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PHOTO-INDUCED ELECTRON PARAMAGNETIC RESONANCE IN MUTANT PHOTOSYNTHETIC SPECIES LACKING CAROTENOIDS OR CHLOROPHYLL

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SUMMARY

The association of the photo-induced electron paramagnetic resonance signal in photosynthetic tissue with the necessity for the presence of chlorophyll is demonstrated with a variety of mutant organisms. This is taken as additional evidence of the direct electron-transfer reaction from and/or to chlorophyll as the primary quantum conversion step.

I. INTRODUCTION

The two pigment molecules found in all photosynthetic organisms are the chlorophylls and the carotenoids. We have studied mutant strains of photosynthetic organisms in which one or the other of these ubiquitous molecules was missing in order to determine their possible connection with the EPR which can be photo-induced in photosynthetic materials generally. A strong dependence of the photo-induced EPR on the presence or absence of these molecular species should shed some light on the role in photosynthesis of the unpaired electrons responsible for the resonance.

The carotenoid-less strain studied was a blue-green mutant of *Rhodopseudomonas spheroides* which was supplied to us by Dr. R. Y. STANIER. It has been designated UV 31, and is obtained by subjecting the wild type (241 c) to ultraviolet radiation. Although carotenoids, as such, are absent in the mutant, there is present a molecule of similar structure. This is a colorless C_{40} polyene, phytoene[1]. The mutant grows photosynthetically, and exhibits physiological behavior which differs from that of the wild type[2,3].

The chlorophyll-less strain was a yellow mutant of *Chlamydomonas reinhardi* supplied to us by Dr. R. SAGER. Several different mutant strains were used (see Section III). This mutant does not grow photosynthetically. It grows in the dark, synthesizing only a few per cent of its normal chlorophyll complement. When exposed to light, chlorophyll synthesis is accelerated, and after several hours the mutant is completely greened.

Abbreviation: EPR, electron paramagnetic resonance.

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In Section II we discuss the differences in the EPR behavior noted between the wild type and the carotenoid-less mutant of *R. spheroides*. In Section III the chlorophyll-less mutant of Chlamydomonas is considered. Our experiments are concerned with the period during which this mutant greened. The EPR amplitude, as well as the behavior of several other physiological variables, was followed during the time of greening. The conclusions which follow from these experiments are discussed in Section IV.

II. CAROTENOID-LESS MUTANT OF *R. spheroides*

A comparison of the EPR in the wild type and the ultraviolet mutant of *R. spheroides* is difficult to make when the cells are in good physiological condition in aqueous suspension. The difficulty arises from the fact that the EPR amplitude is a complicated function of light intensity, oxygen concentration (when present in trace amounts), time, and perhaps other as yet undetermined variables. This situation does not arise from properties peculiar to these two bacterial strains, but is common also to *Chlorobium thiosulfatophilum* and to *R. rubrum* (the bacteria we have studied most under these conditions).

The observed behavior is represented schematically in Fig. 1. When light energy is absorbed at a rate which is fast compared to subsequent recovery reactions in the cell, the equilibrium concentration of unpaired electrons becomes small. At sufficiently high light intensities the unpaired-electron concentration is reduced, in a time which depends on the light intensity, to a level which is not detectable. Behavior at any given light intensity can be approximately reproduced after a dark period of about 0.5 h.

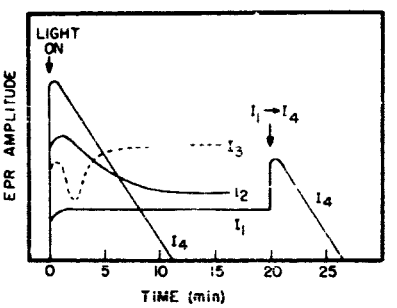

Fig. 1. Schematic representation of the behavior of the photo-induced EPR in whole *R. rubrum* in good physiological condition as a function of light intensity and time. $I_1 < I_2 < I_4$. Dashed curve (I_3) shows induction phenomena frequently observed after long dark periods. At right intensity I_1 is changed to I_4 with no intervening dark period.

