AN INVESTIGATION OF ELECTRON SPIN RESONANCE IN WILD TYPE *CHLAMYDOMONAS REINHARDI* AND MUTANT STRAINS HAVING IMPAIRED PHOTOSYNTHESIS

R. P. LEVINE *and* L. H. PIETTE

From The Biological Laboratories, Harvard University, Cambridge, Massachusetts, and the Instrument Division, Varian Associates, Palo Alto, California

ABSTRACT The electron spin resonance signals of wild type *Chlamydomonas reinhardi* and three mutant strains having impaired photosynthesis have been investigated. The wild type strain generates two different electron spin resonance signals. Signal I is obtained without illumination (*i.e.*, dark signal) whereas signal II is generated preferentially only by red light. Signal I is missing from wild type cells that have been cultured in the dark, but it returns after these dark-grown cells have been illuminated. Chloroplast fragments obtained from the three mutant strains cannot photoreduce TPN. Two of the strains lack the dark signal I while the third strain has both signal I and signal II. Other studies have revealed that the two mutant strains which lack signal I give no Hill reaction but that they can photoreduce TPN if supplied with an artificial reductant. The mutant strain which has both electron spin resonance signals can carry out the Hill reaction, yet it too will not photoreduce TPN unless reductant is supplied. The electron spin resonance signals generated by the wild type and mutant strains are discussed in terms of the pathway of TPN photoreduction, and it is suggested that signal I is associated with one of the two light-dependent phases of this pathway.

INTRODUCTION

The primary products of photosynthesis are considered to be reduced pyridine nucleotide and ATP[1] (1). Recently, a number of pathways have been proposed to explain the light-dependent sequence of reactions leading to the formation of these products (2-5). There remain, however, major gaps in our knowledge of the

[1] Abbreviations used: ATP, adenosine triphosphate; TPN, triphosphopyridine nucleotide; DPIP, 2,6-dichlorophenol indophenol; ESR, electron spin resonance; and *g*, the measure of the contribution of the spin and the orbital motion of the electron to its angular momentum.

mechanism of photosynthesis, and as a consequence none of the proposed pathways is entirely satisfactory.

The pathway by which reduced pyridine nucleotide and ATP are generated during photosynthesis in the unicellular green alga *Chlamydomonas reinhardi* has been under investigation with mutant strains that have impaired photosynthesis (6-8). Cells of these strains, all of which possess normal amounts of chlorophyll *a* and chlorophyll *b*, are unable to fix carbon dioxide in the light at a rate comparable to that of the wild type strain (6, 7). The mutant strains are maintained in culture by growing them in a minimal medium (9) that has been supplemented with sodium acetate. Three of the strains have been used to investigate the pathway of TPN photoreduction (8).

The photoreduction of TPN is experimentally separable into two phases. The first phase includes the photo-oxidation of water and the production of an unknown reductant. This phase can be demonstrated as the Hill reaction using DPIP or several other compounds as the terminal electron acceptor. The second phase includes the oxidation of the reductant and the reduction of pyridine nucleotide. This can be demonstrated experimentally using ascorbate and the reduced DPIP as the electron donors (10-12). The two phases of TPN photoreduction have been shown to be independent of each other (11, 12).

The photoreduction of TPN has been studied with preparations of chloroplast fragments from wild type *C. reinhardi* and three mutant strains (*ac-21*, *ac-115*, and *ac-141*) according to the technique of San Pietro and Lang (13). Details of the procedure for *C. reinhardi* as well as the results of the studies with the wild type and mutant strains are described elsewhere (8). In brief, it has been demonstrated that chloroplast fragments obtained from two strains, *ac-115* and *ac-141*, cannot carry out the first phase of TPN photoreduction; however, they can carry out the second phase if reduced DPIP and ascorbate are supplied. Chloroplast fragments from the mutant strain *ac-21* cannot photoreduce TPN yet they can carry out both phases of TPN photoreduction. These results suggest that *ac-115* and *ac-141* are blocked in some step in the first light-dependent phase of TPN reduction. On the other hand, since *ac-21* is capable of performing both light-dependent phases it is assumed that the block lies in a step independent of light (8).

