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ELECTRON PARAMAGNETIC RESONANCE SIGNAL II IN SPINACH CHLOROPLASTS

I. KINETIC ANALYSIS FOR UNTREATED CHLOROPLASTS

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SUMMARY

An analysis of electron paramagnetic resonance Signal II in spinach chloroplasts has been made using both continuous and flashing light techniques In order to perform the experiments we developed a method which allows us to obtain fresh, untreated chloroplasts with low dark levels of Signal II. Under these conditions a single 10-μs flash is sufficient to generate greater than 80 % of the possible light-induced increase in Signal II spin concentration. The risetime for this flash-induced increase in Signal II is approx. 1 s. The close association of Signal II with Photosystem II is confirmed by the observations that red light is more effective than is far red light in generating Signal II, and that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) does not inhibit the formation of the radical. Single flash saturation curves for the flash-induced increase in Signal I and Signal II indicate that the quantum efficiency for Signal II formation is close to that for Signal I While one or two flashes (spaced 10 ms apart) are quite efficient in generating Signal II, three or four flashes are much less effective. However, if this spacing is decreased to 100 μs, three or four flashes become as efficient as one or two flashes. From observations of a deficiency of O_2 evolved during the initial flashes of dark-adapted chloroplasts, we conclude that the species which gives rise to Signal II is able to compete with water for oxidizing equivalents generated by Photosystem II. On the basis of these results we postulate a model in which Signal II arises from an oxidized radical which is produced by a slow electron transfer to the specific states S_2 and S_3 on the water side of Photosystem II.

INTRODUCTION

At room temperature O_2-evolving photosynthetic materials generate two free radical species which are detectable using EPR spectroscopy[1,2]. The first, which has

Abbreviations H, magnetic field in gauss, χ, susceptibility; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone.

been termed Signal I, has rapid rise and decay kinetics and has been established as arising from the oxidized reaction center of Photosystem I, $P700^+$ (refs 3,4). The second, Signal II, has been less well characterized. It has been reported to have a g value of 2.0046, a linewidth of about 20 gauss, hyperfine structure resulting from interaction with protons and decay kinetics on the order of hours[5]. The studies of Weaver and Bishop[6,7] have shown Signal II to be absent in photosynthetic bacteria, in algal mutants lacking the ability to evolve O_2 and in algae grown on a manganese-deficient medium. Chloroplast preparations which have lost O_2-evolving capacity through heating or sonication also lack the spin signal[8] Chloroplast particles enriched in Photosystem II activity show an increased Signal II magnitude, whereas Photosystem I particles are deficient in this feature[4]. On the basis of these findings Signal II has been associated with the O_2-evolving Photosystem II in algae and green plants[9]

Kohl and coworkers[10-12], using deuteration, extraction and readdition procedures and *in vitro* studies on model compounds, have presented evidence suggesting that the molecular species giving rise to Signal II may be plastoquinone or a species closely related to it. Kinetic evidence linking this observation with the functional pool of plastoquinone located between the two photosystems is lacking[13,14] There are, however, several different pools of quinone present in the chloroplast, so this observation does not invalidate the assignment of Signal II to a plastoquinone derivative[15]. A review article on the properties of both Signals I and II has recently appeared[4]

Recently, speculations on the functional location of Signal II have centered on the water side of Photosystem II, where long-lived intermediates involved in the water oxidation process have been demonstrated[16]. The basis for this assignment comes from both the long decay time of the radical and its behavior to reagents such as hydroxylamine, anilinothiophene and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), which speed the decay of both Signal II (refs. 17 and 18) and oxidized precursors involved in O_2 evolution[19]

Kinetic analysis of Signal II has been greatly hampered by its slow decay, which is on the order of hours at room temperature Kinetic measurements after such long times are difficult to interpret because of severe aging effects[20]. In the experiments presented in this paper we have established conditions under which fresh chloroplasts with low dark levels of Signal II can be obtained. We have made a kinetic analysis of the light-induced increase in spin concentration using 10-μs flashes from a xenon lamp and have found results which support a model in which Signal II arises via electron transfer to oxidized intermediates between the Photosystem II reaction center chlorophyll and the site of water oxidation. A preliminary report of this work has been presented[21]

MATERIALS AND METHODS

Chloroplast preparation

Spinach (*Spinacia oleracea* var. early hybrid No. 7) was grown in growth chambers under conditions as outlined by Sun and Sauer[22]. Chloroplasts were isolated by grinding for 10 s in a Waring blender using an isolation solution consisting of 0.4 M sucrose, 0.1 M tricine (pH = 7.6), 0.01 M NaCl. The chloroplasts were then

filtered through 8 layers of cheese-cloth, spun for 1 min at 3000 × *g* in a Sorval RC2B centrifuge, and the pelleted chloroplasts were resuspended in the isolation solution. All operations were carried out at 4 °C. Chloroplasts referred to in the text as "dark-adapted chloroplasts" were prepared in the same manner except that the spinach leaves were picked after they had been in the dark for at least 8 h, and all subsequent isolation steps were carried out in the dark. Chlorophyll concentrations in samples used for the EPR measurements were between 2 and 4 mg chlorophyll/ml; for O_2 measurements the chlorophyll concentration was 0.2 mg/ml. For the EPR measurements 10^{-4} M EDTA (final concentration) was present in all experiments to eliminate the Mn^{2+} signal which otherwise would distort the baseline. In control experiments the same results were obtained with or without added EDTA.

Light sources

Xenon flashes were obtained from a flash system similar to that described by Weiss and Sauer[23], except that a capacitor bank was used which gave flashes of 10-μs duration (measured at half height) instead of the 28-μs flashes which they used. The light was filtered through a Corning 1-69 heat filter and a Corning 3-74 ultraviolet filter and was focused on the slotted microwave cavity in EPR experiments or the platinum electrode in O_2 experiments using appropriate lens systems.

Broad band, continuous white light was obtained from a microscope illuminator and was passed through a water filter and the 1-69, 3-74 filter combination. The intensity at the sample for this continuous light was 45 mW/cm^2. Red (650 nm) or far red (700 nm) continuous light was provided by a tungsten lamp, a Bausch and Lomb monochromator (Model 33-86-03, entrance slit = exit slit = 2 mm, dispersion, 6.5 nm/mm), and appropriate Optical Industries interference filters to eliminate higher order diffractions. Light intensity was adjusted using appropriate Balzers neutral density filters and measured with a Hewlett-Packard radiant flux detector (Model 8334A). Illumination was initiated using an electromechanical shutter which has an opening time less than 10 ms.

EPR measurements

A Varian E-3 (X band, 9.5 GHz) EPR spectrometer fitted with a slotted cavity to permit *in situ* illumination was used in recording spectra and kinetic changes in chloroplast suspensions contained in a quartz EPR flat cell (nominal optical path length = 0.2 mm). The cavity was continuously flushed with dry, room temperature N_2 gas. The microwave power in all experiments, except those described in Figs. 1 and 3, was 50 mW, modulation amplitude in recording spectra was 5.0 G, this was increased to 6.3 gauss in kinetic experiments to increase the signal-to-noise ratio. Spectra were recorded by sweeping from low field to high field with the spectrometer time constant and scan rate as noted in figure legends. In kinetic experiments in which signal averaging techniques were applied, the output of the E-3 spectrometer was fed into a 1024 channel Enhancetron signal averager. Timing circuits provided pulses which triggered the averager and, after a preset delay time, initiated the flash lamp pulse. All experiments were carried out at room temperature.

