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Flash Inactivation of Oxygen Evolution

IDENTIFICATION OF S_2 AS THE TARGET OF INACTIVATION BY TRIS[1, 2]

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ABSTRACT

Brief saturating light flashes were used to probe the mechanism of inactivation of O_2 evolution by Tris in chloroplasts. Maximum inactivation with a single flash and an oscillation with period of four on subsequent flashes was observed. Analyses of the oscillations suggested that only the charge-collecting O_2-evolving catalyst of photosystem II (S_2-state) was a target of inactivation by Tris. This conclusion was supported by the following observations: (*a*) hydroxylamine preequilibration caused a three-flash delay in the inactivation pattern; (*b*) the lifetimes of the Tris-inactivable and S_2-states were similar; and (*c*) reagents accelerating S_2 deactivation decreased the lifetime of the inactivable state. Inactivation proved to be moderated by F, the precursor of Signal II_s, as shown by a one flash delay with chloroplasts having high abundance of F. Evidence was obtained for cooperativity effects in inactivation and NH_3 was shown to be a competitive inhibitor of the Tris-induced inactivation. S_2-dependent inactivation was inhibited by glutaraldehyde fixation of chloroplasts, possibly suggesting that inactivation proceeds via conformational changes of the S_2-state.

A previous report (11) on the mechanism of Tris inactivation of O_2 evolution (5, 6, 9, 27, 54-57) yielded evidence that a light-induced state within PSII markedly accelerates the rate of inactivation by Tris and is a determinant of the potential for subsequent reactivation of inactive S-states[5] (11, 55-57). Quantum yield and flash yield measurements of the light-induced, Tris-inactivable state suggested that minimally only one to two hits per PSII trap were required in a DCMU-insensitive process for the inactivation of about half of the O_2 evolving centers.

Based largely on these lines of evidence it was suggested that the S_2- and/or S_3-states of the Kok-Joliot model (30) for O_2 evolution were the target of attack by Tris, leading to an inactive state that could be subsequently partially reversed by compounds having the common property of increasing the abundance of F, the precursor of Signal II_s ($F.^+$) (3, 4, 13, 14, 49, 52). The high quantum yield of inactivation and the at least partial reversible nature of inactivation implies that relatively subtle changes occur within the S_2, S_3-states during the inactivation by Tris and subsequent reactivation. Other chemical agents or physical treatments which also cause loss of S-state function and result in release of thylakoid-bound Mn also disturb protein or membrane structure (hydroxylamine [10, 28], heat treatment [9, 32, 53], chaotropic agents [36], acid [40], or alkaline pH [8, 24, 43]). To our knowledge, the inactivated O_2 centers produced by such treatments, other than Tris, cannot be reversed by simple equilibration of inactivated chloroplasts with specific chemicals.

In recent years, some evidence has been presented indicating possible conformational changes of proteins and/or membranes associated with electron flux through PSII (2, 16, 43, 59). We reasoned that the reversible inactivation of O_2 evolution by Tris possibly was a manifestation of such changes. Accordingly, we here describe attempts to define specifically the light-induced state inactivable by Tris, to determine the effects on inactivation of NH_3 which is believed (48, 49) like Tris (11) to complex with S_2, S_3, and to evaluate effects of a protein cross-linking reagent on inactivation of O_2 evolution by Tris. These experiments were made using short saturating flashes to probe the system.

MATERIALS AND METHODS

Chloroplast Preparation. Broken chloroplasts were prepared essentially as described in (46) from greenhouse-grown spinach *Spinacia oleracea*, var: Duet or Hybrid No. 7 (4- to 8-week-old plants) maintained on a 10-h light regime per day. Unless otherwise noted, leaves were harvested 1-2 h after the beginning of the light cycle. The grinding medium, STM, consisted of 0.4 M sucrose, 50 mM Tricine-NaOH, 10 mM $MgCl_2$, and 10 mM NaCl (pH 7.5) containing 1 mM Na-ascorbate and 0.1% BSA (Fraction V, Sigma). Ascorbate was omitted from the medium used in subsequent isolation steps and for the resuspension of chloroplasts. The isolated chloroplasts routinely were resuspended to yield ≥1 mg Chl/ml then stored in darkness at 4 C for a minimum of 30 min before use.

Photoinactivation Treatments. Flash preillumination of dark-adapted chloroplast suspensions (50 μg Chl/ml) was provided by simultaneously firing two Xenon flash lamps (EG & G model 0C-5) mounted about 0.5 cm on opposite sides of a cuvette of 0.33-cm light path and 2-ml volume. The saturating flashes were obtained with 4 μfarad capacitors charged to 1,000 v for each lamp, thus yielding 4 Joules per flash of about 2 μs half-duration. The dark time (t_d) between flashes was controlled electronically. All operations were done at 4 C and with extreme precautions to exclude stray light from the working area (11).

Procedure A. Dark-adapted chloroplasts (2 ml of 50 μg Chl/ml

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[2] In memory of Bessel, who didn't suffer fools gladly and never compromised his high standards in science.

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[5] Abbreviations: S-state(s): charge-collecting O_2-evolving catalyst of photosystem II; F and $F.^+$: precursor and species yielding EPR Signal II_s, respectively; FeCN: potassium ferricyanide; FCCP: carbonylcyanide-p-trifluoromethoxyphenylhydrazone; $DCIPH_2$: reduced form of 2,6-dichlorophenol indophenol; CCCP: carbonyl cyanide-*m*-chlorophenylhydrazone; ANT-2p: 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; DMQ: 2,5-dimethyl-*p*-benzoquinone; PD: *p*-phenylenediamine.

in STM) were exposed to n saturating flashes then rapidly (<3 s) injected into an equal volume of 1.6 M Tris-Cl^- (pH 7.8) with rapid mixing. Following incubation in darkness for 4.5 min, the chloroplasts were recovered by rapid centrifugation (12,000*g* for 30 s), resuspended in STM, then remaining rates of O_2 evolution were determined using 15 μg Chl/ml.

Procedure B. Dark-adapted chloroplasts were diluted immediately before flashing to yield 50 μg Chl/ml in 0.8 M Tris-Cl^- (pH 7.8). Following *n* saturating flashes and 4.5 min incubation in darkness, the chloroplast suspensions (2 ml) were treated in one of two ways: (*a*) The pH of the suspension was decreased to pH 7.0 by injection of 0.55 ml of 1.0 M KH_2PO_4 with rapid mixing, thereby eliminating the inactivating action of Tris (11, 55) on O_2 evolution; or (*b*) and more routinely, an aliquot (60 μl) of the chloroplast suspension in 0.8 M Tris was injected into reaction mixture B (1.2 ml) contained in the assay vessel. Preliminary experiments indicated that the resulting 40-fold dilution of the 0.8 M Tris also eliminated the inactivating action of Tris on O_2 evolution. Remaining rates of O_2 evolution were determined directly on the "diluted" suspensions or on the neutralized suspensions following addition of an aliquot (3 μg Chl) to 1.2 ml of reaction mixture A contained within the assay vessel.

Dark controls were run in all photoinactivation experiments. Reported values of flash-induced inactivation of O_2 evolution by Tris have been corrected for the small extents of inactivation (≤5%) that occurred in the dark controls.

Solutions of Tris were adjusted to the desired pH values with HCl at 23 C and measured with a combination electrode free from errors that affect measurement of pH of Tris.

Rate Measurements of O_2 Evolution. Rate measurements of O_2 evolution in saturating light were made polarographically in a reaction vessel of 1.2 ml essentially as described previously (9). Reaction mixture A contained in μmol/ml: Sorbitol, 400; Hepes-NaOH (pH 7.5), 50; FeCN, 1.0; methylamine, 30; and chloroplasts equivalent to 3–15 μg Chl/1.2 ml. Reaction mixture B was identical to mixture A except at pH 7.0. The addition of 60 μl of the chloroplast suspension in 0.8 M Tris (pH 7.8) to 1.2 ml of reaction mixture B yielded pH 7.4 for the assays of O_2 evolution. Preliminary experiments showed that the results from the inactivation studies were independent of use of class III *versus* class I electron acceptors in the rate measurements of O_2 evolution.

Glutaraldehyde Fixation of Chloroplasts. Glutaraldehyde (Ultrapure TEM grade, Tousimis Research Co.) was obtained as a 25% solution in ampoules and stored at −15 C until use. Chloroplasts (4 ml of 250 μg Chl/ml) were reacted with 1% glutaraldehyde in STM in darkness for 5 min then washed with an equal volume of 200 mM methylamine and twice with an equal volume of STM before final resuspension in STM (20, 21). Rates of O_2 evolution of chloroplasts carried through this procedure with the exception of incubation with glutaraldehyde were decreased no more than 15%. Such washed chloroplasts served as controls in experiments with the glutaraldehyde-reacted chloroplasts. The glutaraldehyde to Chl ratio used here for fixation has been shown to be sufficient for inhibition of chloroplastic osmotic, configurational changes (38, 58).