The removal of oxygen to approx. 10^{-6} M with purified argon does not alter the general behavior. The presence of oxygen generally enhances the maximum photo-induced EPR amplitude achieved at a given light intensity, and alters the rates of the reactions subsequent to light absorption so that it takes higher light intensities to reduce the unpaired-spin concentration to a level which is not detectable. It is known that the presence of oxygen inhibits the usual light-driven reactions in photosynthetic bacteria[4,5]. It is likely that oxygen reacts with the bacterial apparatus at several points as an electron sink.

Induction phenomena were frequently observed if the sample had been allowed to stand in the dark for some time (order of 0.5 h—dashed curve of Fig. 1). These phenomena were not observed when the light intensity was readjusted after an initial illumination at a different light intensity (right portion of Fig. 1). New light intensities served only to alter the kinetics of the processes internal to the cell.

We have attempted to stabilize the observed EPR in two ways. In the first method whole cells were dried in an oxygen or nitrogen atmosphere. The sample material was a relatively thin film of dried cells on a rod or a flat plate. In these samples the photo-induced EPR light saturates in the usual way[6] and is stable in time. In the second method, chromatophores were prepared from the whole cells and observed in aqueous suspension. (The general method of preparation has been described elsewhere[7]. Both the wild and the mutant *R. spheroides* required 12 min sonication in that step of the procedure.) Relatively stable EPR signals were observed in chromatophores prepared from both the wild type and the carotenoid-less mutant.

In the remainder of this section we shall compare the behavioral differences noted between the two strains in these two stabilized sample materials.

The differences noted are of a secondary nature. Photo-induced EPR's of approximately equal amplitude are observed in dried whole cells or chromatophores of both strains, although the chlorophyll concentration in the wild-type cell was, on the average, at least twice that of the mutant cell[8]. The resonance g-values are the same within experimental error ($g = 2.0026$ was measured). The line widths were the same within experimental error. (After approximate correction for modulation broadening[9] a value of 11.2 ± 0.4 Gauss was obtained.) The behavior of the kinetics of rise and decay of the photosignal as a function of temperature is the same in both strains, and parallels the behavior observed in *R. rubrum*[6].

The forms of the action spectra, taken with chromatophores of the respective strains in suspensions of low absorbancy[7], appear to show one difference, but this is, perhaps, not surprising in view of the differences in their absorption spectra. The absolute form of the action spectra is uncertain—*i.e.*, the relative amplitudes of the peaks observed in different spectra were quite variable. One possible reason for this arises from the fact that the amplitude of the EPR observed at a particular wavelength decreases slowly with time. During the 6–8 h of intermittent irradiation required for the taking of a spectrum, the signal amplitude at a particular wavelength decreased in the chromatophores of both strains by as much as a factor of two. The rate of decrease was not constant in time, so that even by taking every third point at the same wavelength this source of uncertainty could be only partially accounted for. The relative amplitudes in the final spectrum depended quite critically on the precise form assumed for the correction curve. Moreover, only fair agreement was found between the correction curves deduced from following two separate wavelengths with time. We will thus present no complete action spectra.

Keeping these restrictions in mind the action spectra in the two different strains were mutually consistent in two respects. (1) As has been found in other optically dilute suspensions of photosynthetic materials[7], the long-wavelength maxima of the action spectra fell on the long-wavelength maxima of the respective absorption spectra. In the case of the wild type of *R. spheroides* there is evidence of the complex structure of this absorption band in the action spectrum, but, as has been remarked, the relative amplitudes of these maxima do not repeat consistently. (2) The action

spectra were generally different in the carotenoid region of the absorption spectrum. The type of difference noted is shown especially well in the data reproduced in Fig. 2. In the wild type the concentration of unpaired electrons increases as the carotenoid band is entered, while in the carotenoid-less mutant the concentration falls in the same spectral region.

