamine. After designated times, 7 ml of phosphate buffer was blown in, and the cells were recovered by rapid centrifugation. Subsequently, the cells were washed repeatedly (two to four times), with 8 ml of phosphate buffer before determination of Hill activity.

Scenedesmus and Anacystis cells were cultured as described previously (5, 6). C. reinhardi, wild type, was cultured at 25°C on minimal medium supplemented with 0.2% sodium acetate (19). Illumination was provided with a bank of cool-white fluorescent lights supplemented with tungsten lamps providing 600 ft-c incident. Cells were harvested by centrifugation at 25°C and then washed twice in 0.02 M potassium phosphate buffer, pH 6.75.

52Mn-Labeled chloroplasts were prepared from 5Mn-labeled spinach plants cultured in nutrient solution (4). Mn analyses were made as described previously (4).

Chloroplast chlorophyll determinations were made as described by Arnon (1). Chlorophyll a was determined for Anacystis following extraction with 80% (v/v) acetone. A millimolar extinction coefficient of 82 (13) (663 nm) was used. For chlorophyll determination of Chlamydomonas and Scenedesmus, a modification (4) of the extraction procedure of Milner et al. (14) was used.

Source and Preparation of Solutions of Hydroxyamine and Its Derivatives. Hydroxyamine HCl (A.C.S. certified) was obtained from Fisher Scientific Company, Fair Lawn, New Jersey. Derivatives of hydroxyamine were obtained from K and K Laboratories, Plainview, New York. Solutions of these compounds were adjusted to the pH of the extraction media, rapidly chilled to 4°C, and used immediately. Despite these precautions, decomposition of some of the derivatives, particularly the O-sulfonate derivative, was indicated by gas emanation from the solution and an ever decreasing titr using Mn52 as oxidant.

FIG. 1. Effect of hydroxyamine upon chloroplasts in light and dark. Three milliliters of the viologen Hill assay mixture (see Materials and Methods) containing 2 mM NH₄OH were equilibrated at 15°C in 25 ml Erlenmeyer flasks. Chloroplasts (300 μg chl) then were added. The abscissa represents the elapsed time before addition of 5 volumes of STN and recovery of chloroplasts. Flasks of curves 1', 2', and the open symbols of curves 3 and 3' received no illumination. Flasks of curves 1, 2, and the closed symbols of Curves 3 and 3' were irradiated with light (>460 nm) sufficient to saturate the Hill reaction. The following assays were made: curves 1 and 1', Hill activity; curves 2 and 2', NH₄OH photooxidation; curves 3 and 3', DCMU-DCIPH₅ photooxidation. Hill activity of chloroplasts not exposed to NH₄OH was 1164 equivs/chl-hr.

RESULTS

General Effects of NH₄OH upon Reactions of System II and System I. Figure 1 shows the effect of NH₄OH in the presence and absence of light on O₂ evolution and donor reactions of system II and system I. In this experiment chloroplasts were incubated during the indicated time with or without light in the presence of NH₄OH. After incubation the suspensions were diluted 5-fold with cold STN, centrifuged, and washed twice with STN to remove the NH₄OH. The following activities were then assayed: Hill activity (curves 1 and 1'); DCMU-sensitive NH₄OH photooxidation (curves 2 and 2'); and DCMU-insensitive DCIPH₅ photooxidation (curves 3 and 3').

First, we note that rates of system I sensitized DCIPH₅ photooxidation in low (curve 3) or strong light (curve 3) were totally unaffected by incubation with NH₄OH, irrespective of light (open symbols) or darkness (closed symbols). Second, we noted that NH₄OH induces a progressive loss of Hill activity which proceeds four times faster in light (curve 1) than in darkness (curve 1'). Third, the DCMU-sensitive photooxidation of NH₄OH was unaffected by preincubation in the dark (curve 2') but progressively declined in the light (curve 2').

These results show that (a) NH₄OH does not affect system I, (b) its effect on O₂ evolution is greater in light than in dark-

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Effect of NH$_2$OH Concentration on the Decay Rate and the Immediate Inhibition of $O_2$ Evolution. Though NH$_2$OH has often been used to inhibit photosystem II reactions (2–4, 7, 9–11, 15), in many instances the descriptions of the experiments have not permitted a clear distinction between the dark decay effect and a possible immediate inhibitory effect of this compound on the $O_2$-evolving reactions. Accordingly, the experiments shown in Figure 3 with chloroplasts were made in an attempt to delineate between these two effects upon $O_2$ evolution.