RESULTS

Kinetics of Inactivation Following a Single Flash. Meaningful measurements of the extent of flash-induced inactivation of O_2 evolution by Tris required knowledge of the kinetics of inactivation following a flash (Fig. 1).

In this experiment, chloroplasts were equilibrated in darkness in STM in the presence or absence of 200 μM FeCN, then diluted to yield 50 μg Chl/ml in 0.8 M Tris (pH 7.8) immediately before giving a single saturating flash. Following the flash and incubation in Tris in darkness for times indicated on the abscissa, the inactivation process was stopped by dilution of an aliquot of the chloroplast suspension into reaction mixture B. Results similar to those shown in Figure 1 were obtained when the inactivation process was stopped by addition of sufficient KH_2PO_4 to decrease the pH of the chloroplast suspension in Tris to pH 7.0. Rates of O_2 evolution remaining were then determined. Nonflashed chloroplasts were treated similarly and provided corrections for the small amount(s) of inactivation (≤5%) that occurred in darkness.

The data of Figure 1 show that with increasing dark time in the presence of Tris or Tris containing 200 μM FeCN following the flash, the extent of inactivation increased in a biphasic manner in both cases. The biphasicity is seen more clearly in Figure 1 inset where the inactivation occurring over the first 150 s is plotted on an expanded time scale. The presence of the low concentration of FeCN preceding and following the flash yielded two effects: (*a*) it decreased the time for half-maximal inactivation observed in Tris alone from about 23 to 10 s; and (*b*) it increased the maximal extent of inactivation induced by the flash from about 40 to 55% after 10 min incubation in the dark. Somewhat similar experiments

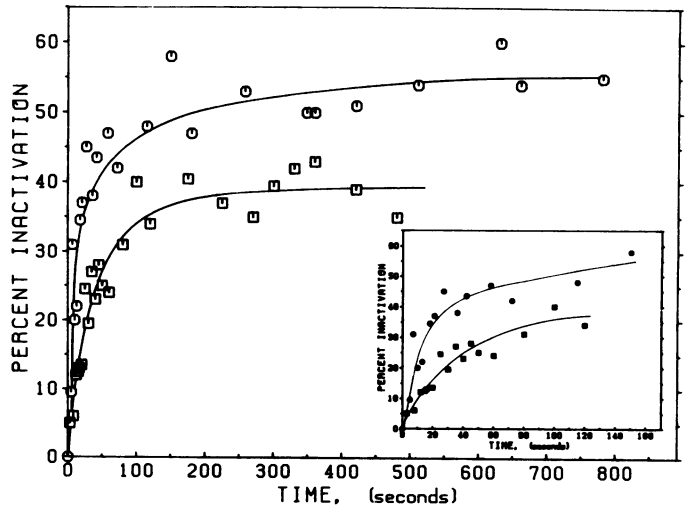

FIG. 1. Time Course of flash-induced inactivation of O_2 evolution by Tris. Chloroplasts (2 mg Chl/ml) were dark equilibrated (1 h) in STM in the presence (○) or absence (□) of 200 μM FeCN then diluted (50 μg Chl/ml) with 0.8 M Tris-Cl^- (pH 7.8) (maintaining the FeCN concentration) just prior to presentation of a single flash. Following the flash and incubation in darkness for times indicated on the abscissa, 60 μl of the suspension was injected into reaction mix B and remaining rates of O_2 evolution were determined. Inset: data (first 150 s) of main Figure 1 replotted on an expanded time scale.

by Briantais *et al.* (8) on the flash-induced alkaline (pH 9.3) inactivation of O_2 evolution in the absence of FeCN yielded results showing monophasic kinetics with a $t_{1/2}$ of about 10 s. The $t_{1/2}$ for flash-induced inactivation by Tris and alkaline pH are therefore similar, and the kinetics of both inactivation processes are fast compared to the spontaneous dark deactivation of S_2 (15).

The $t_{1/2}$ of inactivation obtained from the data of Figure 1 are considerably less than the $t_{1/2}$ (200 s) for inactivation deduced from measurements of first order rate constants of inactivation in weak, continuous 650-nm light (11). This apparent discrepancy obtained from the two different conditions may not be serious if one assumes inactivation occurs via second order kinetics dependent on S_2 population and that some turnover of S-states occurs in the presence of Tris in weak continuous light (see later section). The effects of FeCN on the inactivation kinetics shown in Figure 1 also may relate to relative differences in S_2 abundance arising from a double hit on the first flash (34) and/or possibly a decreased rate of deactivation of S_2 (see, however, ref. 42).

Though the data of Figure 1 indicate that maximum flash-induced inactivation required 10 min incubation following the flash, in most of the subsequent experiments an incubation time of 4.5 min was used for technical reasons. This time was sufficient, however, for obtaining 90% of the maximum flash-induced inactivation.

Lifetime of Flash-induced Inactivable State. A single flash given to dark-adapted normal O_2-evolving systems results in the advancement of the S_0- and S_1-states with a $t_{1/2}$ of 200–400 μs (33). Assuming the initial distribution of S_0/S_1 to be 25/75 and, for the moment, ignoring the effects of double hits and misses (15), the S-state distribution following a flash will be: $S_0(0)$: $S_1(25)$: $S_2(75)$: $S_3(0)$: $S_4(0)$. In subsequent darkness, S_2 decays to S_1 via kinetics and $t_{1/2}$ that vary somewhat depending on the material and its condition (31, 41).

Figure 2 compares the lifetime of the flash-induced Tris-inactivable state with the lifetime of the S_2-state as determined by Forbush *et al.* (15). In these experiments, chloroplasts were equilibrated in darkness in STM containing 200 μM FeCN, given a single flash, then after the time in darkness given on the abscissa, the chloroplasts were injected into Tris and incubated for a fixed duration to allow inactivation to occur. Rates of remaining O_2-evolving capacity were then determined. In this way, a meaningful comparison between the lifetime of the flash-induced Tris-inactivable state and the lifetime of the S_2-state could be made, uncomplicated by the stabilization of S_2 by Tris (11). The results shown are from a number of different experiments with different batches of chloroplasts.

At the shortest time period (3 s) between the flash and injection into Tris, an average value of 55% of O_2-evolving capacity was inactivated. This value was used to normalize decay of the flash-induced inactivable state to the decay of the S_2-state as determined by Forbush *et al.* (15). The data show at least over the initial 100 s between the flash and injection into Tris, a fairly close correspondence between decay of the inactivable state and the S_2-state. At this time, about 70% of the flash-induced inactivable state had decayed. At times >100 s, considerable variability from experiment to experiment was encountered and in many of the experiments, the decay of the inactivable state appears to be considerably slower than S_2 decay. After 6 min, the flash-induced inactivable state had decayed to the level of dark controls. The results of Figure 2 are interpreted to suggest that the inactivable state might be identifiable with S_2.

This possibility was explored further by examining the effect of FCCP on the lifetime of the flash-induced inactivable state. This reagent accelerates the rate of deactivation of S_2 by an order of magnitude such that after 10 s almost all (98%) of the S_2 is deactivated (44, 45). The data of Figure 2 show that the presence of 2.0 μM FCCP with chloroplasts during the flash and subsequent incubation before injection into Tris essentially abolished the

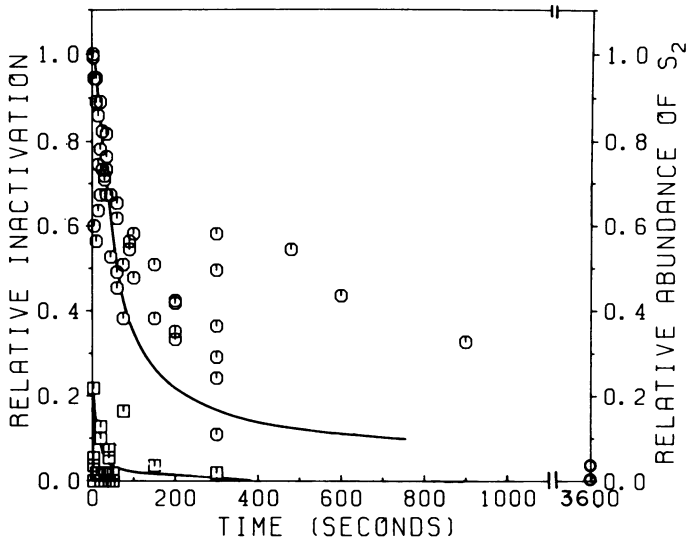

FIG. 2. Comparison of the lifetime of the flash-induced Tris-inactivable state to the lifetime of the S_2-State. Chloroplasts (50 μg Chl/ml) were dark-adapted (~60 min) in STM containing 200 μM FeCN in the presence (□) and absence (○) of 2 μM FCCP. After a single flash and incubation in darkness for times given on the abscissa, the chloroplast suspension was injected into an equal volume of 1.6 M Tris-Cl^- (pH 7.6) then incubated for 4.5 min in darkness. Chloroplasts were recovered by rapid centrifugation, resuspended in STM then assayed for remaining O_2 evolution in reaction mix A. Symbols are data of six separate experiments normalized at the shortest time period (≤3 s) where an average of 55% inactivation was observed. (——): Decay of S_2 (15).

single flash-induced inactivation. We postulate that this effect of FCCP reflects only an increased rate of deactivation of S_2; in a later section an additional effect of FCCP on single flash-induced inactivation is described. Nevertheless, Figure 2 in conjunction with the previously described inhibition by FCCP of inactivation induced by continuous light (11) suggests that a major inhibitory effect of FCCP on inactivation is a result of increased rates of deactivation of S_2.

