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ELECTRON PARAMAGNETIC RESONANCE SIGNAL II IN SPINACH CHLOROPLASTS

II ALTERNATIVE SPECTRAL FORMS AND INHIBITOR EFFECTS ON KINETICS OF SIGNAL II IN FLASHING LIGHT

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SUMMARY

Linewidth and hyperfine structure measurements of the EPR spectrum of Signal II in spinach chloroplasts show that the signal reflects two alternative states. One state is characterized by a 16-G linewidth and four partially resolved hyperfine components. The other state has 19 G linewidth and five partially resolved hyperfine components. It is possible to interconvert these two states by changing the ionic strength of the chloroplast suspension. Both states of Signal II show similar light-induced increases in dark-adapted chloroplasts and respond to 10-μs white light flashes with identical kinetics.

In chloroplasts at room temperature, Signal II dark decays to 50 % of its total light-induced level in about 1 h. Single flashes increase the spin concentration in these aged chloroplasts but with decreased effectiveness compared with fresh, dark-adapted chloroplasts. Carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) decreases the decay time of Signal II from hours to seconds without appreciably altering the level of Signal II formed in saturating continuous light. However, both the formation time constant and the extent of Signal II increase stimulated by a single saturating flash are decreased in CCCP-treated chloroplasts.

These results are interpreted in terms of the model, proposed in the preceding paper, in which Signal II is generated by oxidation–reduction reactions on the water side of Photosystem II.

INTRODUCTION

In the preceding paper we presented evidence to support a kinetic model for the light-induced generation of Signal II in O_2-evolving photosynthetic organisms[1]. In this communication we present further evidence for this model and discuss certain

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Abbreviations: ANT, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

aspects of the hyperfine structure and linewidth of the EPR spectrum of Signal II.

Signal II was first observed by Commoner *et al.*[2], who reported a linewidth of 19 G. (The linewidth is defined as the difference, in gauss, between the positive and negative extrema in the 1st derivative spectrum.) This linewidth has subsequently been confirmed by numerous other workers[3-5] and, in addition, several features of the partially resolved hyperfine structure of Signal II have been characterized. Kohl, in a recent review article, designated two of these partially resolved peaks in the EPR spectrum of Signal II as A and B (*cf.* ref. 6, Fig. 6-5), and he reported that the ratio of signal amplitude at field point B to that at field point A is 3/4. In the preceding paper we report a linewidth for Signal II of 15–16 G and a much lower ratio for the hyperfine Peaks B and A. We have undertaken to resolve this discrepancy and in this report present evidence indicating that the species giving rise to Signal II exists in two alternative states. The EPR spectrum of one of these states is similar to that reported by other workers; the EPR properties of the second state are similar to those which we reported in the preceding paper[1]. We have carried out preliminary experiments exploring the factors which control the interconversion of these two states. The kinetic behavior of Signal II in response to flashes is the same for both states

Lozier and Butler[7] showed that a number of reagents, including carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and hydroxylamine, decrease the decay time of Signal II from hours in untreated chloroplasts to seconds at high concentrations of these compounds. The effects of these reagents on a number of other reactions involved in photosynthesis have been studied in detail. Vredenberg[8] showed that CCCP and nigericin increase the rate of decay of variable fluorescence following illumination, while Kimimura *et al.*[9] showed that the level of variable fluorescence during illumination decreased with increasing CCCP concentration. These same authors also showed that the inhibition of the Hill reaction which they observe with CCCP is caused by a decreased quantum efficiency for the light reaction and not by an inhibition of a dark step. Renger[10,11] studied the action of CCCP and related compounds on intermediates on the water side of Photosystem II (the S states in the Kok *et al.*[12] model for O_2 evolution) and found a decrease in the lifetime of these intermediates with increasing CCCP concentration. While one effect of hydroxylamine is likewise to decrease the lifetimes of these intermediates, it appears that the modes of action of hydroxylamine and CCCP in achieving this are different. Bennoun and Joliot[13] showed that hydroxylamine reduces the S states chemically, while Renger[14] ruled out this possibility for CCCP on the basis of stoichiometric arguments Instead, Renger *et al* [15] proposed a model for the action of CCCP and similar reagents involving a more efficient re-reduction of the S states by reduced components of the electron transport chain on the acceptor side of Photosystem II. This model is consistent not only with their results on lifetimes of oxidized equivalents in the water splitting process but also with the fluorescence and Hill reaction inhibition data

In the model which we presented for light-induced Signal II generation[1], we postulate an interaction between the Signal II precursor, F, and oxidized intermediates on the water side of Photosystem II In the experiments described in this report we studied the effect on the kinetics of Signal II caused by CCCP and similar reagents which accelerate the decay of these oxidized intermediates. We also investigated the effect on the decay and flash-induced generation of Signal II caused by aging, heating, and similar treatments which are known to disrupt the water-splitting process[16].

MATERIALS AND METHODS

1. Chloroplast preparation and reagents

Spinach was grown as described previously. Chloroplasts were isolated by the procedures described in the preceding paper[1], using either 0.4 M sucrose, 0.1 M tricine (pH 7.6), 0.01 M NaCl (unwashed sucrose chloroplasts) or 0.35 M NaCl, 0.02 M Tris (pH 8.0) (unwashed salt chloroplasts)[17] as the isolation solution. Washed chloroplasts were prepared by resuspending either of these types of chloroplasts in fresh isolation medium and subsequently centrifuging at 1000 × *g* for 10 min. Chlorophyll concentration in all experiments was between 2 and 4 mg chlorophyll/ml.

