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SEPARATION OF TWO LIGHT-INDUCED ELECTRON-SPIN-RESONANCE SIGNALS IN SEVERAL ALGAL SPECIES*

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SUMMARY

Two overlapping electron-spin-resonance signals are observed when algal cells are illuminated with light. This paper describes two methods of algal cell pretreatment, heating and sonic oscillation, which effectively eliminate one of the signals while enhancing the other signal.

Using *Chlorella pyrenoidosa* cells pretreated by these procedures, electron-spin-resonance measurements are correlated with Hill-reaction activity and fluorescence. In addition, light-saturation studies for both electron-spin-resonance signals are reported for whole cells and sonicated cells of *Scenedesmus obliquus*, *Plectonema boryanum*, *Chlamydomonas reinhardi*, *Anacystis nidulans*, and *Ochromonas danica*.

On the basis of these experiments it appears that one signal is due to free radicals involved in some manner in a functional O_2 evolution system. The other appears to be due to a photophysical response of chlorophyll to light.

INTRODUCTION

COMMONER *et al.*[1,2] first reported two overlapping ESR signals, obtained when algal cells are illuminated. Now confirmed at several laboratories, one signal is a narrow fast rising and fast decaying signal of about 11 gauss line width at $g = 2.002$, which we shall call Signal I following COMMONER's original designation. The second signal is a broad, fast rising but slow decaying signal of about 19 gauss line width and $g = 2.005$ which we shall refer to as Signal II. The origin of neither of these signals has been identified positively to date, although several theories have been presented[2-6].

We have found two methods of algal cell pretreatment which effectively eliminate Signal II and at the same time enhance Signal I. These procedures, which may aid in the eventual identification of the two signals, consist of heat treatment and sonication of the cells.

METHODS AND MATERIALS

Growth conditions

The algae were grown in low-form 3-l culture flasks with continuous bottom daylight fluorescent illumination at light intensities which have been found to be

Abbreviations: ESR, electron-spin resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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optimum for metabolic activity. The optimum light intensities expressed in ft-candles for each species were: *Anacystis nidulans*, 500; *Chlamydomonas reinhardi*, 400; *Chlorella pyrenoidosa*, 900; *Ochromonas danica*, 500; *Plectonema boryanum*, 500; and *Scenedesmus obliquus*, 350. These intensities were determined through growth at various intensities with subsequent measurements of photosynthesis, respiration, Hill-reaction activity and pigments. Anacystis, Chlamydomonas, Chlorella, Plectonema, and Scenedesmus were grown autotrophically in completely inorganic media while Ochromonas was cultured on a partially organic medium. Growth temperature was 25° in all cases with horizontal agitation being provided. Ochromonas was cultured under sterile conditions without gassing, while the other algae were gassed with 5% CO_2 in air. Since the work necessitated heavy cell suspensions, harvests were made somewhat past the log phase of growth, the density ranging from 4–7 μl packed cells per ml culture. Details of culture and growth determinations were similar to those described previously[7].

ESR methods

ESR studies were made using a Varian Model 4500 EPR spectrometer (100 kcycles field modulation) equipped with aqueous sample cell and slotted cavity for illumination. The amplitude of the field modulation was 3 gauss. The light source was a 1000-W G. E. No. DRS-T20 tungsten lamp projected through 4 cm of water with 2.5% $CuCl_2$ as a heat filter and focused on the cavity window. Light-intensity measurements were made with Eppley thermopile No. 4856 standardized against a National Bureau of Standards calibrated light source. Appropriate corrections for light intensity were made to take into account the effective area of sample illuminated in the cavity. Wire mesh screens were used as neutral density filters to adjust the light level. This minimized spectral changes in the light source such as would occur if voltage on the lamp were changed to adjust intensity. The experiments involving light-intensity measurements were conducted by advancing from the lowest to higher light intensities, thus compensating for the slow decay characteristic of Signal II.

Amplitude measurements referred to in this paper actually are measurements of the amplitude of the first derivative of the absorption curve since this is the form in which data is obtained from the ESR spectrometer. For presentation of such data in a precise quantitative manner, the observed spectra must be integrated to give the total number of spins corresponding to each signal.