Inactivation in a Sequence of Flashes: Preflashed in STM. The preceding experiments described the effectiveness of a single flash for inducing a state of the O_2-evolving mechanism which is inactivated by Tris, thus verifying and extending a previously reached conclusion; namely, that S_2 was a state sensitive to inactivation by Tris (11). However, no data were available to exclude S_3 or S_4 and thereby show specific sensitivity of S_2 to Tris inactivation analogous to the dependency of alkaline inactivation of O_2 evolution on the S_2-state (8).

According to the Kok-Joliot model for O_2 evolution (30), peak populations of S_2 and S_3 occur on flashes 1, 5, 9, etc., and 2, 6, 10, etc., respectively, until the oscillations damp and steady-state conditions are attained.

The experiments in Figure 3 were designed to determine if S_3 in addition to S_2 was a target for inactivation by Tris. In these experiments dark-equilibrated chloroplasts suspended in STM were given a sequence of saturating flashes (t_d = 1 s) then injected into Tris immediately after the *n*th flash of the flash sequence. After incubation in Tris, the remaining O_2 evolution capacity was determined. The inactivation observed after the *n*th flash reflects the dependency of inactivation on the specific S-state present following the *n*th flash.

Figure 3 shows the average values along with standard deviations of the extent of inactivation induced by the number of flashes given on the abscissa. The data show maximal extents of inactivation occurring after the first and fifth flashes with minima after the third and seventh flashes.

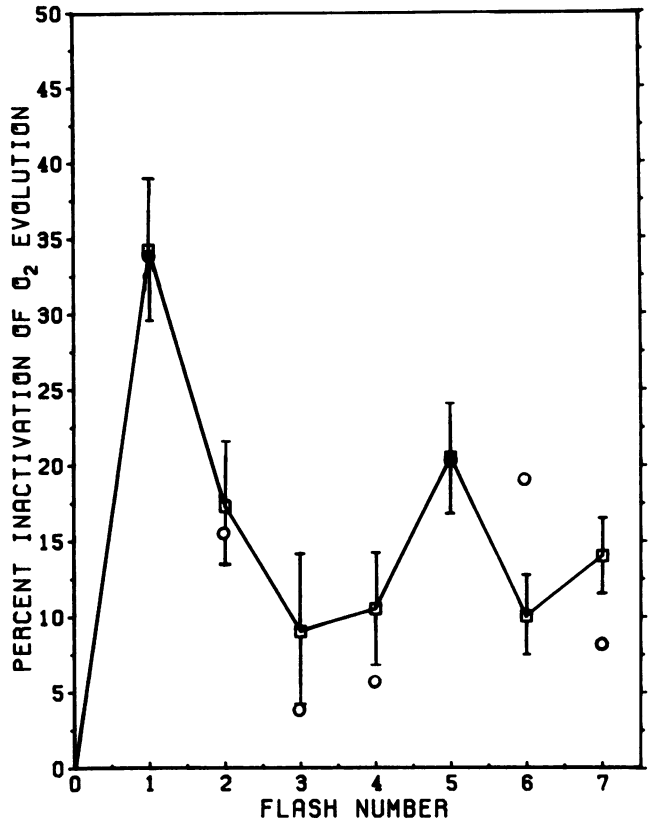

FIG. 3. Yields of inactivation of O_2 evolution in a sequence of flashes (t_d = 1 s). Dark-adapted chloroplasts in STM were given n flashes as indicated on the abscissa. After the nth flash, the chloroplasts were immediately injected into Tris, incubated, recovered by centrifugation, and assayed. (□): Yields of inactivation with SD. (○): Theoretical populations of S_2 (15) in the flash sequence (assuming S_0:S_1 = 25:75, α = 0.10, and β = 0.05) normalized to first-flash-induced inactivation.

We assumed an initial distribution of S_0/S_1 = 25/75 (15), calculated abundances of the S-states after each flash using various values for misses, α, and double hits, β, on each flash, then attempted to curve fit the theoretical distribution of individual S-states, or combinations thereof, with the experimental data on flash-induced inactivation. Of the numerous attempts, a "best fit" was obtained using only the S_2-state population calculated with values for α = 0.10 and β = 0.05, values commonly used to get computed Y_{0_2} values to match experimental Y_{0_2} values (15) obtained with flash durations very similar to those employed here.

In Figure 3, the theoretical S_2 populations normalized to the extents of inactivation obtained in the sequence of flashes are shown. Normalization was made to the first flash data by assuming that 52% of the theoretical S_2 population was inactivated. Comparison of the experimentally obtained inactivation *versus* the theoretical distribution of S_2 states in the flash sequence indicates a reasonably close agreement except on the sixth flash where the inactivation yield was about 10% lower and out of phase from the predicted. No cogent explanation for this departure can be offered; nevertheless, we interpret the flash-induced pattern of inactivation in Figure 3 to indicate that the S_2-state is the principal target for attack by Tris with subsequent inactivation of O_2 evolution.

Flashed in Tris. Some evidence has been presented (11) that the initial events of the Tris inactivation effect on O_2 centers was related to the action of some other unprotonated amines on O_2 evolution, namely, a formation of a complex between the unprotonated amine and S-states greater than S_1 (48, 50). In the case of NH_3, Velthuys (48) has presented evidence suggesting a rapid ($t_{1/2}$ = 0.5 s) and a slow ($t_{1/2}$ = 10 s) complex formation with S_2 and S_3, respectively. The S_2- and S_3-NH_3 complexes may be advanced to the S_4-NH_3 complex, but further transition to the S_0-state cannot occur and O_2 evolution is inhibited in a nondestructive manner (48).

Chloroplasts flashed in the presence of Tris at long spacing (t_d = 10 s) between flashes showed yields of inactivation (~60%) on both the first two flashes with a 40% yield on the third flash (11). It was tacitly assumed that the inactivation effect(s) by Tris were mechanistically analogous to NH_3 effects on S-states. This assumption was supported by the observation that Tris-like NH_3 (11, 48, 49) inhibited recombination of charges in DCMU-poisoned chloroplasts:

$$Q - S_1 \overset{h\nu}{\rightleftarrows} Q^- - S_2.$$

The results of the inactivation(s) observed in the sequence of 3 flashes were tentatively interpreted to indicate the following susceptibility of inactivation of O_2 evolution by Tris: $S_2 = S_3 > S_4$. The preceding results here, however, seem to suggest that only the S_2-state is a target for Tris attack. We therefore reexamined yields of inactivation in a sequence of flashes of chloroplasts suspended directly in Tris (0.8 M [pH 8.13]) (Fig. 4) rather than preflashed in STM then injected into Tris (Fig. 3). In these experiments, we used a flash spacing of 1 s rather than the 10 s spacing employed by Cheniae and Martin (11).

