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STUDIES ON THE MECHANISM OF TRIS-INDUCED INACTIVATION OF OXYGEN EVOLUTION

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Summary

A study was made of the inactivation by Tris of O_2 evolution in chloroplasts and the subsequent reactivation of O_2 evolution. We conclude:

1. At concentrations of Tris sufficient to inhibit O_2 evolution directly, a slow rate ($t_{1/2} \approx$ 20—25 min) of inactivation occurs;

2. Inactivation is accelerated ($t_{1/2} \approx$ 2 min) by weak light absorbed by system II and is rate limited by a dark step with a half-time of about 200 s;

3. Minimally one quantum event within System II is sufficient to inactive 50—70% of the O_2 evolving centers;

4. This process is 3-(3,4-dichlorophenyl)-1,1-dimethylurea insensitive but is inhibited by reduced dichlorophenol indophenol and phenazine methosulfate, carbonylcyanide-p-trifluoromethoxyphenylhydrazone, 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene and tetraphenylboron;

5. Partial reactivation of inactive O_2 evolving centers is effected by the use of the same reagents inhibiting the light induced inactivations;

6. The life-time ($t_{1/2} \approx$ 1 to 3 h) of the activable state is correlated with diffusion across thylakoids of the larger manganese pool released from binding sites and remaining in thylakoids following inactivation of O_2 evolution.

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Abbreviations: ANT-2p, 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCIP and $DCIPH_2$, oxidized and reduced forms of 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F and F^+, the precursor and the species yielding EPR Signal II; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; F_0, the invariable fluorescence yield; F_{max}, the maximum level of fluorescence; Sig. II_f and Sig. II_{vf}, the fast and very fast decaying species of EPR Signal II; V, quantum yield; V_{max}, uncoupled rate at saturating intensity. Buffer 1, 0.4 M sucrose/0.05 M Tricine/0.02 M NaCl, pH 7.4.

Introduction

Tris washing of chloroplasts [1—7] has been used extensively to interrupt the reactions linking the S states of oxygen evolution to the System II reaction center. It is generally believed that the inactivation of oxygen evolution by this treatment is correlated with a perturbation of about two-thirds of the bound manganese of System II as observed by a loss of chloroplast manganese from the thylakoids [4,7] (see, however, refs. 8, 9), or the appearance of EPR detectable manganese within the thylakoids [5], or a decrease of the proton relaxation rate of water in chloroplast suspensions [9].

Other techniques for rather specific inactivation of oxygen evolution, such as temperature shock [4,7,11,12] and hydroxylamine washing [4,10,13], similarly affect the bound manganese of System II. However, the inhibitory effect on chloroplasts of Tris washing appears to be distinct from these other System II inhibitory treatments in that the inactivated O_2 evolving centers can be partially 14—16] or completely reactivated [6] by washing the Tris-inactivated chloroplasts with compounds such as reduced DCIP. According to Blankenship et al. [6], such reactivation results in a rebinding of a major portion of the free, uncomplexed Mn^{2+} within the thylakoids, disappearance of the Tris-induced Sig. II_f species with reappearance of Sig. II and Sig. II_{vf} [17,18], and a reappearance [6] of normal kinetics of the S-state transitions [19].

To date, however, little information exists concerning the mechanism of the Tris-induced inactivation of the S-states. Results have indicated that this inactivation process is highly pH dependent, suggesting that the rate of inactivation is a function of the concentration of the unprotonated species of Tris [14]. In addition, Ikehara and Sugahara [2] have noted that the Tris-induced inactivation of O_2 evolution is markedly enhanced by light. This light effect was attributed to a photoinhibition process even though only weak intensities of light were required for obtaining enhanced rates of inactivation.

On the other hand, Velthuys and Amesz [20] and Velthuys [21] have indicated that the non-destructive [22] inhibition of O_2 evolution by another unprotonated amine, NH_3 [23], is associated with formation of a complex between NH_3 and the S_2 and S_3 states but not with the dark-stable S_1 state. We reasoned that the weak light-enhanced inactivation of O_2 evolution by Tris first noted by Ikehara and Sugahara [2] might not reflect a typical photoinhibition process [24] but rather a mechanism involving formation of a complex of the amine function of Tris to an S-state (S_{1+n}) with subsequent release of manganese from its native binding site. Here we report studies of the kinetics and quantum requirements for the Tris-induced inactivation of O_2 evolution, effects of DCMU, flashes, and chemicals known to destabilize the S_2, S_3 states. Evidence is presented indicateing that any reactivation of O_2 evolution in Tris-treated chloroplasts is dependent on prior inactivation via a process involving one charge separation within System II. Chemical destabilizing S_2 and S_3 states were found to inhibit inactivation and to promote reactivation of O_2 evolution. The life-time of the activable state and its relationship to thylakoid bound manganese is reported.

Methods

Chloroplast preparations and Tris washing

Chloroplasts were prepared routinely as described previously [4] from greenhouse grown spinach (4 to 8 week old plants). In some experiments, chloroplasts uniformly labeled with ^{54}Mn were employed [4]. Entirely similar results to those reported here were obtained with chloroplasts prepared from market spinach or garden peas using a number of isolation media and the chloroplast isolation procedures descrived by Yamashita et al. [14] or Blankenship and Sauer [5].

The isolated chloroplasts routinely were suspended in grinding medium to yield 4 mg chloroplyll/ml then stored in darkness at 4°C for a medium of 30 min before incubation with Tris. Unless otherwise stated, all subsequent operations were made at 4°C with extreme precautions to exclude stray light. These precautions included: (1) taping of microliter syringes and pipettes; (2) use of aluminum foil wrapped test-tubes and flasks; and (3) the elimination of artificial and natural light from the working area.

For incubations with Tris (Reagent Grade, Sigma Chemical Co.), the chloroplasts were added with rapid mixing to 0.8 M Tris-Cl^- (pH 7.6 and pH 8.0 measured at 23° and 4°C, respectively) to yield 200 μg chlorophyll/ml unless otherwise noted. Any additions to the suspension were made just prior to addition of the chloroplasts. After incubation in Tris, the chloroplasts were recovered by centrifugation at 5000 × *g* for 2 min. The resulting pellet was then resuspended (200 μg chlorophyll/ml) in buffer 1 or in buffer 1 containing "reactivating" agents. After 15 min incubation in darkness, the chloroplasts were recovered as above and finally resuspended in buffer 1 for assay of O_2 evolution capacity.

Preillumination and light measurements

The suspensions of chloroplasts in Tris were stirred during preillumination in a cuvette of 0.33 cm light path and 2 ml volume. Light from a 750 W tungsten lamp was passed through 10 inches of water, appropriate neutral density and interference filters before being focussed onto the cuvette. Absorption measurements of chloroplast suspensions in Tris buffer were made in a split-beam integrating sphere. The same interference filters used in the preillumination of the chloroplast suspension were employed in the absorption measurements. These filters had a 5 nm half-hand width blocked to infinity on both sides of the specific wavelength. Light measurements were made with a silicon photocell calibrated versus a standardized bolometer. Rates of quantum absorption were corrected for differences of geometry between the photocell and the vessel.

In experiments employing flash illumination, saturating flashes (2 μs half-duration) were obtained by simultaneously firing two Xenon flash lamps (E.G. and G. OC-5) mounted on opposite sides of a cuvette of 0.33 cm light path. The chloroplast concentration in such experiments was equivalent to 50 μg chlorophyll/ml.

In some experiments, chloroplast suspensions (800 μg chlorophyll in 4 ml of the Tris extraction 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 chloroplast suspension and the tungsten light source. The intensity at the base of the flask in such experiments was $8 \cdot 10^3$ ergs/cm^2/s. Under these conditions fresh chloroplasts, assayed in the reaction mixture described below, yielded a rate of FeCN reduction equivalent to only 3 percent of the rate obtained in saturating light.

Rate measurements of O_2 evolution

Rate measurements of O_2 evolution were made polarographically as described previously [4]. The standard reaction mixture contained in μMol: Tricine/NaOH (pH 7.5), 50; FeCN, 1.0; methylamine, 30; and chloroplasts equivalent to 5 or 10 μg chlorophyll in a total volume of 1.13 ml. Unextracted chloroplasts assayed in this mixture routinely yielded rates about twice that obtained in the mixture described by Blankenship et al. [6].

In some experiments (see specific figure legends) on the kinetics of inactivation of O_2 evolution by Tris, 50-μl aliquots of chloroplast suspensions (200 μg chlorophyll/ml) were removed from the above described cuvette at specific times and added directly to the polarograph vessel for assay of O_2 evolution. Preliminary experiments employing this procedure with dark-equilibrated chloroplasts showed that less than 10% decrease in rates of O_2 evolution occurred at zero time relative to control chloroplasts suspended in buffer 1.