CCCP, valinomycin and sodium ascorbate were obtained from Calbiochem, Tris and tricine from Sigma. 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT) was prepared according to methods outlined by Büchel and Shäfer[18]. Stock solutions of CCCP, ANT and valinomycin were prepared in 95 % ethanol. The final ethanol concentration in all chloroplasts samples was less than 1 %. Aging and heating were carried out as described in the text

2. Light sources and EPR measurements

10-μs white light flashes and continuous white light were obtained from sources as described in the preceding paper[1]. EPR experiments were carried out under conditions described previously[1] or in the text.

Signal averaging was performed using a 1024 channel Enhancetron signal averager. The output of the Varian E-3 spectrometer, with a time constant as noted in the text, was fed into the averager. Appropriate timing circuits synchronized the initiation of the averager sweep and the lamp discharge in kinetic experiments or the field sweep for recording spectra. Experiments involving a flowing sample suspension were carried out using an EPR flat cell connected in a closed loop *via* tygon tubing to a peristaltic pump. The flow rate was adjusted so that a new sample was pumped into the flat cell in the interval between scans. Total volume of chloroplasts necessary to fill the system was 7 ml; each 7-ml sample was used for 20 min and discarded. Low temperature EPR experiments were performed using 3 mm cylindrical (I.D.) quartz sample tubes. A Varian low temperature accessory (Model No. E-4557-9) was used to maintain the temperature within ± 5 °C of the nominal value.

RESULTS

EPR spectra of Signal II

Fig. 1 presents EPR spectra of spinach chloroplasts measured in the dark under various conditions. Magnetic field positions corresponding to partially resolved hyperfine peaks and throughs in the spectra have been labeled with the letters A–F. Fig. 1a shows the EPR spectrum for Signal II which we observe routinely from unwashed sucrose chloroplasts. There are four partially resolved components and the linewidth, measured as the magnetic field difference between the low field maximum at B and the high field minimum at D, is 16 G. Under these conditions the shoulder at A is prominent, the amplitude of the Peak C is small compared to that at B, and there is no obvious shoulder or peak at field position E.

If these unwashed sucrose chloroplasts are subsequently washed with the sucrose

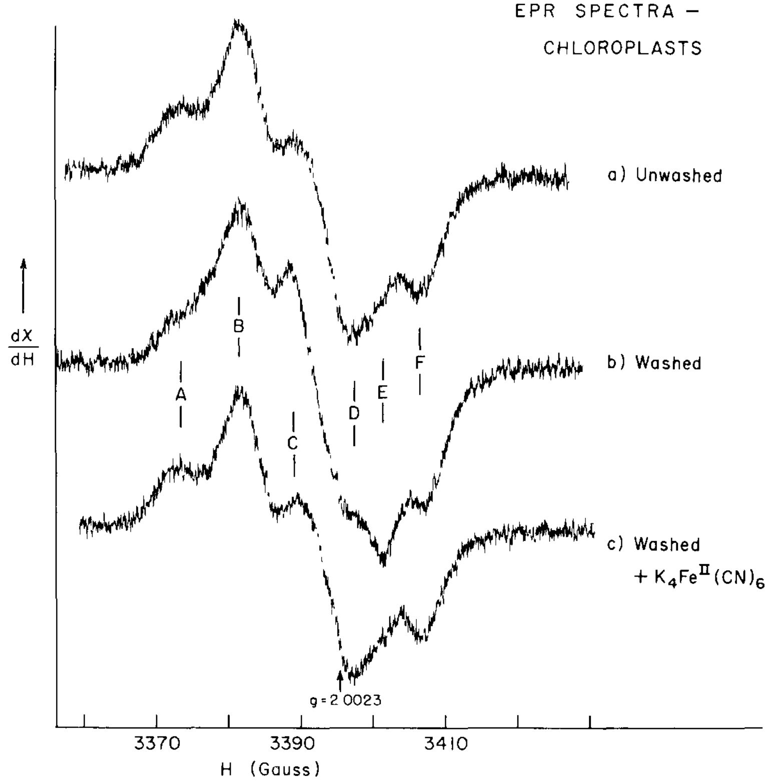

Fig. 1. EPR spectra (1st derivative) of spinach chloroplasts, isolated in sucrose isolation medium, (a) resuspended in isolation medium, (b) washed once in sucrose isolation medium and resuspended in isolation medium, (c) as in (b) with 10 mM $K_4Fe^{II}(CN)_6$ added following resuspension. All samples were illuminated with continuous, broad band white light for 30 s before the spectra were recorded. The microwave power was 16 mW, the instrument time constant was 0.3 s, and the scan rate was 12.5 G/min

isolation medium, the spectrum shown in Fig. 1b results. This spectrum is now similar to that reported by other workers[6]. The low field shoulder at A is less well resolved; the peak at C is much larger; there is a shoulder at D and a high field minimum at E. The apparent linewidth, measured between B and E, is 19 G. For the remainder of this report we shall refer to the 16-G spectrum of Signal II in Fig. 1a as Signal II (16) and to the 19-G spectrum in Fig. 1b as Signal II (19).

Signal II (19) in washed sucrose chloroplasts can be converted to Signal II(16) by increasing the ionic strength. For example, we have found that the addition of 30 mM $MgSO_4$, 40 mM K_2SO_4, 30 mM $MgCl_2$, 30 mM Na_2MoO_4 or 10 mM $K_4Fe^{II}(CN)_6$ to washed sucrose chloroplasts converts Signal II(19) to Signal II(16). Fig. 1c shows the EPR spectrum of Signal II in washed sucrose chloroplasts in which the ionic strength has been increased by the addition of 10 mM potassium ferrocyanide. This trace shows structure very similar to that recorded for Signal II(16) in unwashed sucrose chloroplasts (Fig. 1a). The shoulder at A is again more apparent, the amplitude at C is reduced, and the high field minimum has shifted from E to D, resulting in a linewidth of 16 G.