Figure 4 shows an oscillatory pattern of inactivation for chloroplasts flashed even in the presence of 0.8 M Tris. This oscillation also has a period of four flashes with distinct maxima following the first and fifth flashes and possibly another after the ninth flash. Orthophenanthroline completely abolished the oscillation but had no effect on the yield of inactivation on the first flash. Since this inhibitor blocks electron transport at the Q-A locus, this result indicates that the oscillation of inactivation even in the presence of Tris is related to charge accumulation by the S-states. Excluding the possibility that Tris rather than water is oxidized in

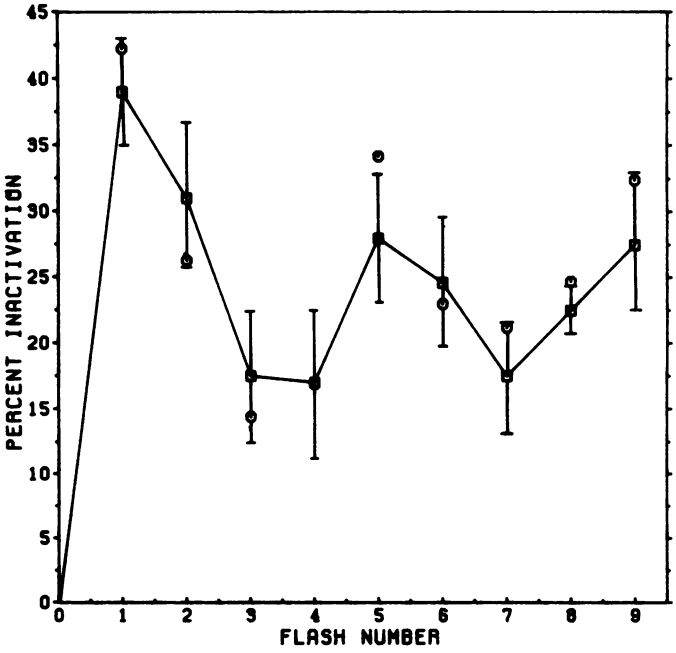

FIG. 4. Oscillatory pattern of inactivation of chloroplasts flashed in Tris. Chloroplasts (50 μg Chl/ml in 0.8 M Tris-Cl^- [pH 8.13]) were flashed as in Figure 3 and incubated (4.5 min) in darkness. After incubation, 60 μl of the suspension was injected into assay reaction mix B then assayed for O_2 evolution capacity. (□): Yields of inactivation with SD. (○): Theoretical S_2 populations.

the transition of $S_4 \rightarrow S_0$, the results would suggest that Tris is poorly effective in blocking this transition, at least at the flash repetition rate used (t_d = 1 s).

The interpretation we give to the data of Figure 4 is based principally on the observations that the yield of inactivation (38% measured and 55% of theoretical S_2) on the first flash of Figure 4 was essentially equivalent to first flash data of Figure 3 (34% measured and 52% of theoretical S_2). On subsequent flashes, however, the yields of inactivation per flash were consistently greater in Figure 4 (Tris) than in Figure 3 (STM). We assumed that this additional increment of inactivation reflected a small fraction of S_2 that was inactivated when flashed in the presence of Tris within the time of the flash spacing (1 s) but which would not be observed with STM-flashed chloroplasts.

We reasoned that the total inactivation observed after a series of flashes given to chloroplasts in Tris would be:

$$S_{intact} = 0.52\,[S_2]_n + \sum_{i=0}^{i=n-1} B\,[S_2]_i$$

where B equals the fraction of the S_2-state inactivated by each flash (n) in the series of i = 0 to i = n − 1 flashes. We calculated S_2-state populations as described earlier but after each flash in the flash sequence subtracted some fraction, B, of the S_2 population before proceeding with calculations of the S-state distributions following another flash. We also assumed that 52% of the S_2 population present after the final flash was inactivated.

The yields of inactivation in a sequence of flashes calculated using B = 0.10 and the experimental values are shown in Figure 4. A reasonably close fit between calculated and experimental data was obtained. Other attempts to curve fit the theoretical S_2-state distribution with the experimentally determined inactivation pattern included calculations based on α and/or β values differing from those commonly employed in Y_{O_2} curve fits. These attempts yielded less satisfactory fits than shown in Figure 4; however, with other assumptions, possibly a better fit can be obtained than shown.

According to the hypothesis advanced above in equation form, the yield of inactivation following presentation of three flashes, *e.g.* to chloroplasts suspended in Tris, would be dependent on repetition rate (t_d) of the flashes. Results of preliminary experiments (data not shown) tend to support this hypothesis and to lend some credence to the interpretation given to the data of Figure 4. In experiments where yields of inactivation after three flashes were measured as a function of t_d in the flash regime, we observed that the yield of inactivation, relative to that observed with t_d = 1.0 s, increased by about 45% when t_d = 10 s and decreased by 50% when t_d = 0.3 s. We argue that the flash pattern of inactivation observed here for chloroplasts in Tris, or that obtained previously (11) can be explained via specific sensitivity of S_2 without postulating a sensitivity of S_3 or S_4 to Tris. We also estimate from studies of yields of inactivation (third flash) as a function of t_d between flashes that Tris binding to S_2 (at 0.8 M Tris-Cl^- [pH 7.8]) occurs with a $t_{1/2}$ of about 1 s. This estimate is similar to the $t_{1/2}$ (0.5 s) determined for NH_3 complexing with S_2 (at 50 mM NH_4Cl [pH 7.8]).

Figure 5 shows results of an additional experiment that also argues for unique sensitivity of S_2 to Tris. In this experiment the effect of preincubation of chloroplasts with a low concentration of NH_2OH was determined. Such preincubation causes a delay in the yields of O_2 in a flash sequence (7) which now is generally accepted (34) to indicate a conversion of the S_1 and S_0 states of dark-adapted chloroplasts by NH_2OH to the S_{-1} and/or S_{-2} states.

If the S_2-state is the principal target for inactivation by Tris, we might expect a 3- to 4-flash delay before maximum yields of inactivation would be obtained. Such a delay would also give additional arguments for insensitivity of S-states less than S_2 to Tris attack. Thus far we have tacitly assumed that the slow inactivation of dark-adapted chloroplasts reflects an insensitivity of the S_0- and S_1-states to attack by Tris.

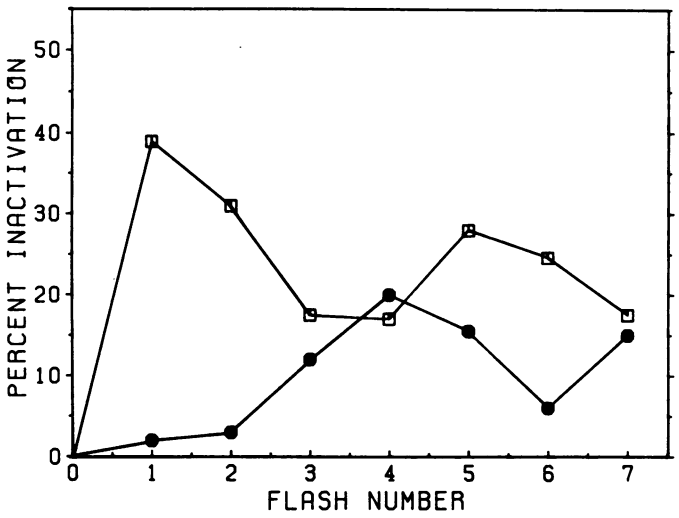

FIG. 5. Effect of hydroxylamine preequilibration on the oscillatory pattern of inactivation of chloroplasts flashed in Tris. Data shown by (○) were obtained with chloroplasts preequilibrated (60 min) at 1 mg Chl/ml in STM containing 200 μM hydroxylamine, then diluted to 50 μg Chl/ml in 0.8 M Tris-Cl^- (pH 7.8) just prior to the flash regime. Such preincubation resulted in a 20% loss of O_2 evolution capacity. Data shown by (□) were obtained similarly except for omission of hydroxylamine.

A comparison of the flash-induced pattern of inactivation in Tris alone with the NH_2OH (200 μM) preequilibrated chloroplasts flashed in the presence of Tris and only 10 μM NH_2OH shows that the normally observed high yield of inactivation observed on the first flash was abolished and maximal yield was not obtained until the fourth flash. We suspect that the diminution in maximum extents of inactivation obtained with the NH_2OH preequilibrated chloroplasts reflects the presence of a very low concentration (10 μM) of NH_2OH during the flash regime. We believe the data of the effect of NH_2OH preincubation on the pattern of inactivation in a sequence of flashes (Fig. 5) is consistent with current interpretations given to effects of low concentrations of NH_2OH on S-state distribution as well as the conclusion that S_2 is the principal target in the inactivation of O_2 evaluation by Tris.

Evidence for Moderation of Inactivation by F. Yamashita *et al.* (55, 56) first showed that $DCIPH_2$ inhibited the Tris destructive effect on O_2 evolution. With recognition of the light effect on the inactivation mechanism, other compounds such as CCCP, FCCP, and ANT-2p, as well as $DCIPH_2$, were identified to be inhibitors of light-induced inactivation (11). All these inhibitors of inactivation increase rates of decay of Signal II_s (3, 4, 13, 14, 50, 51), thereby increasing abundance of F, and, with the exception of $DCIPH_2$, all such compounds are known to increase rates of deactivation of S_2 and S_3 (44, 45).