One further difference in behavior was observed. The photo-induced EPR in the mutant is more easily saturated with microwave power than is the resonance in the wild type, indicating that the immediate environment of the electron is affected by the absence of carotenoids[10].

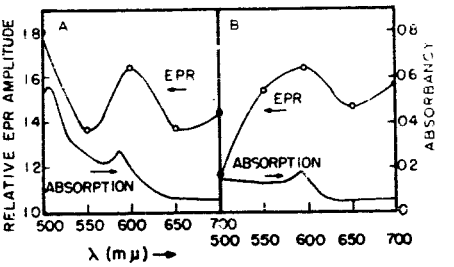

Fig. 2. Portions of the action spectra determined for chromatophores from the wild type (A) and the ultraviolet mutant (B) of *R. spheroides*, showing the different forms observed in the carotenoid region of the absorption spectra. $8 \cdot 10^{15}$ quanta/sec; 100 Å band width.

III. GREENING OF CHLAMYDOMONAS MUTANT

This experiment was difficult to carry out in that the mutant reverted several times. In all, three different yellow Chlamydomonas mutants were used, Strains 2052 C 6303 D, and 6415 C, each obtained from Dr. R. SAGER's laboratory. Results obtained from the three strains were consistent with each other.

Two procedures were followed in readying the cultures for the start of the greening experiment. At first, 6 2.5-l culture flasks containing 1 l of acetate growth medium[11] were inoculated. The cultures were then allowed to grow in the dark until the cell suspension was sufficiently dense for the requirements of the experiment (usually about 2 weeks). The 6 flasks were then exposed to uniform illumination (about 700 ft candles), and the synthesis of chlorophyll commenced. At the time of exposure to light each flask had been developing independently for approx. 2 weeks. In order to obviate culturing differences in the later repetitions of the experiment the dark growth took place in 1 large volume. At the end of the developmental period this volume was divided into six and exposed to uniform illumination. At the time of exposure to light the chlorophyll concentration was on the order of 5 % of its final value in the fully greened cells.

One of the 6 volumes was harvested immediately upon exposure to light. The remaining 5 flasks were harvested at 3-h intervals following the first one. This interval spread the harvesting periods equally over the time necessary for the greening to go to completion under the conditions used. Portions of each volume of harvested cells were used in several separate experiments. Chlorophyll concentration (*a* plus *b*) was determined spectrophotometrically[12]. The ratio of chlorophyll *a*/chlorophyll *b* was constant (of the order of 1) during the greening. The rate of oxygen evolution was

determined for two aliquots from each stage of greening by manometry. The cell suspensions for this determination were very dilute (0.032 ml of wet packed cells in 3 ml of Na_2CO_3–$NaHCO_3$ buffer), and the light intensity was 5–10 times greater than that used for greening. The temperature was 22°. The maximum oxygen evolution rates recorded were of the order of 130 μmoles O_2/mg chlorophyll/h

A rate of carbon fixation was also determined at each stage of greening by measuring the amount of $^{14}CO_2$ uptake in 2 min[13]. Again the cell suspension was dilute (0.032 ml wet cells in 1 ml of Na_2CO_3–$NaHCO_3$ buffer), and the light intensity and temperature the same as that used in determining oxygen-evolution rates. On one occasion the pattern of carbon fixation into the various products of photosynthetic reactions was followed by methanolic extract of the cells and chromatographic analysis[13]. The cells harvested at time zero—*i.e.*, with minimum chlorophyll content—fixed carbon only into compounds typical of dark carboxylation reactions. The subsequent samples fixed carbon principally into carbon-reduction cycle intermediates[13].

Finally, an equilibrium EPR amplitude was determined. In contrast to the above metabolic experiments, this determination was made using dense cell suspensions. In the early stages of greening the density was limited by the volume of cells available; in the latter stages the cells were only diluted from the wet packed condition to the extent necessary to insert them with ease into the aqueous suspension cell. White light was used in all phases of the experiment, and in this latter phase it was of saturating intensity (equivalent to 10^{17}–10^{18} quanta/sec of 700-mμ light). Approx. 60% of the white-light energy was absorbed in the 0.025-cm thickness of the first sample. This increased to about 80% in the fully greened samples (same thickness). Light in the chlorophyll absorption bands was essentially completely absorbed in the third and subsequent cultures harvested.