In these experiments the ionic strength of the added salt was the critical factor For example, 10 mM $K_4Fe^{II}(CN)_6$ ($I = 0.1$ M) was sufficient, whereas 10 mM $MgCl_2$ (I = 0.03 M) was without appreciable effect on the conversion process. However, if 30 mM $MgCl_2$ ($I = 0.09$ M) was added to washed sucrose chloroplasts, Signal II(16) was observed. In these experiments we found, in general, that the ionic strength of the added salt had to be greater than $I = 0.08$ M to convert Signal II(19) to II(16)

This conversion of Signal II(19) to Signal II(16) is not facilitated by 30 mM sodium ascorbate, indicating that the mechanism for this conversion does not involve reduction. Further evidence supporting the conclusion that oxidation–reduction reactions are not involved comes from the observation that 10 mM potassium ferricyanide added to washed sucrose chloroplasts converts Signal II(19) to Signal II(16) as effectively as does 10 mM potassium ferrocyanide. At this concentration ferricyanide is a strong enough oxidant to cause appreciable oxidation of P700 and hence the presence of Signal I obscures Signal II in the region around field positions C and D. However, the shoulder at A in ferricyanide-treated chloroplasts is as well resolved as it is in Fig. 1a or 1c, indicating that Signal II(16) is present This experiment also demonstrates that Signal II(19) does not result from an addition of Signal I and Signal II(16), in agreement with the interpretation of Kohl[6].

In a second set of experiments designed to test the effect of ionic strength on the structure of Signal II, we prepared unwashed and washed salt chloroplasts. The final NaCl concentration in both types of chloroplasts was 0.35 M ($I = 0.35$ M) and in each case Signal II(16) was observed These experiments indicate that Signal II(19) results in washed sucrose chloroplasts from an ionic strength decrease caused by the washing procedure and not, for example, by the removal of soluble factors contributing directly to Signal II(16). This suggests that the interconversion of the two states of Signal II is mediated by an alteration in the structure of the radical species giving rise to the signal.

In order to determine the relative number of spins in Signal II(19) and Signal II(16), we performed double integration for Signal II(19) in samples of washed sucrose chloroplasts Using chloroplasts from the same preparation, we then converted Signal II(19) to Signal II(16) and carried out double integration for Signal II(16). Comparing these two values we found that the ratio of spins in Signal II(19) to spins in II(16) was 1.0 ± 0.05 for three different preparations of washed sucrose chloroplasts. We conclude from these experiments that the species giving rise to Signal II can exist in two alternative states and that converting from one state to the other by ionic strength changes does not alter the number of spins detected.

Preliminary results suggest that the interconversion between the two states of Signal II is temperature sensitive. Unwashed sucrose chloroplasts which exhibit Signal II(16) at room temperature show Signal II(19) when frozen to −60 °C or to −196 °C. However, as discussed in greater detail below, illumination does not play a role in the interconversion of these two states of Signal II.

We observed both Signal II(16) and II(19) in chloroplasts prepared from market and greenhouse spinach and in pea chloroplasts. In these samples it is possible to convert Signal II(19) to II(16) by ionic strength increase. In whole leaves of growth chamber spinach we observed a 19-G linewidth for Signal II, indicating that the predominant *in vivo* state is that giving rise to Signal II(19) We also investigated the structure of Signal II in the algae *Chlorella pyrenoidosa* and *Chlamydomonas reinhardi* In agreement with published spectra by Weaver[19], Kohl[6] and others we observe essentially Signal II(19). The ratio of peak intensities at field point C to that at field point B in these algae varies between 0.50 and 0.80, compared to the C/B ratio in Signal II(16) which is less than 0.25.

Light-induced increases in Signals II(16) and II(19)

In the preceding paper[1] we presented data on the light-induced increase in Signal II in dark-adapted chloroplasts. The experiments were done with spinach chloroplasts which exhibited Signal II(16). We have repeated these experiments with chloroplasts which show Signal II(19) and with chloroplasts in which Signal II(19) has been converted to Signal II(16) by ionic strength increase.

The left side of Fig. 2 shows spectra of Signal II recorded for dark-adapted chloroplasts before (Curves 1) and in the dark after (Curves 2) illumination. On the right in the figure are the spectra of the light-induced increase in Signal II which is obtained by subtracting Curve 1 from Curve 2 for each of the three experiments.