Signal II_s has been shown to arise ($t_{1/2} \sim$ 1 s) via the reaction: $S_2, S_3 + F \rightarrow S_1, S_2 + F.^+$, where F is the precursor of the molecule, $F.^+$, responsible for Signal II_s (3, 4). According to the above reaction and conclusions reached in earlier sections, the abundance of F in dark-equilibrated chloroplasts should moderate the yield of inactivation observed on the first flash. To test this hypothesis and to delineate more clearly the effects of FCCP shown in Figure 2, we examined yields of inactivation in a sequence of flashes using chloroplasts containing high abundance of F. The previously described results were obtained with chloroplasts under conditions yielding low abundance of F.

In Figure 6 a comparison was made between chloroplasts isolated in darkness from leaves maintained in darkness for 22 h

as against chloroplasts from leaves 1 h within the light growth cycle. Such prolonged maintenance of leaves in darkness assured virtually complete decay of Signal II_s ($F.^+$) to F (3, 4, 13, 14).

Figure 6 shows that chloroplasts containing high abundance of F showed a 1-flash delay and slightly higher yields of inactivation on the second and third flashes compared to the flash pattern observed with chloroplasts having low abundance of F. We calculate from the known abundance of F for Signal II_s (3, 4), its reaction time with S_2, S_3, and the reaction times of inactivation that such behavior is predicted in the flash regime employed in Figure 5. However, quantitative comparisons between predicted and observed data cannot be justified from our data.

Figure 6 also shows that the 64% inhibition of inactivation observed on the first flash with chloroplasts having high abundance of F was abolished by a brief preillumination of these chloroplasts to convert F to $F.^+$. This is shown by Figure 6. We conclude from the results of Figure 6 that F can moderate flash-induced inactivation via reactions consistent with the reactions: (*a*) $S_2 + F \rightarrow S_1 + F.^+$; and (*b*) $S_2 + \text{Tris} \rightarrow S_{\text{inactive}}$.

The inhibition of single-flash-induced inactivation (Fig. 2) was tentatively ascribed to an FCCP-induced increased rate of deactivation of S_2. However, the recognition that F moderates first-flash-induced inactivation suggested an alternate explanation; namely, the inhibition reflected a high abundance of F arising from FCCP-induced accelerated decay of Signal II (4, 14).

To extend the conclusions reached from data of Figures 2 and 6, the experiments shown in Figure 7 were made. Here we show the effect of inactivation in the presence of 2 μM FCCP during and following flashes during preequilibration but removed by washings before flashing *versus* controls without FCCP treatment. First, we noted that the presence of FCCP during and following flashes largely abolished the inactivation induced by any of the flashes in the three flash sequence shown in Figure 7. Such inhibition was observed over more extended flash regimes and thus these results confirm the inhibitory effect of FCCP on inactivation induced by continuous, weak 650 nm light (11).

Studies of the dependence of such inhibition on FCCP concentration showed that 0.05 and 1.8 μM FCCP were required for half-maximal and maximal inhibition, respectively (Fig. 8). Though these results were obtained from measurements of the inhibitory effect of FCCP on only single-flash-induced inactivation, we believe the concentration dependencies to apply to any illumination regime used for induction of inactivation (11). A comparison of the results of Figure 8, obtained with FCCP, with CCCP concentration dependence studies on decay of Signal II_s (4) and deactivation of S_2, S_3 (44, 45) showed that the concentration requirements for affecting these three processes were similar when compared on a Chl/FCCP(CCCP) basis, thus giving us some assurance that the effects of 2 μM FCCP on the flash-induced inactivation were meaningful.

Figure 7 shows results obtained when chloroplasts were preincubated with FCCP but then repeatedly washed to remove FCCP before subjecting them to flash-induced inactivation. In this man-

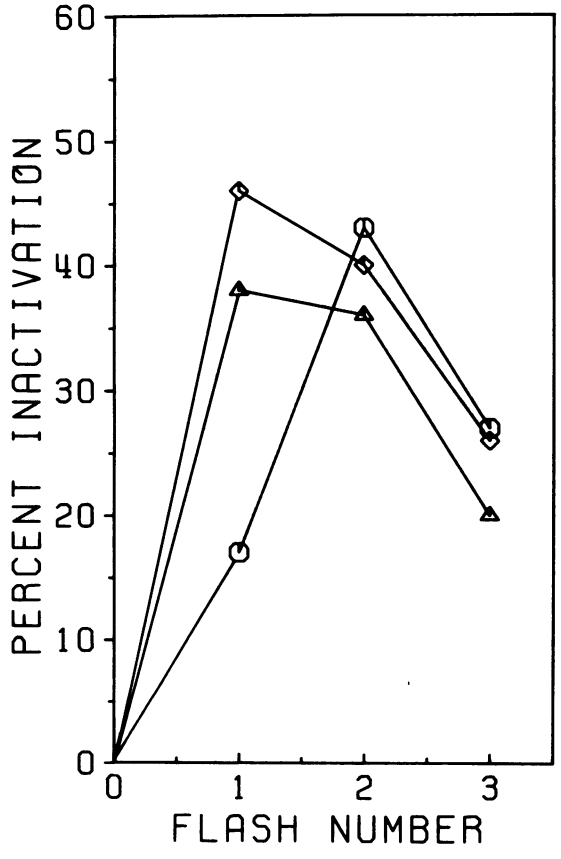

FIG. 6. Effect of long dark storage of leaves on flash-induced inactivation of chloroplasts. Flash-induced inactivation of chloroplasts suspended in Tris was measured essentially as described in Figure 4. (△): chloroplasts from leaves of plants 1 h into growth light cycle; (○): chloroplasts isolated in darkness from leaves of plants maintained in darkness for 22 h; (◇): as for (○) except that the chloroplasts (1 mg Chl/ml in STM) were illuminated (600 $\mu E/m^2 \cdot s$) for about 2 min then dark-adapted (20 min) before the flash regime.

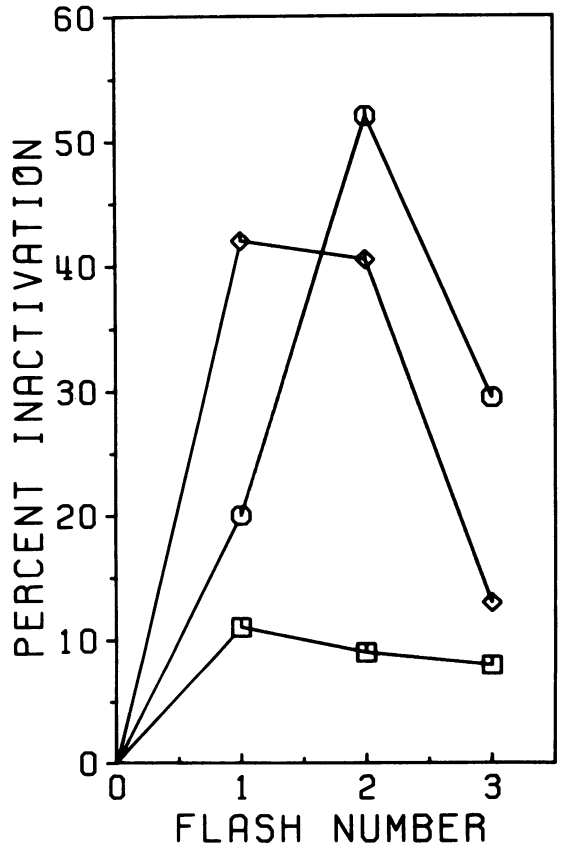

FIG. 7. Effects of FCCP on yields of inactivation in a sequence of flashes. Flash-induced inactivation in Tris was measured essentially as described in Figure 4 on chloroplasts treated in the following ways. (□): preincubated in STM and flashed in Tris containing 2 μM FCCP and 1 mM ascorbate; (○): preincubated (30 min) in STM containing 2 μM FCCP and 1 mM ascorbate but washed with STM before flashing in Tris containing 1 mM ascorbate; (◇): preincubated in STM then flashed in Tris.

