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Effects of Hydroxylamine on Photosystem II

II. PHOTOREVERSAL OF THE NH_2OH DESTRUCTION OF O_2 EVOLUTION[1]

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ABSTRACT

The inactivation of O_2-evolving centers by NH_2OH extraction was shown to be reversible. This reversal required light and manganese. This light-induced restoration of active O_2-evolving centers was analyzed using three green algae and the blue-green alga, *Anacystis nidulans*. The following results were obtained:

1. In any given illumination, the time course proved first order, the rate being proportional to the number of *inactive* O_2 evolution centers.

2. The action spectrum proved to be identical to that of system II. The process was independent of system I and occurred in *Scenedesmus* mutant No. 8 devoid of this system.

3. The quantum efficiency was optimal over a small range of intensities and declined at both lower and higher intensities. The efficiency was half maximal at either 1 or 10 hits per system II trap per second.

4. Kinetic data obtained with flashing or continuous light showed that the activation of O_2 evolution centers is a multi-(minimally two) quantum process: the product of the first photoact relaxes to a new photosensitive state of limited stability which is converted by light into a stable O_2 evolution center.

5. The rate constants involved were very simlar to those observed previously with algae depleted of Mn by growth in deficient media.

6. The reappearance of O_2 evolution capacity was correlated with the reappearance into system II of the bound Mn pool which we correlate with the O_2-yielding catalyst.

7. Micromolar concentrations of compounds known to reduce chemically Mn valency states $> +2$ and/or to reduce the light-generated photooxidant of the system II-trapping center proved effective reversible inhibitors.

8. It is suggested that the photoactivation of the manganese catalyst of O_2 evolution is a general phenomenon in all photosynthetic O_2-evolving tissue.

Depletion of Mn from the photosynthetic apparatus by growth in Mn free medium results in a decreased amount of O_2-evolving centers without gross alterations of system II and system I trapping centers, the interconnecting electron transport chains and the associated phosphorylative mechanism (6). The reappearance of active O_2 centers in such Mn-depleted tissue has been shown to require Mn specifically; other metals tested at equivalent or higher concentrations are ineffective (6). Such Mn-depleted cells accumulate Mn intracellularly via dark processes in amounts more than sufficient to satisfy the pool of Mn required in O_2-evolving centers; however, no active O_2 centers are formed without light (4). The Mn-dependent formation of active O_2 centers in such tissue is strictly light-dependent. Our previous kinetic analysis of this photoactivation of O_2 evolution revealed a multi-(minimally two) quantum process driven by the system II trapping centers (8, 22).

More recently, a number of chemical and/or physical agents have been employed to inactivate O_2 centers of chloroplasts without affecting other parts of the photosynthetic apparatus. One such agent is hydroxylamine (5, 7, 14). This inhibitor causes destruction of O_2 centers in algae as well as isolated chloroplasts. With spinach chloroplasts, the loss of O_2 evolution capacity is accompanied by a loss of the manganese we associated with the O_2 center (5, 7).

In isolated chloroplasts the hydroxylamine-induced inactivation of O_2 centers appears to be irreversible. With whole algae, however, the effect can be completely reversed by light after removal of NH_2OH (5, 6). It appeared that the previously noted photoreversal of the effect of NH_2OH "extraction" might be similar to the photoactivation of O_2 centers in tissue depleted of Mn by growth (4, 8).

In this communication we describe a kinetic analysis of the photoactivation of inactive O_2 centers of NH_2OH-extracted algae. The effect of NH_2OH extraction and subsequent photoactivation on the Mn associated with the O_2 centers is described.

MATERIALS AND METHODS

Algal Culture. *Anacystis nidulans* and wild-type *Scenedesmus* cells were cultured as described previously (3, 4). *Scenedesmus* mutant No. 8 (1) was grown on a glucose-yeast extract medium (17) and *Chlamydomonas reinhardi*, wild type, was cultured in a low salt medium (23), supplemented with 0.2% sodium acetate. After 40 to 48 hr of growth, cells were harvested by centrifugation at 25 C, washed and resuspended (about 2 mg chl/ml) in their respective growth media unless otherwise noted. Mutant No. 8, however, was washed and resuspended in wild type *Scenedesmus* medium. The cell suspensions were subsequently aerated with water saturated 5% CO_2 in air until assay of O_2 evolution or incubation with NH_2OH.

Incubation with and Subsequent Removal of NH_2OH. Preliminary experiments indicated that NH_2OH autooxidized rapidly in any of the growth media containing metal micronutrients. Accordingly, for extraction with NH_2OH the cells were washed, then diluted (200 μg chl/ml) with 20 mM potas-

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sium phosphate buffer, pH 6.75. Neutralized NH_2OH then was added to yield 2 mM NH_2OH (details are given in figure legends), and the cells were incubated for 5 or 10 min at 20 to 25 C. The incubation and all other subsequent manipulations were done in darkness. Following addition of 10 volumes of 20 mM phosphate buffer, the cells were recovered by centrifugation, washed repeatedly (4–5 times) with complete growth medium, and resuspended (about 1 mg chl/ml) in complete growth medium. The kinetics of the light-induced appearance of O_2-yielding centers of such incubated and washed cells showed no time lag and were indistinguishable from those of similarly incubated cells which were dialyzed *versus* growth medium at 25 C for 4 hr. Without the repeated washings, finite time lags in the photoinduced appearance of O_2-evolving centers were observed. It was concluded that the repeated washing effectively removed NH_2OH from the cells. Such treatment decreased both V[2] and V_{max} of O_2 evolution to 5 to 10% of the original rate but did not alter respiratory rates.

Assay of O_2 Evolution Capacity. Reaction mixtures for assay of O_2 evolution capacity (Hill reaction) of *Anacystis* (4) and *Scenedesmus* (3) have been described. The assay mixture for *Anacystis* proved optimal for the assay of *Chlamydomonas*.