O_2 measurements

O_2 evolution from chloroplasts in response to individual flashes was measured using an arrangement similar to that described by Weiss and Sauer[23]. We have

modified the teflon-covered electrode described in their work so that it is possible to perform experiments without the teflon membrane, thus decreasing the response time of the electrode to approx. 10 ms. The Ag/AgCl reference electrode is located 4 cm down-stream from the platinum electrode in a reservoir of electrolyte. The current increase resulting from chloroplast O_2 evolution is represented as the voltage output from a current-to-voltage transducing operational amplifier. This voltage is subsequently amplified and recorded using a Sanborn recorder (rise time = 5 ms). In the experiments described in this work flash lamp pulses were spaced 1 s apart and were of saturating intensity. All experiments were carried out at room temperature

RESULTS

Effect of dark adaptation on Signal II decay

Fig. 1 shows EPR spectra of chloroplasts in the light and in the dark after illumination. In the light, both Signal II and Signal I are observed, although the magnitude of Signal I is low because no electron acceptor system, such as ferredoxin/$NADP^+$, has been included in the chloroplast suspension. Upon darkening, Signal I decays quickly whereas the extent of Signal II decay is slight. In his recent review article, Kohl[4] mentions that in the dark Signal II has hyperfine structure in the region labeled "I" in Fig. 1, such that the ratio of the peak in this region to the peak at the position labeled "II" in Fig. 1 is 3/4. We have found that this ratio varies considerably and is dependent on the method of chloroplast isolation. In the following

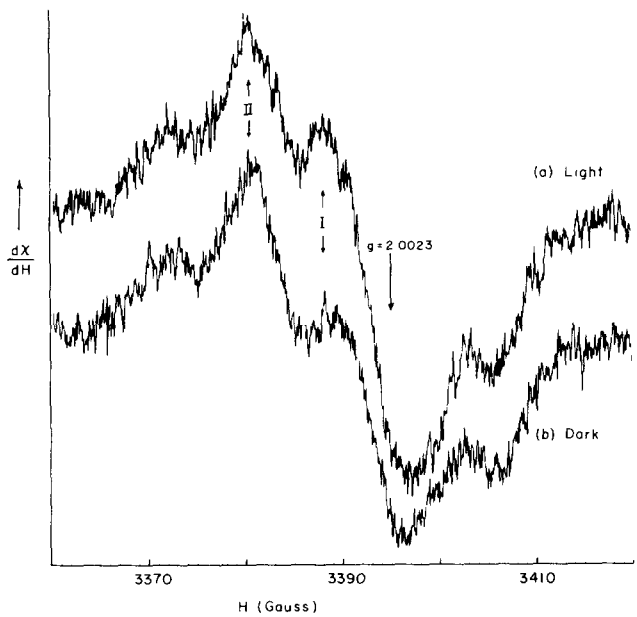


Fig 1 EPR spectra (1st derivative) of chloroplasts resuspended in isolation solution in the light (a) and in the dark immediately after illumination (b). Broad band white light was used to illuminate the sample The instrument time constant was 0 3 s, the scan rate was 25 G/min and the microwave power was 16 mW Low field maximum for Signal II labeled as "II", for Signal I as "I".

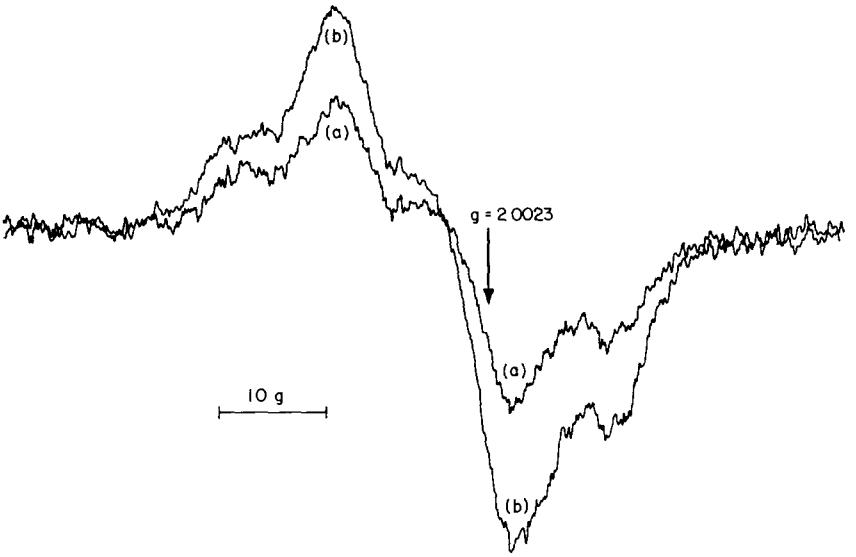


Fig 2. EPR spectra of dark-adapted chloroplasts in the dark before (a) and immediately after (b) illumination. The sample was illuminated with broad band white light for 2 min before spectrum (b) was recorded. The instrument time constant was 1 0 s and scan rate was 25 G/min.

article we consider the sources for this variation in the structure of Signal II in detail[20]

Cycles of red and far red light have been shown to have no effect on the decay of Signal II (ref. 13), and exogenous redox systems appear to be excluded from the site of Signal II formation. Fresh chloroplasts prepared from spinach picked during the light cycle show high dark levels of Signal II and exhibit little additional light-induced increase. We have found that incubation of these chloroplasts for 2–4 h at 0 °C in a darkened ice bucket leads to a 20–30 % decrease in the signal, which is regained upon illumination It appears that a soluble endogenous factor facilitates this decay, since washed chloroplasts show very little (less than 10 %) Signal II decrease even after 5 or 6 h of dark incubation.

We have found, however, that if spinach leaves are picked toward the end of the dark period of their growth cycle and the chloroplast isolation procedure is carried out in the dark, the magnitude of Signal II prior to illumination is reduced. Fig 2 shows EPR spectra of such dark-adapted chloroplasts before and after illumination. The chloroplasts in this experiment, prepared from leaves which had been in the dark approx. 8 h, show a 45 % increase in Signal II upon illumination.

The effect of the microwave power level on the Signal II amplitude and on the ratio of Signal II before and after illumination in dark-adapted chloroplasts is shown in Fig. 3. Curve (a) indicates that Signal II saturates at fairly low power in agreement with Kohl[4], and decreases slightly at higher powers. However, the ratio of Signal II in dark-adapted chloroplasts to Signal II in these same chloroplasts following illumination is not influenced by the microwave power as shown in Curve (b) in Fig. 3. This ratio remains constant at about 0 60 in this set of experiments for microwave powers between 1 0 and 125 mW.

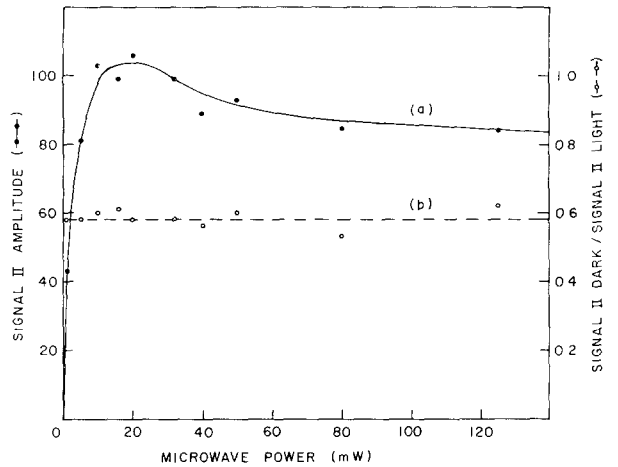


Fig 3 EPR Signal II amplitude (Curve a, ●–●) and the ratio of Signal II before and after illumination (Curve b, ○–○) as a function of microwave power A fresh sample of untreated, dark-adapted chloroplasts was used for each experimental point The spectrum of the dark-adapted chloroplasts was recorded Following a 2-min illumination with broad band white light, a second spectrum was recorded for each power setting. The magnitude of Signal II was measured as the difference between the low field maximum at 3380 G and the high field minimum at 3396 G in Fig 1 This value for the spectrum of Signal II recorded following illumination is plotted in Curve a, the ratio of the magnitude of Signal II prior to illumination to this value following illumination is plotted in Curve b The instrument time constant and scan rate were as described in Fig. 2