Manganese analyses

Manganese analyses were made by atomic absorption (Perkin Elmer, Model 303, acetylene flame). Samples (chloroplasts equivalent to 3—8 mg chlorophyll and Mn standards) for analyses were digested in conical centrifuge tubes overnight at 170°C in a sand bath with 2 ml of a mixture of 17 vols. conc. HNO_3 per 3 vols. of 70% $HClO_4$ [25]. Following digestion, 4 ml of water was added and the tubes centrifuged to remove any precipitate. The supernatant and two distilled water rinses of the tubes were combined and the volume adjusted to 5 ml before analyses. Linearity of absorption vs Mn concentration was obtained up to at least 20 μM Mn. ^{54}Mn analyses were made as described previously [4].

Fluorescence measurements

Fluorescence measurements were made at room temperature essentially as described by Malkin and Kok [26] employing a fluorimeter with an electronically timed electromechanical shutter. The time-course of fluorescence ($\lambda > 700$) upon admission of a blue exciting light was recorded on a Fabri-Tek 1052 signal averager with subsequent recording on a Clevite 250 recorder. Chloroplasts, equivalent to 3 μg chlorophyll/ml, were equilibrated in darkness for 5 min in the designated media containing 5 μM DCMU before measurements of fluorescence.

Results

Fig. 1 compares the effect of light and dark on the rate of the Tris-induced inactivation of O_2 evolving centers of spinach chloroplasts. In these experiments, chloroplasts were incubated in Tris in darkness or in the presence of

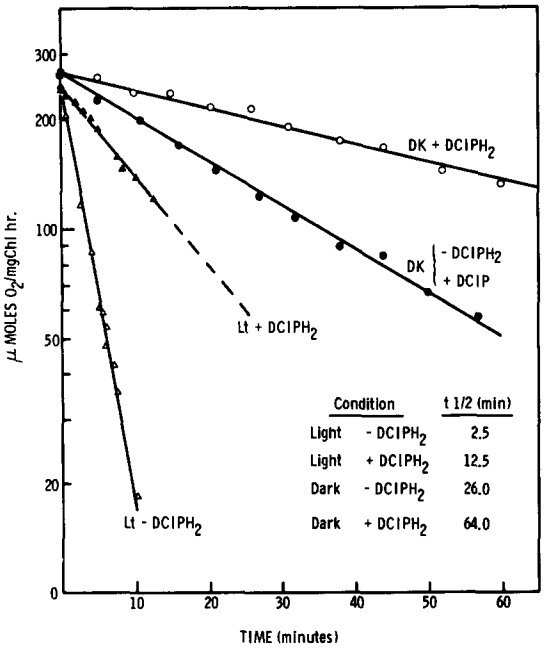


Fig. 1. Rates of Tris-induced inactivation of O_2 evolution in darkness and in weak light. Chloroplasts (200 μg chlorophyll/ml) in 0.8 M Tris at 4°C and containing additions as noted, were incubated on a shaker bath for durations indicated on the abscissa. Following incubation, chloroplasts were recovered by centrifugation, washed once with buffer 1, then rates of O_2 evolution were determined. The concentration of $DCIPH_2$ (with 1 mM ascorbate) or DCIP, where used, was 200 μM. For other details see Methods. DK, dark; Lt, light.

light of an intensity sufficient to yield only three percent of the maximum uncoupled rate of O_2 evolution obtainable with uninhibited chloroplasts. As shown later, however, this light intensity was sufficient to yield maximum rates of inactivation. Following incubation on a shaker bath for the durations indicated on the abscissa, the chloroplasts were recovered by rapid centrifugation then washed once with buffer 1 before assay of remaining O_2 evolution capactiy. Under all of the conditions employed in these experiments, the rate of inactivation of O_2 evolution exhibited apparent first-order kinetics, thus permitting direct comparisons of the effectivity of the various conditions described in Fig. 1 for promoting or inhibiting the inactivation process.

Although we attempted to exclude all light during the course of the experiments for obtaining the data for the Curve labeled Dark, we cannot totally exclude contributing effects from some stray light. With such qualification, we observed a slow ($t_{1/2}$ = 26 min) rate of inactivation of O_2 evolution by Tris in darkness. This rate was unaffected by addition of DCIP to the suspension but was inhibited in the presence of $DCIPH_2$ ($t_{1/2}$ = 64 min). The exposure of chloroplasts to weak light during the incubation with Tris caused a more rapid ($t_{1/2}$ = 2.5 min) rate of inactivation of O_2 evolution which was only partially inhibited ($t_{1/2}$ = 12.5 min) by $DCIPH_2$. This "protective" effect of $DCIPH_2$ against inactivation of O_2 evolution by Tris was previously noted by Yamashita et al. [14]. However, in their experiments the effect of light on the inactivation process was not noted.

Irrespective of the conditions (light vs. dark) for the inactivation of O_2 evolution, equivalent rates of photo-oxidation of artificial electron donors via either Photosystem II and I [4,20] or via Photosystem I [20] were obtained with chloroplasts having 90—95% inactivated O_2 evolving centers. Moreover, we could not detect, using sensitive difference spectroscopy, photobleaching of pigments [27] of chloroplasts inactivated by Tris in weak light. Ignoring the uncoupling action Tris has upon chloroplasts [28], the results of Fig. 1 suggest: (1) The specific inactivation of O_2 evolution by Tris either requires or is enhanced at least 10-fold by weak intensities of light; and (2) $DCIPH_2$ partially inhibits inactivation, its effectiveness dependent on light vs. dark regime during the inactivation process.

Results similar to those of Fig. 1 were obtained with concentrations of Tris ranging from 0.4 to 0.8 M and over a pH range of 7.6 to 8.3, measured at room temperature. With increasing pH, however, we observed increasing rates of inactivation in "darkness" and a corresponding increase in rates of inactivation via the light process. At the more alkaline conditions, $DCIPH_2$ was less effective in either light or dark for inhibiting the inactivation of O_2 evolution, and, in agreement with others [6,14—15], less effective in the reactivation of inactivated O_2 evolving centers. Since our interests here were to define conditions yielding inactive O_2 evolving centers that could be maximally reactivated, all subsequent experiments reported here were limited to use of 0.8 M Tris, pH 7.6 [6].

Intensity dependence of the inactivation process

The saturation of the photoinactivation of O_2 evolution by the relatively weak light intensitites used in the experiments of Fig. 1 (about 3% of the intensity required for saturation of the uncoupled rate of Hill activity) implies that one or more slow dark steps are involved in the overall apparent inactivation process. If the rate of light absorption is sufficiently low to permit rate-limiting steps to reach completion, maximum quantum efficiency will be observed. A decline of quantum efficiency with increasing rate of quantum absorption will reflect the rate-limiting dark step(s). In later sections evidence is presented indicating that the inactivation process is sensitized by the reaction centers of System II. We therefore measured rates of inactivation of O_2 evolution as a function of intensity of 650 nm light in order to evaluate the limiting dark step.

In these experiments, stirred suspensions of chloroplasts (200 μg chlorophyll/ml) in 0.8 M Tris were irradiated with a 650 nm beam over a 50 fold range of intensity. At designated times 50-μl aliquots were removed and assayed directly for remaining O_2 evolution capacity. Typical results are shown in Fig. 2 where the relative rate of O_2 evolution is plotted as a function of time of exposure to 650 nm light of the relative incident intensities shown within Fig. 2.

The results indicate again a slow rate ($t_{1/2}$ = 20 min) of inactivation in darkness that increases with increasing intensity of the 650 nm beam. At the highest intensity (I = 5.2, corresponding to a rate of quantum absorbtion of 17.1 m/einsteins absorbed/min/mol chlorophyll) shown in Fig. 2, the half-time for inactivation was 2.6 min. A 19 fold further increase of intensity resulted in

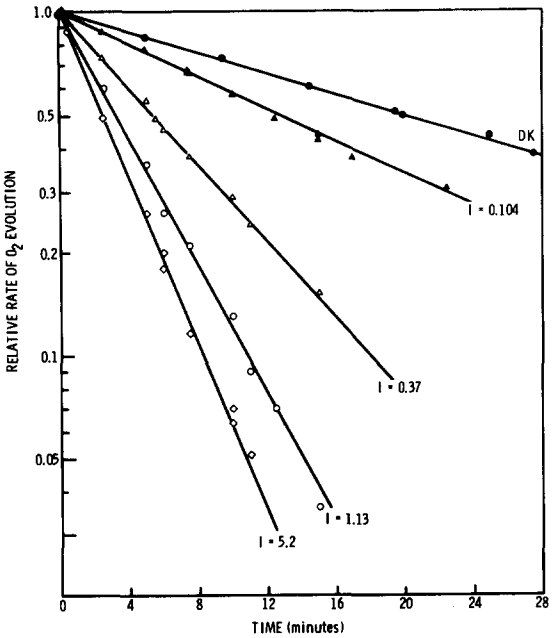


Fig. 2. Rates of Tris-induced inactivation of O_2 evolution with increasing intensity of 650 nm light. Relative intensities are given by the numbers by each curve. I = 5.2 corresponding to a rate of quantum absorbtion of 17.1 meinsteins absorbed/min/mol chlorophyll. Rates of inactivation obtained at I = 100 were only 10 percent greater than at I = 5.2 and, for purposes of clarity, are not shown. For other details see Methods and text. DK, dark.

only about a 10% increase in rate of activation.