Chlorophyll and Mn Analyses. Chlorophyll was determined (19) for *Anacystis* following extraction with 80% acetone. A millimolar extinction of 82 (19) (663 nm) was used for determination of chlorophyll. For green algae, the total chlorophyll was determined (3) following extraction with a 1:1 (v/v) mixture of 5% (w/v) methanolic KOH and 10% (w/v) Triton X-100. Procedures for Mn determinations and ^{54}Mn counting have been described (5).

Preillumination Conditions and Assay of the Photoreversal of the Effect of NH_2OH Incubation. The conditions for preillumination of *Anacystis* with flash or continuous monochromatic light have been described (8). With the other algae, the concentration of the cell suspensions was chosen such that the flashes were saturating and the absorption of the monochromatic light was less than 30%. An integrating sphere was used for absorption measurements.

In some instances algae (300 μg chl in 3 ml of growth medium in 25 ml Erlenmeyer flasks) were preilluminated from below on a shaker bath. A yellow filter (No. 46-Cinemoid filter, Kliegl Brothers, Long Island City, N.Y.) interceded between the cell suspension and the tungsten light source. The intensity at the base of the flask was 25 ft-c.

After removal of NH_2OH, the O_2 evolution capacity was determined in strong light to yield a value of R_1. Following preillumination of cells, the O_2 evolution capacity was redetermined. The increase of O_2 evolution capacity from preillumination over and above R_1(Δ specific activity of V_{max}) was a measure of the effect of preillumination. In no instance did we observe any increase of O_2 evolution capacity during incubation (up to 12 hr) in darkness; moreover, the effect produced by preillumination was independent of the dark time between the preillumination and assay regimes.

RESULTS

The following diagram outlines the sequence of steps used here for the assessment of the photoinduced appearance of active O_2-evolving centers in NH_2OH-"extracted" cells:

NH_2OH-extracted cells → 1 preillumination → 2 dark → [addition of BQ, FeCN, KCN] → 1, 2 → [assay of rate of O_2 evolution in strong light]

The difference between specific activity of rates of O_2 evolution from regimes 1 and 2 was taken as a measure of the effectiveness of the preillumination regime for photoinducing the appearance of active O_2-evolving centers.

Previous results with NH_2OH-extracted *Anacystis* (8) and preliminary experiments with the other algae established that the light-induced increase of V_{max} was accompanied by a proportionate increase in V. Such results ruled out the possibility that the preillumination simply affected k_d, the over-all dark rate-limiting step of the Hill reaction.

The above sequence for determination of new O_2 centers rests on earlier studies of photoactivation of O_2 evolution with Mn-deficient algae. Its validity rests upon the peculiar synergistic inhibitory effect by benzoquinone and high quantum flux on the appearance of new active O_2 centers (4). This same synergistic effect was verified for the NH_2OH-extracted cells and thus permitted determination solely of the effect of preillumination for inducing new active O_2 centers. The evidence that no new O_2 centers are formed *during* the actual assay of O_2-evolving capacity rests on the observation that no significant *increases* in rates of O_2 evolution are observed during alternate 1-min light-dark regimes with either extracted, partially or fully photoactivated cells.

In contrast to the studies on the photoinduction of O_2 centers with Mn-deficient cells (4, 8), the kinetics and quantum yield for photoreversal of the effect of NH_2OH extraction proved totally independent of prior dark equilibration duration with Mn^{2+}. Preliminary experiments with ^{54}Mn-labeled algae showed that the NH_2OH incubations did not alter the intracellular Mn concentration (~1 Mn/10–20 chl). This intracellular amount of Mn is in excess of the amount required for Mn-dependent photoactivation of O_2-evolving centers (4).

Effect of the Number of Inactive O_2-evolving Centers on the Time Course of the Photoreversal of NH_2OH Extraction. Studies of the photoactivation (8) of the Mn-deficient *Anacystis* cells obtained by growth yielded evidence which indicated: (*a*) the time course was first order and the quantum yield invariant in any given illumination and (*b*) the rate of photoactivation was proportional to the number of *inactive* O_2 centers. In these experiments the number of inactive O_2 centers was varied by culturing cells at different suboptimal concentrations of Mn. The pigment alterations which accompany Mn depletion of *Anacystis* by growth necessitated corrections for differences in absorption and possibly complicated conclusions made from these experiments.

With NH_2OH extraction the concentration of inactive O_2 centers can be varied without altering the pigments. Accordingly, we re-examined the relationship between the rate of photoactivation as a function of the initial concentration of inactive O_2 centers (Fig. 1). *Anacystis* cells (V_{max} = 376 μmoles O_2/mg chl·hr) were partially extracted to yield cells with a R_1 value of 37.6 (closed circles, Fig. 1). Then a portion of such cells were preilluminated on a shaker bath to yield cells with R_1 value of 160 (open circles, Fig. 1). If we neglect correction for possible photon transfer between units (15, 21), the 4-fold difference in R_1 reflects a 4-fold difference in initial concentration of O_2-evolving centers. The closed and open circles of Figure 1 describe the increase of O_2 evolution capacity of cells (R_1 = 37.6 and 160, respectively) as a function of time of preillumination with 620 nm light. Open circles

[2] Abbreviations: V_{max}: rate of O_2 evolution at saturating intensity; V: rate of O_2 evolution in linear portion of rate *versus* intensity curve; R_1: value of V_{max} following partial extraction with hydroxylamine; STN: 0.4 M sucrose-50 mM Tricine-50 mM NaCl, pH 7.4.

(R_1 = 160) are displaced on the abscissa by 125 sec. These data show that despite a 4-fold difference in initial concentration of O_2 centers, the photoinduced appearance of active O_2 centers followed a simple exponential curve. Similar results were obtained in studies with extracted *Scenedesmus, Chlamydomonas,* and *Chlorella* cells.