In these experiments chloroplasts were equilibrated directly at 24 C in the reaction vessel with NH$_2$OH. FeCN was then injected into the vessel and initial rates ($V_{	ext{max}}$) of FeCN Hill activity in saturating light were measured.

First, we note that the loss of $O_2$ centers, as indicated by the slopes of the curves of Figure 3 increases with increasing concentration of NH$_2$OH. Curve 1, Figure 3 inset, shows that under the conditions employed, the rate constant of the first order decay was proportional to the concentration of NH$_2$OH. If we assume that $V_{	ext{max}}$ is proportional to the concentration of $O_2$ centers, then the rate of decay of $O_2$ centers is $dx/dt = k(a - x)$ [NH$_2$OH] where $a = \text{initial concentration of } O_2$ centers and $x$, the decrease in concentration of $O_2$ centers in time $t$.

Evaluation of the "immediate" inhibitory effect of NH$_2$OH upon $O_2$ evolution in saturating light was made by extrapolation of curves of Figure 3 to $t = 0$. By comparison of the so-

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**Fig. 2.** Time course of decay of $O_2$ evolution of Chlamydomonas, Anacystis, and spinach chloroplasts. Curves 1 and 1': Anacystis nidulans assayed at high and low light, respectively, after preincubation with 1 mM NH$_2$OH at 24 C for times designated on abscissa; curve 2: Chlamydomonas after preincubation with 2.5 mM NH$_2$OH at 24 C; curve 3: spinach chloroplasts after preincubation with 1 mM NH$_2$OH at 24 C. All tissues were washed twice before assay of Hill activity. For other details, see "Materials and Methods."

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**Fig. 3.** Effect of NH$_2$OH concentration on the rate of destruction of $O_2$-yielding centers. Spinach chloroplasts (10 $\mu$g chl) were suspended in the polarograph vessel with the Hill reaction mixture except for omission of FeCN. NH$_2$OH was then injected to yield concentrations indicated. FeCN was added 15 sec before the end of the preincubation followed by strong illumination to determine the rate of $O_2$ evolution.
obtained intercept values with the original value, estimates of the extents of immediate inhibition were obtained. Such determinations yielded results as shown by curve 2, Figure 3 inset. With five different such determinations a maximal immediate inhibition of 60 to 70% was obtained with 5 mM NH₂OH and half-maximal inhibition at 0.5 mM.

The results presented in Figure 3 and Figure 3 inset suggest at least two modes of action of NH₂OH in darkness upon the O₂-yielding reactions: (a) a first order destruction of the O₂-evolving complex; and (b) an immediate inhibition of O₂ evolution.

Effect of Temperature, pH, and Redox Buffering upon the Rate of Decay of O₂ Evolution. We examined the effect of temperature, pH, and redox buffering upon the rate of decay in darkness of the NH₂OH-induced loss of O₂ evolution.

Temperature. Figure 4 shows typical data relating the effect of temperature to the rate of decay. At 24°C the half-time of the decay was 5.9 times greater than at 4°C. For a 20°C change the results indicate a Q₁₀ of 2.43, which corresponds to an activation energy of 14.5 kcal for O₂ center destruction by NH₂OH.

pH. Hydroxylamine, a weak base (pKₐ = 6.04 at 20°C (18)), exists in solution either protonated or unprotonated. The effect of pH was studied to determine which of the two species invokes loss of O₂ evolution.

In the experiments of Figure 5, spinach chloroplasts were extracted at 4°C with 2 mM NH₂OH in sucrose media buffered at various pH values. After removal of NH₂OH by washing, the rates of O₂ evolution with FeCN as electron acceptor and of NH₂OH photooxidation with violoen as electron acceptor were determined.