The present paper reports the results of an investigation of wild type and mutant *C. reinhardi* using the technique of ESR spectroscopy. ESR spectroscopy has been used to investigate light-dependent reactions in photosynthesis (14-18), and Allen, Piette, and Murchio (17, 18) have suggested that the two ESR signals detected in *Chlorella pyrenoidosa* may be attributed to two different light-dependent reactions. *C. reinhardi* wild type also shows two ESR signals (19). The results presented here show that the two mutant strains which cannot carry out the first phase of TPN photoreduction have lost one of these two ESR signals while the mutant strain which can carry out both phases has both signals.

MATERIALS AND METHODS

Wild type strain 137c of *C. reinhardi* was used in the experiments described here. The mutant strains were obtained from it by irradiation with ultraviolet light (20). These strains, as well as the wild type strain, are capable of chlorophyll synthesis in both the light and the dark. Cells were grown at 27°C in shake cultures of high salt minimal medium (21) supplemented with sodium acetate and at a light intensity of 2500 lux from daylight fluorescent lamps. For one experiment the wild type cells were cultured in the dark. Cells from cultures in the logarithmic phase of growth were concentrated by centrifugation and resuspended in their original culture medium before being placed in the cuvette of the ESR spectrometer. Cell concentration ranged from 9.4×10^7/ml to 11.8×10^7/ml.

The ESR spectra were measured according to the technique described by Allen, Piette, and Murchio (18) using a Varian V4501 spectrometer having a field modulation of 100 kc. Samples of *C. reinhardi* were placed in a cuvette (Varian V4546 Aqueous Sample Cell) having an effective volume of 0.05 ml, and the spectra were recorded on a Moseley 2D X-Y recorder. Diphenylpicryl hydrazyl ($g = 2.0036$) was used to determine g values. This was accomplished by placing a small, solid sample of the compound, contained in a capillary tube, into the cavity but outside of the cuvette. Magnetic field sweep rates were determined by calibrating the field scan against an aqueous solution of peroxylamine disulfonate having a hyperfine splitting of 13.0 gauss. Field modulation amplitude was adjusted for optimum resolution and signal-to-noise (2.5-5.0 gauss). Cells in the cavity of the spectrometer were illuminated with a collimated beam of light from a 500 watt projection lamp. Monochromatic light of 540 mμ was obtained with a narrow band interference filter and red light (640 mμ and above) was obtained with a Corning 2-64 filter. The intensity of light obtained using the Corning 2-64 filter was 30 times greater than that of the 540 mμ as measured with a calibrated thermopile and ammeter. The Corning filter is a broad band filter passing light above 640 mμ whereas the 540 mμ filter is a narrow band filter (~10A). The red light was found to be saturating but not the 540 mμ light.

The ESR spectra for cells cultured and handled as described above were found to be highly reproducible. The spectra presented for light-grown cells are typical of those obtained from cells of at least four independent cultures of wild type and each of the mutant strains. Cells from only one culture of dark-grown wild type were tested.

RESULTS

The ESR spectra obtained with wild type *C. reinhardi* grown in the light are shown in Fig. 1*a*, *b*. The spectra were obtained using an X-Y recorder by first scanning the appropriate magnetic field with no illumination followed by a second scan of the same magnetic field during illumination of the cells with 540 mμ light and finally a third repetitive scan during illumination with red light using the Corning 2-64 filter. The recorder Y-axis was then vertically displaced and the same magnetic field scanned once again with no illumination, Figure 1*b*.

A broadline signal I was observed during the scan in the absence of illumination. This signal I increased slightly during the second scan with 540 mμ light but did not change during the third scan with red light from the Corning 2-64 filter even

