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KINETIC STUDY OF OXYGEN EVOLUTION PARAMETERS IN TRIS-WASHED, REACTIVATED CHLOROPLASTS

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SUMMARY

Tris-washed chloroplasts which have lost the ability to evolve oxygen can be reactivated by the procedure of Yamashita, T., Tsuji, J. and Tomita, G. ((1971)Plant Cell Physiol. 12, 117–126) [7] to give 100 % of the rate of control chloroplasts in continuous illumination. Furthermore, in flashing light the reactivated chloroplasts exhibit oxygen-yield oscillations of period four that are characteristic of the control. Similar kinetic parameters for intermediate steps in the water-splitting process are observed for the two preparations. We conclude that the reactivation procedure restores the native oxygen evolution mechanism to Tris-washed chloroplasts.

A relatively rapid and reversible (0.5 s decay) light-induced component of EPR Signal II is observed upon inhibition of O_2 evolution by Tris washing (Babcock G. T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328) [10]. Reactivated chloroplasts are similar to untreated chloroplasts in that this Signal II transient is not observed. Manganese, which is released by Tris treatment to the interior of the thylakoid membrane in an EPR-detectable state, is returned to an EPR-undetectable state by reactivation. The reactivation procedure does not require light to restore O_2 evolution and EDTA has no effect on the extent of reactivation. These results are discussed in terms of possible mechanisms for manganese incorporation into photosynthetic membranes.

INTRODUCTION

Inhibitory treatments have proved very useful in the study of the mechanism of O_2 evolution in green plant photosynthesis. Tris washing is a widely used treatment that provides insight into the function and location of several components of Photosystem II not observable in untreated systems.

The elegant studies of Yamashita and Butler [1, 2] established the site of Tris

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenol indophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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action to be on the water side of Photosystem II. Tris treatment is similar, in many respects, to a number of other Photosystem II inhibitory techniques which include heat [3], washing with chaotropic agents [4] and hydroxylamine extraction [5, 6]. Chloroplasts which have been subjected to these treatments show decreased chlorophyll a fluorescence in the light, little or no oxygen evolution capacity and higher concentrations of EPR-detectable manganese. DCMU-sensitive electron flow to Hill oxidants can be restored by the addition of exogenous electron donor systems such as phenylenediamine/ascorbate, manganese, or diphenylcarbazide. However, Yamashita et al. [7, 8] showed that Tris inhibition is distinct from the other Photosystem II inhibitory treatments in that oxygen evolution in Tris-washed chloroplasts can be reactivated by rewashing with buffer containing reducing agents such as DCIP/ascorbate.

Blankenship and Sauer [9], following the initial observations of Lozier et al. [4], showed that Tris inhibition leads to the appearance of a manganese EPR signal not present in untreated chloroplasts. This EPR-detectable manganese, which comprises about 60 % of the total chloroplast manganese, is localized within the interior space of the chloroplast thylakoids and only slowly ($t_{\frac{1}{2}} = 2.5$ h) diffuses across the thylakoid membrane into the suspending medium. Babcock and Sauer [10] used a number of inhibitory treatments, including Tris washing, which act on the water side of Photosystem II and showed that a relatively rapid and reversible (0.5 s decay) light-induced component in EPR Signal II appears upon inhibition of O_2 evolution. They established that in Tris-washed chloroplasts the component responsible for Signal IIf serves as an electron donor to $P680^+$, the oxidized Photosystem II reaction center chlorophyll, and proposed that the Signal IIf species may function as the physiological donor, Z, in oxygen evolving chloroplasts (Babcock, G. T. and Sauer, K., (1975) Biochim. Biophys. Acta 376, 329–344).

We have investigated the effect of Tris washing and subsequent reactivation on oxygen evolution in continuous and flashing light, on the levels of EPR-detectable and total chloroplast manganese and on the extent of Signal IIf formation. Our results indicate that reactivated chloroplasts resemble untreated controls in exhibiting each of the kinetic characteristics that accompany oxygen evolution. Manganese released by Tris washing is reincorporated into the thylakoid membrane upon reactivation, and Signal IIf is no longer seen.