To evaluate the rate-limiting step(s) of the inactivation process, first order rate constants were calculated from the half-times of the curves shown in Fig. 2. With correction of the inactivation occurring in darkness, such determined rate constants were plotted vs. System II trap hits per second (Fig. 3). We assumed an abundance of System II traps of 1/400 chlorophyll and that 60% of the absorbed 650 nm quanta were distributed to System II [29]. This evaluation of the rate limiting step of apparent inactivation of O_2 evolution indicates that the quantum efficiency is half-maximal at about 0.005 hits per System II trap/s, corresponding to a dark rate limiting step of about 200 s. The data of Fig. 3 also allow us to estimate that the inactivation process is light saturated at about 1 hit per System II trap per 10 s.

The results shown were typical for many such experiments; however, in a few instances we sometimes observed a short time lag (1 to 2 min) before the onset of inactivation in the light. The chloroplasts exhibiting this behavior also showed extended half-times (40—50 min) of decay of O_2 evolution in darkness. Washing of such chloroplasts with buffer 1 or the addition of 200 μM FeCN abolished both the time lag of inactivation in the light and the extended half-times in darkness. Apparently, unknown soluble reductants within the chloroplasts contributed to these anomalous behaviors of the inactivation process.

Inactivation is sensitized by System II with high quantum yield

In other experiments, the dark equilibrated chloroplasts suspended in Tris

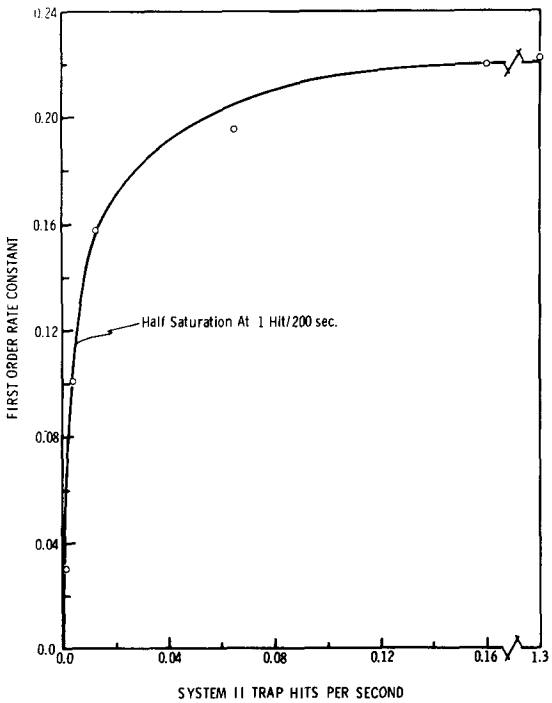


Fig. 3. Plot of first-order rate constants of inactivation versus System II trap hits per second. First-order rate constants were calculated from the date of Fig. 2, corrected for dark-decay rates then plotted versus System II trap hits per s. See text for other details and the assumptions made in calculations of quantum absorbtion by System II traps.

were irradiated with limiting rates of quantum fluxes absorbed primarily either by System II (650 nm) or System I (710 nm) in order to determine the quantum requirement at these wavelengths for the inactivation process. A rate of 650 nm quantum absorption, corresponding to 0.49 neinsteins per min into 360 nmol of chlorophyll, yielded a half-time for decay of O_2 evolution of 4.1 min with correction for the slow decay occurring in darkness. To obtain similar rates of decay of O_2 evolution with the 710 nm bean, a 4.2 fold greater rate of quantum absorption was required. A similar comparative effectiveness of these wavelengths (650 vs 710 nm) also is reflected in the efficiency spectrum of O_2 evolution by chloroplasts [29,30]. Experiments with blue light (447 nm) indicated that the quantum efficiency for inactivation of O_2 evolution was 70—80% of that in 650 nm light. Since the efficiency spectrum of photosynthesis in Chlorella [31] shows a similar inefficiency of 447 vs. 650 nm light, we can exclude any special effect of blue light on the light induced inactivation of O_2 evolution by Tris. These results therefore suggest that the inactivation of O_2 evolution by Tris is driven by quantum events within System II.

With assumptions regarding trap abundance and distribution of 650 nm quanta between the two photosystems (see previous section), we calculated from the results of a number of such experiments that 50% inactivation of O_2 evolution was obtained with 1.1 to 1.9 hits per System II trap and 1.3 to 2.0 hits per System II trap using chloroplast concentrations giving 50% and 94%

absorption of the incident 650 nm light, respectively. These results indicate that the light induced inactivation of O_2 evolution by Tris is a high quantum yield process sensitized by quantum events within the System II complex and involving a very slow dark rate-limiting step.

Effects of a sequence of flashes

Direct inhibition of O_2 evolution by Tris, largely independent of the inactivation process itself, has been noted previously [28]. We observed, however, no direct effects of 0.8 M Tris on either the kinetics of the rise-curve or yield of variable fluorescence in DCMU poisoned chloroplasts, thus suggesting that primary charge separation within the System II trap was unaffected by the concentrations of Tris yielding direct inhibition of O_2 evolution.

Since both the inactivation process and the direct inhibition of O_2 evolution by Tris proved highly pH dependent, it seemed likely that both Tris effects on O_2 centers were related to the action of other unprotonated amines on O_2 evolution [23,32]. According to Velthuys [21], the inhibition of O_2 evolution by NH_3 is a consequence of formation of a complex between NH_3 and S-states greater than S_1 (S_2 and S_3 states) with subsequent inhibition of the transition of S_4 to S_0 and release of O_2. Thus in the context of the generally accepted model for O_2 evolution [19], dark equilibrated chloroplasts can accumulate in the presence of NH_3 three charges within the S-states. The observed high quantum yield for the inactivation of O_2 evolution by Tris, under conditions not affecting primary charge separation within System II, led us to reason that the inactivation process might be a consequence of interaction of Tris with an S state greater than S_1. According to this hypothesis, the inactivation process would be mechanistically analogous to Velthuys' model [21] for the inhibition of O_2 by NH_3.

To test this hypothesis and extend the conclusions reached in the previous sections, we equilibrated chloroplasts in the dark with Tris for a few minutes then gave either 0, 1, 2 or 3 brief saturating flashes (t_d = 10 s). Such flashed chloroplasts were subsequently recovered by centrifugation within 5 min after the flash, resuspended in buffer 1, and then assayed for remaining O_2 evolution capacity. The results of such experiments are recorded in Fig. 4.

We note that a 5 min equilibration of the chloroplasts in Tris in darkness yielded only 9.8% (average value) inactivation. If, however, the Tris suspended chloroplasts received a single saturating flash, a large increase in the extent of apparent inactivation of O_2 evolution was observed. Compared to the controls, the single flash experiments yielded an average extent of inactivation of about 67%, a 7-fold increase in inactivation relative to dark controls. Very similar extents of inactivation (68.5%, average value) were obtained in the experiments employing two flashes and somewhat less (50.5%, average value) was obtained in experiments employing a sequence of three flashes. The results of Fig. 4 confirm and extend the conclusions obtained from the determinations of quantum yields for inactivation and indicate, assuming no double hits, that minimally one quantum event within the System II complex is sufficient to yield a maximum of 67% apparent inactivation of O_2 evolution.