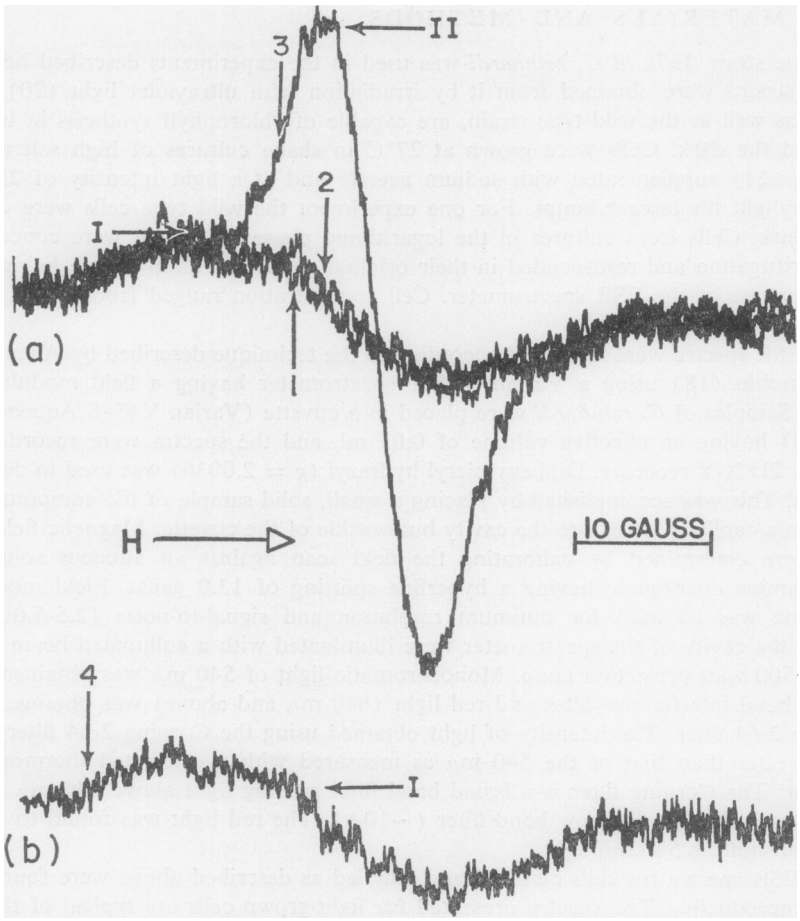

FIGURE 1 ESR spectra of wild type *C. reinhardi*. (*a*): *1*, Magnetic field scan in the absence of illumination; *2*, Magnetic field scan with 540 mμ light; *3*, Magnetic field scan with red light (Corning 2-64 filter); I, Multicomponent resonance at $g = 2.0036$; II, Narrow resonance, line width = 8.2 gauss and $g = 2.0023$. (*b*): 4, Retrace of magnetic field scan in the dark after light experiment. Only signal I remains.

though this filter gives 30 times more light. Signal I was observed to be an unresolved multiplet with a g value of 2.0036. The decay of this signal I in the dark was very slow; no appreciable decay was detected for wild type cells when they were allowed to remain in the culture flask in the dark for 6 hours.

A second signal, signal II, was observed during the scan with red light illumination. This signal was a single narrow line of width 8.2 gauss and a g value of 2.0023. It decayed in approximately 80 msec. when the red light was extinguished. In general signal II could be generated only by the longer wave length irradiation; however, a weak signal associated with signal II could be induced by 540 mμ il-

lumination if the cells had been allowed to become anaerobic by storing them in the cuvette in the dark for 1 hour or more. Under these conditions the magnitude of signal II was also greatly enhanced by irradiation with red light.

Fig. 2 shows an action spectrum for the induction of the ESR signal II. The action spectrum was obtained by plotting the ESR signal II intensity (corrected for constant energy of light illumination) as a function of wave length as obtained

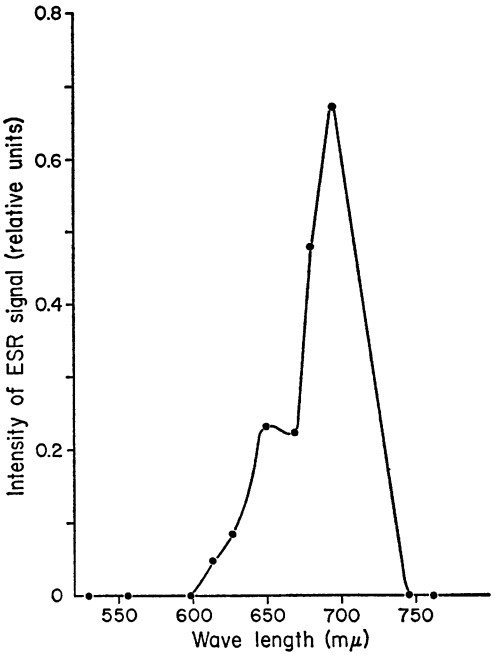

FIGURE 2 Action spectrum for the induction of the ESR signal II. The action spectrum was obtained by plotting the intensity of the ESR signal II (corrected for constant energy of light illumination) as a function of wave length as obtained with a series of narrow band interference filters.

from a series of narrow band interference filters. The spectrum shows a maximum at 694 mμ and another at 650 mμ. The absorption maximum for chlorophyll *a* in whole cells is at 680 mμ.