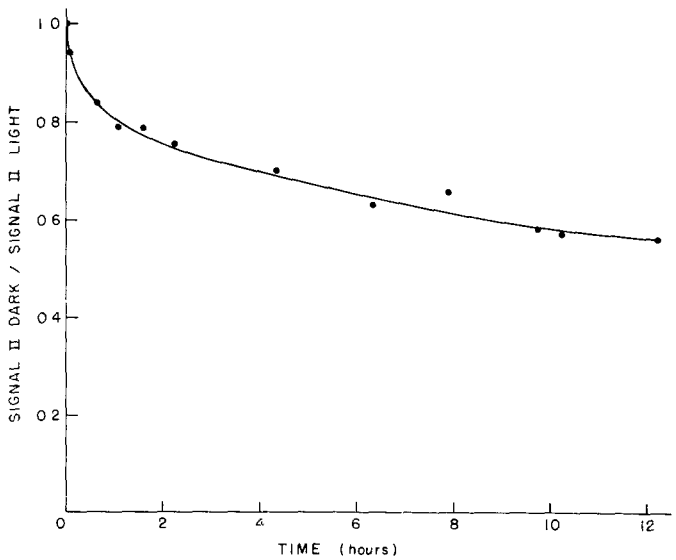


Fig 4 The decay of Signal II *in vivo* Spinach plants in the growth chamber entered the dark period of their growth cycle at time zero. At various times following this, chloroplasts were isolated under rigorously dark conditions Spectra were recorded before and after illumination with broad band white light, using the time constant and scan rate described in Fig. 2. The magnitude of Signal II was measured as described in Fig 3 The ratio of Signal II before illumination to Signal II following illumination is plotted as a function of the time the spinach leaves were picked.

Fig. 4 summarizes a series of experiments in which chloroplasts were prepared from leaves picked at various times after initiation of the dark period. While there is some scatter in the data, we can discern several general features of the *in vivo* dark decay of Signal II. Within the first 2 h after darkening there is a decrease of about 25 % in the signal, which may correspond to the 20–30 % decrease (see above) that has been found to be associated with dark incubated chloroplasts at 0 °C. Following this initial decrease there is a slower decrease to about 50–60 % of the light-induced signal after 12 h in the dark. We have consistently found that Signal II appears to decay only to this 50 % level in the dark *in vivo*. Lozier and Butler[18] have reported a similar 50 % plateau in the decay of Signal II following illumination in isolated spinach chloroplasts at room temperature. The possible significance of this effect will be discussed in detail below.

Effect of single flashes on Signal II induction in dark-adapted chloroplasts

By setting the magnetic field of the spectrometer at the low field position labeled "II" in Fig. 1 we are able to monitor the kinetics of light-induced changes in Signal II. The effect of a series of 10-μs flashes on radical concentration in dark-adapted chloroplasts is shown in Fig. 5. In this experiment Signal II before illumination was about 50 % of the signal found after the flashes. As can be seen in Fig. 5, a single flash is sufficient to induce about 80 % of the increase. Subsequent flashes increase the signal only slightly and, significantly, there are no oscillations with these later flashes such as those observed in experiments monitoring O_2 evolution as a function of flash number[16]. The characteristic slow decay of Signal II is apparent in this experiment.

During a single 10-μs flash, at most a single electron can be transferred through each of the photosystems[23], yet under these conditions we find that 80 % of the light-induced increase in Signal II occurs. This observation implies that the species which gives rise to Signal II is present in relatively small concentrations

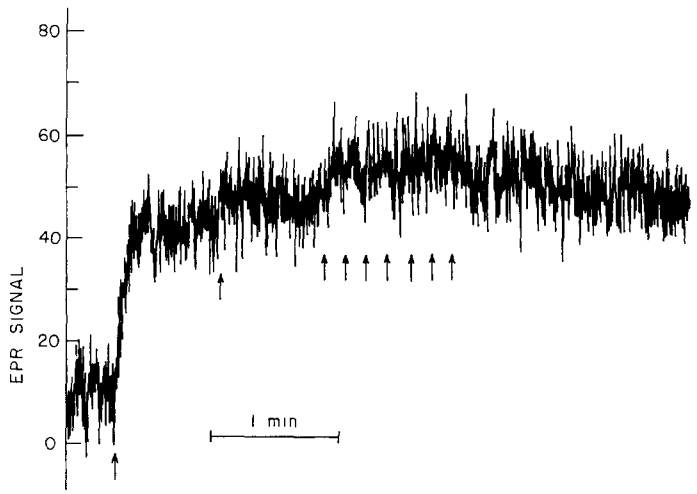


Fig. 5. Response of Signal II in untreated, dark-adapted chloroplasts to light flashes 10 μs in duration. A single saturating flash was given at each of the arrows. Magnetic field was set at the low field peak of Signal II labeled "II" in Fig. 1. Instrument time constant was 0.3 s.

compared to the total amount of chlorophyll in the chloroplast. We have confirmed this hypothesis by determining the ratio of total spins in Signal II to the number of spins in Signal I in saturating light. We used the method of double integration as outlined by Chang and Johnson[24] and found a value for this ratio which is close to unity in fresh chloroplasts.

Quantum efficiency for Signal II formation in flashing light

We have determined single flash saturation curves for both Signal I and Signal II. These results are plotted in Fig. 6 as the fraction of Signal I or II formed as a function of the light intensity of a single flash. In these experiments dark-adapted chloroplasts, to which the acceptor system ferredoxin/$NADP^+$ had been added, were used. The extent of Signal II formation resulting from a single flash of intensity J was divided by the extent of Signal II formation after 10 saturating flashes to obtain the fraction of Signal II formed at intensity J. Then, by changing the magnetic field from position II to the position labeled "I" in Fig. 1 without changing either lamp or sample placement, we determined the saturation behavior of Signal I. Since Signal I decays rapidly, the average of 36 flashes was used in these experiments. The extent of Signal I formation for a flash of intensity J was divided by the extent of Signal I formation for a saturating flash to obtain the fraction of Signal I formed at intensity J.

Half saturation for both Signals I and II occurs at the same light intensity, which together with the results described above indicates that the quantum efficiency for Signal II formation in dark-adapted chloroplasts approaches that for Signal I formation. These results appear to be at variance with data reported by Treharne and Vernon[25] which indicated that Signal II saturated at an intensity at least an order of magnitude lower than Signal I in whole *Chlorella* cells. However, from their experimental description it appears as if their work was done under steady-state conditions which, because of the long decay time for Signal II, would yield a saturation intensity

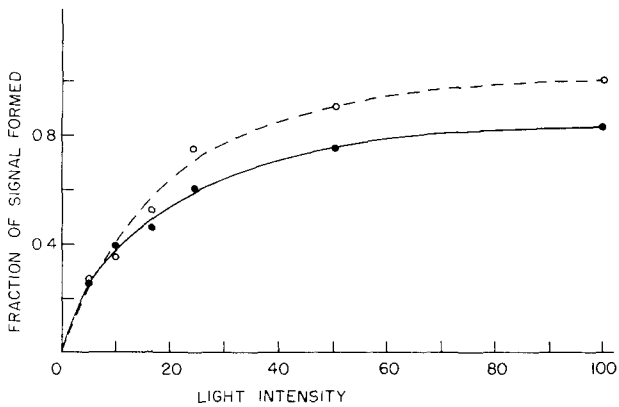


Fig. 6 Single flash saturation curves for Signal I (○- - -○) and Signal II (●–●) in untreated chloroplasts. A fresh sample of dark-adapted chloroplasts (2 mg chlorophyll/ml) *plus* 2 10^{-3} M $NADP^+$ and 60 μg ferredoxin/ml was used for each light intensity The instrument time constant in the Signal II determinations was 0 3 s This was decreased to 10 ms for the Signal I determinations. Experimental procedure is described in the text A light intensity of 100 corresponds to the unattenuated flash lamp output, neutral density filters were used to lower the incident intensity.

significantly lower than initial rate or single flash saturation values. In experiments which we have performed with *Chlorella* we find that a single flash is less effective in generating Signal II than in spinach chloroplasts.