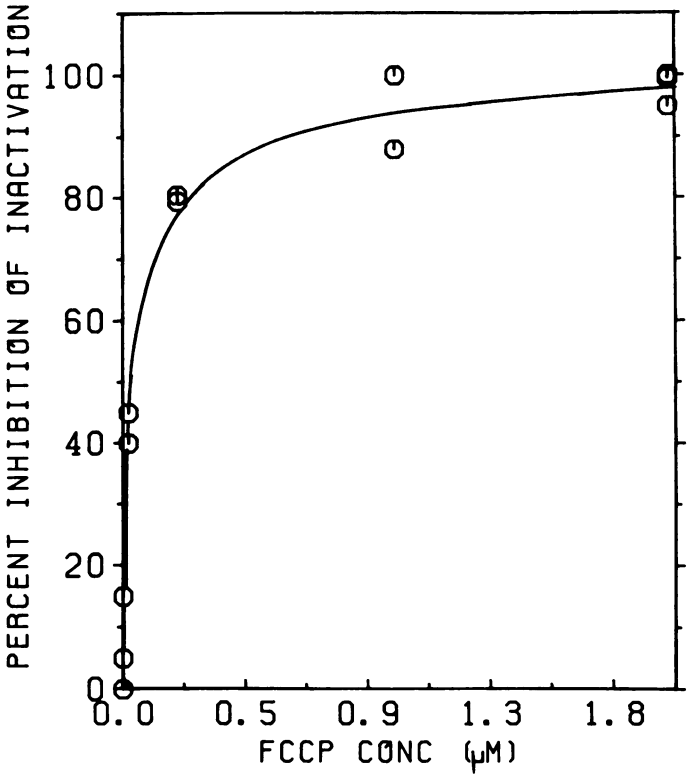

FIG. 8. Concentration dependency of FCCP for inhibition of single flash-induced inactivation. Flash-induced inactivation was done as described in Figure 2 except for the omission of FeCN. The concentrations of FCCP on the abscissa were present in the preincubation with STM and the flash and incubation in Tris.

ner, any effect of FCCP on the inactivation process arising from FCCP-induced accelerated rates of deactivation of S_2, S_3 can be separated from a FCCP effect arising from an increased abundance of F.

The results show an inhibition (~53%) of inactivation on the first flash only with extents of inactivation on subsequent flashes greater than those obtained with control chloroplasts. The results are qualitatively similar to those of the chloroplasts of Figure 5 with high abundance of F but generated under entirely different conditions. In data not shown, results similar to those of Figure 7 also were obtained in experiments employing $DCIPH_2$ preincubation (with subsequent removal by washes) to generate high abundance of F (49).

We attribute the one flash delay effect of FCCP and $DCIPH_2$ preincubation to be a consequence of the following: (*a*) $F.^+ + e \rightarrow F$, formed during preincubation; and (*b*) $S_2 + F \rightarrow S_1 + F.^+$. We attribute the inhibitory effect of the presence of FCCP and $DCIPH_2$ on inactivation on all flashes, or continuous light (11), to be a consequence of the following: $S_2 + e \rightarrow S_1$ (deactivation) and/or $S_i + Mn^{+2} \rightarrow S_{act}$ (reactivation, where S_i is a Mn-free inactive S-state (11) formed by inactivation of S_2 and S_{act} is the unknown active S-state species resulting from recomplexing the hexaquo Mn^{2+} released during inactivation (5, 6, 9, 27). The rationale for these interpretations appear to be supported from the following lines of evidence: (*a*) S_2 is a "substrate" for the inactivation process and its abundance is determined either by rates of deactivation or by abundance of F; (*b*) FCCP (CCCP) induced increased rates of deactivation of S_2 are observed in the presence of these compounds but not following preequilibration and their subsequent removal (B. Kok, personal communication); (*c*) F formed in response to long dark storage of leaves or preincubation of chloroplasts with FCCP or $DCIPH_2$ is of low abundance ($\leq S_2$), stable in darkness after formation and slowly reactive ($t_{1/2}$ ~ 1 s) with S_2 (3, 4); and (*d*) reactivation of S_i can be induced by FCCP, $DCIPH_2$, and other chemicals (11).

Evidence for Competition between Ammonia and Tris at S_2. We postulated previously (11) that the inactivation of O_2 evolution by Tris is mechanistically analogous to Velthuys' model (48) for the nondestructive inhibition of O_2 evolution by NH_3. This model suggests formation of complexes of NH_3 with S_2 and S_3 with $t_{1/2}$ of 0.5 and 10 s, respectively. With the recognition of S_2 as the target of inactivation by Tris, we reasoned that NH_3 and the unprotonated species of Tris possibly complexed with S_2 at the same site and that NH_3 might inhibit the inactivation of S_2 by Tris.

We examined the possible inhibitory effect of NH_3 on single-flash-induced inactivation, where, from all lines of evidence, interactions between Tris and NH_3 at the S_2-state would be determined.

Figure 9 shows the concentration dependency for NH_4Cl inhibition of single-flash-induced inactivation of O_2 evolution by Tris. We observed that the extent of single-flash-induced inactivation decreased markedly with increasing concentration of NH_4Cl until a minimum was obtained at about 200 mM NH_4Cl. As shown, a 5-fold higher concentration of NH_4Cl failed to inhibit inactivation completely in these experiments.

Figure 9, inset shows the data of main Figure 9 plotted as per cent inhibition *versus* NH_4Cl concentration. This plot shows that NH_4Cl inhibits inactivation maximally by about 80%. We estimate from this plot that maximal and half-maximal inhibition of inactivation is obtained at 200 and 25 mM NH_4Cl, respectively.

We assumed that NH_3 and not NH_4Cl is the effective inhibitor species of the inactivation of S_2. This assumption is based on measurements of the Hill reaction (26, 29) and luminescence (48, 49, 58) which indicate that NH_3 rather than NH_4Cl affects the oxidizing side of PSII. Based on the pK_a (NH_4^+/NH_3) = 9.24, the concentrations of NH_3 for maximal and half-maximal inhibition of inactivation are about 7 and 0.9 mM, respectively. The concentrations of NH_3 for maximal and half-maximal effect on the 40 ms-enhanced luminescence are about 3.5 and 0.4 mM, respectively

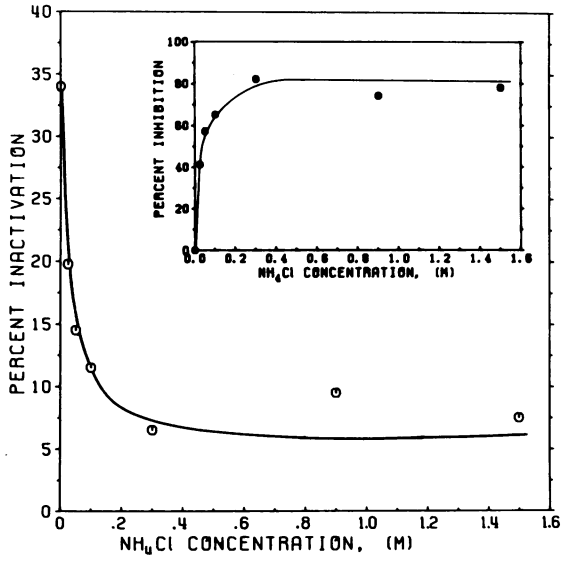

FIG. 9. Concentration dependency of NH_4Cl for inhibition of single flash-induced inactivation. Following preincubation (20 min) in STM containing concentrations of NH_4Cl given on the abscissa, the chloroplasts (1 mg Chl/ml) were diluted to yield 50 μg Chl/ml in 0.8 M Tris Cl^- (pH 7.8) while maintaining the NH_4Cl concentrations. After a single flash, extents of inactivation were measured after the recovery of the chloroplasts (see Fig. 10, legend). Inset: data of main Figure 9 expressed as per cent inhibition of inactivation by NH_4Cl.

(48). The approximate similarities of concentration dependency for NH_3 on the two processes gave us some assurance that the inhibitory effects of NH_3 on inactivation were related to known effects of NH_3 on the S-states.

The inhibitory effect of NH_3 on single-flash-induced inactivation could be the result of competition with Tris for a common binding site on the S_2-state or by an increased rate of deactivation of S_2 induced by NH_3 (12).

We directed experiments towards the possibility that the inhibitory effect of NH_3 on inactivation by Tris reported in Figure 9 reflected possible competition between these two amines at the S_2 target. Curve 2 of Figure 10 shows the dependency of inactivation induced specifically by a single flash on the concentration of unprotonated Tris. The data were obtained by measurements of flash-induced inactivation of chloroplasts suspended in Tris at the concentrations of unprotonated Tris given on the abscissa. Preliminary experiments indicated that over a wide range of unprotonated Tris concentrations the relationship shown by curve 2 was independent of the procedure employed (constant pH with variable concentration *versus* constant concentration with variable pH) to vary the amine concentration. In obtaining this curve, we corrected for amounts of dark-induced inactivation occurring at each of the concentrations of the unprotonated Tris.

Curve 2 of Figure 10 shows sigmoidal characteristics revealing that essentially no flash-induced inactivation was observed until the concentration of unprotonated Tris reached about 80 mM (equivalent to 240 mM Tris [pH 7.8]). Thereafter, the flash-induced inactivation increased reaching half-maximal and maximal values at about 135 and 380 mM unprotonated Tris, respectively.