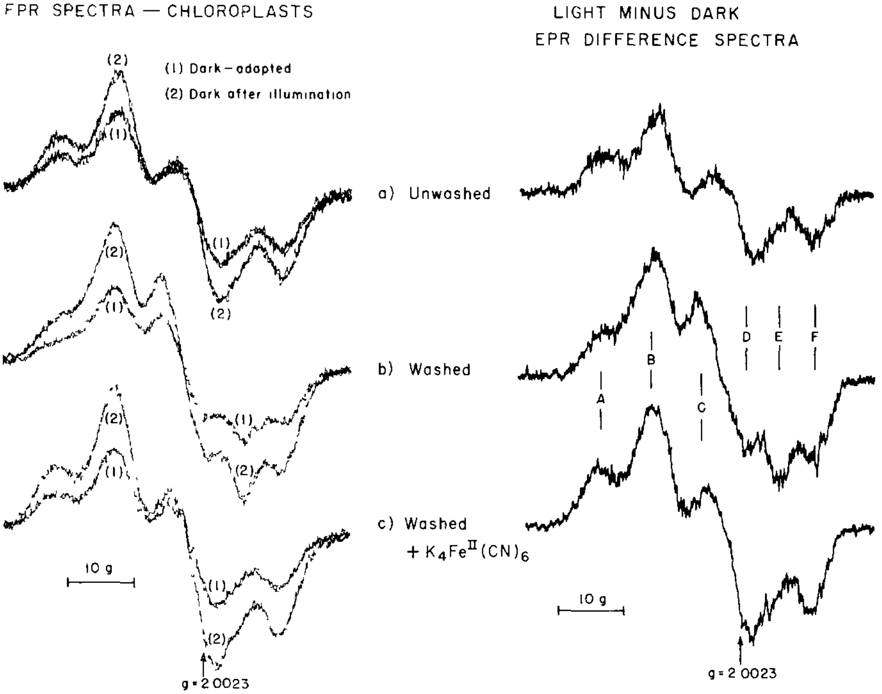

Fig 2 Left EPR spectra of dark-adapted spinach chloroplasts, isolated in sucrose isolation medium, in the dark (1) before and (2) after illumination, two scans are shown for both (1) and (2) Right Light *minus* dark EPR difference spectra obtained by subtracting spectra (1) from spectra (2) for each of the samples on the left (a) Unwashed, (b) washed, and (c) washed + $K_4Fe^{II}(CN)_6$ sucrose chloroplasts were prepared as described in Fig 1 Broad band white light was used for illumination The subtractions were performed electronically with the signal averager. The microwave power was 5 mW, instrument time contant was 0 3 s, and the scan rate was 25 G/min.

Fig. 2a was done with unwashed sucrose chloroplasts, Fig. 2b with washed sucrose chloroplasts, and Fig. 2c with washed sucrose chloroplasts to which 10 mM $K_4Fe^{II}(CN)_6$ was added. Data show that in unwashed chloroplasts and in washed chloroplasts *plus* ferrocyanide the spectrum of the light-induced increase is that of Signal II(16). Washed sucrose chloroplasts (Fig 2b), however, exhibit Signal II(19) before and after illumination and, as expected, the difference spectrum is also that of Signal II(19). The results indicate that Signals II(16) and II(19) undergo light-induced increases without change in spectral characteristics

The chloroplasts used in the experiments in Fig. 2a were prepared from spinach plants which had been in the dark about 4 h. The ratio of the amplitude of Signal II before illumination to Signal II following illumination is 0.7, in agreement with data presented in the preceding paper[1] on the *in vivo* decay of Signal II. In Figs 2b and 2c a second preparation of chloroplasts, isolated from spinach plants which had been in the dark for 10 h, was used. In both Figs 2b and 2c the amplitude of Signal II before illumination accounts for about 55 % of the amplitude of Signal II following illumination. This result agrees with the *in vivo* decay of Signal II in spinach plants which we measured earlier using unwashed sucrose chloroplasts and indicates that the state in which the Signal II precursor, F, exists has little effect on the decay or light-induced increase in Signal II

We have explored the hypothesis that the state of F has little effect on the kinetics of Signal II in more detail by studying the light-induced increase in Signal II(19) in flashing light. The results of these experiments are shown in Table I. One or two flashes spaced 10 ms apart are quite effective in increasing Signal II(19). Three or four flashes 10 ms apart are much less effective; however, if the spacing between four flashes is decreased to 100 μs, more than 80 % of the maximum light-induced increase occurs. These results are the same as we report in the preceding paper[1] for the flash-induced increase in Signal II(16) in unwashed spinach chloroplasts. We performed analogous flashing light experiments in washed sucrose chloroplasts in which Signal II(19) has been converted to Signal II(16) by ionic strength increase and find parallel results. In both sets of experiments we observed a 1-s half-time for the rise of Signal II

TABLE I

FLASH-INDUCED INCREASE IN SIGNAL II(19)

A fresh sample of dark-adapted sucrose chloroplasts was used for each experiment The flash-induced increase in Signal II(19) was monitored at the low field peak B in Fig 1 with an instrument time constant of 1 s and microwave power of 5 mW The dark-adapted sample was given the designated number of flashes with a spacing, t_d, between flashes Following this initial set of flashes, single flashes were given to complete the induction of Signal II(19) The increase in Signal II(19) resulting from the initial set of flashes divided by the total Signal II(19) increase is tabulated in the right column.

Number of flashes	t_d *(ms)*	$\frac{\textit{Flash-induced Signal II(19) increase}}{\textit{Total Signal II(19) increase}}$
1	10	0.77
2	10	0.88
3	10	0.51
4	10	0 40
4	0 1	0.84

following a flash, which is the same as reported in the preceding paper[1]. These data strengthen the hypothesis proposed above that both states of the Signal II precursor exhibit the same light-induced kinetic behavior.

Effects of CCCP on Signal II decay and induction

Lozier and Butler[7] have shown that CCCP dramatically increases the rate of decay of Signal II. Fig 3 presents the concentration curve for the effect of CCCP on both the decay time and light-induced response of Signal II. The decay of Signal II in untreated chloroplasts in the EPR cavity at room temperature is on the order of 1 h. Fig. 3a shows that with increasing concentrations of CCCP this decay time decreases to seconds. In contrast, however, the magnitude of Signal II formed in saturating light (Fig. 3b) is only slightly decreased at high CCCP concentration. For example, at 3 10^{-4} M CCCP the magnitude of Signal II, measured at field point B in Fig. 1, is only 20 % less than in untreated chloroplasts. The chlorophyll concentrations used in these EPR experiments are much higher than those used in experiments monitoring O_2 evolution or fluorescence changes. Calculating the ratio of CCCP to chlorophyll for these systems, however, we find that CCCP exhibits its effects on Signal II in the same CCCP/chlorophyll range as observed in experiments on O_2 evolution or fluorescence changes.