If we assume that the flashes advance the S-states in the Tris suspended chloroplasts in the experiments shown in Fig. 4 [21], then the percent distri-

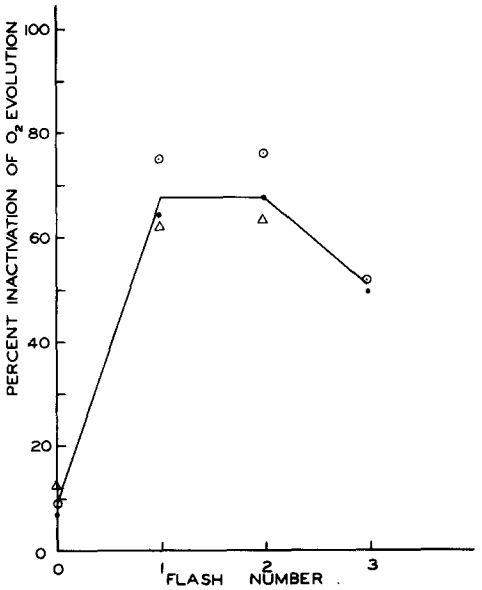


Fig. 4. Induction of the inactivation of O_2 evolution by brief saturating flashes of light. Chloroplasts (50 μg chlorophyll/ml) in 0.8 M Tris were exposed to 0, 1, 2, or 3 saturating flashes of light (t_d = 10 s) at 4°C, recovered 5 min after the last flash then resuspended in buffer 1 and assayed for remaining O_2 evolution capacity. Values on the ordinate have been corrected for about a 5% loss of O_2 evolution capacity that occurred with injection of chloroplasts into Tris or buffer 1 and recovered immediately (≈2 min) at "zero time". The "zero time" rates of O_2 evolution in the experiments of closed circles, open circles and open triangles were 283.5, 200.1 and 162.2 μmol O_2 (mg chlorophyll)$^{-1}$ · h^{-1}, respectively.

bution of S-states (neglecting corrections for double hits and misses) after the various flash treatments are [19]: zero flash, 25/75 as S_0/S_1; one flash, 25/75 as S_1/S_2; and two flashes, 25/75 as S_2/S_3. If Tris, like NH_3 [21], prevents the transition of $S_4 \rightarrow S_0 + O_2$, then after 3 flashes in the experiments of Fig. 4, S_4 would be the predominant species.

With these assumptions, the data of Fig. 4 obtained with Tris suspended chloroplasts indicate the following susceptibility of inactivation of O_2 evolution by Tris: $S_2 = S_3 > S_4 >> S_1$. The observed average value of 67% inactivation at S_2 (Fig. 4) is somewhat less than the 75% predicted value. A partial deactivation of the S_2 state could contribute to this disparity; also, in the experiments involving more than one flash (10 s spacing), we can not rule out some contribution to the inactivation process of the S_2 state arising from deactivation of S_3 or S_4. It is clear, however, that minimally only one charge separation within the System II trap is required in the Tris-induced inactivation of O_2 evolution.

Effects of DCMU and FCCP on inactivation

In the presence of DCMU it is generally believed that in otherwise unpoisoned chloroplasts, System II can cycle slowly

$$Q\text{-}S_1 \underset{\text{dark}}{\overset{h\nu}{\rightleftharpoons}} Q^-\text{-}S_2 .$$

Normally, this dark back reaction shows a half-time of 0.6—1.9 s [33], a half-time some 100-fold less than the deactivation of S_2 when the Q-A pools are oxidized [19]. The slow rate-limiting reaction ($t_{1/2}$ = 200 s) revealed from quantum efficiency measurements of the inactivation process conceivably could indicate a reaction between S_2 and Tris. If this were the case, then the comparatively rapid back-reaction between S_2 and Q^- in DCMU poisoned chloroplasts would exclude the reaction between S_2 and Tris and little effect of light on the inactivation process would be observed. If on the other hand, the reaction, S_2 + Tris → S_2-Tris, were more rapid than the back reaction between S_2 and Q^- and resulted in a charge stabilization of S_2 analogous to effects of NH_3 [21], then the inactivation process might not be inhibited in the presence of DCMU.

Typical results that were obtained on the effect of DCMU on the light-induced inactivation of O_2 evolution are shown in Table I. In these experiments, we irradiated suspensions of chloroplasts in Tris for sufficient durations at two intensities of a 650 nm beam to yield approximately 50 percent inactivation. The intensities used in Experiments 1 and 2 were sufficient to give half-maximal and maximal rate of inactivation, respectively. After correction for the extent of inactivation occurring in darkness in Experiments 1 and 2,

TABLE I

EFFECT OF DCMU AND FCCP ON THE LIGHT INDUCED INACTIVATION OF O_2 EVOLUTION BY TRIS

Suspensions of chloroplasts (1.8 ml of 200 μg chlorophyll/ml) in 0.8 M Tris, containing the additions as noted, were incubated at 4°C in intensities of 650 nm light sufficient to yield the noted rates of quantum absorption by System II traps (see Fig. 3). The suspensions were diluted 8-fold with buffer 1 and the chloroplasts recovered by centrifugation. Following 3 washes with buffer 1 (16 ml) to remove DCMU and FCCP, the chloroplasts were resuspended in buffer 1 and rates of O_2 evolution determined.

Experimental conditions	Rate of 650 nm quantum absorption by system II traps during preillumination	Rate of O_2 evolution (μmol O_2 (mg chlorophyll)$^{-1} \cdot h^{-1}$)	Inactivation of O_2 evolution (%)
Experiment 1			
1. Zero time	—	231.8	—
2. Dark, 10 min	—	185.4	20.0
3. Light, 10 min	1 hit/200 s	77.8	66.4
4. Light, 10 min plus 5 μM DCMU	1 hit/200 s	79.3	64.8
5. Light, 10 min plus 5 μM DCMU and 2 μM FCCP	1 hit/200 s	180.2	23.3
Experiment 2			
1. Zero time	—	233.2	—
2. Dark, 3 min	—	222.1	4.8
3. Light, 3 min	1.4 hits/10 s	121.7	47.8
4. Light, 3 min plus 10 μM DCMU	1.4 hits/10 s	131.7	43.5
5. Light, 3 min plus 10 μM DCMU and 2 μM FCCP	1.4 hits/10 s	219.7	6.2

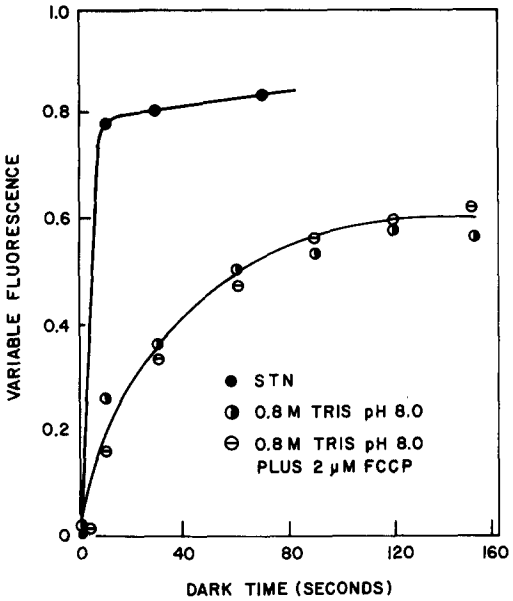


Fig. 5. Inhibition by Tris of the recovery of variable fluorescence of DCMU-poisoned chloroplasts. Any differences in the half-time (150 ms) of the fluorescence rise and F_{max}/F_0 (4.9) of chloroplasts in buffer 1 (STN), Tris, or Tris containing 2 μM FCCP were indistinguishable. Suspensions were illuminated for 10 s then a dark time (given on the abscissa) was interposed before re-illumination. Variable fluorescence is expressed as F_{max} minus the fluorescence level observed immediately upon readmission of the exciting light. Values have been normalized.

the results indicate that approximately equivalent extents of light induced inactivation were obtained under the two different light regimes. At either light intensity the addition of DCMU did not result in any significant decrease in the extent of inactivation. Such results give additional evidence for identifying the S_2 state to be susceptible to inactivation by Tris and seem to exclude identification of the slow rate-limiting reaction ($t_{1/2}$ = 200 s) of the inactivation process with a reaction between S_2 and Tris.