The Light-induced Reversal of NH_2OH Inactivation is Sensitized by System II. In experiments of Figure 2, extracted, washed *Anacystis* cells were irradiated with light absorbed primarily either by system II (620 nm) or system I (700 nm), then assayed for O_2 evolution capacity to determine the relative effectivity of system II *versus* system I for inducing appearance of O_2 centers. In 620 nm light the O_2 evolution capacity increased eventually some 18.3-fold and attained a value equivalent to the original unextracted cells after about 15 min of irradiation. As noted (Fig. 2) the time course of appearance of O_2 centers in 620 nm light proved to be identical at either 15.1 nanoeinsteins absorbed per min or a 3- to 10-fold higher quantum flux. This result indicated that a 620 nm quantum flux of 47.8 nanoeinsteins absorbed per min was more than sufficient for saturation of the process without yielding photoinhibitory effects.

As shown in a later section, the lower intensity of 620 nm light used in the experiments of Figure 2 was nearly optimal for maximum quantum efficiency. This information plus the constancy of kinetic order at any intensity (see later section) therefore permitted a meaningful evaluation of the effectiveness of light at other wavelengths.

In the experiments of Figure 2, we compared the effectiveness of system I *versus* system II light by setting the rate of absorption of system I light (700 nm) equivalent to the maximum rate of absorption of system II light yielding near optimal quantum efficiency. The initial slopes of the curves of Figure 2 therefore yield the effectivity of system II *versus* system I

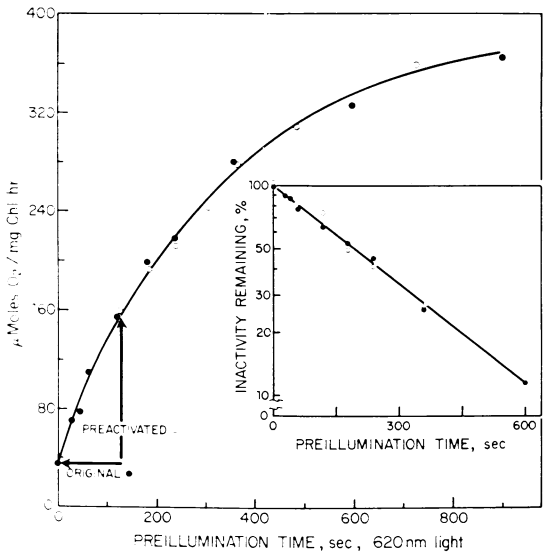

FIG. 1. Time course of photoactivation as affected by the initial concentration of O_2-yielding centers. *Anacystis* cells (200 μg chl/ml) were extracted with 2 mM NH_2OH for 10 min at 23 C, washed repeatedly, and then resuspended (50 μg chl/ml) in 20 mM potassium phosphate buffer, pH 6.75. A portion of the extracted cells were preilluminated on the shaker bath for 1 min before dilution to 4 μg chl/ml. The extracted, and extracted and "preactivated" cell suspensions (4 μg chl/ml; 1.8 ml) were then preilluminated with 620 nm light (12.9 nanoeinsteins absorbed per min) for times given on the abscissa. Subsequently, the O_2 evolution capacity was determined using 2 μg chl/ml. The Hill activity of the original and extracted cells was 376 and 37.6 μmoles O_2/mg chl·hr, respectively.

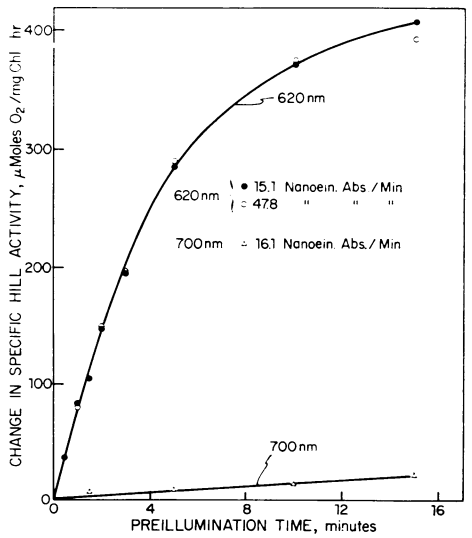

FIG. 2. Wavelength sensitization of the photoreversal of the effect of NH_2OH extraction. *Anacystis* cells (200 μg chl/ml) were extracted with 2.5 mM NH_2OH for 10 min at 20 C, then washed repeatedly (see "Materials and Methods") and finally resuspended in 20 mM potassium phosphate buffer, pH 6.75. Cells then were diluted to 4.6 μg chl/ml and 30 μg chl/ml for preillumination with 620 nm and 700 nm light, respectively. The suspensions (1.8 ml; 23.5% absorption in 0.34 cm light path) were preilluminated with the quantum fluxes noted in the figure. Following preillumination the O_2 evolution capacity was determined using 3.6 μg chl/ml. The original and extracted cells yielded rates of O_2 evolution of 530 and 19.6 μmoles O_2/mg chl·hr.

light for photoreversal of the NH_2OH extraction effect. We estimate from the data of Figure 2 that system I light is maximally only 3% effective as system II light. Similar results were obtained in experiments where the intensity of the system I beam was adjusted to yield a rate constant equivalent to that obtained under conditions of optimal quantum efficiency of system II light.

Experiments with blue light (447 nm) showed that the quantum efficiency in blue light for the appearance of active O_2 centers was only 40% of that in 620 nm light. This comparative effectiveness of these wavelengths (620 *versus* 447 nm) for appearance of O_2-evolving centers also is reflected in earlier reports of the efficiency spectrum of photosynthetic O_2 evolution (9). Such results allow us to exclude any special blue light effect on the light-induced reversal of the NH_2OH extraction.

The above results suggested that quantum events in system II reversed the NH_2OH effect; however, they did not completely eliminate the possibility that both system II and I are required for the reversal. If strictly a system II process, photoreversal also should be observed with *Scenedesmus* mutant No. 8 (1), an organism essentially lacking Photosystem I. This supposition proved valid as shown by the results in Figure 3. We conclude from the results of Figure 2 and 3 that the light induced appearance of active O_2 centers in NH_2OH extracted cells is driven only by quantum events within the system II trapping center.