Two overlapping EPR signals have been observed in chloroplasts and green algae[7,14,15]. Resolution of these two signals requires narrow magnetic-field modulation amplitude (about 3 Gauss). This lowers the spectrometer sensitivity. In order to observe a photo-induced EPR at minimum chlorophyll concentration we have used a field modulation of 10 Gauss which obliterates resonance line structure. Thus, we can make no statement concerning the relative rate at which the two lines grow in. We can remark that the observed EPR-line shape did not change during greening, that the ratio of chlorophyll *a*/chlorophyll *b* stayed constant during greening, and that the two overlapping lines, although never observed at the end of 15 h, were resolved in fully greened Chlamydomonas cultures a day or more after completion of greening. The EPR amplitudes were in general larger in such cultures, presumably due to a slowing of kinetics with age.

In all, three greening experiments were performed in which all of the above named determinations were made, and three were performed in which the measurement of oxygen-evolution rate and a carbon-fixation rate were omitted.

Our experimental results are shown in Fig. 3. In order to present them in one composite plot we have normalized each parameter measured, making the maximum value observed for each parameter in each greening experiment equal to 1. In none of the three variables did the maximum necessarily occur at maximum chlorophyll concentration. We have, in addition, omitted the experimental points for the curves representing the rates of oxygen evolution and CO_2 fixation. The experimental

variation in these determinations varied sufficiently to make the plot confusing. Thus, average curves were constructed using the arithmetic mean of the curves obtained from the individual greening experiments. These curves, the rate of oxygen evolution, and the rate of carbon fixation are consistent with the results of similar experiments on the greening of etiolated leaves of higher plants[16,17].

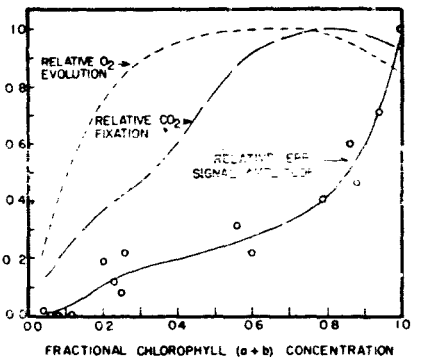

Fig. 3. The EPR amplitude, oxygen-evolution rate and $^{14}CO_2$-fixation rate as a function of chlorophyll (a + b) content during the greening of the yellow mutant of *Chlamydomonas reinhardi*.

In Table I we list chlorophyll (a + b) concentration for two experiments as a function of time. In both experiments the cells were grown in 1 large volume in the dark, and carbon-fixation and oxygen-evolution rates were determined. This data allows the transferral of the information in Fig. 3 to a time basis, and also gives some indication of the experimental variation encountered.

The results shown in Fig. 3 may be expressed in the following statements: (1) No EPR of any kind (dark or photo-induced) is observed in the chlorophyll-less mutant. (2) As chlorophyll is synthesized it becomes possible to photo-induce an EPR signal in the cells. (3) The equilibrium EPR amplitude is not linear with chlorophyll concentration; half of the EPR amplitude growing in with the last 15% of the chlorophyll synthesized. It is worth emphasizing here that we are looking at the steady-state concentration of unpaired electrons. This observed concentration of unpaired electrons will vary with any variation of the kinetics of the processes by which they are utilized in the cell, as well as with changes in the amount of light energy absorbed. As mentioned above, the light-absorption step is saturated. (4) Photosynthesis, as measured by the rate at which the carbon cycle is turning over and as measured by the rate of oxygen evolution, is maximum long before the EPR

TABLE I

CHLOROPHYLL CONCENTRATION AS A FUNCTION OF TIME

	Greening time (h)					
	0	*3*	*6*	*9*	*12*	*15*
Relative chlorophyll (a + b)	0.05	0.08	0.20	0.80	0.84	1.00*
Concentration	0.04	0.23	0.55	0.86	0.88	1.00**

* Corresponds to 2.7 mg chlorophyll (a + b) per ml wet packed cells.
** Corresponds to 3.2 mg chlorophyll (a + b) per ml wet packed cells.

amplitude is maximum. (5) The steepest rise in the EPR-amplitude curve occurs in a region where both the oxygen evolution and carbon-fixation rates seem to be decreasing.