The dependency of inactivation on unprotonated Tris differs markedly from the concentration dependency of "direct" inhibition of O_2 evolution on unprotonated Tris (Fig. 10, curve 1). The data of curve 1 were obtained from assays (<20 s duration) in which chloroplasts were injected directly into the assay vessel containing reaction mixture A (pH 7.8) and the unprotonated Tris concentrations given on the abscissa. With increasing concentrations of Tris, pronounced bend-off of rates of O_2 evolution were observed. We estimate, in agreement with previous data (17) that about 57 and 160 mM concentrations of unprotonated Tris are required for half and maximal direct inhibition of O_2 evolution. Comparable experiments as those of curve 1, but with NH_3, showed that only about 0.8 mM NH_3 was required to yield half inhibition of O_2 evolution (data not shown). The results suggest that the affinity of NH_3 to S-states is some 70 times greater than Tris.

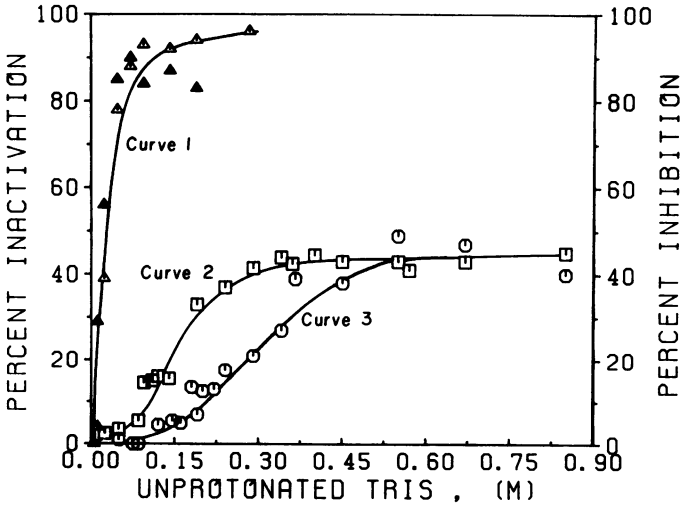

FIG. 10. Effect of NH_4Cl on concentration dependency of Tris for inactivation of S_2. Curve 2 was obtained from single flash experiments in which chloroplasts (50 μg Chl/ml) were suspended in Tris yielding the unprotonated Tris concentrations given on the abscissa. Curve 3 was obtained similarly except for addition of 100 mM NH_4Cl. Following the flash and a 4.5 min incubation, the chloroplasts were recovered and assayed in reaction mix A. Curve 1 shows the concentration dependency of unprotonated Tris for inhibition of Hill activity measured in reaction mix A at pH 7.8.

If Tris complexes with S_2, the sigmoidal shape of curve 2 (Fig. 10) might suggest (35): (*a*) a binding of more than one molecule of Tris to a site on S_2 with site interaction; (*b*) a binding of one molecule of Tris on S_2 which facilitates a binding of another molecule of Tris to S_2 at a different site; and (*c*) a "classical" allosteric effect (37). Alternately, the sigmoidal shape might simply reflect a concentration dependency frequently observed with chaotropic reagents (25). In the latter case, we would not expect NH_3 to inhibit inactivation directly; if NH_3 should compete with Tris for a common site on S_2, we might expect competitive inhibition kinetics.

The results of curve 3 describe the effect of addition of 100 mM NH_4Cl (3.5 mM NH_3) to chloroplast suspensions subjected to flash-induced inactivation. Comparison of curve 2 (no. NH_3) with curve 3 (plus NH_3) shows that NH_3 effectively inhibited inactivation at low but not high concentrations of unprotonated Tris. We note that significant inhibition (47%) by 3.5 mM NH_3 was obtained, for example, at 300 mM unprotonated Tris, a concentration some 86 times greater than NH_3. We are not aware of such inhibitory behavior of another molecule on action of a chaotrope and conclude that the inhibition of inactivation by NH_3 is more direct than a simple inhibition of a possible chaotropic action by Tris.

Figure 11 shows the data of curves 2 and 3 of Figure 10 plotted as a double reciprocal plot. This plot shows clearly the nonhyperbolic relationship between inactivation and unprotonated Tris concentration. This analysis suggests: (*a*) NH_3 competitively inhibits Tris-induced inactivation; and (*b*) an involvement of a cooperative effect in the Tris-induced inactivation. These tentative conclusions are made realizing the complexity of inhibitor-inhibitor interactions.

Effects of Glutaraldehyde Fixation. Glutaraldehyde fixation of chloroplasts protects PSII activities against inactivation by aging (20–23), enzymatic iodination (2), Tris-washing (59), but not the alkaline inactivation of S_2 (8). Presumably, inactivation of PSII by such treatments, as well as others (42) that disturb membrane and/or protein structure, reflects dependency of PSII on a high order of structural organization. The protection by glutaraldehyde, a bifunctional cross-linking reagent (52), has been interpreted to suggest that protein/membrane conformational changes are involved in these inactivation processes.

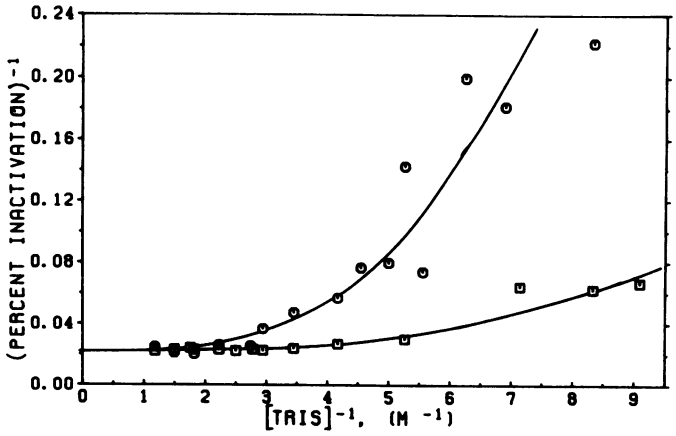

FIG. 11. Lineweaver-Burk plot of data of curves 2 and 3 of Figure 12. (□): data of curve 2, Figure 12 (no NH_4Cl); (○): data of curve 3, Figure 12 (plus 100 mM NH_4Cl).

Zilinskas and Govindjee (59) previously showed that inactivation of O_2 evolution by Tris was inhibited approximately 50% by preincubation of chloroplasts with glutaraldehyde at concentrations sufficient to inhibit macroconfigurational or large structural changes but not microconformational changes. These results were obtained before recognition of the marked acceleration of Tris-induced inactivation of O_2 evolution by a high quantum yield process (10). We therefore questioned whether the inhibition observed by Zilinskas and Govindjee (59) pertained to the dark-induced, slow inactivation process or the light-induced S_2-dependent rapid inactivation process, and whether the S_2-dependent inactivation involved possible conformational changes.

In these experiments we used DMQ_{ox} (18) as the electron acceptor in our rate assays of O_2 evolution in order to avoid possible confusion resulting from inactivation of plastocyanin by glutaraldehyde (22, 23). With DMQ as electron acceptor, secondary effects of glutaraldehyde fixation on the electron transport chains relate only to reactions with the PSII complex (18, 47). The glutaraldehyde to Chl ratio generally used in our experiments was equal to or greater than those found by Zilinskas and Govindjee (59) for "complete" fixation of chloroplasts.

Figure 12 shows the effect of fixation of chloroplasts with 1% glutaraldehyde on a 3-flash pattern on inactivation of chloroplasts flashed in Tris. In the experiment shown, the pattern of inactivation observed for control chloroplasts was somewhat different than usually observed; nevertheless, this atypical pattern does not prohibit making the conclusion that glutaraldehyde fixation markedly inhibits (74% on the first flash) inactivation induced by any of the three flashes. Glutaraldehyde concentration dependency studies showed 50 and 98% inhibition of the first flash inactivation was obtained at 0.47 and 5% glutaraldehyde, respectively.

These same glutaraldehyde concentrations caused 30 and 80% inhibition of DMQ-coupled O_2 evolution of control chloroplasts assayed in strong light. However, inhibition by glutaraldehyde of O_2 evolution coupled to another class III acceptor (PD_{ox}) (47) has been reported to be more pronounced in strong than in weak continuous or flashing light (22, 23). We believe that the rather large "direct" inhibition(s) by glutaraldehyde of our rate assays does not cause serious complications in the interpretation of data on glutaraldehyde inhibition of flash-induced inactivation.