MATERIALS AND METHODS

Chloroplast preparation

Sucrose-washed and Tris-washed broken spinach chloroplasts were prepared as previously described [9]. Tris-washed, reactivated chloroplasts were prepared according to the method of Yamashita et al. [7] by rewashing Tris-washed chloroplasts in 15 ml of the sucrose isolation buffer which had been made 0.3 mM in DCIP and 2 mM in ascorbate. Chlorophyll content during the reactivation procedure was between 200 and 400 μg/ml. The presence or absence of room light during the reactivation procedure did not affect the extent of reactivation. Since it has been our experience that chloroplasts lose manganese upon each washing, even with isolation buffer, all samples were washed the same number of times. The only differences were the solutions used or the order of washing with them. All procedures were performed at 0 °C.

Oxygen measurements, EPR measurements, and manganese analysis

The techniques and instrumentation for measuring O_2 evolution in continuous and flashing light have been described previously [9, 11]. The reaction mixture for continuous light measurements contained 50 mM HEPES (pH 7.6), 20 mM NaCl, 1 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$. Chlorophyll concentration was 25–50 μg/ml. For flashing light experiments, the reaction mixture contained 200 μg chlorophyll/ml, 50 mM HEPES (pH 7.6), 10 mM NaCl, 20 mM $MgCl_2$, 0.2 mM NADP and 10 μg/ml ferredoxin. NADP and ferredoxin were obtained from Sigma.

EPR spectra were obtained using a Varian E-3 (X band, 9.5 GHz) spectrometer. Instrument settings are given in the figure captions. All spectra were taken at room temperature. EPR spectra of Signal II were taken using a flat aqueous sample cell (nominal internal diameter = 0.2 mm) obtained from J. F. Scanlon Co., Costa Mesa, California. Manganese EPR spectra were taken in 1.0 mm internal diameter quartz tubes, which permitted more reproducible sample positioning.

Manganese analyses were performed with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer. Samples and standards were prepared as previously described [9].

RESULTS

Using the techniques developed by Yamashita et al. [7], we have been able to reactivate O_2 evolution in Tris-washed chloroplasts to 100 % of the control rate.

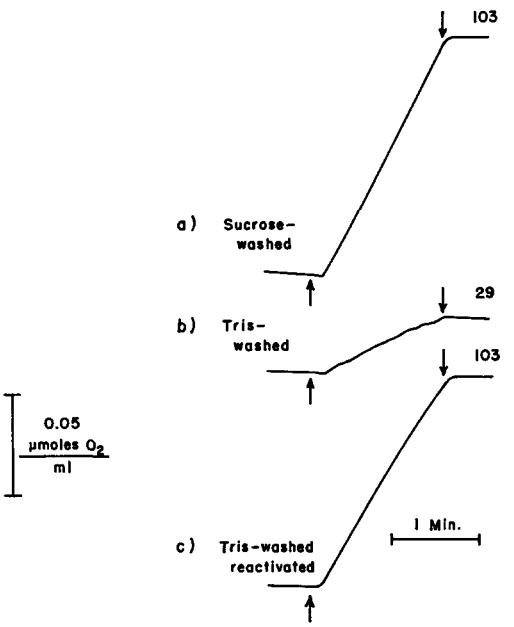

Fig. 1. O_2 evolution in continuous light measured as described in Materials and Methods. Upward-pointing arrows signify when the light was turned on and downward-pointing arrows signify when the light was turned off. Numbers appearing to the right of the downward-pointing arrow are the rates of O_2 evolution in μmol O_2(mg chlorophyll)$^{-1} \cdot h^{-1}$. Chlorophyll content, 48 μg/ml.

This is shown in Fig. 1 where the control rate (a) is seen to be inhibited by Tris washing (b) and then restored to the control level by reactivation (c).

We have found that the pH of the Tris solution used in the Tris washing step is crucial in determining both the extent of inhibition and the rate of O_2 evolution upon subsequent reactivation. At a pH higher than 8.0 we were able to obtain greater inhibition, but not 100 % restoration of oxygen evolution upon reactivation.