While the six line spectrum of manganese has been observed in ESR spectra of *Chlorella pyrenoidosa* (18), it was not detected here except when cells were allowed to remain in the dark in the cuvette for several hours. When the cells were treated in this way it was observed that the intensity of the six-line hyperfine of Mn++ increased with time from a negligible value to one of several orders of magnitude greater than the initial concentration of the Mn++ placed in the original medium. This result indicates that *C. reinhardi* is capable of concentrating Mn++ in the cell in either a strongly complexed form or different oxidation state (as evidenced by lack of ESR Mn++ signal in freshly prepared cells).

Fig. 3*a*, *b* shows the results of measurements made with wild type cells cultured in the dark and transferred to the cuvette in the dark. The recording in Fig. 3*b* was

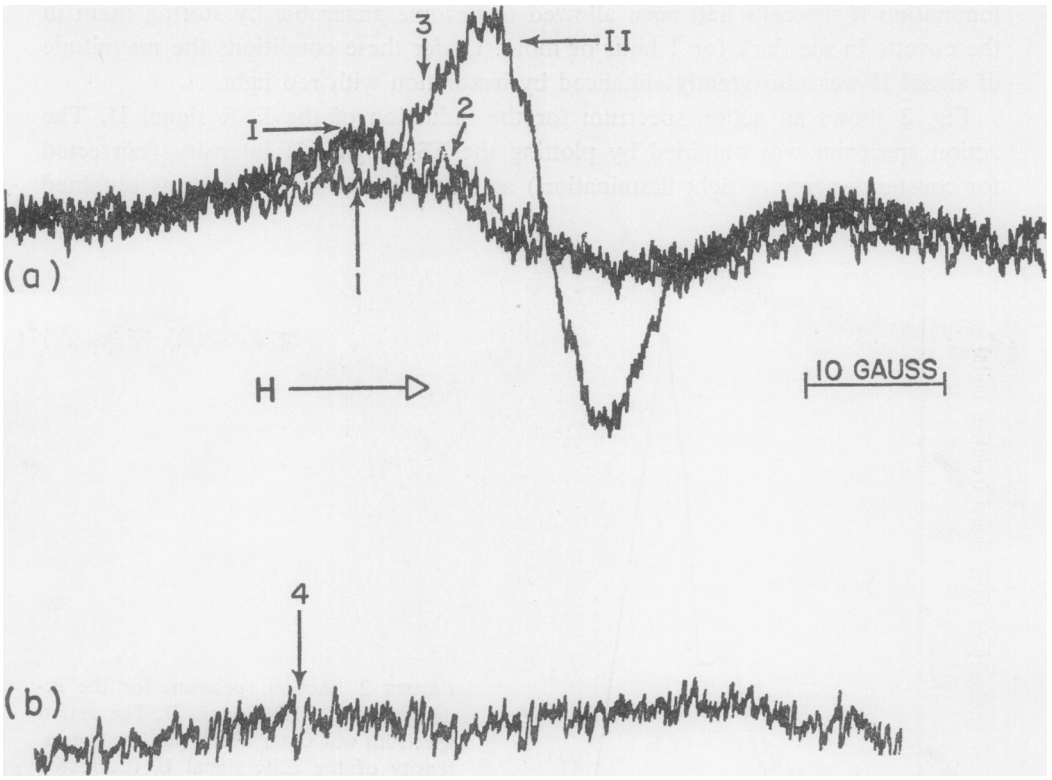

FIGURE 3 ESR spectra of dark-grown wild type *C. reinhardi*. (*a*): *1*, Magnetic field scan with 540 mμ light; *2*, Magnetic field scan in absence of light after having been irradiated with 540 mμ light; *3*, Magnetic field scan with red light after having been irradiated with 540 mμ light. (*b*): *4*, Magnetic field scan with dark-grown cells before any exposure to light.

obtained during the magnetic field scan without illumination. Under these conditions the dark-grown cells gave no spectrum characteristic of signal I. Figure 3*a* shows the spectra obtained when the field was scanned first with 540 mμ light followed by red light and then a third trace with no light. In the presence of 540 mμ light a signal characteristic of I returns immediately and also a weak signal characteristic of II. In the presence of red light the signal II increases, probably because of the difference in light intensity. When the light was extinguished only signal II disappeared; signal I remained and was comparable in intensity to that observed for wild type grown in the light. Both signals I and II reappear when the sequence of light irradiation is reversed and the cells appear to behave as normal, light-grown, wild type.