Charge stabilization by NH_3 of the S-states in chloroplasts is reflected by stimulation of the intensity of luminescence in the time range around 40 ms and a decreased rate of decay in darkness of the variable fluorescence measured in the presence of DCMU [21]. In the experiments of Fig. 5, we measured the effect of Tris on variable fluorescence recovery in DCMU poisoned chloroplasts in an attempt to ascertain whether charge stabilization of S_2 occurred in the presence of Tris. In these experiments chloroplasts were suspended in darkness in the designated media for a few minutes, then DCMU added (5 μM final concentration) and the suspension irradiated while measuring the increase of fluorescence level (F_0 to F_{max}) over a time-interval of 10 s. After the dark times given on the abscissa, the amount of quencher remaining in the reduced state, Q^-, was indicated by the level of fluorescence observed immediately upon readmission of the exciting beam. Under the conditions employed, neither F_0, F_{max} nor the half-time of the fluorescence rise was altered by the various designated media thus permitting normalization of the data. The results shown in Fig. 5 indicate that the recovery of variable fluorescence in DCMU-poisoned chloro-

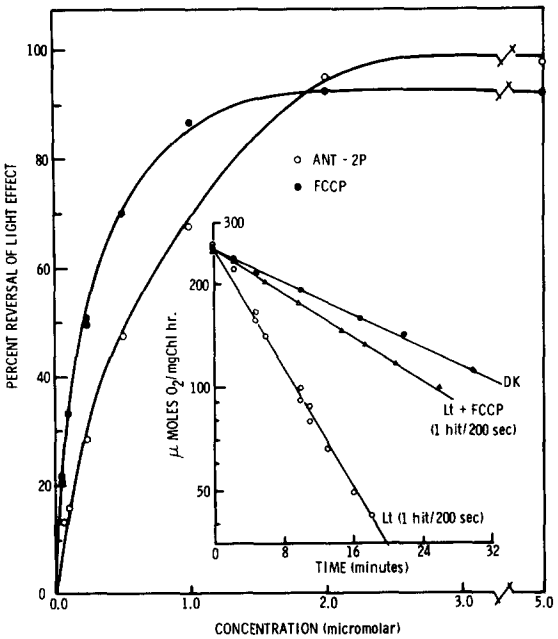


Fig. 6. (Main figure) concentration dependence of FCCP and ANT-2p for the inhibition of the induction of inactivation of O_2 evolution by 650 nm light. (Inset) time course of inactivation in dark and in weak light in the absence and presence of 2 μM FCCP. The data of the main figure were obtained using a 12 min illumination (1 hit per System III trap/200 s) of chloroplasts suspended in 0.8 M Tris containing concentrations of ANT-2p of FCCP given on the abscissa. DK, dark; Lt, light.

plasts is markedly inhibited in the presence of 0.8 M Tris, pH 8.0. Such inhibition by Tris was markedly diminished at pH 7.0. We tentatively suggest that the unprotonated species of Tris, like NH_3 [21], results in charge stabilization of the S_2-state via formation of a complex, S_2-Tris, and that its formation occurs in times equal to or less than 3 s, the time required to raise the fluorescence level from F_0 to F_{max} under our experimental conditions.

We note in the experiments of Table I that the addition of FCCP to the irradiated DCMU-poisoned chloroplast suspensions in Tris essentially abolished the light induced inactivation of O_2 evolution but did not alter the kinetics of decay of variable fluorescence (Q^- oxidation) of Tris-suspended, DCMU-poisoned chloroplasts (Fig. 5). Very similar inhibition of the inactivation by FCCP was also observed in the absence of DCMU (Fig. 6 Inset). Under these latter conditions (1 hit/System II trap/200 s), Q was in the oxidized state as determined from fluorescence measurements. Thus the inhibitory effect of FCCP on inactivation appears to be independent of the redox state of Q.

Previous studies on the effect of FCCP on S-state stability viewed either by flash yields of O_2 or by luminescence indicate that FCCP markedly decreases the stability of S_2, presumably by facilitating a reaction between S_2 and an unknown endogenous reductant other than Q^- [34,35]. The similarity in behavior of the decays of variable fluorescence (Q^- oxidation) observed in the presence of Tris, with and without FCCP (Fig. 5), presumably reflect two dif-

ferent fates of S_2 both resulting in inhibition of the normal back reaction between S_2 and Q^-: (1) charge stabilization of S_2 in the presence of Tris alone; and (2) an FCCP facilitated reduction of S_2 to S_1 by an endogenous reductant other than Q^- [34,35].

We conclude from the experiments of Table I and Figs. 5 and 6: (1) the Tris induced inactivation of O_2 evolution is not inhibited by DCMU thus providing additional evidence for minimally a one quantum process; (2) at alkaline pH the unprotonated Tris species results in charge stabilization of S_2 via formation of a S_2-Tris complex; and (3) in the presence of DCMU, FCCP inhibits the inactivation process.

Inhibition of inactivation by compounds affecting the S_2, S_3 states

The inhibition by FCCP of the inactivation of O_2 evolution in the illuminated, DCMU poisoned, Tris suspended chloroplasts (Table I) can be explained by the known effects this compound has on decreasing the stability of the S_2 state of O_2 evolution. This compound is known, however, to have a number of other effects on chloroplast reactions thereby excluding any definitive conclusion concerning its mode of action on the inactivation process. A number of chemically unrelated compounds other than FCCP affect the S_2, S_3 states either by increasing their rates of deactivation [34,35] or by discharging them via reactions coupled to the species giving rise to Sig. II [36,37]. To further test the hypothesis that the Tris-induced inactivation of O_2 evolution was dependent on S_2, S_3 states, we examined the effect on inactivation of a number of compounds that discharge S_2, S_3 by the above mechanisms. In these experiments, in contrast to those of Table I, DCMU was omitted from the suspensions during irradiation in order to simulate more closely the experimental conditions (Q in the oxidized state) employed for measuring the effects of such compounds on yields of O_2 in a sequence of flashes.

Fig. 6 Insert shows typical time courses for the inactivation process in darkness and response to a weak 650 nm beam of an intensity near maximal quantum efficiency (1 hit/System II trap/200 s) for the inactivation. A 12 min incubation of chloroplasts in Tris alone in dark versus this light regime generally resulted in 20—25 and 75—80% inactivation of O_2 evolution, respectively. This three to four-fold difference in extent of inactivation observed between a dark and a light regime near maximum quantum efficiency permitted analyses of the effect on inactivation of a number of chemicals affecting S_2, S_3 stability.

In the experiments shown in Fig. 6, we compared concentrations of FCCP and ANT-2p, reagents increasing rates of deactivation of S_2, S_3 [34,35], for their effectivity for inhibiting the inactivation process. In only a few experiments did we observe an effect of FCCP or ANT-2p on the dark decay rates (see Fig. 6 Insert), thus Fig. 6 records only the effect of these compounds on the light induced inactivation. The data show that 2 μM concentrations of either FCCP or ANT-2p yielded virtually complete inhibition of the light induced inactivation of O_2 evolving centers. Half-maximal inhibition was obtained at 0.16 and 0.5 μM for FCCP and ANT-2p, respectively. Similar concentrations of these reagents induce maximum rates of deactivation of the S_2, S_3 states of O_2 evolutiion [34,35] and decay of Sig. IIs [37].

Tetraphenylboron (10 μM), CCCP (3 μM), reduced phenazine methosulfate

(5 μM), and reduced DCIP (100—200 μM) also virtually completely inhibited the light induced inactivation of O_2 evolving centers at this low rate of quantum absorbtion (data not shown). In contrast, picric acid [38] was ineffective over a wide range of concentrations (0.2 to 50 μM). With this exception and reduced phenazine methosulfate, for which no published information exists regarding specific effects on S_2, S_3, all the other above reagents have been shown either to (1) increase rates of deactivation of S_2, S_3 (ANT-2p, CCCP, FCCP [34,34]), or (2) to increase rates of decay of Sig. IIs ($DCIPH_2$ [39], CCCP [37,40—41], ANT-2p [40]) thereby promoting discharge of S_2, S_3 via oxidation of F, the precursor of Sig. IIs [36] or (3) to rather directly reduce positive charges generated by the System II trap (tetraphenylboron [42—43]).

We therefore ascribe the inhibition of the inactivation of O_2 evolution by FCCP CCCP, ANT-2p and the other above reagents to be a consequence of a decreased stability of the S_2, S_3 states.

Reactivation of inactivated O_2 centers is dependent on prior light induced inactivation

Equilibration of Tris-washed chloroplasts with $DCIPH_2$ in isoosmotic buffer leads to partial [14—16] or complete reactivation [6] of the O_2-evolving centers inactivated during Tris washing. We previsouly had attempted to obtain magnitudes of reactivation as reported by Yamashita et al. [14—16] and Blankenship et al. [6] but were unsuccessful. With the recognition that the Tris-dependent inactivation of O_2 evolution either required or was markedly enhanced by weak light, we inquired whether light vs. dark conditions during the inactivation process subsequently influenced the extent of reactivation of the inactivated O_2-evolving centers.

In the experiments of Fig. 7, suspensions of chloroplasts were incubated in Tris at 0°C on a shaker bath in either dark or weak light for various durations to obtain a wide range of inactivation. Following incubation, each sample was divided in half and centrifuged to recover the chloroplasts. One half of each sample was resuspended and incubated in buffer 1 (inactivated) and the other half was resuspended and incubated in buffer 1 containing $DCIPH_2$ (reactivated). The chloroplasts were subsequently recovered by centrifugation, resuspended in buffer 1, and then rates of O_2 evolution were determined.