The flashing light experiments of Joliot et al. [12], Kok et al. [13], and Weiss and Sauer [14] demonstrated that oxygen evolution from dark-adapted chloroplasts illuminated by a series of closely spaced, short (20 ns to 10 μs) flashes has a distinct period-four oscillatory pattern. Fig. 2 presents a comparison of oxygen evolution in flashing light for sucrose-washed chloroplasts with that for Tris-washed, reactivated chloroplasts. Both chloroplast samples show period-four oscillations with maximal amounts of oxygen on the third flash. The ratio Y_3/Y_4 (yield of O_2 on the third flash to O_2 on the fourth flash) is higher in the sucrose-washed chloroplasts than in the reactivated chloroplasts. This can probably be attributed to a decrease in the S_1/S_0 ratio caused by the reductants DCIP/ascorbate used in the reactivation procedure [15]. We have also noticed that in the reactivated chloroplasts the first flash after dark adaption produces a positive-going polarographic signal. The dependence of this signal on polarizing voltage is different from that of oxygen, and we have concluded that it arises from oxidized DCIP. In the results for reactivated chloroplasts shown in Fig. 2 this component has been subtracted from the O_2 yield on the first flash.

Following each flash on O_2-evolving chloroplasts, a dark relaxation time is required before quanta from a second flash will be effective in producing further Photosystem II charge accumulation. In untreated chloroplasts this reset time is less than 1 ms [12–15]. We have made comparisons of this kinetic parameter in sucrose-washed and Tris-washed, reactivated chloroplasts under a variety of conditions. In the experiments shown in Fig. 3, flashes spaced 1 s apart were given until O_2 evolution from each flash was uniform. Having reached this steady state, we then varied the

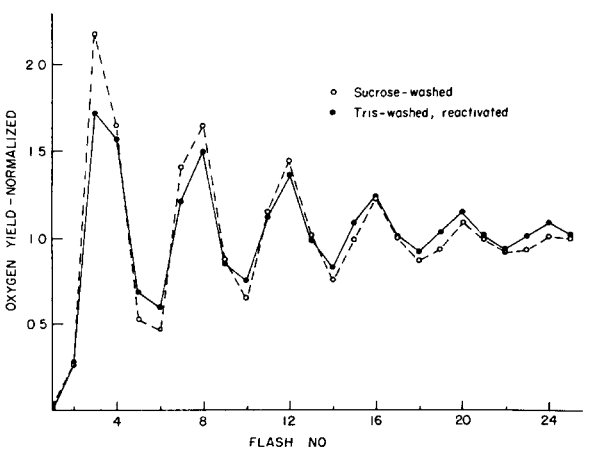

Fig. 2. Pattern of O_2 evolution in flashing light measured as described in Materials and Methods. The average yield of O_2 on the 20th through the 25th flash was used to normalize the two curves. ○, sucrose-washed chloroplasts; ●, Tris-washed, reactivated chloroplasts. The Xenon flashes were 10 μs long and were spaced 1 s apart.

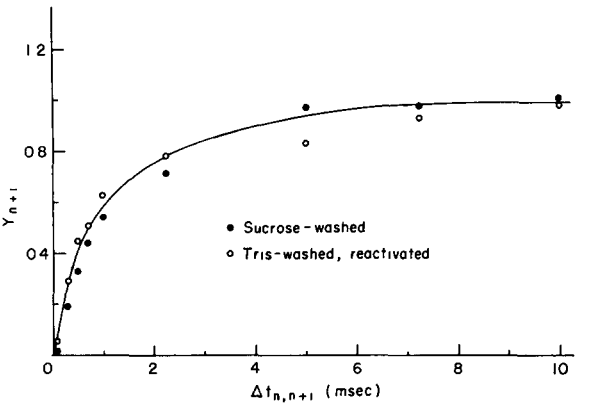

Fig. 3. Steady-state flash kinetics of O_2 evolution. Flashes spaced 1 s apart were given until the amount of O_2 evolved following each flash was uniform. A pair of flashes separated by a time, $\Delta t_{n,n+1}$, was then given to the system. The yield of O_2 from the 2nd flash of the pair, Y_{n+1}, is plotted versus the dark time between the two flashes, $\Delta t_{n,n+1}$. ●, sucrose-washed chloroplasts; ○, Tris-washed, reactivated chloroplasts.

time between two successive flashes. The ordinate in Fig. 3 is the yield of O_2 on the following flash Y_{n+1}, as a function of the time, $\Delta t_{n,n+1}$, between the two flashes. In sucrose-washed chloroplasts, Y_{n+1} reaches 50 % of the steady-state yield after 800 μs dark time; in reactivated chloroplasts this halftime is 650 μs. In Table I we summarize the results of analogous experiments on dark-adapted chloroplasts. In one set of experiments we varied the time between the first and second flashes (which measures the halftime for the $S'_1 \rightarrow S_2$ reaction in the Kok et al. [13] model for O_2 evolution), and in a second set we varied the time between the second and third flashes. This measures the $S'_2 \rightarrow S_3$ halftime.