Effect of DCMU on Signal II formation in dark-adapted chloroplasts

Lozier and Butler[18] and Weaver and Weaver[26] have reported that DCMU does not inhibit the light response of Signal II We repeated these experiments using dark-adapted chloroplasts and, as shown in Fig. 7, confirmed the finding that DCMU does not inhibit the formation of Signal II in continuous light. The DCMU concentration in this experiment was $2 \cdot 10^{-4}$ M with a molar ratio of DCMU to chlorophyll of 0.1, which is sufficient to inhibit O_2 evolution in chloroplasts completely. However, when these dark-adapted, DCMU-treated chloroplasts are subjected to a series of 10-μs saturating flashes we find that the first flash evokes only one-third of the maximal light-induced response and approx. 10 flashes are needed to induce Signal II fully. This is to be contrasted with untreated chloroplasts (Fig. 5) in which a single flash produces more than 80 % of the light-induced signal and no further increase is observed following the third flash. Thus the effect of DCMU is to lower the quantum efficiency of Signal II formation without inhibiting the maximal extent of its response.

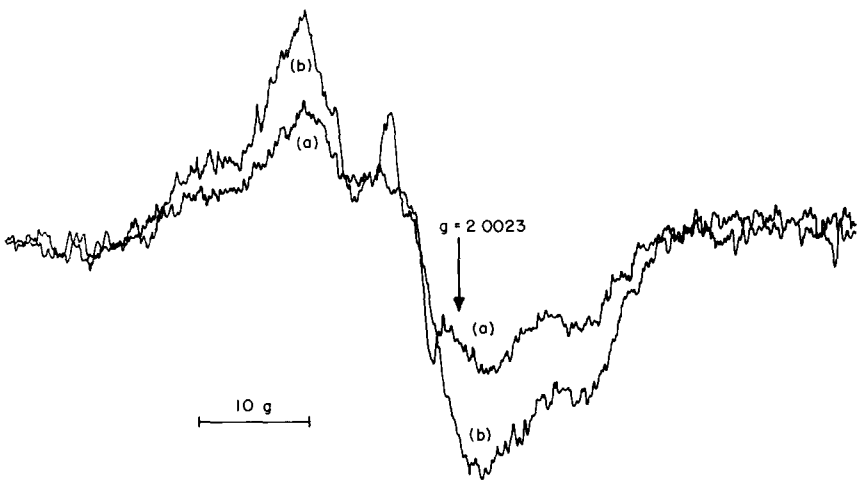


Fig. 7 EPR spectra of dark-adapted, DCMU-treated chloroplasts before (a) and immediately after (b) illumination with broad band white light Chlorophyll concentration in the experiment was 2 2 mg chlorophyll/ml, DCMU concentration was $2 \cdot 10^{-4}$ M, ethanol was 1 5 % in the final reaction mixture The instrument time constant and scan rate were as described in Fig 2. The narrow signal in the center of the spectra is due to ascorbate-free radical which is present in variable concentrations in spinach leaves[27]

Effect of red vs far red illumination on Signal II formation

The insensitivity of the extent of Signal II formation to DCMU indicates two possible sites for its location. One places Signal II on the Photosystem I side of the DCMU block, in which case far red light should be more effective than red light in stimulating its formation; the other possibility would locate Signal II on the Photosystem II side of the block with red light more stimulatory than far red. In order to

test these two possibilities we have done studies of the rate of Signal II formation in 650- and 700-nm continuous light. The experiments were done at low incident light intensities, since rates of formation yield more precise information than steady-state Signal II levels, for reasons mentioned above. At the high absorbances used in this study ($A_{650\ nm} = 4$; $A_{700\ nm} = 0.8$) essentially all of the light is absorbed at either wavelength, so that no corrections involving the extinction coefficients at 650 and 700 nm are applied. The results of the experiments are shown in Fig. 8. They indicate that for approximately equal incident photon fluxes, the initial rate of Signal II formation in 650-nm light is more than twice the initial rate in 700-nm light. This is in agreement with the results of Allen *et al.*[28] which indicate that Signal II is preferentially excited by shorter wavelengths of light, while the Signal I action spectrum persists to longer wavelengths. It is also consistent with the evidence cited in the Introduction associating Signal II with Photosystem II.

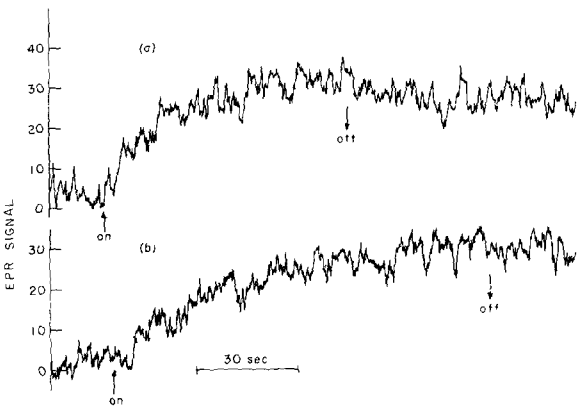


Fig. 8. Time course of Signal II generation in untreated, dark-adapted chloroplasts in 650-nm (a) and 700-nm (b) light. Light on and off as indicated. The instrument time constant was 0.3 s. light intensity at the sample was 65 μW/cm^2 for the 650-nm light, 70 μW/cm^2 for the 700-nm light

O_2 evolution in flashing light in dark-adapted and preilluminated chloroplasts

The results described above suggest that Signal II arises from a species located on the Photosystem II side of the DCMU block. DCMU is known to act very close to the primary Photosystem II photochemistry by blocking electron transfer from the primary acceptor to secondary acceptors in the chain between Photosystem II and Photosystem I. However, fluorescence induction studies of Joliot and Joliot[29] indicate that the primary acceptor pool may be inhomogeneous. Therefore, there exists the possibility that Signal II arises from either the oxidizing side or reducing side of Photosystem II. Since we have shown in Fig. 5 that Signal II arises *via* an electron transfer step that occurs largely on the first flash, we have carried out a series of experiments in which we monitored O_2 evolution in response to individual flashes in order to investigate these possibilities in more detail. Briefly (see Discussion), we expect dark-adapted (low Signal II) chloroplasts to show equal or higher yields of O_2 on the third flash compared to preilluminated (high Signal II) chloroplasts if the species giving rise to Signal II were a potential electron acceptor supplementing the primary acceptor on the reducing side of Photosystem II. The opposite effect would be