IV. CONCLUSIONS

The obvious conclusion to be drawn is that, at least in the organisms studied here, chlorophyll must be present if the photo-induced EPR signal is to be observed, while the absence of carotenoids has little effect on the observed EPR. However, the action spectrum for EPR production indicates that energy absorbed by carotenoids may be transferred to chlorophyll for electron separation[18].

The experiments yield little specific information concerning the site of the unpaired electrons. However, the results of the Chlamydomonas experiment suggest that the observed unpaired electrons may lie directly in the pathway of photosynthesis. Light energy is absorbed by the chlorophyll. Assuming that, aside from chlorophyll, the photosynthetic apparatus of the mutant cells is complete, one might expect that during the early stages of greening the rate of photosynthesis, as measured by the rate of oxygen evolution, would be linear with chlorophyll concentration. This is the case in etiolated barley leaves[17] and appears to be the case in Fig. 3. Following this stage of greening a state exists in which the rate at which energy is absorbed is greater than the rate at which it can be used in subsequent reactions.

Photosynthesis in the cell is limited by factors other than the cell's ability to absorb energy[16]. The unpaired electrons in the various biological pools, presumably along the route of energy transformation, are only slightly filled when the rate-limiting step precedes them in the sequence, and become considerably more populated when the rate-limiting step follows them in the energy-transformation sequence. Several facts support this argument. (1) After approx. 80% of the chlorophyll has been synthesized the oxygen-evolution and carbon-fixation rates fall off slightly, for reasons undiscovered in this study. This is just the region in which the EPR amplitude rises most steeply. (2) On several occasions the oxygen-evolution rate of a sample, usually in the latter stages of greening, was particularly low. These points were considered as anomalous, and have not been used in constructing the curves of Fig. 3. However, in these cases the EPR amplitude was particularly high. (3) Experiments have been performed on *Chlamydomonas reinhardi* in which oxygen evolution was completely inhibited using 3-(3,4-dichlorophenyl)-1,1-dimethylurea[19]. In these experiments the photo-induced EPR amplitude of the narrow signal with fast kinetics observed in fully greened cells (see above) was considerably enhanced. Thus, at least a portion of the observed, photo-induced unpaired electrons seems to be in the photosynthetic pathway.

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THE EFFECT OF ULTRAVIOLET LIGHT ON PHOTOPHOSPHORYLATION AND THE HILL REACTION

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SUMMARY

1. Photophosphorylation and Hill-reaction activity of swiss-chard chloroplasts or chloroplast fragments were decreased by irradiation with short-wavelength ultraviolet light.

2. Photophosphorylation was more resistant to irradiation than the Hill reaction.

3. The presence of ascorbate during irradiation prevented the decrease in Hill reaction and photophosphorylative activity. Protection was more pronounced for the Hill reaction than for photophosphorylative activity. Compounds containing SH-groups could not replace ascorbate; it is suggested that ascorbate protects mostly by reducing the effective ultraviolet intensity, and in addition by exerting a specific effect on the chloroplasts themselves.

4. By ultraviolet irradiation, it was possible to obtain chloroplast preparations which had lost the ability to perform the Hill reaction, but still possessed high photophosphorylative activity. Such preparations may be useful in further research.

5. The endogeneous plastoquinone of chloroplasts was shown to be destroyed by irradiation.

6. The activity of irradiated chloroplasts was stimulated by the addition of plastoquinone. However, a similar stimulation was obtained on addition of plastoquinone to non-irradiated chloroplasts.