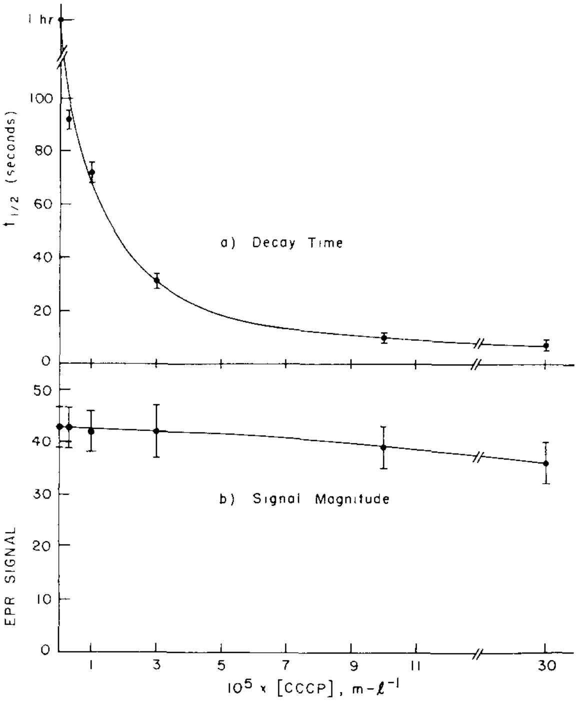

Fig. 3 EPR Signal II decay time (a) and signal magnitude (b) as a function of CCCP concentration in spinach chloroplasts. Decay time ($t_{1/2}$) was measured as the time elapsed between cessation of illumination and the half decay of Signal II. Signal magnitude was measured as the amplitude of the low field peak B in Fig 1 in saturating broad band white light Microwave power was 20 mW, instrument time constant was 1.0 s

We have taken advantage of the rapid decay of Signal II in CCCP-treated chloroplasts to study the flash-induced increase in radical concentration. Previously we showed that in rigorously dark-adapted chloroplasts Signal II is present at about 50 % of its fully induced magnitude. Using CCCP, however, it is possible to obtain chloroplasts with no Signal II spins in the dark

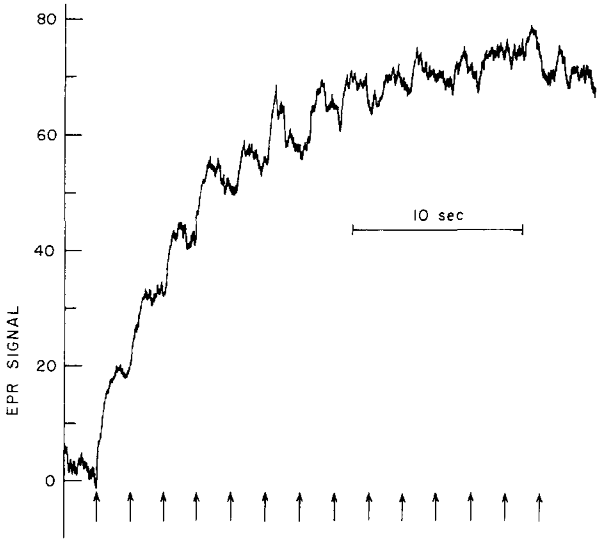

Fig 4 Response of Signal II in chloroplasts treated with 3 10^{-5} M CCCP to 10-μs flashes A single saturating flash was given at each arrow. The light-induced increase in Signal II was measured as in Fig 3 Microwave power was 20 mW, instrument time constant was 0 3 s

A typical experiment showing the rise of Signal II in CCCP-treated chloroplasts stimulated by 10-μs flashes is shown in Fig. 4. The CCCP concentration is $3 \cdot 10^{-5}$ M and the interval between successive flashes is 2 s. The flash-induced increase in Signal II is measured with the magnetic field set at the low field maximum B in Fig. 1. Signal averaging techniques were used in this experiment; the data shown are the average of 90 scans. At this CCCP concentration the first flash induces only about 25 % of the total light-induced increase in Signal II Increasing the intensity of this flash does not lead to the generation of more spins. This result is markedly different from our previous experiments[1] with untreated dark-adapted chloroplasts, where a single saturating flash generated about 80 % of the light-induced increase in Signal II Thus, it appears that CCCP lowers the effectiveness of a single flash in increasing the Signal II spin concentration. A second feature of this experiment is the much faster rise of Signal II following the flash compared to the 1-s half-time in untreated chloroplasts[1].

Fig. 5 shows a concentration study for the CCCP-induced decrease in the effectiveness of a single flash in generating Signal II. The experiments were carried out in a manner similar to those shown in Fig. 4. The increase in Signal II resulting from the first flash in a series divided by the total increase generated by 20 flashes is plotted as a function of CCCP concentration. With increasing CCCP, the first flash becomes progressively less effective, indicating that CCCP decreases the fraction of Photo-