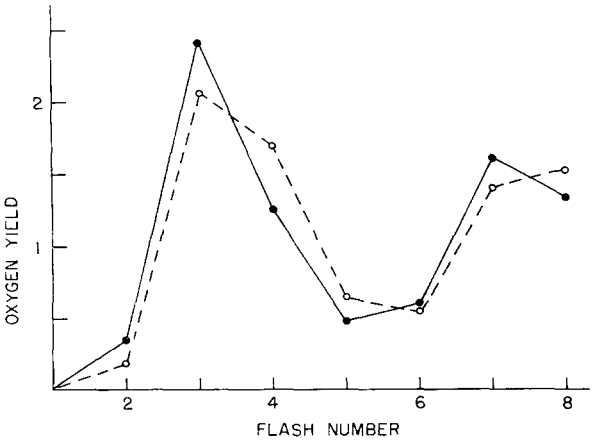


Fig 9 O_2 evolution in response to a series of saturating light flashes 10 μs in duration from dark-adapted (○- - -○) and preilluminated (●–●) chloroplasts Individual flashes in each series were spaced 1 s apart O_2 yield in response to each flash was normalized with respect to a steady-state value of 1, which is reached after approx 25 flashes

expected if Signal II arose as a result of electron donation to species on the water side of Photosystem II. The results of the experiments are shown in Fig. 9. In the thoroughly dark-adapted chloroplasts the level of Signal II was 55 % of the signal after the train of pulses Preilluminated chloroplasts were obtained by exposing chloroplasts to room light for 2 min, which served to induce Signal II fully, prior to injection into the electrode. Each sample was allowed 10 min dark time on the O_2 electrode before the flash sequence was initiated. A comparison of the two curves indicates that in fully dark-adapted chloroplasts the O_2 yield resulting from the third flash is lower and the yield of the fourth flash higher than in preilluminated chloroplasts. Dividing the O_2 yield of the third flash, Y_3, by that for the fourth flash, Y_4, we find values for the Y_3/Y_4 ratio of 1.2 for the dark-adapted chloroplasts and 1.9 for the preilluminated sample. According to the analysis described above and in the Discussion, these results favor a model in which Signal II originates as a consequence of electron donation on the water side of Photosystem II.

Risetime of Signal II in response to a single flash

In constructing models locating Signal II on the water side of Photosystem II we have found two which adequately explain the data thus far. The first of these places Signal II as an intermediate between the site of water oxidation and the reaction center chlorophyll P680. This model associates Signal II directly with the oxidized intermediates, which the experiments of Joliot *et al.*[30], Kok *et al.*[16], and Weiss and Sauer[23] have demonstrated in the water oxidation process. These oxidized intermediates correspond to the S states in the Kok *et al.*[16] model for O_2 evolution. The second model places the species giving rise to Signal II off this electron transport pathway, but its formation would occur through interaction with intermediates in the chain between water and Photosystem II.

The experiment described above (Fig 5) in which we monitored the response of Signal II to single flashes argues against the assignment of Signal II to one of the

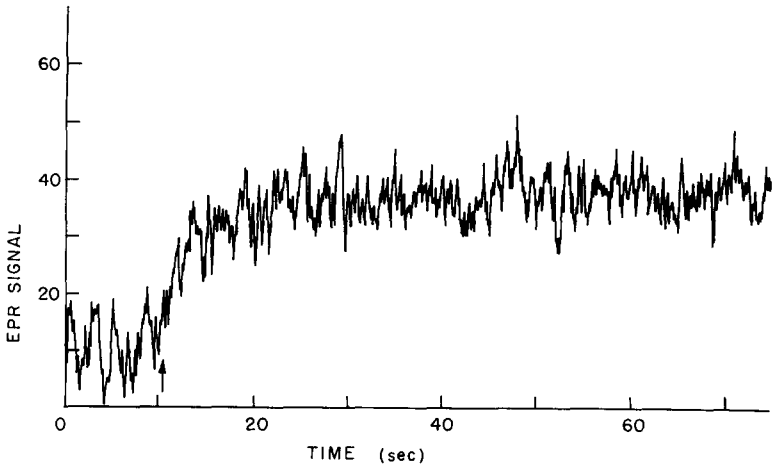


Fig. 10. Time course of the response of Signal II in untreated, dark-adapted chloroplasts to a single saturating light flash. The arrow designates the time at which the flash lamp was fired. Instrument time constant was 0.3 s.

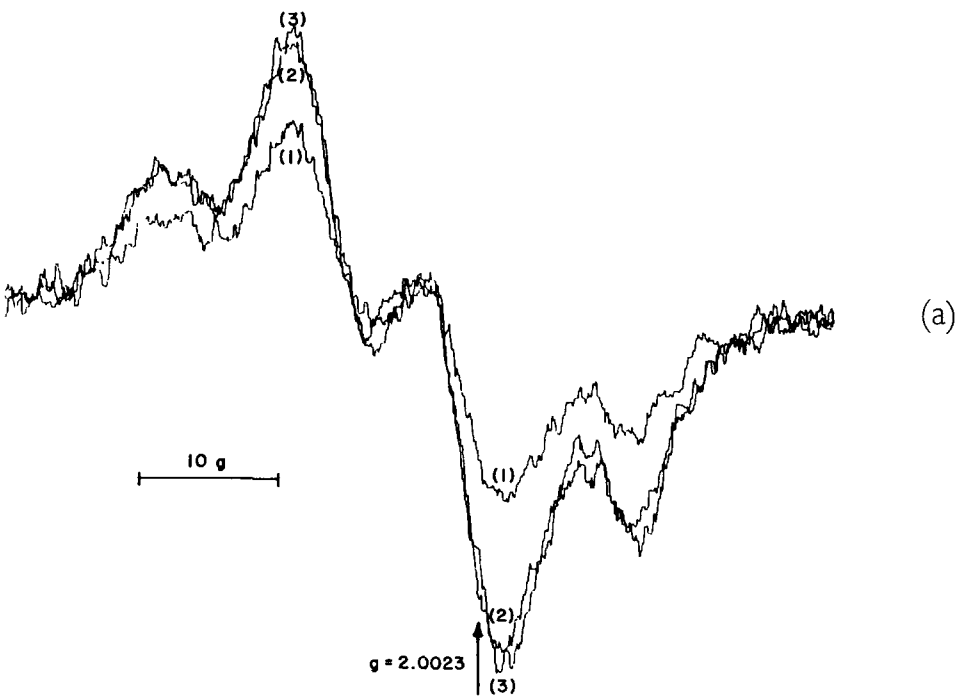
S states in the electron transport chain between P680 and the water splitting site. This experiment shows that the concentration of the radical does not vary with flash number, whereas there should be marked oscillations in the concentrations of the oxidized intermediates involved in water splitting[16]. We have obtained further evidence against the identification of Signal II with an S state directly on the pathway from the water oxidation site to P680 by determining the risetime of Signal II in response to a single flash. The results of this experiment, shown in Fig. 10, indicate that Signal II is formed rather slowly after a flash. The half-time for its rise is approx. 1 s, which is three orders of magnitude greater than the values found for the rise-times of the intermediates involved in the water splitting process in experiments measuring O_2 evolution[16]. Therefore both its response to a series of flashes and its risetime in response to a single flash argue against the direct assignment of Signal II to one of the S states involved in water oxidation.

Multiple flash studies of Signal II formation

If Signal II arises indirectly via interaction with oxidized intermediates on the pathway from the water oxidation site to P680, we expect its extent of formation to be related to the concentrations of one or more of these oxidized species formed on a flash or in a series of flashes. As mentioned above, a number of workers have shown that following a flash the risetime for concentration changes in these oxidized species is less than 10 ms. On the other hand, Fig. 10 indicates that the rate of formation of Signal II is much slower following a flash. We have taken advantage of this disparity in rate constants to test the second model mentioned in the previous section. In a series of closely spaced flashes, with the dark time between flashes short compared to the risetime for Signal II, the species which generates the radical should be sensitive to the concentration of oxidized intermediates present at the conclusion of the flash series. The pattern of O_2 evolution shown in Fig. 9 has been most successfully explained by postulating a build-up of oxidized intermediates on the first and second

flashes which are subsequently discharged in the water splitting process on the third and fourth flashes. Thus, after two flashes we expect a large concentration of highly oxidized intermediates, and after four flashes a much lower concentration.