The results from the experiments shown in Fig. 3 and Table I show that both the individual step and steady-state kinetic parameters of O_2 evolution in reactivated chloroplasts are comparable to those observed for sucrose-washed chloroplasts.

TABLE I

S STATE KINETICS IN SUCROSE-WASHED AND TRIS-WASHED, REACTIVATED CHLOROPLASTS

O_2 evolution in flashing light measured as described in Materials and Methods.

Sample	$t_{\frac{1}{2}}$ ($S'_1 \rightarrow S_2$) (μs)	$t_{\frac{1}{2}}$ ($S'_2 \rightarrow S_3$) (μs)
Sucrose-washed	210	420
Tris-washed, reactivated	170	370

Manganese content and EPR characteristics

Table II lists O_2 rates and manganese content of sucrose-washed, Tris-washed, and Tris-washed, reactivated chloroplasts. Tris-washed chloroplasts have lost only about 15 % of their total manganese pool, but 63 % of their O_2 evolving capacity

TABLE II

O_2 RATE AND MANGANESE CONTENT OF SUCROSE-WASHED, TRIS-WASHED, AND TRIS-WASHED, REACTIVATED CHLOROPLASTS

O_2 evolution in continuous light and manganese content by atomic absorption were measured as described in Materials and Methods. Chlorophyll content, 26 μg/ml.

Sample	O_2 evolution		Manganese content	
	μmol O_2 / (mg chlorophyll) · h	%	Manganese/400 chlorophyll	%
Sucrose-washed	78	100	6.65	100
Tris-washed	29	37	5.61	85
Tris-washed, reactivated	79	101	4.33	65

has been inhibited. Tris-washed, reactivated chloroplasts have completely regained the O_2 evolution rate of the sucrose-washed control, but have lost 35 % of the manganese of the control.

It appears that total manganese content is not a reliable indicator of O_2 evolving capacity in chloroplasts that have been Tris washed. One-third of the chloroplast manganese can be removed without noticeable effect on the rates of O_2 evolution, compared with untreated controls. The possibility exists that part of the chloroplast manganese is accounted for by a manganese-containing superoxide dismutase, and that this manganese can be lost without affecting O_2 evolving capacity. A cyanide-insensitive superoxide dismutase has been found in chloroplast lamellae by Lumsden and Hall [16]. It was not determined whether this superoxide dismutase was Mn-type or Fe-type (both are cyanide insensitive while the CuZn-type is cyanide-sensitive).

Analysis of total manganese gives no information about its chemical environment in the chloroplast. EPR spectroscopy, however, is extremely sensitive to the local environment of manganese. It was shown previously that upon Tris treatment about 60 % of the chloroplast manganese is converted to an EPR-detectable state.

Fig. 4 shows results of an EPR experiment on the same chloroplast samples used in the O_2 rate experiments shown in Fig. 1. The sucrose-washed sample shows little manganese EPR signal, while the Tris-washed sample shows the six-line pattern characteristic of manganese. The Tris-washed, reactivated sample has little of the six-line manganese signal and its EPR spectrum is very similar to that of the sucrose-washed sample. Upon reactivation of O_2 evolution capacity, the major portion of the manganese liberated by Tris treatment is returned to an EPR-non-detectable state. It is not possible to determine from this experiment whether the environment of manganese in reactivated chloroplasts is identical to that of manganese in sucrose-washed chloroplasts. However, from the spectra presented in Fig. 4, it is apparent that the environment of manganese in reactivated chloroplasts is quite unlike that present in Tris-washed chloroplasts.