KINETICS OF THE PHOTOREVERSAL OF THE EFFECT OF NH_2OH EXTRACTION: A MULTI-QUANTUM PROCESS.

Evidence From Quantum Yield Measurements. The saturation of the photoreversal of the effect of NH_2OH extraction by moderate light intensities (about 10–20% of the intensity required for saturation of photosynthesis) implies that one or

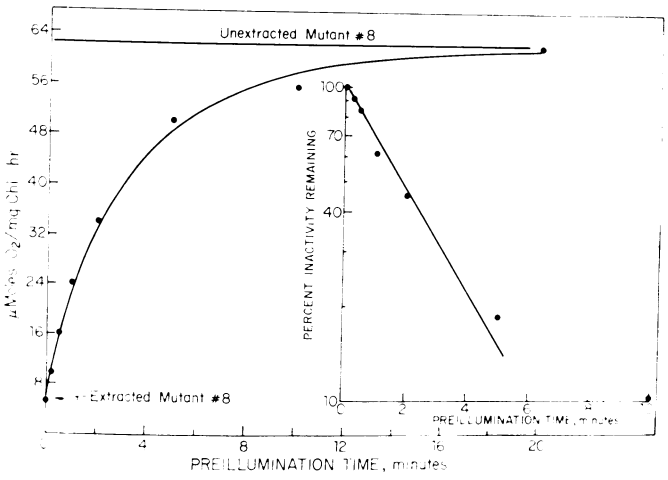

FIG. 3. Photoreversal of the effect of NH_2OH extraction on the O_2 evolution capacity of *Scenedesmus* mutant No. 8. Cells were centrifuged from growth medium, washed once with 20 mM potassium phosphate, pH 6.75, and resuspended at 200 μg chl/ml. The suspension was extracted with 2 mM NH_2OH for 20 min at room temperature, then 10 volumes of phosphate buffer were added. Cells were recovered by centrifugation, washed three times, and finally resuspended at 100 μg chl/ml in phosphate buffer. Three ml of the suspension in 25 ml Erlenmeyer flasks were illuminated (25 ft-c) from below on a shaker bath (see "Materials and Methods") for the times indicated on the abscissa. The unextracted mutant No. 8 cells yielded a rate of photosynthesis (in Warburg No. 9 buffer) in saturating light of only 8 μmoles O_2/mg chl·hr.

more slow (> 100 msec) dark steps are involved in the over-all process. As long as the rate of system II light absorption is sufficiently low to permit the rate-limiting dark steps to reach completion, the quantum efficiency for the process will be maximal. Any decline of quantum efficiency with increasing rate of quantum absorption will reflect the limiting dark step(s). To evaluate the limiting dark step(s), we measured the quantum efficiency for the appearance of active O_2-evolving centers over a 20-fold range of intensities yielding optimal quantum efficiency as well as saturation of the process.

Typical results of such experiments are shown in Figure 4 where the change in specific activity of O_2 evolution is plotted *versus* the absorbed light dose. In this plot the initial slopes of the curves are a measure of the quantum efficiency for the appearance of O_2-evolving centers.

Within the narrow range of 0.97 to 2.1 einsteins absorbed per minute per mole chl (closed squares and closed circles, respectively) the quantum efficiency was constant, and the rate of appearance of O_2 centers was proportional to intensity. With increasing intensity the quantum yield declined until eventually the rate of appearance of O_2 centers became constant, *i.e.*, the process was saturated. Curves 1 and 2 (quantum efficiency and rate, respectively) of Figure 4 (inset) reveal the effect of intensity on these parameters. Ignoring for a moment the descending portion of curve 1 of Figure 4 (inset) at very low intensities, we observe a "flat" portion of curve 1 (constant quantum yield) which is reflected by a linear portion of curve 2 of Figure 4 (inset) (rate *versus* intensity). In this range of intensity, quantum efficiency is maximal (I × t), and the observed rate of appearance of O_2-evolving centers is a linear function of intensity. With increasing intensity, the quantum efficiency declined as a consequence of the dark rate-limiting step coming into play. From the descending portion of curve 1 at the higher intensities, an estimate of the rate-limiting dark step can be made. The data show that the quantum efficiency was decreased to one-half maximal at about 4.2 einsteins absorbed per minute per mole chl. Assuming an abundance of system II traps of 1/300 chl and that half of the 620 nm quanta are directed towards system II, we calculate that the quantum efficiency is half-maximal at about 10 hits per trap per sec, corresponding to a dark rate-limiting step of about 100 msec.

Figure 4 and Figure 4 (inset) also show the effect of very weak intensities on the quantum efficiency and rate of appearance of O_2-evolving centers. At 0.35 einstein absorbed per min per mole chl (open triangles of Fig. 4), the quantum efficiency is only 26% of maximal and the rate of appearance of O_2 centers shows an intensity "lag" (Fig. 4, inset). This result suggests that in the process leading to the appearance of an O_2 center, the absorption of the first quantum produces a product which decays and is lost from the process unless processed by the absorption of another quantum. An estimate of the half-life of the unstable intermediate can be made from the ascending portion of curve 1, Figure 4 (inset). Using the same assumptions employed above, we estimate from these data the half-life of the intermediate to be about 1 sec.

Evidence from Flashing Light Experiments. Preliminary experiments showed that properly spaced brief (2 μsec) saturating light flashes were effective with various algae for reversal of the effect of NH_2OH extraction. This observation permitted an extension of the kinetic studies reported in Figure 5 and a comparison with similar studies (8) previously made with Mn-deficient *Anacystis*.

The effect of dark time (t_d) between flashes on the yield of O_2-evolving centers induced by 75 repetitive flashes is recorded in Figure 5. We observed that the yield of O_2-evolving centers induced by the flashes was minimal at short t_d values but increased with increasing t_d values reaching a maximum at t_d of 0.5 to 1 sec. The yield then markedly declined with increasing dark time between flashes. Yields of 13.8 and 3.5% of maximal were observed even with flash spacing of 5 and 8 sec, respectively.