The effects of these two flash patterns on the extent of Signal II formation are shown in Fig. 11. The spectra of dark-adapted chloroplasts were obtained (Curves 1). Then, either two (Fig. 11a) or four (Fig. 11b) saturating flashes were given and the second spectra (Curves 2) were taken. At the conclusion of this scan ten saturating flashes were given and the third spectra (Curves 3) were recorded. Different samples from the same chloroplast preparation were used for the two experiments because of the long decay of Signal II. In each experiment the dark signal (Curve 1) was about 58 % of the fully induced signal (Curve 3). However, two flashes, 10 ms apart, generated 90 % of the light-induced signal whereas four flashes 10 ms apart increased the signal only 40 %. 10 ms was chosen as the dark time between flashes because four flashes spaced 10 ms apart yield maximal amounts of O_2 per flash, *i.e* the intermediates in the water splitting process are fully advanced within 10 ms after a flash Three flashes, 10 ms apart, behave in a manner similar to four flashes, whereas a single flash has effects similar to two flashes. These results are shown in Fig. 12a, in which we summarize the data from the four experiments. The results are presented in histogram form to emphasize that each experiment was performed with a different sample and that the effects we see are not oscillations; for example, two flashes followed by a 1-s dark period and then two flashes 10 ms apart does not decrease the level of Signal II. In all experimental approaches we have explored we have found no method involving light which decreases the concentration of Signal II spins.



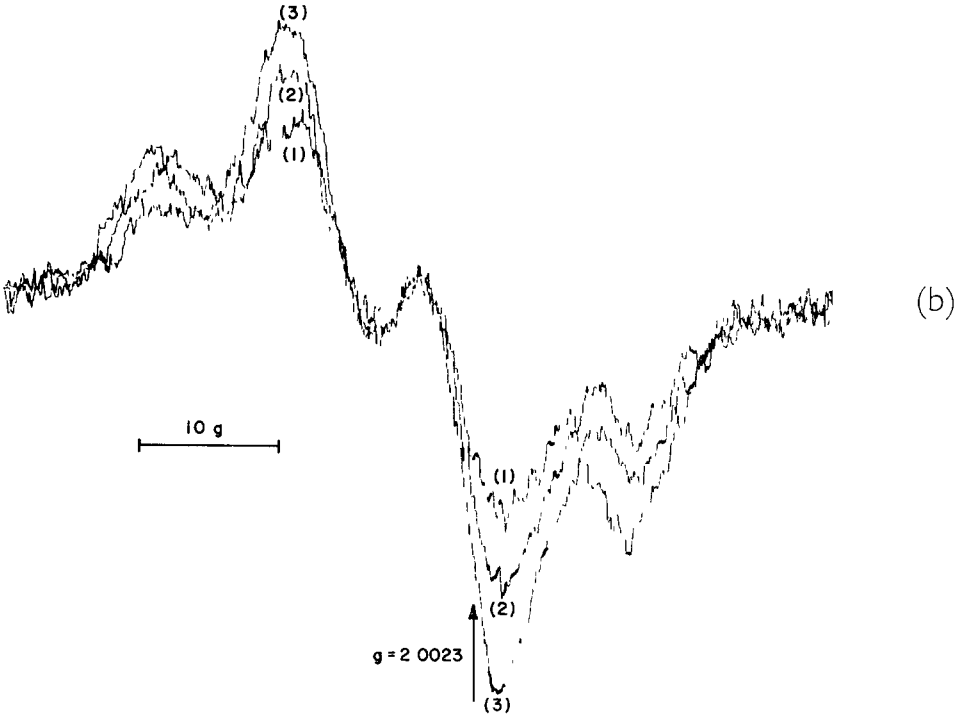


Fig 11 Response of Signal II spectra in untreated, dark-adapted chloroplasts to 10-μs saturating flashes (a) two flashes separated by 10 ms, (b) four flashes separated by 10 ms. Each experiment was performed with a fresh sample of dark-adapted chloroplasts as described in the text. Instrument time constant and scan rate as described in Fig 2 Total time between initiation of Curve 1 and completion of Curve 3 was 10 min

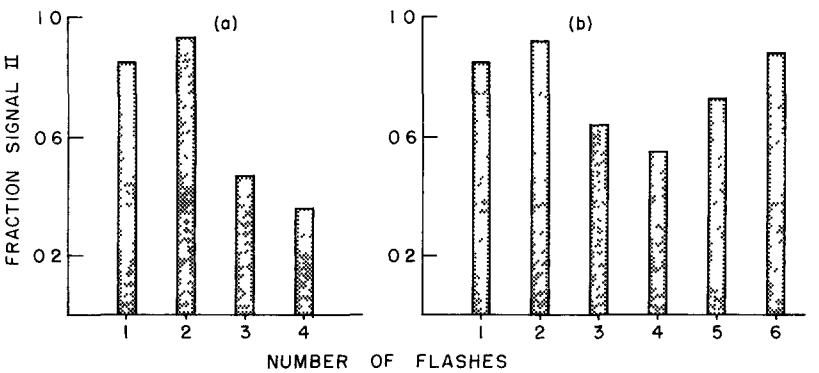


Fig 12. Response of Signal II in untreated, dark-adapted chloroplasts to various numbers of 10-μs saturating flashes spaced (a) 10 ms or (b) 370 ms apart. Each bar corresponds to an experiment on a fresh sample of dark-adapted chloroplasts in which the sample was given the designated number of flashes followed by single flashes to complete the induction of Signal II The ratio of the increase in Signal II stimulated by this set of flashes to the total light-induced increase in Signal II is plotted as a function of the number of flashes in each set Response to the flashes was measured at the low field peak of Signal II with an instrument time constant of 1.0 s

After four flashes the O_2-evolving system has been largely discharged and, to a first approximation, is similar, with respect to the concentration of oxidized intermediates, to the situation before the first flash. The fifth and sixth flashes yield little O_2 but serve to restore a pool of oxidized species which are discharged on the seventh and eighth flashes. Therefore Signal II should react to five flashes 10 ms apart as it did to a single flash and to six flashes as it did to two flashes Because of limitations in the flash apparatus, it was necessary to increase the time between flashes to 370 ms to give five or six flashes in a sequence. The results of these experiments are shown in Fig. 12b. Again, one or two flashes yield greater than 80 % of the light-induced increase of Signal II For this longer dark time between flashes, the distinction between the effects of one or two flashes and three or four flashes is somewhat less pronounced. With five flashes the fraction of Signal II formed is increased, and with six spaced 370 ms apart this increase is even more substantial, in accord with the model.