It was shown previously [9] that EDTA is able to complex manganese released into the interior of the thylakoid upon Tris treatment. It was therefore of considerable interest to determine the effect of EDTA incubation on the reactivation of O_2 evolution and the manganese content of Tris-washed chloroplasts. The results of this

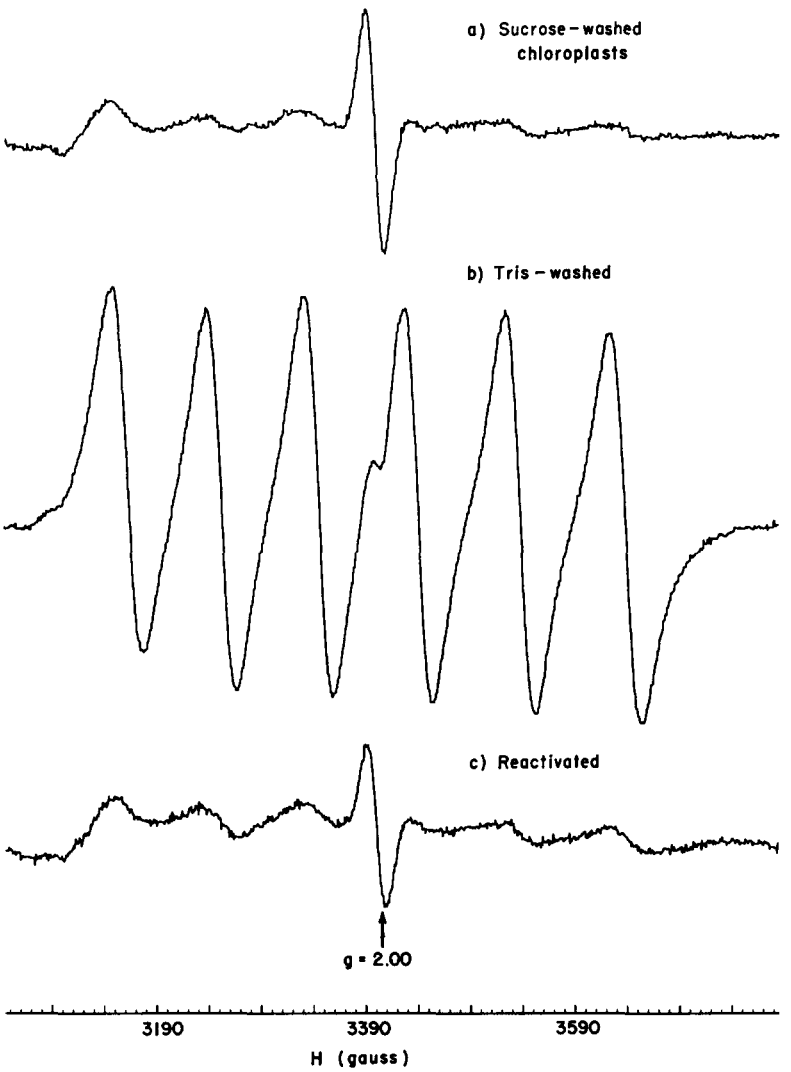

Fig. 4. Room temperature EPR spectra (1st derivative) of chloroplasts prepared as described in Materials and Methods. Instrumental conditions: microwave power, 100 mW; modulation amplitude, 10 G; time constant ,0.3 s; scan rate, 250 G/min. Receiver gain was the same for all three spectra. Chlorophyll content, 4.8 mg/ml.

experiment are presented in Table III. The presence of 10^{-3} M EDTA during the Tris washing had no significant effect on the rate of O_2 evolution for any of the samples, even those which had been reactivated. Nor was the level of chloroplast total manganese affected by EDTA. Similar results were obtained with EDTA concentrations ranging from 10^{-5} to 10^{-2} M: no change was observed, regardless of when the EDTA was added during the chloroplast preparation procedure.

TABLE III

EFFECT OF EDTA ON REACTIVATION OF O_2 EVOLUTION AND CHLOROPLAST MANGANESE CONTENT

O_2 evolution in continuous light and manganese analysis by atomic absorption were measured as described in Materials and Methods. EDTA concentration was 10^{-3} M and was added during the Tris-washing step of chloroplast treatment. Chlorophyll content, 40 μg/ml.