Figure 5 shows first that conditions leading to a considerable loss of O₂ evolution do not seriously impair the system II sensitized DCMU-sensitive photooxidation of NH₂OH (8), and second, the NH₂OH-induced loss of O₂ evolution appears to be strongly pH dependent. Whereas extraction at pH 5.7 yielded essentially no loss (about 5%), extractions at pH above 6.9 yielded a loss of 60 and 70% loss of O₂ evolution in weak and strong light, respectively. Half of the maximal effect occurred at about pH 6.3 where the populations of the protonated and unprotonated species of NH₂OH are about equal.

These results suggest that either the unprotonated form of NH₂OH or an oxidation product formed from the alkali labile NH₂OH induces the loss of O₂ evolution. These alternatives were tested by the inclusion of Mn⁴⁺-pyrophosphate in the extraction medium to accelerate the chemical oxidation of NH₂OH. This abolished the destructive effect of NH₂OH upon O₂ evolution. We therefore conclude that the unprotonated species of NH₂OH is responsible for the effects described above.

Redox Poising. A previous report (4) indicated that the loss of one of the Mn pools of system II induced by NH₂OH is correlated with the loss of O₂ evolution capacity. The oxidation state of this Mn pool in dark equilibrated tissue is unknown. We therefore examined the effect of oxidizing or reducing reagents on the rate of decay of O₂ evolution. Figure 6 records results of the effect of prior equilibration of chloroplasts with either FeCN or DCPIP₃ before the addition of NH₂OH.

Curve 1 shows the effect of equilibration of chloroplasts in the presence and absence of FeCN, (closed and open squares) on rate of decay of O₂ evolution. This rate proved to be independent of the pre-equilibration.

The results of curve 2, Figure 6, were obtained by NH₂OH extraction at pH 6.5 of chloroplasts in STN medium (closed circles) or in STN medium containing ascorbate-DCIP (open

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**Fig. 4.** Effect of temperature on the rate of decay of O₂ evolution induced by hydroxylamine. Chloroplasts (400 μg chl) were suspended in 2 ml of STN containing 1 mM NH₂OH. After designated times 6 ml of cold STN were added, and the chloroplasts were recovered by centrifugation. The FeCN Hill activity was assayed with saturating light after two washes with STN. The short durations at 24°C in STN alone did not result in any loss of Hill activity.
Similar results were obtained with NH$_2$OSO$_3$, except that, in general, slightly higher concentrations were required to yield the same effect as NH$_2$OH. Since this derivative proved extremely labile and the results of Figures 7 and 8 were not obtained with the same solution, precise comparison of the effect of NH$_2$OSO$_3$ with NH$_2$OH cannot be made.

N-Methyl and O-methyl substitution of NH$_2$OH resulted in a much lower effectiveness for inducing decay of O$_2$ centers (Fig. 7) as well as loss of Mn (Fig. 8). Very high concentra-

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**Figure 6.** Effect of oxidizing and reducing agents on the rate of NH$_2$OH-induced decay of O$_2$ evolution. Data of curve 1 were obtained as follows: open squares: chloroplasts were preincubated in the Hill reaction mixture (minus FeCN) containing NH$_2$OH, FeCN was added 15 sec before the end of preincubation and assay of O$_2$ evolution; closed squares: chloroplasts were preincubated for 3 min in the Hill reaction mixture (plus FeCN) then incubated with NH$_2$OH. Exposure time to NH$_2$OH is given on abscissa. Data of curve 2 were obtained as follows: chloroplasts were suspended for 3 min at pH 6.5 in STN medium (closed circles) or in STN medium containing 2000 μM ascorbate and 200 μM DCIP (open circles), then incubated with NH$_2$OH for designated times. Chloroplasts subsequently were recovered, washed, and assayed for Hill activity (see "Materials and Methods").

(circles). The results show that ascorbate and DCIPH$_2$ do not alter the effect of NH$_2$OH upon O$_2$ evolution. This same conclusion was reached from extraction experiments at pH 7.4.

Equilibration of the NH$_2$OH-extracted chloroplasts with ascorbate-DCIPH$_2$ did not "restore" the O$_2$ evolution of NH$_2$OH-extracted chloroplasts. Such a restoration has been reported recently by Yamashita (personal communication) for tris-extracted chloroplasts (4, 22). It appears that extraction with NH$_2$OH may be relatively more specific than tris extraction for the destruction of O$_2$ centers. We conclude from results of Figure 6 that the rate of destruction of O$_2$ centers by NH$_2$OH is unaffected by redox potential within the limits set by FeCN or ascorbate-DCIPH$_2$.