The ESR spectra obtained with *ac-21* (Fig. 4*a, b*) were found to be similar to

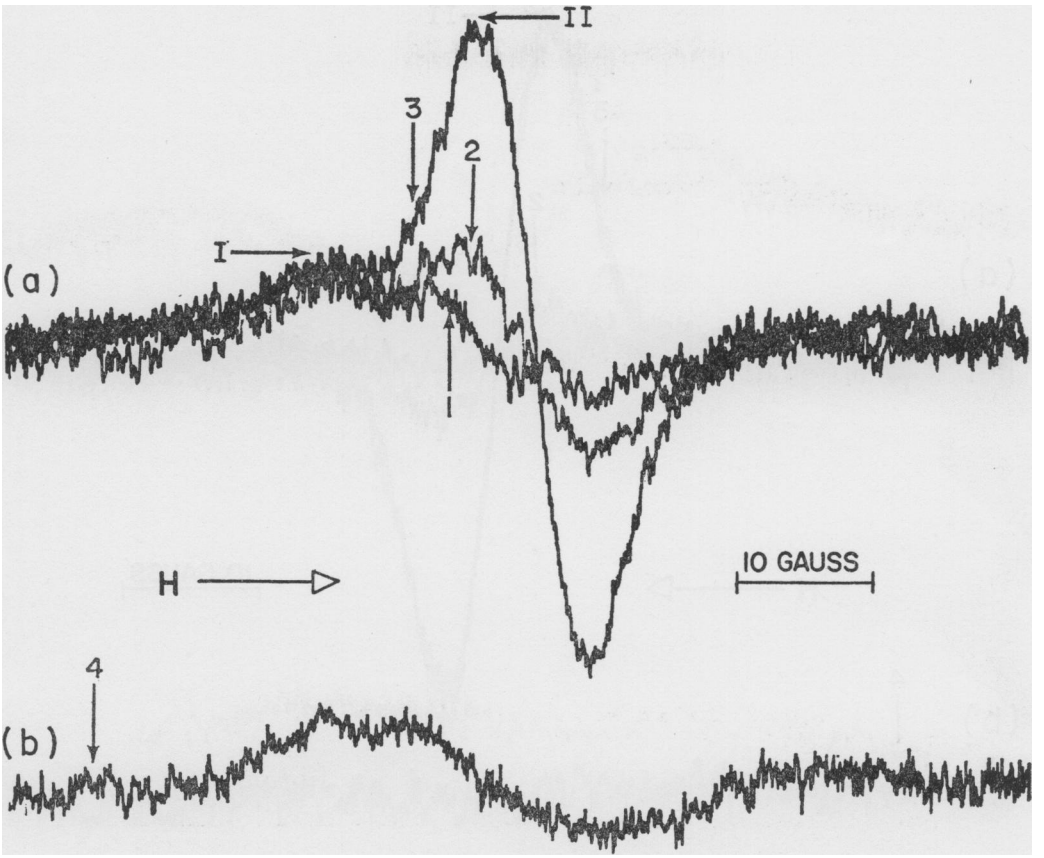

FIGURE 4 ESR spectra of *ac-21*. (*a*): *1*, Magnetic field scan in absence of light; *2*, Magnetic field scan with 540 mμ light; *3*, Magnetic field scan with red light. (*b*): *4*, Retrace of magnetic field scan in the dark after light experiments.

those of wild type with the exception that in addition to signal I, a small signal II could be generated during illumination with 540 mμ light. The nature of this response is not clearly understood. It may be non-specific and due to a lack of oxygen in the cuvette as was the case with wild type cells which had been kept in the cuvette for an hour or more. The magnitude of signal I in the dark was analogous to that for wild type.