Three or four flashes spaced 100 μs apart yield only small quantities of O_2 compared to the case in which the spacing is 10 ms. This observation has been taken as evidence that the relaxation time for concentration changes in the water splitting process is somewhat longer than 100 μs but shorter than 10 ms[16]. We have performed experiments of this nature for Signal II formation in response to two, three and four flashes in which we varied the time between flashes from 100 μs to 10 s. The results of these experiments are shown in Fig. 13. Again, each point represents an experiment with a fresh sample. In order to increase signal-to-noise we performed the experiments kinetically by monitoring the signal level as shown in Fig. 5, except that the instrument time constant was increased to 1.0 s. In these experiments the dark signal was 55–60 % of the fully induced Signal II. Fig. 14 shows typical data; this experiment was done with four flashes spaced 3 7 ms apart followed by single flashes to complete the induction of Signal II. The fraction of Signal II formed is then calculated by dividing the extent of Signal II formation resulting from the initial set of flashes by the fully generated light-induced signal. Referring to Fig. 13, two flashes, regardless of the dark time between the two, always generate greater than 80 % of the light signal. Between 100 μs and 1 ms there is a slight increase in the effectiveness of the two flashes, which

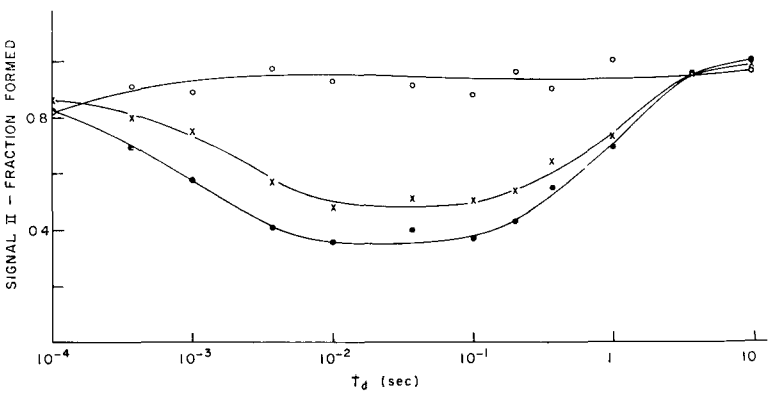


Fig. 13 Response of Signal II in untreated, dark-adapted chloroplasts to sets of 2 (○–○), 3 (–), or 4 (●–●) 10-μs saturating flashes in which the time dark (t_d) between flashes in the set was varied. Each point corresponds to an experiment on a fresh sample of dark-adapted chloroplasts. Normalization of the response of Signal II was carried out as described in Fig 12

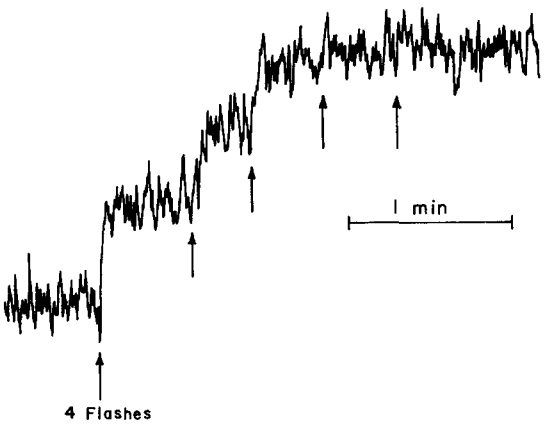


Fig. 14. Response of Signal II to 4 saturating 10-μs flashes spaced 3.7 ms apart, followed by single flashes to complete the photoconversion. At the first arrow the four flashes were given, at the subsequent arrows only a single saturating flash was given. The instrument time constant was 1 0 s.

probably indicates that at the shorter time the chloroplasts are able to process only the first flash whereas at the longer time both flashes are effective in producing oxidized intermediates. The effect of three or four flashes is remarkably different. Between 100 μs and 10 ms the fraction of Signal II generated by the flashes decreases, reflecting the increasing effectiveness of the set of three or four flashes in discharging the pools of oxidized intermediates formed during the flashes. Between 10 and 100 ms is a plateau region for both three and four flashes followed by a region from 100 ms to about 4 s in which the fraction of Signal II increases. This rising section of the curve reflects the observed rise of Signal II, which we showed (Fig. 10) to have a half-time on the order of 1 s. As the time dark between flashes approaches this half-time, proportionately more of the Signal II precursor reacts with the intermediate(s) formed after each flash and not, as with the shorter times, only with the intermediates present following the final flash. At times greater than 4 s, Signal II is fully generated after the second flash and additional flashes have no further effect. At all times less than 4 s three flashes are slightly more effective in generating Signal II than four, indicating that four flashes more completely discharge the pool of oxidized intermediates formed during the sequence.

DISCUSSION

Previous work on Signal II has concentrated on its molecular identity and its general location with respect to the two photosynthetic light reactions. The main conclusions from this earlier work has been the identification of Signal II with either plastoquinone or a plastoquinone derivative and the general association with the O_2-evolving photosystem. Very few kinetic experiments have been reported, primarily because of the difficulty associated with the long decay of the radical; consequently, more specific information regarding its location and mode of generation has been lacking.

We have developed a procedure that allows us to obtain fresh untreated chloro-

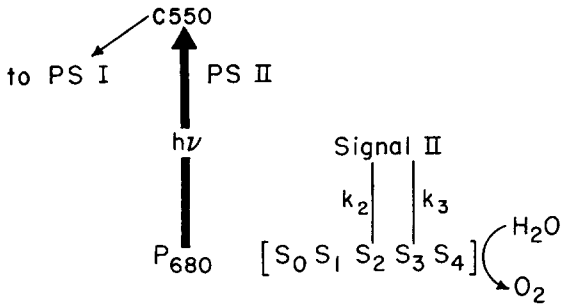


Fig 15. Model for Signal II generation in untreated, dark-adapted chloroplasts. Details described in text.

plasts with low dark levels of Signal II radicals and have made a kinetic analysis of the light-induced increase in Signal II spin concentration primarily using flashing light techniques. Our results can be explained in terms of the model shown in Fig. 15. C550 represents the primary acceptor as described by Erixon and Butler[31]. P680 is the reaction center chlorophyll[32], and S_0 through S_4 represent successively more oxidized intermediates involved in the water splitting process. These states have been described in detail by Kok[16]. Briefly, S_0 and S_1 are stable states persisting in the dark. S_4 is a strong enough oxidant to oxidize water and, once formed, does so in less than 1 ms[33]. S_2 and S_3 are oxidized states formed rapidly after a flash and are stable for 10–20 s in the dark after formation. Signal II arises from a radical which is formed *via* electron transfer to the intermediates S_2 and S_3. The rate constants, k_2 or k_3, for this reaction are approx. 1 s^{-1}, which is low compared to the rates of advance of the S states following a flash.

The evidence from our experiments supporting this model has been briefly discussed in the Results section. Thus, the model explains the greater stimulation of Signal II by red than by far red light observed both by us and by Allen *et al.*[28], and the failure of DCMU to inhibit the formation of the radical. The fact that in DCMU-treated chloroplasts more than a single flash is required to saturate the signal probably reflects a competing back reaction between the reduced primary acceptor and an oxidized intermediate on the water side of Photosystem II which is stimulated by DCMU. The stimulation of this back reaction has been postulated to account for the increased delayed fluorescence observed in chloroplasts treated with DCMU[34]. Bennoun[35] has carried out a detailed study of the kinetics of the back reaction in DCMU-treated chloroplasts and found that the time constant for this process is comparable to the 1-s half-time we have observed for Signal II formation. Since S_2 and S_3 have lifetimes on the order of 10–20 s in untreated chloroplasts, it appears that DCMU decreases the effectiveness of a single flash in generating Signal II by decreasing the lifetimes of the intermediates which give rise to the radical. However, because the reaction center is regenerated by the back reaction and is therefore able to be reexcited, subsequent flashes eventually fully induce Signal II. Bennoun[35] has shown that hydroxylamine inhibits the back reaction in DCMU-treated chloroplasts by a rapid re-reduction of the oxidized intermediates on the water side of Photosystem II and Lozier and Butler[18] have shown that under these conditions the light response of

Signal II is completely inhibited. Similarly CCCP, which stimulates the re-reduction of S_2 and S_3, has been shown to inhibit the light response of Signal II in DCMU-treated chloroplasts[18]. Therefore the formation of Signal II is quite sensitive to the lifetimes of oxidized intermediates, and treatments which destabilize the S states serve to reduce the efficiency of Signal II generation[20].