In Fig. 7, we plotted the ratio of specific rates of O_2 evolution (activated/inactivated) versus the fraction of inactivated O_2 evolving centers normalized to zero time controls. For the purposes here, we assumed a linear relationship between V and abundance of O_2 evolving centers. Curve 1 (no symbols) of Fig. 7 represents the "theoretical" curve for complete activation of O_2 evolution. We note that O_2-evolving centers inactivated by Tris in darkness could not be reactivated by subsequent incubation with $DCIPH_2$, irrespective of the extent of inactivation. On the other hand, inactivation of O_2-evolving centers by the light-driven process led to an inhibited state which was partially reversed or activable by $DCIPH_2$. Such activation was reflected in similar increases of V and V (data not shown). Comparison of Curve 1 ("theoretical complete reactivation") with the experimentally obtained reactivation suggests that about 40 and 100% of the O_2 evolving centers were irreversibly inactivated by Tris in the light and dark, respectively.

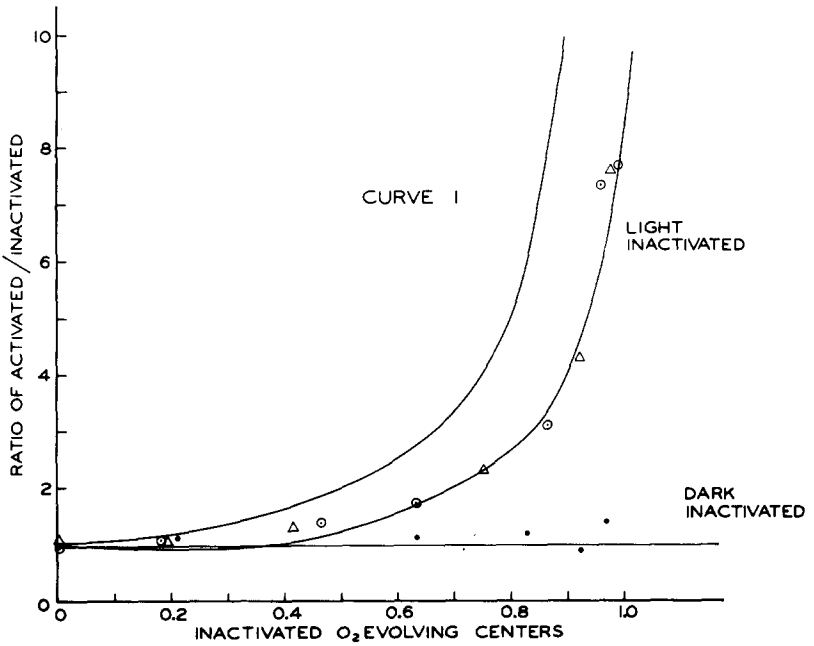


Fig. 7. Reactivation of O_2 evolution in Tris-inactivated chloroplasts is dependent on previous inactivation in light. Curve 1 (no symbols) represents the "theoretical" curve for complete reactivation of O_2 evolution. Data for the other curves were obtained from incubations of chloroplasts in Tris in either weak light or in darkness for durations sufficient to yield the fractions of inactivated O_2-evolving centers plotted on the abscissa. See Text for other details.

Many such experiments over the course of several years employing various light intensities during the inactivation process never yielded chloroplasts showing greater reactivation than shown in Fig. 7. Similarly, chloroplasts prepared from market or greenhouse spinach, or peas by several procedures never were completely activable following inactivation (20 to 90%) by Tris in weak light within the pH range of 7.5—8.3, and in no instance did we observe reactivation of O_2 evolution with chloroplasts inactivated by Tris in darkness. Thus, although we attempted to duplicate the conditions that enabled Blankenship et al. [6] to obtain complete reactivation as judged from their measurements of V_{max}, we could not confirm their results. In a later section, we record data that bear on this apparent disparity. We conclude, however, from the results of Fig. 7 that light absorption during inactivation is a major determinant of the extent of subsequent reactivation of the inactivated O_2 evolving centers by $DCIPH_2$.

Reactivation of O_2 evolution by reagents affecting stability of the S_2, S_3 states

Data presented in previous sections seemed to indicate the involvement of the S_2 state in the Tris-dependent formation of the inhibitory state. In the experiments of Table 2, we compared the effectivity of FCCP, ANT-2p, tetraphenylboron, picric acid, and reduced phenazine methosulfate with reduced DCIP, the most commonly used reagent for affecting reactivation. In these experiments, the duration (15 min) of inactivation in weak light was sufficient to

decrease rates of O_2 evolution from 335 to 41.3 μmol O_2 (mg chlorophyll)$^{-1}$ · h^{-1}, a decrease of about 88%. Subsequent equilibration of the inactivated chloroplasts with reduced DCIP yielded about a 3-fold increase in O_2 evolution capacity as shown by the ratio of activities for activated/non-activated chloroplasts. This increase in rate, though substantial, still represented only 35.6% of the activity of chloroplasts suspended in buffer 1 but otherwise treated as the chloroplasts suspended in Tris. Nevertheless, the difference in specific activities between activated and nonactivated chloroplasts in the experiments of Table II was sufficiently large to permit determination of the effectivity of the other listed chemicals for obtaining reactivation of O_2 evolution. These comparisons clearly show that 2 μM FCCP and ANT-2p, which are not generally considered as reducing agents, are equally as effective for the reactivation of Tris-inactivated O_2 evolving centers as 150-fold greater concentration of reduced DCIP. Also, 5 μM concentrations of reduced phenazine methosulfate and tetraphenylboron proved as effective as 300 μM $DCIPH_2$. Picric acid was ineffective; however, this reagent also did not inhibit the inactivation process as shown in a previous section. Entirely similar results as those recorded in Table II were obtained also in instances of less extensive inactivation and more complete reactivation (50—60%).

In a few experiments such as those of Table II, we observed that the immediate addition of FCCP or ANT-2p following inactivation yielded only 70—80% of the reactivation obtained with $DCIPH_2$. Moreover, the effectivity of

TABLE II

COMPARISON OF THE EFFECTIVITY OF COMPOUNDS AFFECTING STABILITY OF THE S_2, S_3 STATES FOR THE REACTIVATION OF O_2 EVOLUTION IN TRIS-TREATED CHLOROPLASTS

Chloroplast suspensions (4 ml of 200 μg chlorophyll/ml in 0.8 M Tris) were incubated for 15 min in weak light on a shaker bath then centrifuged. The pellet was then resuspended and incubated for 10 min in 4 ml buffer 1 containing, where noted, concentrations of the various reagents listed. The listed concentrations of the various reagents were optimal for obtaining maximal activation. 1 mM ascorbate was included with samples containing DCIP and phenozine methosulfate (PMS). The chloroplasts then were recovered by centrifugation, washed with buffer 1, and assayed. Picric acid was used at 0.2 to 50 μM and was ineffective for affecting reactivation in this range of concentrations. TPB, sodium tetraphenyl boran.

Sample	Rate of O_2 evolution (μmol O_2 (mg chlorophyll)$^{-1}$ · h^{-1})	Percent of control	Activated nonactivated
1) Washed with buffer 1	335.0	100	—
2) Tris extracted	41.3	12.3	—
3) Tris extracted, reactivated (300 μM $DCIPH_2$)	119.3	35.6	2.89
4) Tris extracted, reactivated (2 μM FCCP)	127.5	38.1	3.09
5) Tris extracted, reactivated (2 μM ANT-2_p)	132.5	40.0	3.21
6) Tris extracted, reactivated (5 μM $PMSH_2$)	118.3	35.3	2.86
7) Tris extracted, reactivated (5 μM TPB)	112.6	36.6	2.97
8) Tris extracted, reactivated (picric acid) *	42.6	12.7	1.03

* 0.2 to 50 μM.

FCCP and ANT-2p relative to $DCIPH_2$ for inducing reactivation decreased further with increasing time in darkness. In such instances, the addition of ascorbate (2 mM), which by itself yielded no reactivation [14], increased the reactivating effectivity of FCCP and ANT-2p to that obtained with $DCIPH_2$. Such results imply that the FCCP and ANT-2p mediated reactivation of O_2 evolution is dependent on some unknown, auto-oxidizable chloroplast constituent which is reducible by ascorbate.

With the exception of picric acid and possibly reduced phenazine methosulfate, the results of Table II indicate that reagents destabilizing S_2, S_3 states or reagents (tetraphenylboron) adding electrons to the System II complex [42, 43] are effective reagents for the reactivation of O_2 evolution. Since reactivation is dependent on prior inactivation in the light and, even in the case of FCCP and ANT-2p, requires reducing equivalents, our results imply that reactivation is the reductive discharge of a state formed in the light during Tris treatment which directly inhibits O_2 evolution, or alternately, a reversal of a perturbation within the O_2-evolving catalyst.