The results obtained with *ac-115* and *ac-141* are shown in Figs. 5*a, b* and 6*a, b*. The sequence of illuminations was as before; dark, 540 mμ, red, and a displaced trace again in the dark. The distinct feature of these spectra is the absence of signal I, regardless of radiation used. A weak response of signal II to 540 mμ light was observed for *ac-115* and *ac-141*.

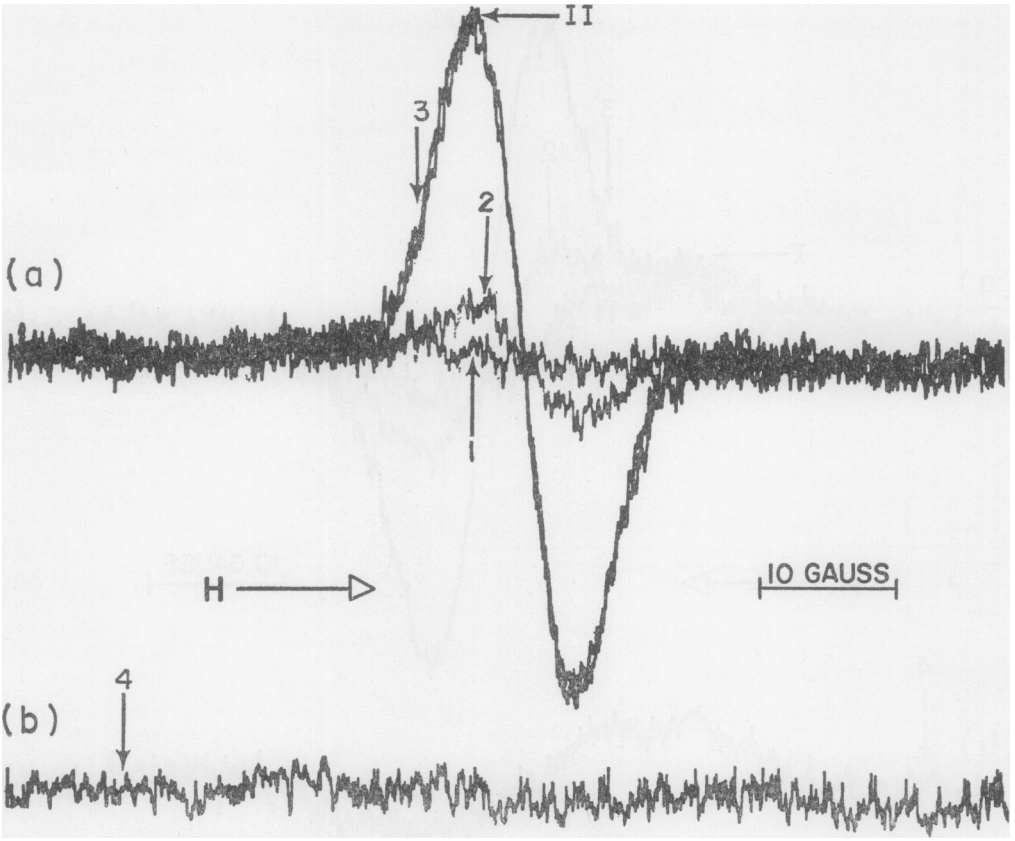

FIGURE 5 ESR spectra of *ac-115*. (*a*): *1*, Magnetic field scan in absence of light; *2*, Magnetic field scan with 540 mμ light; *3*, Magnetic field scan with red light; (*b*): *4*, Retrace of magnetic field scan in the dark after light experiments.

DISCUSSION

ESR spectra obtained from illuminated unicellular algae or isolated higher plant chloroplasts presumably reflect free radicals produced during photosynthesis. The nature of these free radicals is unknown and at best ESR spectra can only be correlated with photosynthetic reactions and not casually related to them. It has been suggested for *Chlorella pyrenoidosa* (18) that signal I is associated with a reaction involving chlorophyll *b* while signal II is associated with a reaction involving chlorophyll *a*. In other words, two different light reactions of photosynthesis can be detected by ESR spectroscopy.