Chloroplast treatment	EDTA	O_2 evolution		Manganese/400 chlorophyll
		μmol O_2 / (mg chlorophyll) · h	%	
Sucrose-washed	−	183	100	7.64
Sucrose-washed	+	184	100	7.30
Tris-washed	−	53	29	6.54
Tris-washed	+	41	22	6.38
Reactivated	−	193	105	5.12
Reactivated	+	191	104	5.53

EPR Signal II behavior

Signal II displays only a small, reversible light-induced kinetic response in broken chloroplasts that are capable of O_2 evolution [11]. However, Tris washing and a number of other treatments which inhibit O_2 evolution on the water side of Photosystem II produce dramatic changes in Signal II response [10]. Upon inhibition of O_2 evolution, a rapid kinetic component of Signal II, designated Signal IIf, is observed in addition to the Signal II present before inhibition [10]. This distinction is demonstrated in Fig. 5 for sucrose-washed (Fig. 5a) and Tris-washed (Fig. 5b) chloroplasts. Upon reactivation of O_2 evolution (Fig. 5c) in Tris-washed chloroplasts, Signal II behavior returns to that exhibited by the sucrose-washed control. The chlorophyll content of the three samples in Fig. 5 was adjusted to the same concentration. The ratio of maximum Signal II spin concentrations in the light is 1.1 (Fig. 5a) : 1.9 (Fig. 5b) : 1.0 (Fig. 5c). The large Signal I observed in Tris-washed and in reactivated chloroplasts could result from a blocked reduction of P700. This would occur if a soluble electron-transfer component (e.g. plastocyanin) is lost during the Tris-washing procedure.

DISCUSSION

Yamashita et al. [7, 8, 17] showed that oxygen evolution in Tris-washed chloroplasts can be reactivated by rewashing with isoosmotic buffer containing reductants such as DCIP/ascorbate. They also demonstrated that reactivation restored chlorophyll fluorescence to levels exhibited by untreated controls, and that Cl^- stimulated oxygen evolution in both untreated and reactivated chloroplasts in a similar manner. In the experiments shown in Figs 1–3 and Table I in this paper, we have extended these observations through a more detailed kinetic analysis. The pattern of oxygen evolution in flashing light and the kinetic parameters of the S state intermediates associated with charge accumulation in Photosystem II are similar for reactivated and sucrose-washed chloroplasts. These results, plus those of Yamashita

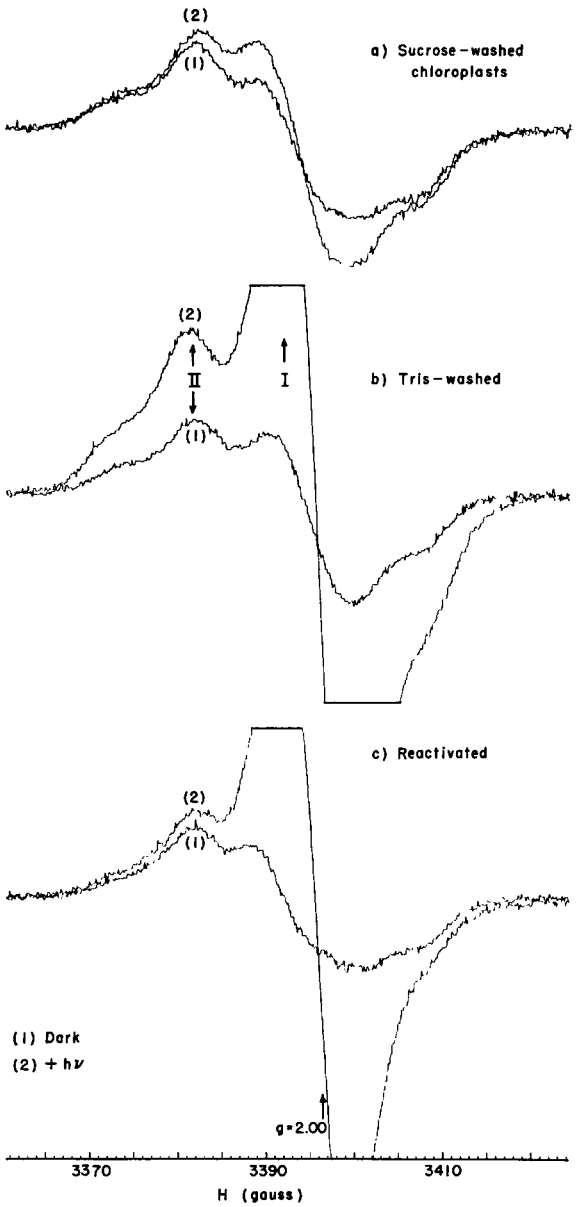

Fig. 5. Room temperature EPR spectra of chloroplasts prepared as described in Materials and Methods. In each of the three samples Spectrum 1 is recorded in the dark, followed by Spectrum 2 recorded in continuous light. 10^{-4} M EDTA was added to each sample to eliminate the manganese EPR signal. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 2.0 G; time constant, 0.3 s; scan rate, 50 G/min. Receiver gain was the same for all spectra. Chlorophyll content, 4.8 mg/ml.

and coworkers, argue strongly that oxygen evolution in sucrose-washed and in reactivated chloroplasts proceeds by the same mechanism.