The high quantum efficiency for Signal II formation following a single flash (Fig. 6) is a consequence of the relative stability of the S states in untreated chloroplasts. The experiments of Kok *et al*[16] and Joliot *et al.*[30] have shown that the S states are advanced with high quantum efficiency by a single flash and that following the flash their decay time is on the order of 10–20 s. This time is long compared to the 1-s onset time for Signal II generation, and it allows for efficient formation of the radical. A corollary to this argument is that with four flashes spaced 10 ms apart the quantum efficiency for Signal II formation is decreased since the lifetime for the S_4 state is only on the order of a millisecond. This analysis also explains the lowered quantum efficiency we observe for Signal II formation in *Chlorella* since Joliot *et al.*[36] have shown that the lifetimes for S_2 and S_3 are about five times shorter in this alga than in spinach chloroplasts. Similarly we have found with CCCP-treated chloroplasts that the quantum efficiency for Signal II formation is decreased[20], which is a consequence of the action of CCCP in decreasing the lifetime of the oxidized intermediates following a flash.

In the model proposed in Fig. 15, Signal II arises by an interaction with S_2 or S_3, in which the species giving rise to Signal II is oxidized by an S state. This interaction may be represented as

$$S_n \xrightarrow[P680]{h\nu} S_{n+1} \tag{1}$$

$$S_{n+1} - F \xrightarrow[k_{n+1}]{} S_n + F^{+}\cdot \tag{2}$$

where S_n is an S state with $n = 1$ or 2, S_{n+1} is one equivalent more oxidized than S_n and F represents the species which, when oxidized, to the radical $F^{+}\cdot$, gives rise to Signal II The behavior of the state S_n in this scheme is such that at the conclusion of the process its final oxidation state is the same as its initial state even though a photon has been absorbed by Photosystem II. This situation in which an S state is left unchanged by a flash has been termed a "miss" in Kok's model[16] for O_2 evolution in flashing light[37]. Thus the process of Signal II formation viewed *via* O_2 evolution results in an increased number of misses on the first three or four flashes. This increased number of misses accounts for the lowered yield of O_2 on the third flash and increased O_2 yield on the fourth flash shown in Fig 9. Computer programs to fit O_2 evolution curves such as those generated by the preilluminated chloroplasts in Fig. 9 usually contain a "miss parameter" to account for S states which are not advanced by a flash. Our results indicate that this parameter may be larger for the first few flashes than it is for later flashes (Ley, A. C. and Babcock, G. T., unpublished results).

We postulate that the Signal II precursor, F, can be oxidized by either the state S_2 or S_3 on the basis of the results presented in Fig 12. Following a single roughly 75 % of the Photosystem II centers are in the state S_2 and 25 % in the state S_1. Under these conditions we observe 80 % of the light-induced increase in Signal II. Following two flashes 10 ms apart 75 % of the centers are in the S_3 state and 25 % in the state S_2.

With this flash pattern we observe greater than 95 % photoproduction of Signal II. Therefore the concentration of $[S_2+S_3]$ following a flash sequence parallels the extent of the light-induced generation of Signal II.

The results of Fig. 13 present the strongest evidence in support of our proposed model. The concentration of $[S_2+S_3]$ is always high after two flashes regardless of the time dark between the two and correspondingly the fraction of Signal II formed under these conditions is high. After three or four flashes $[S_2+S_3]$ are formed to an appreciable extent only if the flashes are spaced less than about 5 ms apart. At times longer than this the O_2 system is able to process each of the flashes individually and the high concentrations of S_2 and S_3 produced by the first two flashes are discharged on the third and fourth. Similarly, the fraction of Signal II formed in response to three or four flashes is high at very short flash intervals and declines as the time between flashes is increased, until a plateau at about 0.4 in the fraction of Signal II formed is reached from 10 to 100 ms for four flashes and at about 0.5 through this time range for three flashes This plateau region is non-zero due to the fact that not all of the S states have been completely cycled during the four flashes; the concentrations of S_2 and S_3 following the fourth flash are non-zero as indicated by the finite O_2 yields of the fifth and sixth flashes.

The kinetic behavior of Signal II identifies it as the first endogenous species besides water (or reduced primary acceptor, in a back reaction) that is able to interact directly with the O_2-evolving complex in photosynthesis at physiological temperatures. The work of Knaff and Arnon[38], Erixon and Butler[31,39] and others has shown that at low termperatures cyt b_{559} is able to donate electrons to an intermediate on the oxidizing side of Photosystem II, probably P680, but the effect is lost upon increasing the temperature above -100 °C (ref. 40). We have also shown that the Signal II precursor is able to interact with the specific intermediates S_2 and S_3, but not with S_0 and S_1. Bennoun and Joliot[41] have shown that hydroxylamine is able to override O_2 evolution, but this most likely occurs by a direct interaction with the reaction center chlorophyll or its primary donor. Similarly, we have studied the oxidation of phenylenediamine and hydroquinone in Tris-washed chloroplasts in flashing light and have found neither the oscillations nor the two flash induction period found in O_2 evolution, indicating that in this system these reductants interact by a mechanism similar to that exhibited by hydroxylamine.

In this study we have focused on the kinetics and location of the Signal II species. Consequently our experiments yield no new information as to the molecular identity of the radical. Kohl and coworkers[11,12] have presented evidence implicating a derivative of plastoquinone as the source for the Signal II spin, mainly on the basis of extraction, deuteration and readdition experiments with chloroplasts and model compound studies *in vitro*. If this assignment proves correct, the experiments reported here provide the first evidence for a known species other than chlorophyll located on the oxidizing side of Photosystem II Our model postulates that Signal II characterizes an oxidized radical, however, in experiments where we have treated chloroplasts with hydroquinone and ascorbate we note only a slight increase in its rate of decay; treatment with an oxidant, ferricyanide, has the same effect. Recently Lozier and Butler[18] reported that in Tris-washed chloroplasts the decay of Signal II is greatly enhanced by ascorbate and we have found similar effects for ascorbate on the decay of the radical in System II particles prepared as described

by Malkin[42]. Signal II appears to share with the O_2-evolving system the characteristic of being normally unavailable to exogenous redox couples, and only under fairly extreme conditions does this complex become accessible. Since Photosystem II generates very strong oxidants it operates much more efficiently when the access of potential reductants other than water is limited.

Our results shed little light on the functional role of Signal II Its stability following formation precludes an integral role in electron transport Cyt b_{559} is similar to Signal II in that light-induced electron transfer through this component at room temperature in untreated chloroplasts is also not observed. The purpose for which chloroplasts maintain these components in these stabilized states is unclear at present.

The integrity of the environment in which the radical is located does appear to be related to the ability of the chloroplasts to evolve O_2. Treatments which decrease the stability of Signal II, such as heating, aging and sonication, have also been shown to impair O_2 evolution. Furthermore, early workers demonstrated that in mutants or manganese-deficient algae the inability to evolve O_2 was accompanied by an inability to generate Signal II We have shown in Fig. 4 that even after 12 h dark *in vivo* the signal decays to only half its value, while Lozier and Butler[18] found a room temperature decay to the 50 % level in approx. 1 h in the dark in isolated chloroplasts. Therefore, it appears that Signal II is inhomogeneous, exhibiting a fraction which decays slowly in the dark and a fraction which is much more stable. It may be that one or both of these components are involved, perhaps in a structural capacity, in O_2 evolution.

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