The wild type and mutant strains of *C. reinhardi*, which have both chlorophyll *a* and *b*, reveal an important relationship between ESR spectra and the two light-dependent phases of TPN reduction. Two different ESR signals have been detected

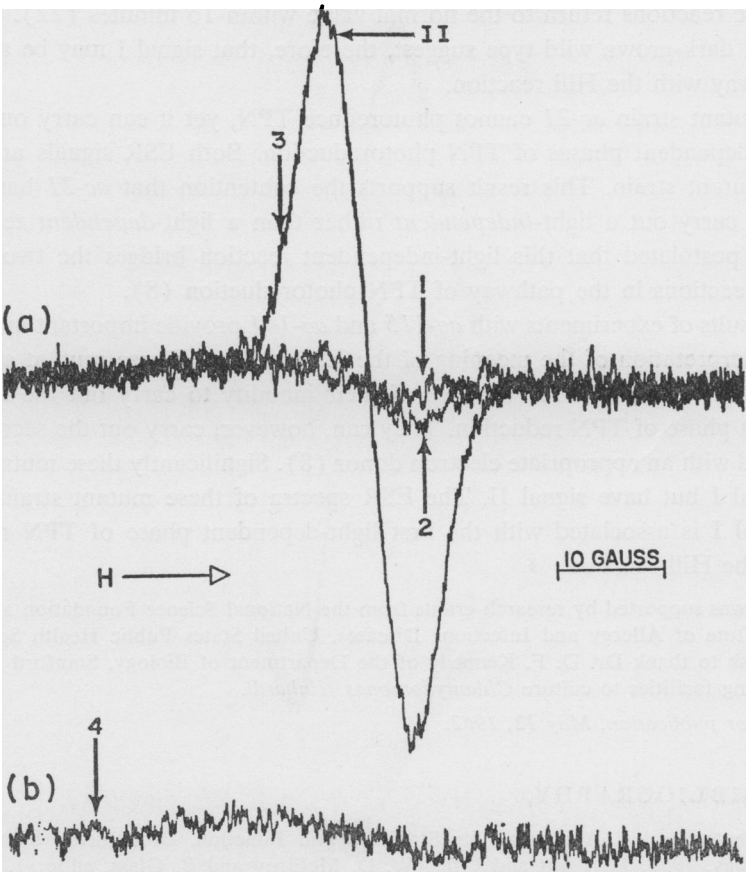

FIGURE 6 ESR spectra of *ac-141*. (*a*): *1*, Magnetic field scan in absence of light; *2*, Magnetic field scan with 540 mμ light; *3*, Magnetic field scan with red light. (*b*): *4*, Retrace of magnetic field scan in the dark after light experiments.

with wild type *C. reinhardi*. Cells grown in the light or the dark give a narrow, rapidly decaying signal (signal II) upon illumination with red light. Cells grown in the light also give a broad, complex signal in the absence of illumination or with illumination with 540 mμ light (signal I). Signal I is retained even after the cells are transferred to the dark for 6 hours, but it is missing in cells cultured in the dark. However, signal I reappears upon illumination with either 540 mμ light or with red light for those cells cultured in the dark. This suggests that signal I depends upon light either for its generation or, more likely, that light is required for the synthesis of a substance associated with this signal. Dark-grown wild type cells have a low rate of photosynthetic oxygen evolution, and their chloroplast fragments have a low Hill reaction rate with potassium ferricyanide. After exposure to light, how-

ever, these reactions return to the normal value within 16 minutes (22). The ESR spectra of dark-grown wild type suggest, therefore, that signal I may be associated in some way with the Hill reaction.

The mutant strain *ac-21* cannot photoreduce TPN, yet it can carry out both of the light-dependent phases of TPN photoreduction. Both ESR signals are present in this mutant strain. This result supports the contention that *ac-21* has lost the ability to carry out a light-*independent* rather than a light-*dependent* reaction. It has been postulated that this light-independent reaction bridges the two light-dependent reactions in the pathway of TPN photoreduction (8).

The results of experiments with *ac-115* and *ac-141* provide important information for an interpretation of the meaning of the ESR spectra. These mutant strains are unable to photoreduce TPN because of their inability to carry out the first light-dependent phase of TPN reduction. They can, however, carry out the second phase if supplied with an appropriate electron donor (8). Significantly these mutant strains lack signal I but have signal II. The ESR spectra of these mutant strains suggest that signal I is associated with the first light-dependent phase of TPN reduction; namely, the Hill reaction.

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