It was shown previously that an increase in Signal IIf magnitude accompanies the decline in oxygen evolution with heating time (at 51 °C). The experiments in Fig. 5 correspond to the complementary situation: a decline in Signal IIf magnitude accompanies the increase in oxygen evolution upon reactivation of Tris-washed chloroplasts. These results confirm the inverse relationship between oxygen evolution and Signal IIf observation. We have proposed two models to account for the observation of Signal IIf upon inhibition of oxygen evolution (Babcock, G. T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 329–344). In one, Signal IIf is an alternate electron donor to Photosystem II which is activated as oxygen evolution is inhibited. In the second, the Signal IIf species is Z, the physiological donor to $P680^+$; upon inhibition of oxygen evolution its rate of reduction is decreased and the radical ($Z \cdot ^+$) becomes EPR detectable. The Signal II results reported here are consistent with either model, i.e. either a deactivation of Signal IIf or an increase in the rate of rereduction of $Z \cdot ^+$ upon reactivation of oxygen evolution. However, we recently observed a very fast component (rise < 100 μs, decay 700 μs) of Signal II in untreated chloroplasts capable of O_2 evolution (Blankenship, Babcock, Warden, and Sauer (1975) FEBS Lett 51, 287–293). This result favors the second of the two proposed models: that Signal IIf arises from the physiological electron donor to Photosystem II, and that its kinetics of rereduction are slower after Tris washing.

The data of Table I demonstrate, in agreement with similar experiments by Yamashita et al. [8], that only a modest decrease in total manganese concentration is observed upon Tris inhibition. Subsequent reactivation of these chloroplasts to 100 % of the control rate occurs even though 35 % of the total manganese has been lost during the treatments. These data indicate that not all of the chloroplast manganese is essential to oxygen evolution; some may be involved in other non-oxygen-evolving chloroplast functions.

A common feature of treatments such as Tris washing, mild heating and treatment with chaotropic agents is the release of manganese into an EPR-detectable state. Blankenship and Sauer [9] showed that with Tris washing this hexaquo manganese is released inside the thylakoid and diffuses out only slowly. The experiments in Fig. 4 show that upon reactivation of Tris-washed chloroplasts the major fraction of this manganese is reincorporated into the chloroplast membrane in an EPR undetectable state. This reincorporation is dependent upon the presence of a reductant but does not require light. Yamashita and Tomita [17] also observed that Tris-washed chloroplasts can be reactivated in the dark without the addition of exogenous manganese. On the other hand Tris/acetone-washed (0.8 M Tris, pH 8.0, 20 % acetone) chloroplasts, which are more extensively depleted of manganese, require both light and externally supplied manganese for reactivation. Similarly, Cheniae and Martin [18] showed that manganese incorporation into manganese-deficient algae proceeds via a multiquantum process. These results suggest that the uptake of manganese from the external medium is a light-driven reaction, but once within the thylakoid, the divalent manganese is incorporated into the membrane in the dark.

EDTA chelates the hexaquo manganese in Tris-washed chloroplasts. However, the data in Table III show that this chelation affects neither the extent of reactivation nor the total manganese content in Tris-washed chloroplasts. These results allow us

to conclude that manganese, chelated by EDTA, is not free to leave the thylakoid. Excluding the less likely explanation that manganese remains as the EDTA complex throughout the reactivation procedure, the manganese binding site restored by the reactivation treatment has a higher affinity for manganese than does EDTA ($K_d = 10^{-14}$) [19]. Yamashita and Tomita [17] showed that EDTA inhibits the light reactivation of Tris/acetone-washed chloroplasts by exogenous manganese. This result, together with the observed lack of EDTA inhibition on the dark reactivation of Tris-washed chloroplasts (Table III), strengthens the conclusion reached above: the incorporation of manganese is a two-step process; a light driven transport into the thylakoid and a dark binding to the site responsible for activity.

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