**Figure 7.** Comparison of the effectiveness of hydroxylamine and some of its derivatives for inducing loss of O$_2$ evolution. Chloroplasts (200 μg chl) were suspended in 3 ml of STN, pH 7.4, at 4°C. The compounds noted in the figure were added to yield concentrations given on the abscissa. After 10-min incubation in darkness 3 volumes of STN were added, and chloroplasts were recovered (see "Materials and Methods"). FeCN Hill activity then was determined in saturating light. Original Hill activity was 1056 eq/chl/hr.

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**Figure 8.** The effectiveness of hydroxylamine and related derivatives for inducing loss of chloroplast Mn. For details see legend to Figure 7 and text. Original chloroplast Mn content was 5.95 g-atoms Mn per 400 moles chl total which was not decreased by two washes with STN alone. Original Hill activity was 1124 eq/chl/hr.
tions (100 mM) of the N-methyl derivative produced the same effect as low concentrations of NH₂OH both with respect to Mn and O₂ evolution. NH₂OCH₃, however, even at high concentrations (100 mM) did not have appreciable effects.

In other extraction experiments (15 min at 4 °C in STN, pH 7.4), the following compounds (10 mM) caused the loss of neither chloroplast Mn nor O₂ evolution: KCN, N₃⁻, NO₂⁻, and NH₄· NH₄· NH₄ was ineffective even in 100 mM concentration and in the presence of a mild reductant such as ascorbate (5 mM). The oxidation potentials (12) for converting hydrazine or NH₂OH to NO are about 0.25 and -0.04 v, respectively. Nevertheless, hydrazine which is isoelectronic to NH₂OH proved much less effective than NH₂OH; 15-min extraction with 20 mM hydrazine at pH 7.5 yielded a 38% loss of O₂ evolution and 36% loss of chloroplast Mn.

**Effect of NH₂OCH₃ in the Light.** Although O-methylhydroxylamine proved only slightly effective in darkness, in the light this compound yielded irreversible decreases of O₂ evolution. Curves 1 and 2 of Figure 9 show the rate of O₂ evolution in the absence of inhibitor at two intensities both in the region of linearity of rate with intensity. Upon admission of strong light, a high and sustained rate was observed with both samples (curves 1' and 2').

In the presence of NH₂OCH₃ (10 mM), irradiation with the same intensities yielded rates of O₂ evolution as described by Curves 3, 3', 4, 4', and 5'. In each instance, the O-methyl derivative was injected into the dark equilibrated vessel 30 sec before illumination. Comparisons of curve 3 with curve 1 (I = 1.13%, with and without inhibitor, respectively) and curve 4 with curve 2 (I = 3.5%, with and without inhibitor, respectively) show that the rates of O₂ evolution are not inhibited initially by O-methylhydroxylamine but decline slowly during the illumination with low light. In subsequent strong illumination, the initial rates (curves 3' and 4') reflected the preceding illumination, i.e., the higher intensity of preillumination resulted in a lower rate in subsequent strong light. Curves 3' and 4' show that continued irradiation with strong light resulted in a rapid abolishment of rate; moreover, in subsequent light-dark cycles no O₂ evolution was seen. In curve 5', no preillumination with weak light was used. In this case the rate of O₂ evolution initially was equivalent to the control rate (curves 1', 2') and declined exponentially with a decay half-time identical to that for curves 3' and 4'. We have also observed that chloroplast Mn is lost (60-70%) with irradiation of chloroplasts in the presence of NH₂OCH₃. These effects upon the loss of O₂ evolution were essentially independent of dark preincubation (a 4 min dark equilibration at 24 °C with 10 mM NH₂OCH₃ yielded only 27% inhibition of the initial rate). We conclude that this compound affects system II primarily in the light. It thus reacts quite differently from NH₂OH.