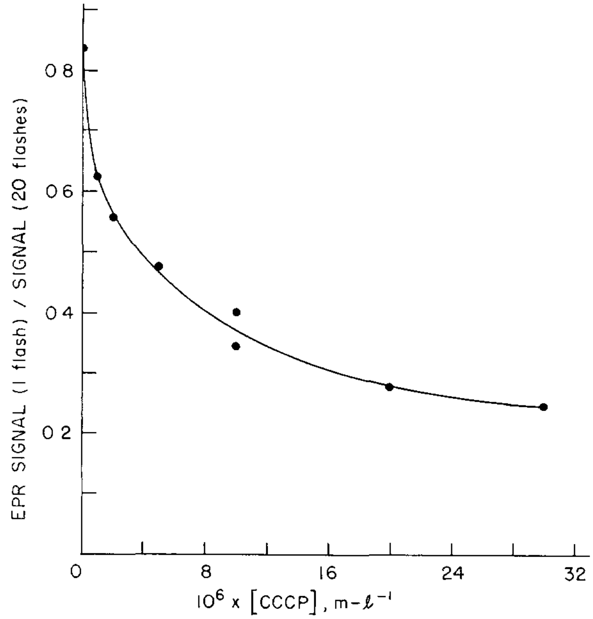

Fig 5. Response of Signal II to 10-μs flashes in spinach chloroplasts as a function of CCCP concentration The extent of Signal II formation stimulated by the first flash divided by the total increase in Signal II evoked by 20 flashes spaced 2 s apart is plotted on the ordinate The flash-induced increase in Signal II was measured with the EPR settings as described in Fig. 4.

system II reaction centers capable of generating Signal II from a single flash.

Renger[14] has performed extensive experiments on a class of compounds including CCCP which decrease the lifetimes of the S states in the Kok *et al* [12] model for O_2 evolution. Recently Renger *et al.*[15] have shown that these compounds exert their effect by increasing the reaction rate between these oxidized intermediates and an unidentified reduced component of the photosynthetic electron transport chain on the acceptor side of Photosystem II His model provides an explanation for the effect of CCCP on Signal II generation, since such a back flow of electrons would decrease the effectiveness of a single flash in any reaction dependent upon long-lived oxidized intermediates We have proposed that Signal II arises via an oxidation–reduction reaction between the states S_2 and S_3 on the water side of Photosystem II and F (ref 1). Furthermore, we have shown that following a flash this oxidation proceeds fairly slowly, the half-time for its rise is about 1 s. Therefore, any factor which appreciably decreases the lifetime of the oxidized intermediates S_2 and S_3 would be expected to decrease the extent of Signal II formation evoked by a single flash. We have already shown that this is the case in DCMU-treated chloroplasts[1].

In untreated chloroplasts the lifetimes of the states S_2 and S_3 are on the order of 10–20 s[20]. Since these lifetimes are long compared with the 1-s half-time for the rise of Signal II, this 1-s time constant reflects the intrinsic rate of reaction between the Signal II precursor and the states S_2 and S_3. Our model predicts, however, that the CCCP-induced decrease in the lifetimes of the S states to less than 1 s would result in a decrease in the time constant for Signal II formation. This decrease in time con-

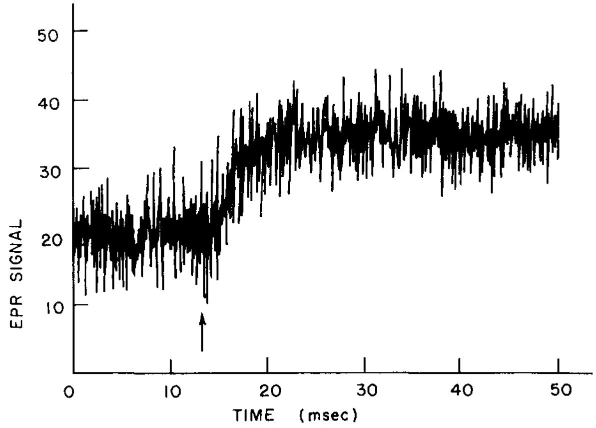

Fig 6. Time course of the response of Signal II to a single saturating 10-μs flash in chloroplasts treated with $1 \cdot 10^{-4}$ M CCCP. The arrow designates the time which the lamp was discharged The flash-induced increase was measured at the low field peak B in Fig. 1 with an instrument time constant of 5 ms. The flow system was used in this experiment, the data are the average of 6000 scans The microwave power was 50 mW

stant would accompany the CCCP-induced decline in the extent of Signal II generated in response to a flash. The experiment shown in Fig. 4 implies that this is the case. There we noted that the time constant for Signal II formation had decreased significantly compared to its 1-s half-time in untreated chloroplasts. We present further evidence to support this conclusion in Fig. 6. In this experiment we have monitored the rise of Signal II following a single 10-μs flash in chloroplasts treated with $1 \cdot 10^{-4}$ M CCCP. Signal averaging techniques and the flow system were used in this experiment A single saturating flash was given once per second; data in Fig 6 are the average of 6000 scans. Clearly the extent of Signal II formation is significantly lowered compared to chloroplasts in the absence of CCCP. However, the flash-induced increase in spin concentration in Fig. 6 occurs in a time limited by the 5-ms instrument time constant, indicating that the rise of Signal II under these conditions is much more rapid than in untreated chloroplasts. This experiment also demonstrates that, with 5 ms time resolution, we observe no fast decaying transients in Signal II induction in CCCP-treated chloroplasts.

We have carried out experiments with a number of other compounds, including NH_4Cl, valinomycin, and ANT, to study their effects on Signal II. Neither NH_4Cl nor valinomycin influenced the decay of Signal II; the radical behaved as it does in untreated chloroplasts. The effects of ANT are similar to those reported for CCCP in that it increased the rate of decay of Signal II and decreased the effectiveness of a single flash in increasing Signal II spin concentration. Renger[14] has shown that ANT is similar to CCCP in that both accelerate the deactivation of intermediates on the water side of Photosystem II, whereas NH_4Cl and valinomycin have been shown not to have such an effect.

Aging effects on Signal II

The O_2-evolving system in chloroplasts is fragile; mild heating or aging for short periods at room temperature leads to an inactivation of the system[16]. We investigated the effects of these treatments on the decay of Signal II and its generation in response to flashes.