The ascending portion of the curve of Figure 5 reflects the same dark rate-limiting step causing the decline of quantum efficiency in weak to moderate 620 nm light (curve 1, Fig. 4,

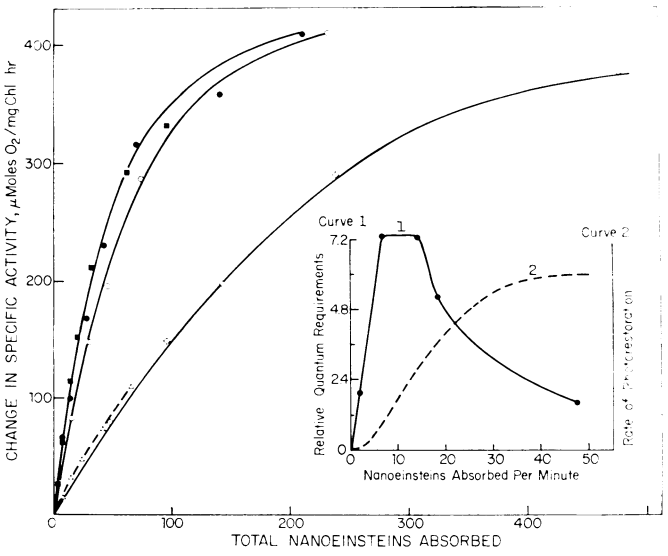

FIG. 4. Effects of rates of quantum absorption on the relative quantum yield of the photoreversal of effects of NH_2OH extraction. Rates of 620 nm light absorption were varied with neutral density filters. Other details are given in Figure 3 legend. Rates of quantum absorption (nanoeinsteins/min) by 6.6 μg chl (total) were: ●: 14.1; ■: 6.4; ○: 15.5; △: 2.3; ◇: 47.8.

inset). Both results therefore show that quanta cannot be effectively utilized until the dark rate-limiting step has approached completion. From Figure 5 we estimate the half-time of the dark rate-limiting step to be about 130 msec, a value in general agreement with results obtained from the quantum efficiency measurements of Figure 4 (100 msec) and those previously obtained (110–200 msec) with Mn-deficient cells (8).

On the other hand, the decreasing yields with increasing t_d between flashes (Fig. 5) reflect the ascending portion of curve 1, Figure 4 (inset) and confirm the results obtained with continuous light. These results imply that more than one photoevent is needed to reverse the effect of NH_2OH extraction and that the effect of the first photoevent decays and disappears. From the descending portion of the curve of Figure 5 we estimate the decay half-time to be 1.3 sec, whereas from Figure 4 we calculated about 1 sec. Flash experiments with extracted *Chlorella* cells yielded results similar to those obtained with extracted *Anacystis* cells. With *Chlorella*, however, the decay rate of the presumed unstable intermediate was somewhat slower ($t_{1/2}$ = 3–4 sec).

These results with NH_2OH-extracted cells proved entirely similar to results previously obtained for photoactivation of O_2 evolution in Mn-deficient cells (8). With the latter cells, evidence also was obtained from paired flash experiments for a relaxation step much faster (completion in 150 msec) than the 100 to 200 msec half-time component mentioned above. Preliminary experiments showed that this more rapid relaxation step also existed in NH_2OH-extracted algae. In the comparison of the effectiveness of single and paired flashes for inducing O_2-yielding centers (Table I), a dark time of 150 msec between the single and paired flashes was used to allow the more rapid relaxing step to reach completion and to maximize any observed effect from the delay flash.

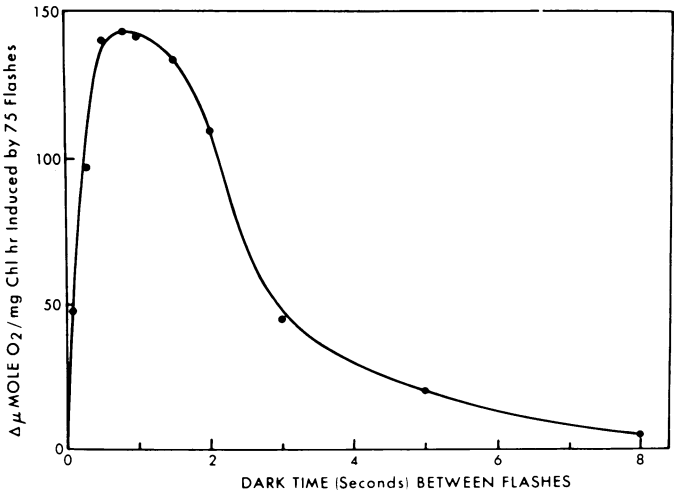

FIG. 5. Effect of dark time between flashes on the yield of new active O_2-evolving centers of NH_2OH-extracted *Anacystis*. *Anacystis* cells were washed and resuspended (200 μg chl/ml) in 20 mM potassium phosphate buffer, pH 6.75. The cell suspension was extracted for 5 min at room temperature with 1 mM NH_2OH. After addition of 6 volumes of growth medium, the cells were recovered by centrifugation, washed, and finally resuspended (50 μg chl/ml) in growth medium. The cell suspension was aerated with 5% CO_2 in air for 2 hr before dilution to 4 μg chl/ml for preillumination by saturating 2-μsec flashes. Hill activity rates of unextracted, extracted, and extracted and fully restored cells were 640, 57.7, and 647 μmoles O_2/mg chl·hr, respectively. Values on the ordinate represent the increase of specific activity of O_2 evolution induced by 75 repetitive flashes.

Table I. *Comparison of the Effectivity of Single versus Paired Flashes on the Yield of New Active O_2-evolving Centers of NH_2OH-extracted Anacystis*

NH_2OH-extracted, washed *Anacystis* cells were exposed to either 75 repetitive single or paired flashes of Δt_d = 150 msec. Hill activity rates of unextracted, extracted, and extracted and fully restored cells were 650, 94.6, and 660 μmoles O_2/mg chl·hr. For other details, see "Materials and Methods" and Figure 6 legend.