**Quantum Yields of O₂ Evolution and NH₂OH Photooxidation by Extracted and Unextracted Spinach Chloroplast.** Previous results (4) and those in the preceding sections showed that Mn-extracted chloroplasts photooxidized NH₂OH at appreciable rates in broad band red light, thus yielding suggestive evidence that system II trapping centers are not seriously damaged by extraction with NH₂OH. To examine this further we studied the effect of NH₂OH extraction of chloroplasts upon the quantum yields of H₂O and NH₂OH photooxidation with monochromatic light. Figure 10 shows typical results of such measurements with monochromatic light sensitizing primarily system II (640 nm) and system I (710 nm).

Curves 1 and 2 describe the rates of H₂O photooxidation by unextracted chloroplasts with either FeCN or viologen as electron acceptor as a function of the rate of absorption of 640 and 710 nm quanta, respectively. From the linear portion of curves 1 and 2, we computed quantum requirements for O₂ evolution of 10.4 and 58 hv/O₂ at 640 and 710 nm, respectively.

Following 10-min extraction at 4 °C with STN containing 10 mM NH₂OH, rates of H₂O photooxidation at 640 nm were obtained as described by Curve 3, Figure 10. The partial extraction resulted in a 13-fold increase in quantum requirement (640 nm) of O₂ evolution, which was independent of electron acceptor (FeCN or viologen).

Though such extracted chloroplasts photooxidized H₂O very inefficiently, they photooxidized NH₂OH with high quantum efficiency. With seven different extracted chloroplast preparations we obtained at 640 nm quantum requirements for NH₂OH photooxidation of 2.2 hv per electron (with a standard deviation of ±0.34).

That electrons photochemically derived from NH₂OH pass through both photosystems, as previously concluded by Bennoun and Joliot (3), is proven as follows: (a) Viologen is required for maximal quantum yield; omission of viologen resulted in a 1.5- to 2-fold decrease of quantum yield; (b) for the photo-oxidation of H₂O (unextracted chloroplasts) and NH₂OH (extracted chloroplasts), we found the ratio of φ710/φ640 to be...
5.6 and 4.2, respectively. Thus the “red-drop” associated with NH₂OH photooxidation is very similar to that seen with H₂O photooxidation; (c) 2 μM DCMU caused 95% inhibition of quantum yield as well as of the rate in strong light. This latter result contrasts with previous observations (4) with ascorbate or ascorbate-PDA (22) as artificial electron donors. Apparently, NH₂OH is a more specific electron donor to system II than either ascorbate or PDA.

The results of Figure 10 affirm our previous suspicion (4) that NH₂OH extraction induces loss of O₂ evolution without affecting the primary charge separation in photosystem II or any other site in the electron transport chain between system II and viologen reduction. An interesting side observation was made with chloroplasts prepared from spinach grown in the greenhouse in summer. Such chloroplasts invariably show very high quantum requirements for O₂ evolution (20-30 hν/O₂ at 640 nm or 5-6 hν/eq) and also a low Mn content (4-5 Mn/400 chl₁₉₃₃). For NH₂OH photooxidation we routinely observed require-

ments of 2 to 2.3 hν/eq at 640 nm. Evidently, the labile O₂ evolving enzyme is affected in such chloroplasts.

**Discussion**

In this report, we have extended previous observations (4) on the complex action of NH₂OH on system II and the associated O₂-yielding centers. The results clearly allow a clear distinction between the effects of NH₂OH in darkness and during irradiation. Exposure to NH₂OH in the dark causes the loss of O₂ evolution capacity while the capacity to photoxi-

dize an artificial electron donor (NH₂OH) through system II is not impaired. However, irradiation in the presence of NH₂OH accelerates the loss of O₂ evolving capacity and, in addition, destroys the capacity of system II to photoxidize artificial donors (NH₂OH, HQ). In neither instance, however, is there any effect upon a system I sensitized photooxidation (DCIPH₂-viologen-DCMU). Such results seem pertinent to the stabilizing effect of NH₂OH upon the system I sensitized photoreduction by H₂-adapted algae (7).