Life-time of activable state and the relationship to thylakoid manganese abundance

Depending on the methodology of Tris-treatment, and apparently the integrity of the thylakoids, the manganese perturbed from its native binding sites within System II during inactivation of O_2 evolution is either lost to the extracting medium [4,7] or is largely retained as hezaquo manganese within the thylakoids [5,6]. With inactivated chloroplasts in which the hexaquo manganese was retained, Blankenship et al. [6] were able to obtain complete reactivation of O_2 evolution. This complete reactivation was accompanied with virtually complete disappearance of the EPR hexaquo manganese signal and with 35% loss of total thylakoid manganese abundance. Assuming bound manganese is an integral part of the O_2evolving catalyst and that reactivation promotes rebinding of intra-thylakoid manganese at native binding sites within the O_2-evolving catalyst, such data suggest that the reactivation of Tris-inactivated O_2 evolving centers reflects a manganese dependent repair of perturbed, inactive O_2-evolving centers.

In the experiments of Curve 1, Fig. 8, we measured the decay of the perturbed, activable state and the decrease of chloroplast manganese abundance following inactivation in weak light in attempts to ascertain the life-time of the activable state and the possible relationship between this state and chloroplast manganese abundance. The relative activable state represents the difference in O_2 evolution capacity of reactivated and inactivated chloroplasts expressed in percent of the value obtained at zero time. We corrected values of total manganese abundance for the samll manganese pool (2 manganese/400 chlorophyll) not correlating with O_2 evolution and not readily released by Tris-treatment [4,6] and plotted the resulting values relative to those at zero time reactivation. Thus, we examined in the experiments of Curve 1 the possible relationship between the loss of the activable state and the loss of any intra-thylakoid manganese [5,6] remaining after the inactivation.

The two sets of data summarized by Curve 1, Fig. 8 were obtained with chloroplasts yielding comparable rates of O_2 evolution [313 and 345 μmol O_2

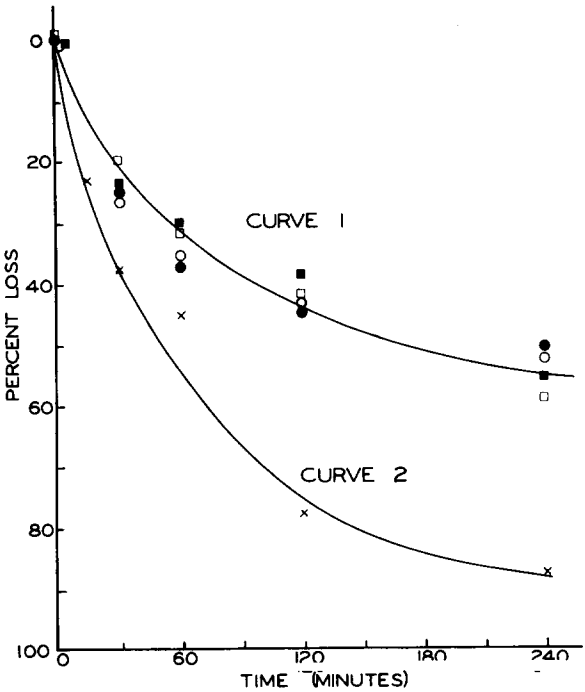


Fig. 8. Life-time of activable state and the relationship to thylakoid manganese abundance. Chloroplasts were treated as described in Table II but following initial centrifugation were resuspended in only 2 ml of buffer 1 and kept in darkness. At times designated on the abscissa, 2 ml of buffer 1 containing 400 μM DCIP and 2 mM ascorbate were added. After 10 min in darkness, the chloroplasts were recovered and assayed for O_2 evolution capacity and ^{54}Mn. Curve 1 summarized data from two experiments done on successive days with chloroplasts prepared from the same batch of spinach leaves: open squares and circles represent thylakoid manganese abundance; closed squares and circles represent the activable state. The thylakoid manganese abundance plotted was corrected for the manganese pool (2 manganese/400 chlorophyll) not correlating with O_2 evolution and not released by Tris treatment [4]. Curve 2 describes loss of activable state in chloroplsts prepared from a different batch of spinach leaves.

(mg chlorophyll)$^{-1} \cdot h^{-1}$, closed circles and squares, respectively] and containing comparable manganese abundance (5.8 and 6.2 manganese/400 chlorophyll, open circles and squares respectively). The inactivation treatment decreased rates of O_2 evolution from 313 to 18 and 345 to 17 μmol O_2 (mg chlorophyll)$^{-1} \cdot h^{-1}$ and total chloroplast manganese abundance from 5.8 to 4.2 and 6.2 to 4.0 manganese/400 chlorophyll, respectively. These values correspond to about a 95% inactivation of O_2 evolution and a 31% decrease in total chloroplast manganese abundance but about a 50% decrease in the manganese pool of O_2 evolution (assuming the limits of n = 6—2 manganese/400 chlorophyll is a measure of this pool [4,13]; see however ref. 6).

Reactivation of the chloroplasts at zero time yielded about a 6-fold increase in rates of O_2 evolution, these rates corresponding to 30—35% of the rates obtained with chloroplasts suspended in buffer 1 but otherwise treated as the Tris-treated chloroplasts. The total manganese abundance in the Tris-treated, zero time reactivated chloroplasts was 4.0—4.2 manganese/400 chlorophyll. Correcting for the small manganese pool not correlating with O_2 evolution, this result implies that 2.0—2.2 manganese/400 chlorophyll was free hexaquo man-

ganese retained within the thylakoids. We estimate, from our previous results [4,13], that 60%, rather than the observed 30—35%, of the original rates of O_2 evolution would have been recovered had all the 2.0—2.2 manganese/400 chlorophyll become bound at native binding sites within the O_2-evolving catalyst.

Increased duration of incubation of inactivated chloroplasts in buffer 1 in darkness before reactivation led to a slow loss ($t_{1/2} \approx 175$ min) of both the activable state and the manganese pool correlating with O_2 evolution. This half-time (Curve 1, Fig. 8) is very similar to the diffusion rate ($t_{1/2} = 150$ min) of intra-thylakoid hexaquo manganese into an isoosmotic suspension medium [5]. In some experiments, however, (Curve 2, Fig. 8), the decay of the activable state was more rapid ($t_{1/2} \approx 50$ min) even though the chloroplasts used in experiments of Curve 2 showed rates of O_2 evolution before and after inactivation (316 and 21 μmol O_2 (mg chlorophyll)$^{-1} \cdot h^{-1}$, respectively) and a magnitude of reactivation (5 fold) similar to chloroplasts used to obtain Curve 1.

The results described in Fig. 8 are interpreted as follows: (1) The observed incomplete reactivation may be partially a consequence of the loss during inactivation of about half of the manganese pool correlating with O_2 evolution [4,14] and an apparent denaturation of 20—30% of the native manganese binding sites; this latter conclusion rests on the difference between the observed reactivation and that predicted from the fraction of the larger manganese pool remaining within thylakoids after inactivation [4]; and (2) loss of the activable state is kinetically correlated with loss of the remaining fraction of the larger manganese pool. Manganese abundance only, however, will not explain the lack of reactivation in chloroplasts inactivated in darkness (Fig. 7), since inactivation in darkness ($t_{1/2} \approx 30$ min) is some six-fold faster than the diffusion rate ($t_{1/2} = 175$ min, Curve 1, Fig. 8) of manganese across the thylakoid.

Additional supporting evidence that intra-thylakoid manganese is required in the reactivation process is derived from the experiments shown in Fig. 9. In these experiments chloroplasts were first subjected to Tris-extraction in weak light for a duration sufficient to decrease O_2 evolution from 200 to 12 μmol O_2 (mg chlorophyll)$^{-1} \cdot h^{-1}$ and total manganese content from 5.1 to 4.1 manganese/400 chlorophyll. Subsequently, the Tris-extracted chlorophyll. Subsequently, the Tris-extracted chloroplasts were extracted for 10 min with the NH_2OH concentrations given on the abscissa then incubated with $DCIPH_2$ to obtain reactivation. The data show that the activable state formed during Tris-extraction is half and fully destroyed by about 0.36 and 1.3 mM NH_2OH, respectively. This result confirms previous observations [13,15] indicating that the O_2 evolving mechanism of NH_2OH extracted chloroplasts can not be reactivated by $DCIPH_2$ treatment.