Flash Regime	First Flash Spacing	Yield of O_2 Centers	Ratio Yield of O_2 Centers
Δt = 150 msec	*Δt in sec*	*Δ μmoles O_2/mg chl·hr*	*paired / single flashes*
Single	0.3	97	
Paired	0.3	135	1.39
Single	1.0	142	
Paired	1.0	245	1.73
Single	5.0	21	
Paired	5.0	106	5.05
Single	8.0	6	
Paired	8.0	90	15.0

This comparison of the effectiveness of single *versus* paired flashes (Table I) showed that the effect from a paired flash is minimal with a first flash periodicity of 0.3 sec, about doubled at a t_d value of 1 sec, and increased up to 15-fold with the longest (8 sec) first flash periodicity. Such results confirm and extend the conclusions made from experiments of Figure 4. The similarity between data of Figures 4 and 5 and Table I with similar data obtained previously (8) with Mn-deficient *Anacystis* is striking. We therefore believe the same kinetic processes deduced for the photoactivation of Mn-deficient tissue also operate in the photoreversal of the effects of NH_2OH extraction.

Effect of NH_2OH Extraction of *Scenedesmus* and Subsequent Photoactivation on the Chloroplast Manganese and O_2 Evolution Rates. The similarity between the kinetics of the Mn^{2+}-dependent photoactivation of Mn-deficient cells and the photoreversal of NH_2OH extraction suggested that Mn perhaps was involved in both processes. In the experiments recorded in Figure 6, attempts therefore were made to relate the effects of NH_2OH extraction of *Scenedesmus* cells and subsequent photoactivation of O_2 evolution to the bound Mn pool(s) (5, 7) of chloroplast particles isolated from such cells.

The unextracted cells used in these experiments yielded rates of O_2 evolution in strong light of 170 and 120 μmoles O_2/mg Chl·hr (open bars, Fig. 6, of experiments I and II, respectively). These rates are less than we generally encounter (225 O_2/mg chl·hr) (3). Such cells yielded chloroplast particles containing 5.2 and 4.8 Mn/400 chl (open bars, Fig. 6, of experiments I and II, respectively). This amount of Mn was not decreased by repeated washings with STN containing 1 mM EDTA and thus represents the bound Mn of the chloroplast particle.

If, however, the same cells were extracted (2 mM NH_2OH for 20 min at 20 C), the rates of O_2 evolution in strong light were decreased in experiments I and II from 170 and 120 to 10 and 5 μmoles O_2/mg chl·hr, respectively, and the bound Mn of isolated chloroplast particles now contained only 1.2 and 0.8 Mn/400 chl (shaded bars, Fig. 6, experiments I and II, respectively). The Mn content of such particles was not diminished by further extraction with 2 mM NH_2OH for 10 min at 4 C. In contrast, NH_2OH extraction of the particles from unextracted cells decreased the Mn content from 5.2 and 4.8 Mn/400 chl to 1.5 and 1.2 Mn/400 chl. Such results reflect that the small Mn pool is relatively resistant to NH_2OH

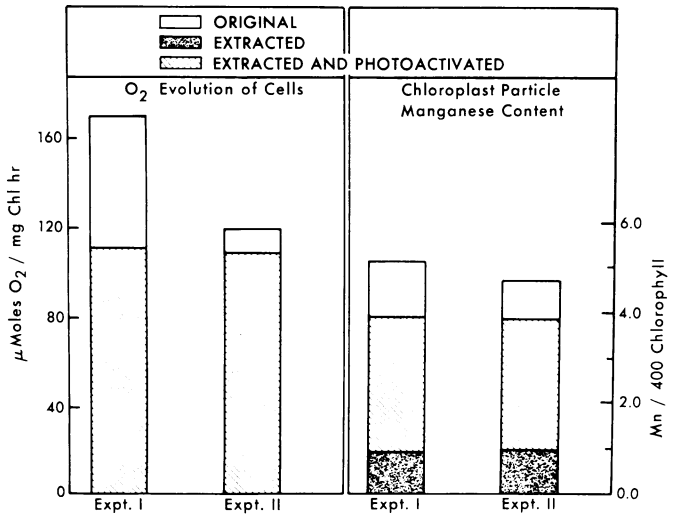

FIG. 6. Effect of NH_2OH extraction and subsequent photoactivation on the Hill activity of *Scenedesmus* cells and the bound manganese of chloroplast particles from the cells. *Scenedesmus* cells (10 μl packed cell volume inoculum per 600 ml medium) were cultured for 48 hr as described previously (3) in a medium containing 30 μc ^{54}Mn/600 ml medium. Cells were collected by centrifugation and divided into two portions. One portion of the cells was assayed for O_2 evolution, then chloroplast particles were prepared (17). The other portion of cells was extracted (2 mM NH_2OH for 20 min), washed, then resuspended (129 μg chl/ml) in the original growth medium. The extracted cells of experiments I and II yielded rates of O_2 evolution in strong light of 10 and 5 μmoles O_2/mg chl·hr, respectively. One-half of the extracted cell suspension was illuminated (400 ft-c for 1 hr) and the other half maintained in darkness for 1 hr. Cells were then assayed and chloroplast particles prepared. The chl*a*/chl*b* ratios of the original, extracted, and extracted and photoactivated cells of experiment 1 were 3.19, 3.25, and 3.14, respectively.

extraction and that the larger Mn pool, correlating with O_2 evolution capacity, is destroyed by NH_2OH (5, 7).