With cells of Anacystis, Chlamydomonas, Scenedesmus, and mutant No. 8 of Scenedesmus, dark equilibration with NH₂OH resulted in “irreversible” loss of O₂ centers in a manner kinetically similar to that in chloroplasts. Unpublished experiments with Scenedesmus cells have shown that like chloro-

plasts, the larger Mn pool of system II is preferentially destroyed by this dark treatment. Thus the totality of the results indicates that this irreversible destruction of O₂ centers and the associated loss of bound Mn by NH₂OH is probably general for all photosynthetic O₂-evolving tissues. We distinguish this “decay” effect from an immediate inhibition of O₂ evolu-

tion by an independent mechanism. Our evidence for an “im-

mediate” inhibitory effect rests only upon the observations that the first order decay of O₂ evolution did not extrapolate to zero inhibition at zero time. With increasing concentration of NH₂OH we found an increasing immediate inhibition, reaching a maximum of 60 to 70% inhibition of the high light rates with 5 to 10 mm NH₂OH, the effect being half maximal at 300 μM. Joliot et al. (10) have shown a transitory inhibition of flash yield of O₂ (about 100% inhibition of the yield of O₂ from the third flash) by 50 μM NH₂OH. To account for these results, it was suggested that NH₂OH complexed with “Z”, a catalyst of the O₂ yielding reactions. Extrapolation of our results on the immediate inhibition (60-70% of O₂ evolu-

tion by NH₂OH [5-10 mm] obtained at high light) to those of Joliot et al. (10) might suggest that the partial inhibition ob-

served by us simply reflects a limiting rate constant for refor-

mation of the proposed Z-NH₂OH complex. Clearly, how-

ever, distinct separation of the immediate and reversible inhibitory effect from the irreversible destructive effect of NH₂OH upon the O₂ centers is difficult, since the latter process is dependent both upon time and concentration of NH₂OH.

With regard to the NH₂OH-induced destruction of O₂ cen-

ters and release of the associated chloroplast Mn by NH₂OH, we observed marked differences in effectiveness depending upon: (a) pH; and (b) substitution or replacement of the N- and O-groups of NH₂OH. Since neither N- or O-methyl substitu-

tion of NH₂OH markedly affects the pKₐ of NH₂OH (18), the specificity observed (pH 7.4) cannot be related to differences in pKₐ values of the various derivatives. We suspect that factors (steric, stability of coordination complexes, etc.) attributable to the Mn catalyst of the O₂ center contribute to the observed specificity. Such factors thus far have proven difficult to evaluate.

Determinations of the total functional Mn pool of System II have yielded values of 5 to 8 Mn/400 chl₁₉₃₃, with Scenedes-

mus cells (5) and 5 to 6 Mn/400 chl₁₉₃₃, with spinach chloro-

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*Fig. 10. Quantum requirements for H₂O and NH₂OH photo-

oxidation by unextracted chloroplasts and chloroplasts partially extracted with NH₂OH. Curves 1 and 2: H₂O photooxidation by unextracted chloroplasts (FeCN or viologen as electron acceptor) with 640 and 710 nm light. Curve 3: H₂O photooxidation by partially extracted chloroplasts (5 min at 4°C with 10 mM NH₂OH) with 640 nm light. Curves 4 and 5: NH₂OH (50 mM) photooxidation with chloroplasts of Curves 3 with 640 and 710 nm light. Quantum requirements were calculated from linear portions of the curves. The uncoupled rate of O₂ evolution of unextracted chloroplasts in saturating light was 310 amoles O₂/mg chl·hr. Chl a/b ratios of unextracted and extracted chloroplasts were 5.12 and 5.20, respectively.*
plasts (4). We should correct a previous error of calculation (using half of both [Mn] and [chl\textsubscript{total}] instead of using half of [chl\textsubscript{total}] only) which assigned only 2 to 3 Mn to each system II trapping center (4). The correct value is 5 to 8 Mn/system II trap in *Scenedesmus* and 4 to 6 Mn/system II trap in chloroplasts. Oxygen evolution is correlated with only two-thirds (~4 Mn/400 chl\textsubscript{total}) of this total Mn pool (Figs. 7, 8 and ref. 4) while capacity for photooxidation of certain artificial electron donors (4) is correlated with the smaller (~2 Mn/400 chl\textsubscript{total}) more tightly bound Mn pool. This leads to the attractive hypothesis that 4 Mn are associated with each O\textsubscript{2} center and that 1 to 2 additional Mn are more intimately associated with the oxidant side of the system II trapping center (1/400 chl\textsubscript{total}) itself.

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LITERATURE CITED