The inhibitions of flash-induced inactivation shown in Figure 12 could be obtained consistently in contrast to similar studies of the effect of glutaraldehyde on rates of inactivation of O_2 evolution by Tris in darkness. Maximally, we observed that glutaraldehyde fixation (1% glutaraldehyde) decreased rates of inactivation by a factor of 2; however, in other experiments the effects were less and sometimes nonexistent. Our results therefore seem to indicate that the flash-induced inactivation is more sensitive to glutaraldehyde fixation than the dark-induced and slow inactivation process.

We conclude that the S_2-dependent inactivation of O_2 by Tris is inhibited by glutaraldehyde fixation and suggest that the fixation either abolishes the postulated site(s) of Tris complexing with S_2 or conformational changes associated with the inactivation process.

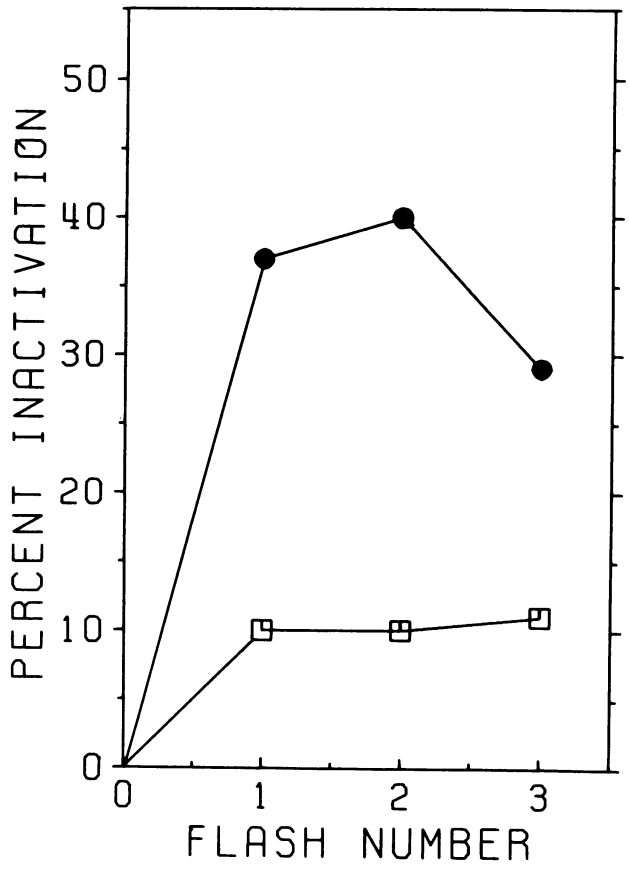

FIG. 12. Effect of glutaraldehyde fixation on inactivation in a sequence of flashes. Procedures of Figure 4 were employed except that assay reaction mix B contained 0.5 mM DMQ. (●): control chloroplasts; (□): chloroplasts fixed with 1% glutaraldehyde prior to the inactivation regime.

DISCUSSION

The following lines of evidences are brought forth here for identifying the S_2-state as the light-induced state inactivated by Tris: (*a*) maximum yield of inactivation is obtained following a single flash even in the presence of orthophenanthroline, an inhibitor allowing one charge separation within PSII; (*b*) the decay of the flash-induced inactivable state in STM approximates the normally observed decay (deactivation) of S_2 (15), and FCCP, a reagent accelerating S_2 deactivation (44, 45) essentially abolishes inactivation induced by a single or a sequence of flashes; (*c*) the inactivable state oscillates with a period of four in a sequence of flashes having maxima on flashes 1 and 5; (*d*) presetting of S_0/S_1 states to S_{-1}/S_{-2} with NH_2OH (7, 34) delays the appearance of the inactivable state from the first to the fourth flash; (*e*) chloroplasts having high abundance of F, set either by FCCP or $DCIPH_2$ (3, 4, 13, 14, 49, 51), or maintaining leaves in darkness for extended times (3, 4), showed a 1-flash delay in attaining maximum yield of the inactivable state; and (*f*) inactivation of S_2 ($t_{1/2}$ = 10 or 25 s depending on conditions, Fig. 1) is some 100–300 times faster than rates of inactivation ($t_{1/2}$ ~ 50 min) of dark-equilibrated chloroplasts (high S_1). These observations coupled with those previously presented (11) argue strongly for identifying S_2 as the principal target of Tris attack in the inactivation of O_2 evolution by Tris.

One of the questions asked here was S_2 the sole target or were S_3 and possibly S_4 also principal targets as suggested previously (11). Despite numerous attempts to improve the accuracy of measurements of yields of inactivation in a sequence of flashes, we had sufficient error that prohibits total exclusion of S_3 as an inactivable state. In data such as those of Figure 3 where yields of inactivation deviate above a specific fraction of theoretical S_2 populations on a given flash (*e.g.* flash 3) high populations of S_3 occur. However, on other flashes (*e.g.* flash 6) where yields of the inactivable state deviate below a specific fraction of theoretical S_2 population, significant theoretical S_3 populations exist. Such lack of systematic deviation of the inactivable state from theoretical S_2 and S_2, S_3 populations prevents precise assessment of S_3 as an additional target. We believe the maximum contribution of the theoretical S_3 population to be no more than 10% (with S_2 contributing about 50%). This estimate is based on curve fit analyses comparing the inactivable state with various sums of contributions from the S_2 and S_3 populations. Contributions of >10% of S_3

yielded increasingly more disparate curve fits to the experimental data. Assuming such analyses are valid, we argue that the possible 10% contribution of theoretical S_3 population to the inactivable state may not be direct but rather reflect $S_3 \rightarrow S_2$ by deactivation.

Likewise, the interpretation we give to the inactivation pattern observed for chloroplasts flashed directly in Tris (Fig. 4) precludes large contributions of S_3 to the inactivable state as postulated previously (11). In the absence of more refined data and more definitive experiments, we suggest that S_2 is the principle, if not the only, target of attack by Tris leading to "inactivated" O_2 centers. If our assays for "inactivated" O_2 centers truly reflect the abundance of centers inactivated, then we observe maximally an inactivation of ~50–55% of the theoretical S_2 population. Possibly this reflects competing rates of deactivation and inactivation of S_2.

We believe that the limiting yield of 50–55% inactivation of the theoretical S_2 is not a consequence of an accelerated initial rate of deactivation of S_2 ($S_2 + Q^- \rightarrow S_1 + Q$) by Tris analogous to the model proposed by Delrieu (12) for inhibition of O_2 evolution by NH_3. Our reasons are: (*a*) in DCMU poisoned chloroplasts, Tris inhibits rather than accelerates charge recombination between S_2 and Q^- (11); (*b*) our results (Fig. 11) suggest that NH_3 and Tris compete for a common site on S_2 in the inactivation process; and (*c*) inhibition of inactivation by NH_3 is reversed by sufficiently high concentrations of Tris. We find it difficult to accept that these two amines which bind at a common site could have opposite effects on the lifetime of S_2. Thus, we favor Velthuys' model for action of NH_3 on S-states (stabilization of S_2) (48) and attribute the inhibitory action of NH_3 on Tris-induced inactivation of S_2 to simple competition between these two amines for a common binding site on S_2.

It seems clear from Figure 10 that the direct inhibition of O_2 evolution by Tris or NH_3 (or other primary amines) is kinetically distinct from Tris-induced inactivation of S_2. Direct inhibition seems to reflect hyperbolic kinetics while inactivation shows distinctive sigmoidal kinetics. We suggest that the sigmoidal kinetics of inactivation reflect a cooperative effect in Tris binding and that inactivation of S_2 by Tris is a process subsequent to direct inhibition, a mechanism, like NH_3, presumably involving only one amine molecule per S_2 (48). The inhibitory effect of glutaraldehyde fixation on a flash-induced inactivation of O_2 evolution by Tris shown here could reflect annihilation of cooperativity of Tris binding in the inactivation process or inhibition of possible conformational changes of S_2 that otherwise result in the release of the bound Mn generally associated with O_2 evolving centers (6, 9, 27).

The chemical nature of S_2 that makes it susceptible to inactivation by Tris or alkaline pH (8) remains unknown. It was speculated previously (11) that the inactivation of S_2 specifically by Tris might be a consequence of: (*a*) a plus three valency state of manganese in the S_2 state; and (*b*) a ligand-field stabilization effect which is observed in complexes of manganic but not manganous ions. Such stabilization effects are larger with amine ligands than with H_2O (1, 39). This postulate appears to be in contradiction to a hypothesis (19) offered for explanation of the oscillatory behavior of Mn-dependent water proton transverse relaxation rates of chloroplasts. The model offered by Govindjee *et al.* (19) suggests that contributions of Mn^{+3} to the S_1 and S_2 states are equivalent yet, as shown here, the inactivation of S_2 by Tris is some 100–300 times faster than S_1.

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