Aeration of such extracted cells in darkness (up to 8 hr) resulted neither in an increase of O_2 evolution capacity nor an increase of the bound Mn of chloroplast particles. However, partial photoactivation of the extracted cells yielded nearly identical rates of O_2 evolution (110 and 107 μmoles O_2/mg chl·hr, experiments I and II; slashed bars, Fig. 6, respectively) and resulted also in an increase of bound chloroplast Mn from 0.8 to 1.2 to 3.9 Mn/400 chl (slashed bars of Fig. 6). This Mn content (3.9 Mn/400 chl) again was decreased to the original Mn content (1 Mn/400 chl) of particles from extracted cells by NH_2OH extraction of either the photoactivated whole cells or the particles isolated from the photoactivated cells.

From such experiments we conclude: (*a*) the effect of NH_2OH extraction on O_2 evolution capacity and chloroplast Mn of whole cells is similar to results obtained with spinach chloroplasts (5, 7); and (*b*) photoreversal (photoactivation) of NH_2OH extracted *Scenedesmus* results in an increase of the chloroplast Mn pool we associate with the O_2-yielding catalyst (7).

Inhibition by Reducing Agents and Artificial Electron Donors to System II. It has been proposed (22) that Mn^{2+} photooxidation (10, 12) by the primary photooxidant of system II is one of a complex series of reactions ultimately leading to formation of an active O_2-yielding center. Components capable of rapidly reducing the system II primary photooxidant and/or the presumed higher valency state of Mn generated in photoactivation therefore should inhibit the photoactivation process. Hydrazine (11, 20) and hydroquinone (24) are known to be photooxidized by system II, and to reduce higher valency states of Mn but to cause essentially no destruction of O_2 centers (7). These properties of the compounds permitted an examination of the effect of reducing agents and/or system II artificial electron donors on photoactivation itself.

The rapidity of oxidation of these compounds by trace metals, accentuated at the alkaline pH values of the growth media, precluded the use of growth media for resuspension of "extracted" cells in these studies. Accordingly, the extracted cells were washed and resuspended in 20 mM potassium phosphate buffer, pH 6.7, prepared in deionized water. With these precautions reproducible results could be obtained.

In the experiments of Figures 7 and 8, using hydrazine and hydroquinone, respectively, the cells were equilibrated in darkness for 20 min at the concentrations given on the abscissas. Cell suspensions then were illuminated long enough (90 sec) to yield in the absence of these compounds $< 50\%$ of the maximum photoactivation. Following four washings of the cells to remove the hydrazine or hydroquinone, the yield of O_2 centers was determined.

As shown in Figure 7, hydrazine at concentrations of 75 and 400 μM yielded 50 and 100% inhibition, respectively, of photoactivation. With 1 mM hydrazine the inhibition was complete even with prolonged illumination regimes (Fig. 7, inset). Similar results also were obtained with hydroquinone (Fig. 8); however 0.6 and 6.6 mM concentrations were required to yield 50 and 100% inhibition, respectively.

The following considerations point to a rather specific effect of these compounds on photoactivation: (*a*) no inhibition of photoactivation was observed after removal of these compounds by repeated washings; and (*b*) active O_2 centers were

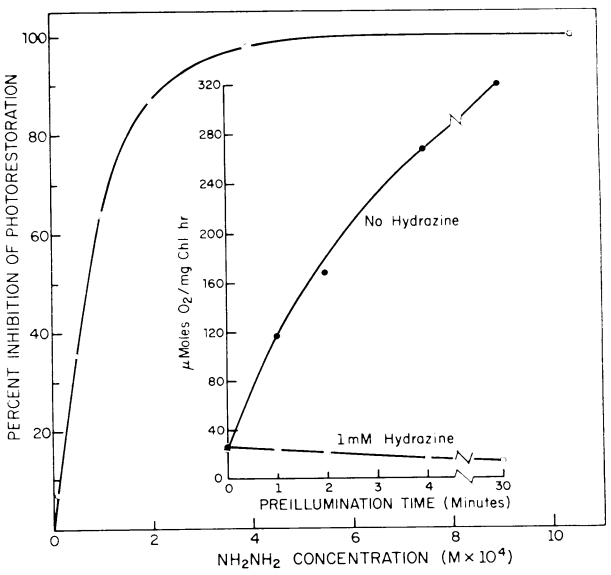

FIG. 7. Inhibition of the light-induced appearance of O_2-evolving centers by hydrazine. *Anacystis* cells were extracted with 2 mM NH_2OH for 10 min at room temperature, then washed and resuspended in 20 mM potassium phosphate, pH 6.75. Three ml of cell suspension (33.3 μg chl/ml) in 25 ml Erlenmeyer flasks were incubated in darkness for 20 min at 20 C in 20 mM potassium phosphate containing the concentration of freshly prepared, neutralized hydrazine given on the abscissa. The suspensions were then illluminated (25 ft-c) from below (see "Materials and Methods") for 90 sec. The flask contents and a 5-ml rinse were combined for recovery of cells by centrifugation (2 min at 20,000*g*). After three washings with 6 ml of 20 mM potassium phosphate buffer, pH 6.75, the cells were suspended and assayed. Hill activity rates of unextracted and extracted cells were 428 and 33.2 μmoles O_2/mg chl·hr.

not diminished by equilibration of cells with hydrazine (1 mM) or hydroquinone (3 mM) in either light or darkness. Several hypotheses can be made to explain these results; at the present time we interpret them to suggest that Mn^{2+} photooxidation is essential in the formation of an active O_2 center.

DISCUSSION

Two rather direct effects of NH_2OH on the O_2-evolving mechanism of green plants have been described: (*a*) the transitory inhibition by "bound" NH_2OH of flash-induced O_2 yield oscillations (2); and (*b*) a dark-irreversible but light-reversible destruction of the O_2 centers (6, 8) which occurs upon incubation of algae or chloroplasts with NH_2OH in darkness. We distinguish these effects of NH_2OH, which at least in algae are reversible, from the irreversible destructive effects on system II observed during illumination of NH_2OH-poisoned photosynthetic tissue (7). This report has described the restoration of O_2 centers of NH_2OH-extracted algae. Since the activation is reflected by increased O_2 evolution capacity in both weak and strong light, we conclude that entire O_2-evolving centers are either made or uncovered during the activation process.