Previously we demonstrated that the decay of Signal II *in vivo* in spinach plants is very slow, reaching a level of about 50 % of the fully generated signal only after 12 h in complete darkness. Recent experiments by Lozier and Butler[7] in which they monitored the decay of Signal II in isolated chloroplasts at room temperature indicated that under these circumstances the spin concentration decays much more rapidly. Their data show that Signal II decays to a level half that of the fully generated signal after only 1 h in the dark.

We have extended this experiment by applying saturating flashes to stimulate the regeneration of Signal II following the dark decay. The results of this experiment are shown in Fig. 7. A sample of chloroplasts in the flat cell was illuminated for 1 min with continuous white light; at time zero this light was extinguished. The magnitude of Signal II, measured as the peak to trough amplitude between B and D in Fig 1, is plotted as a function of the time dark. As shown in Fig. 7, Signal II declines to about 50 % of its light-induced level after 1 h, which agrees quite well with the data of Lozier and Butler[7]. Having allowed the signal to decay to this level, we then applied a single saturating flash and recorded the spectrum immediately following. As shown in Fig. 7, this single flash generated only 25 % of the maximum light-inducible signal in these aged chloroplasts. Subsequently, sets of flashes with the number of flashes and time between flashes indicated in the plot were given and Signal II recorded after each set. It is apparent from the figure that the behavior of Signal II has been altered by the room temperature aging. In untreated chloroplasts, freshly prepared from dark-adap-

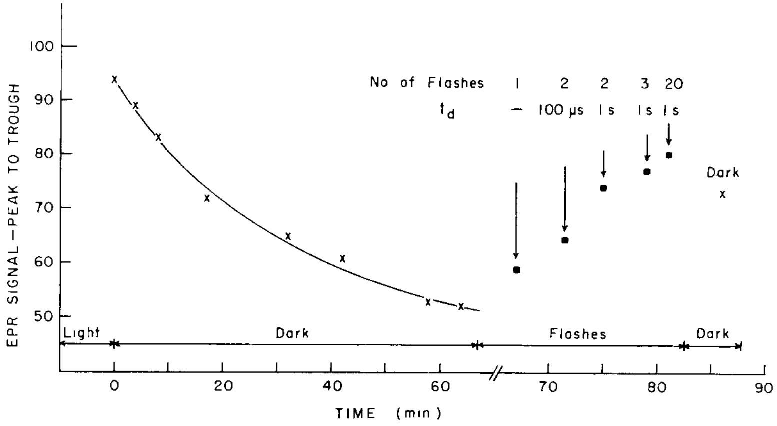

Fig 7. The effect of aging in the dark at room temperature on the decay and flash-induced response of Signal II. A sample of fresh, untreated chloroplasts was illuminated for 30 s with broad band white light and at zero time the lamp was switched off Spectra were recorded in the dark and the magnitude of Signal II, measured as the difference between the low field peak at B and high field trough at D in Fig. 1, is plotted as a function of dark time. When Signal II had decayed to about 50 %, saturating flashes were given. Each arrow corresponds to the flash regime described in the legend, t_d denotes the dark time between flashes in a given set. The filled squares indicate the peak to trough magnitude of Signal II recorded immediately following a flash sequence. Microwave power was 50 mW, instrument time constant was 1 s, and scan rate was 25 G/min

ted leaves, a single flash generates greater than 80 % of the light-induced increase in Signal II, whereas with these aged chloroplasts only 25 % is regenerated by one flash. In addition, the fully induced signal after 20 flashes 1 s apart is about 20 % less than the magnitude of Signal II at the initiation of the aging process.

We have performed similar experiments with heated (51 °C for 2 min) and Tris-washed chloroplasts and with Photosystem II particles prepared as described by Malkin[22]. Under these treatments the behavior of Signal II is similar to that observed with the aged chloroplasts. The decay of Signal II is much more rapid even than with aged chloroplasts, and a single flash is less effective in generating Signal II than is the case for untreated chloroplasts. These systems all share with aged chloroplasts the characteristics of having an impaired O_2-evolving system.

DISCUSSION

As shown in Fig. 1, we have demonstrated that the species which gives rise to Signal II can exist in either of two states. The EPR properties of Signal II in these two states differ both in hyperfine structure and linewidth. The linewidth of Signal II(16) is 16 G, and we observe four partially resolved hyperfine components; the linewidth of Signal II(19) is 19 G with five partially resolved hyperfine components. We have shown that reversible interconversion between these two states can be achieved by changing the ionic strength of the chloroplast suspension. At this time we do not understand the mechanism of the ionic strength induced changes in Signal II structure.

The existence of two different states for the species which gives rise to Signal II is similar to the situation for flavins and flavoproteins. Palmer *et al.*[23] have presented the linewidth of the EPR spectra for 13 flavoprotein free radicals. These flavoproteins fall into two categories: one class has a linewidth of about 15 G, the other has a linewidth of 19 G. In some cases (*e.g.* glucose oxidase) it is possible to convert from one linewidth to the other by a pH change. The optical properties of these two classes of flavoprotein free radicals also show characteristic behavior: the 19 G species is usually blue, the 15 G species is usually red. On the basis of optical, magnetic resonance and model system studies, the blue, 19 G species has been associated with the neutral flavin semiquinone radical of the flavoprotein, while the red 15 G species is the anionic flavoprotein radical. In addition, the cationic flavoprotein free radical appears to have EPR properties similar to the neutral radical species[24], while the metal chelate formed from the neutral flavin semiquinone has an EPR spectrum similar to the anionic flavoprotein radical[25]. Therefore, the difference in both optical and EPR characteristics of the flavoproteins can be attributed to the proton at the N(5) position in the flavin[24]. This proton is present in the blue, 19 G neutral or cationic flavins and absent in the red, 15 G anionic or metal chelated species.