The concentrations of NH_2OH (1—2 mM) yielding complete destruction of the activable state and the residual rate of O_2 evolution after Tris-extraction resulted in a further decrease of thylakoid manganese abundance within the Tris-extracted chloroplasts (4.1 manganese/400 chlorophyll) to a value of only 1.7 manganese/400 chlorophyll. This latter value corresponds closely to the more tightly bound manganese pool of System II that does not correlate with O_2 evolution [4]. We assume that all of the manganese between the limits of

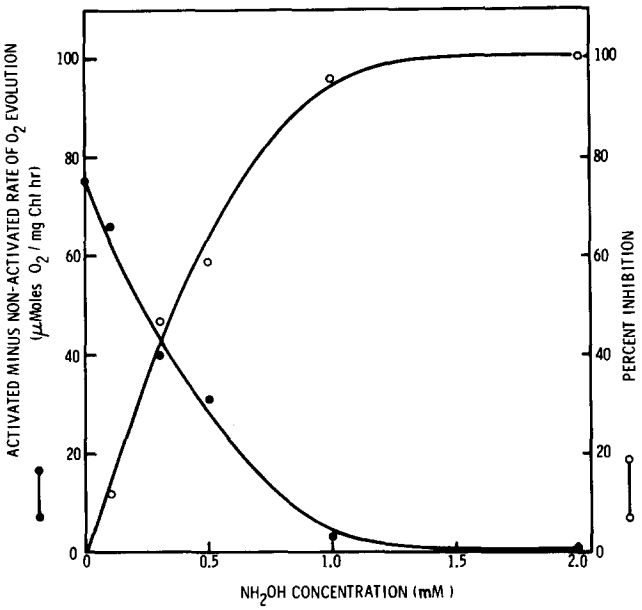


Fig. 9. Effect of hydroxylamine extraction of Tris-treated chloroplasts on the activable state. Chloroplasts were extracted with Tris as described in Table II and resuspended in buffer 1 containing the NH_2OH concentrations given on the abscissa. After a 10 min incubation, each sample was divided in half then centrifuged to recover the chloroplasts. Half of each sample was assayed directly for O_2 evolution (non-activated); the other half was activated then assayed. Plotted on the ordinate (solid circles) is the difference between activated and non-activated rates of O_2 evolution. Percent inhibition, or loss, of the activable state from the NH_2OH extractions is given by the open circles.

4.1 and 1.7 manganese/400 chlorophyll is unbound, EPR-detectable hexaquo manganese [5]. With this assumption, the results of Fig. 9 suggest that NH_2OH induces loss from the thylakoids of this larger EPR detectable manganese pool and the loss of the activable state. This conclusion may relate to the observations of Yamshita and Tomita [16] who observed that Tris/acetone extraction of chloroplasts led to an inhibition of reactivation and more extensive depletion of chloroplast manganese than Tris extraction alone.

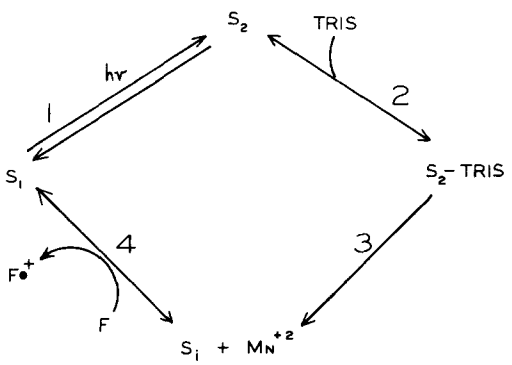
Discussion

The experiments described here clearly indicate that the state of System II during exposure to concentrations of Tris leading to inactivation of O_2 centers markedly affects the rate of inactivation and is a determinant of the potential for the subsequent reactivation of inactive O_2 centers. This conclusion, derived using Tris concentrations and pH values optimal for obtaining maximal reactivation [6], is supported by the following observations reported here: (1) in darkness the rate of inactivation is slow ($t_{1/2} \approx$ 20—25 min) and any inactivated O_2 centers formed from the action of Tris on dark-equilibrated chloroplasts could not be subsequently reactivated with either $DCIPH_2$ or reagents such as FCCP, CCCP and ANT-1p; (2) weak light, at an intensity sufficient to yield only 1 hit/System II trap per 10 s, increased the rate of inactivation some 10 fold ($t_{1/2} \approx$ 2.5 min) and yielded inhibited or inactivated O_2 centers which

were activable partially by $DCIPH_2$, FCCP, CCCP, ANT-2p, reduced phenazine methosulfate and tetraphenylboron. Similarly, one saturating short flash sufficed to inactivate 50—70% of the O_2 centers in a DCMU-insensitive process. This latter observation coupled with determined quantum yield in 650 nm light of 1.1 to 2.0 bits per System II trap for the inactivation of about half of the O_2 evolving centers suggests that minimally one charge separation within System II promotes a state which is susceptible to apparent inactivation by Tris and loss of O_2 evolution capacity.

Though most of the experiments described here were restricted to a Tris concentration and a pH value optimal for obtaining maximal reactivation, the sensitization of the inactivation process by weak light was observed also over the pH range of 7.4—8.6. We did not investigate more extreme conditions (pH 10) that also have been employed [21] for inactivation of O_2 evolution. Significantly, at the more alkaline pH values (>pH 8.0) the rate of inactivation of O_2 evolution in darkness increased relative to rates of inactivation in weak light and the inactivation in weak light in alkaline Tris yielded inactive O_2 centers that were even less activable than those obtained in weak light at pH 7.6. Additionally, $DCIPH_2$ or FCCP was less effective for inhibiting inactivation under either light or dark conditions at pH values >7.6.

We believe the following scheme best explains: (1) the one quantum induced inactivation of O_2 centers by Tris; (2) the inhibition of inactivation by reagents decreasing lifetimes of the S_2, S_3 states; (3) the release of bound manganese with inactivation; and (4) the subsequent partial reactivation of O_2 evolution capacity that is coupled with rebinding of hexaquo manganese [6] and catalyzed by reagents decreasing lifetimes of S_2, S_3 or F^+.



Reaction 1 is, according to the Kok model for O_2 evolution, the advancement of the dark stable S_1 state to the S_2 state with the absorption of a quantum by the System II trapping center. Reaction 2, which is analogous to Velthuys' [21] interpretation of NH_3 inhibition of O_2 evolution, shows formation of the S_2-Tris complex via the amine function of Tris and resulting in a chemical and/or structural modification of S_2. Supporting evidence for such modification of S_2 by Tris is manifested, like NH_3 [21], by an inhibition of the recombination of charges (S_2-$Q^- \rightarrow S_1$-Q) (Fig. 5) in DCMU-poisoned chloroplasts. In this context and the presumed plus three valency state of manganese in the S_2 state [44], we note that ligand-field stabilization effects, which are

manifested in complexes of manganic but not manganous ions, are larger with amine ligands than with H_2O [45,46].

Reaction 3 represents the Tris-induced dissociation of bound manganese from S_2 with formation of inactive S-states (S_i). We attribute the slow ($t_{1/2} \approx$ 200 s) rate limiting step of photoinactivation (Fig. 3) to this reaction. Accordingly, reagents that increase rates of deactivation of S_2 or that increase the abundance of F, thereby facilitating loss of S_2 via formation of Sig. IIs [36], would tend to inhibit and protect against the inactivation induced by light. This interpretation of the protective effect afforded by reagents destabilizing S_2, S_3 does not, however, permit a choice between the two different models [21,47] proposed to explain amine binding to the S_2, S_3-states.

Reaction 4 described the discharge of the postulated chemically and/or structurally modified S_2 (S_i) with accompanying reinsertion of free hexaquo manganese released in Reaction 3. This conclusion rests on the observations that the reactivating reagents cause decay of S_2 or S_3 [34,35,37,40,41] and that the decay ($t_{1/2} \approx$ 175 min) of the potentially activable state is correlated with diffusion of the unbound larger manganese pool of System II within the thylakoids into the suspending medium. We therefore concur with Blankenship et al. [6], for different reasons, that reactivation is manganese dependent. Our failure to obtain levels of reactivation similar to those reported by others [6,14—16] appear to be due, in part, to a loss during inactivation of about half of the larger manganese pool. This loss was not observed by Yamashita et al. [14—16] and Blankenship et al. [6]. Apparently, depending on the chloroplast preparation and the methodologies employed for extraction, the thylakoid membrane can have variable permeability to manganous ions. This may relate to the observed variable but remarkable stability of the potentially activable state (Fig. 8).

We postulate in Reaction 4 that the discharge of S_i with reactivation of O_2 evolution capacity is mediated by the oxidation of F with generation of F^+. Though other hypotheses can be advanced, we believe this hypothesis best explains the effectivity of $DCIPH_2$, FCCP, CCCP, ANT-2p and tetraphenylboron for inducing reactivation. All such reagents would increase abundance of F and thereby provide substrate for Reaction 4. Inherent in this postulate is the assumption that the methodology of Tris treatment employed here yields chloroplasts with Sig. II decay behavior similar to that in untreated chloroplasts ($t_{1/2}$ of Sig. IIs = 1—4 h) [36—37] rather than the much shorter ($t_{1/2}$ = 15 min) and complete decay of Sig. IIs in chloroplasts inactivated by Tris at unspecified conditions (light vs. dark) [41,48].

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