This activation, or photoreversal of the effect of NH_2OH extraction, proved strictly light-dependent with several green algae (*Chlorella, Chlamydomonas, Scenedesmus*) and the blue-green alga, *Anacystis nidulans*. We observed the following similarities between the kinetics of photoreversal of NH_2OH extraction and previously published (8) kinetics of photoactivation of Mn-deficient tissue: (*a*) both processes are sensitized by the reaction centers of system II; (*b*) the rate of appearance of active O_2-evolving centers in both processes is proportional to the number of *inactive* O_2-evolving system II trapping centers, the quantum yield being invariant and low over a finite range of intensity; (*c*) both processes occur via a multi-quantum process in which a product of the first photoact relaxes to a new photosensitive state of limited stability which is converted by another photoact(s) into a stable, active O_2-evolving center; and (*d*) for a given alga (*Anacystis*) the limiting and decay rates of the intermediates involved in the formation of an active O_2 center were essentially the same for Mn-depleted (growth) and NH_2OH-extracted cells. Moreover, the light-induced appearance of O_2 centers in both type tissues is reversibly inhibited (4) by DCMU but is not inhibited by actidione and chloramphenicol, in amounts sufficient for 90% inhibition of ^{14}C-phenylalanine incorporation into protein (13). Results of similar experiments with other extracted algae (*Chlorella, Scenedesmus* wild type and mutant No. 8, and *Chlamydomonas*) were essentially consistent with those obtained with *Anacystis*.

The striking similarity between the kinetics of photoreversal of NH_2OH extraction and the photoactivation of Mn-deficient cells precludes any similarity to the process of photoreversal of "bound" NH_2OH (2). Moreover, the photoreversal of bound NH_2OH (2) minimally is a one quantum process in contrast to the complex low quantum yield, multi-quantum process of photoactivation described here and elsewhere.

We believe that the similarity of the kinetics of the formation of active O_2 centers in NH_2OH-extracted and in Mn-depleted algae strongly suggest an underlying common process with a common factor(s). One of these factors appears to be the system II Mn pool we associate with the S states of Kok et al. (18) or Z states of Joliot *et al.* (16). This supposition seems justified for the following reasons: (*a*) Mn depletion by growth and/or NH_2OH extraction of chloroplasts or algae depletes primarily the system II Mn pool correlating with O_2-evolving capacity, resulting in loss of O_2 evolution in any illumination regime without grossly altering other components of the photosynthetic apparatus; (*b*) the appearance of O_2-evolving capacity and the insertion of the Mn into O_2-yielding centers are both strictly light-dependent; (*c*) photoactivation in Mn-depleted tissue is specific for Mn; other metal ions at equal or 5-fold higher concentration than the optimal concentration of Mn^{2+} are ineffective; and (*a*) the appearance of Mn in the O_2 centers is associated with the appearance of system II to evolve O_2.

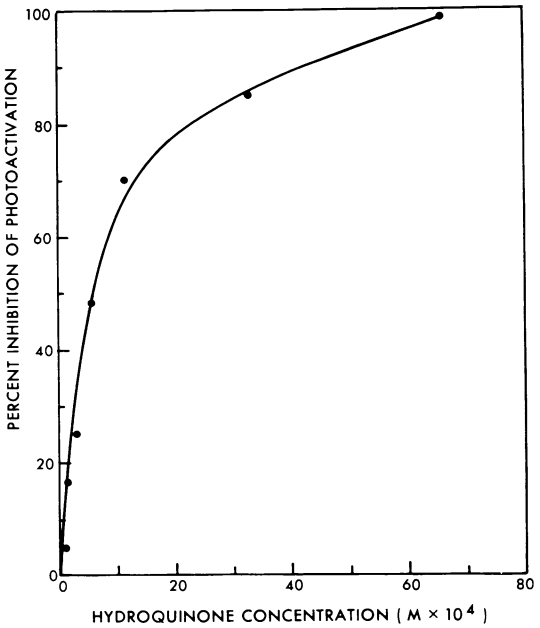

FIG. 8. Inhibition of the light-induced appearance of O_2-evolving centers by hydroquinone. *Anacystis* cells (33.3 μg chl/ml) in 3 ml of 20 mM potassium phosphate, pH 6.75, containing 2 mM ascorbate and the concentration of hydroquinone given on the abscissa were incubated in darkness for 20 min, then illuminated (25 ft-c) from below for 90 sec. Washing and recovery of cells was done as outlined in Figure 7 legend. Hill activity rates of unextracted and NH_2OH-extracted cells were 558 and 44.6 μmoles O_2/mg chl·hr, respectively. The 90-sec illumination in absence of hydroquinone produced a change in specific activity of 116.5 μmoles O_2/mg chl·hr.

From the arguments presented above, the model previously proposed to explain the complex sequence of reactions in the photoactivation of Mn-deficient cells also appears applicable to photoreversal of NH_2OH-extracted cells. The hypothesis (22) has been made that Mn^{2+} photooxidation to Mn^{4+} is required for the insertion of Mn into the O_2-yielding center. The strong and reversible inhibition by compounds (hydrazine, hydroquinone) known to either chemically reduce Mn valency states $> 2+$ and/or to reduce the light-generated photooxidant of system II (11, 20) tend to support this hypothesis. Our results do not necessarily imply that the ground state valency of Mn in such O_2-evolving centers is $> 2+$. It is entirely possible that the presumed photooxidation of Mn^{2+} to Mn^{4+} is required only for the insertion of Mn into its yet unidentified O_2-evolving complex. Clearly, these questions and others pertinent to photoactivation remain to be resolved. Nevertheless, the demonstration of photoactivation with Mn-deficient cells, NH_2OH-extracted cells and heterotrophic cells cultured in darkness (unpublished data) all lead us to conclude that the photoactivation of O_2-evolving centers is fundamental for all O_2-evolving organisms.

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