Model compound studies of flavins indicate that the hyperfine splitting pattern of the neutral flavin semiquinone radical is more complex than that of the anionic flavin radical[24]. This is also the case in the benzoquinone model system. The anionic *p*-benzoquinone radical has five hyperfine lines caused by interaction of the unpaired spin with the four equivalent ring protons[26,27]. The monoprotonated neutral *p*-benzosemiquinone radical has eighteen hyperfine lines, indicating that the hydroxylic proton and two sets of two identical ring protons interact with the spin[28]. Thus the protonation of atoms which share unpaired electron density results in the observation of a more complex hyperfine splitting pattern. This protonation phenomenon may be the basis for the observation of four partially resolved hyperfine components in Signal II-(16) and five partially resolved hyperfine components in Signal II(19)

The work of Kohl and co-workers[5,29] and Weaver[4] have implicated plastoquinone or a plastoquinone derivative as the source of Signal II. The similarities which we find between flavin free radicals and Signal II behavior indicate that this species is also an attractive candidate. The ionic strength-induced changes in the EPR spectrum of Signal II which we report, however, may be accomodated by either molecule. At low ionic strength the Signal II species, which almost certainly is located in the thylakoid membrane, may be the protonated 19 G neutral semiquinone of either a flavin or a plastoquinone derivative As the ionic strength is increased, conformational changes in the membrane may result in loss of a proton to form the 16 G anion or the metal chelate. We are currently exploring these possibilities experimentally.

The similarities in the light-induced changes exhibited by Signals II(16) and II(19) reported in Fig 2, Table I, and the previous paper support the hypothesis presented above. These results preclude the possibility of gross structural changes occurring in the species giving rise to Signal II in converting between Signal II(16) and II(19). Rather, they indicate that both states of F exist in similar environments and react with similar rates with the same reaction partners, the states S_2 and S_3 on the water side of Photosystem II. Only a slight structural perturbation is indicated in the conversion between the two Signal II states.

This conclusion is supported by the inhibition studies that we report. These data indicate that the kinetic behavior of the Signal II precursor is quite labile, since the reasonable mild treatments which we have used, particularly aging at room temperature for 1 h, drastically alter the induction of Signal II in flashing light. Thus, if major structural changes were involved in the conversion between Signal II(16) and Signal II(19), we might expect this to be reflected in the induction kinetics.

The action of CCCP in chloroplasts is complex[8,9,30,31]. It increases the proton permeability of the chloroplast membrane and, at moderate concentrations (10 μM), uncouples phosphorylation[32]. However, the effects of CCCP which we report are not due simply to its uncoupling action since we have shown that neither NH_4^+ nor valinomycin affect Signal II.

Renger[14] has shown that CCCP belongs to a class of reagents which accelerate the decay of the S states This acceleration is mediated by a reaction between these oxidized intermediates on the water side and reduced components on the acceptor side of Photosystem II. In the model we have proposed, Signal II is generated *via* a reaction involving the oxidized intermediates, specifically the two intermediate states, S_2 and S_3 Our model predicts that any treatment which decreases the lifetime of these S states to a time comparable to or less than the time constant for Signal II generation would decrease the extent of formation of Signal II following a single flash. Fig 5 shows that this is indeed the case. With increasing CCCP concentration the effectiveness of a single flash in generating Signal II decreases. A second prediction which our model makes is that, as the lifetimes of S_2 and S_3 are decreased, the time constant for Signal II generation should decrease, *i.e.* as the lifetimes for S_2 and S_3 decrease, only those Signal II precursors that react during this shortened time will produce spins. Data of Figs 4 and 6 show that prediction is also consistent with experiments.

Lozier and Butler[7] reported that neither DCMU nor CCCP alone inhibited the light-induced formation of Signal II. They showed, however, that if chloroplasts were treated simultaneously with these two reagents, the light-induced Signal II response was abolished. Homann[33] has shown that in chloroplasts treated with DCMU, CCCP strongly inhibits the reoxidation of Q^- following illumination. Renger *et al.*[15] have interpreted this result as indicating that CCCP prevents the back reaction between Q^- and oxidized intermediates on the water side of Photosystem II by inducing reduction of the oxidized intermediates. Thus Q^-, S_2 and S_3 remain reduced and further electron flow through Photosystem II is inhibited. Since our model postulates an oxidation of the Signal II precursor by S_2 or S_3, we predict the result observed under these conditions, *i.e.* an inhibition of the light response of Signal II.

The other treatments that we have used: aging, heating, Tris-washing and preparation of System II particles, are less well characterized than the action of CCCP, but all are known to inactive the O_2-evolving system. These effects are of at least three types: a loss of O_2-evolving capacity, a shift toward lower values of the midpoint potential of cytochrome b_{559} (refs 34, 35), and an alteration of the kinetics of Signal II We have proposed that the O_2-evolving system and the species giving rise to Signal II are located in a hydrophobic environment. Under normal circumstances of membrane integrity, aqueous reagents are quite efficiently excluded from this site The treatments which we have described may denature this hydrophobic region allowing access by normally excluded reagents, including exogenous reductants and reduced intermediates on the acceptor side of Photosystem II. The experiments of Lozier and Butler[7] in which they showed that added ascorbate greatly increases the decay of Signal II in Tris-washed chloroplasts support this idea The function of the CCCP-like compounds may be to accelerate this denaturation reaction

The inhibition experiments which we have reported here support the model, proposed both by us[1] and by Butler *et al.*[7,36], in which Signal II is generated by reactions occurring on the water side of Photosystem II.

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