Experimental procedure

For the whole-cell experiments, the algal cells were concentrated by centrifugation and packed in a flat 1 × 5 cm quartz sample holder. The effective volume of the sample irradiated was 0.06 ml. Chlorophyll content in the 0.06-ml volume was calculated to be 0.68 mg for the *Chlorella pyrenoidosa* samples. Heat treatment of the cells was done simply by submerging the packed sample tube in a water bath for 5 min at the desired temperature. Some runs also were made by heating the algal cells at controlled temperatures for 5 min prior to centrifugation and packing in the ESR sample tube.

Chloroplast fragments were obtained by rupturing the cells at low temperature in a 10-kcycles Raytheon sonic oscillator for 20 min. To preserve Hill-reaction activity the cells were broken in a solution of 0.05 M phosphate buffer (pH 7) which contained

0.4 M sucrose. After sonication, the particles which sedimented in the range between 25000 × *g* (20 min) and 140000 × *g* (1 h) were used as the chloroplast fragments. The fragments quickly lost Hill-reaction activity unless kept refrigerated.

Fluorescence measurements

Fluorescence studies were made on some of the cell preparations using an Aminco-Bowman Model No. 4 spectrophotofluorometer. The activating light was held at 440 mμ and fluorescence was observed at 689 mμ. For the heat treatment experiments, the sample was heated directly in a measuring cuvette of 4 ml volume. Cell density for the fluorescence measurements was approx. 2 μl packed cells per ml culture.

RESULTS

Temperature effect

Fig. 1a shows the ESR signal obtained from normal, whole *Chlorella pyrenoidosa* cells. The overlapping broad and sharp signals are evident. The tracing shown in Fig. 1b was obtained from the same sample after heating the cells by submerging the ESR sample tube in a water bath at 68° for 5 min. The sample then was returned to room temperature prior to making the ESR spectrum. Note that Signal II is completely missing from this tracing and only an enhanced Signal I remains. When this process is repeated at 78° (Fig. 1c), Signal I also is decreased markedly and it, too, disappears after heating in a boiling-water bath.

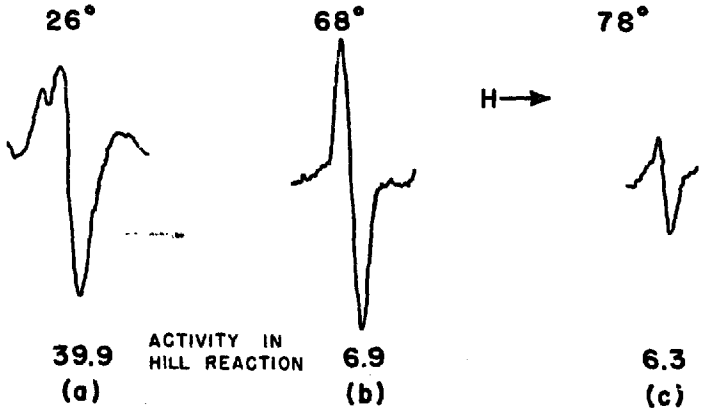

Fig. 1. Effect of temperature on light-induced ESR signals from *Chlorella pyrenoidosa*. Cells were heated for 5 min at the temperatures shown and returned to room temperature for the ESR measurements. Arrow shows direction of increasing magnetic field. Loss of one of the signals is correlated with Hill-reaction activity of the samples expressed in μl O_2/h/10 μl of packed cells.

Hill-reaction-activity measurements also were made on these samples, using *p*-benzoquinone as the Hill reagent. The quinone Hill-reaction activity of the normal cells was 39.9 μl O_2/h/10 μl of packed cells while the heated cells gave rates of 6.9 and 6.3. Thus there appears to be a direct correlation between the loss of Hill-reaction activity and the disappearance of Signal II.

A plot of the data obtained in the heating experiments is shown in Fig. 2. At room temperature both signals were about equal in amplitude at a white light intensity of about $3 \cdot 10^5$ ergs·cm^{-2}·sec^{-1}. As the temperature of algal cell pretreatment was increased, Signal I reached a peak somewhere between 60° and 70°, at

which temperatures Signal II fell off sharply. At higher temperatures Signal I also decreased in amplitude and both signals were missing at 100°.

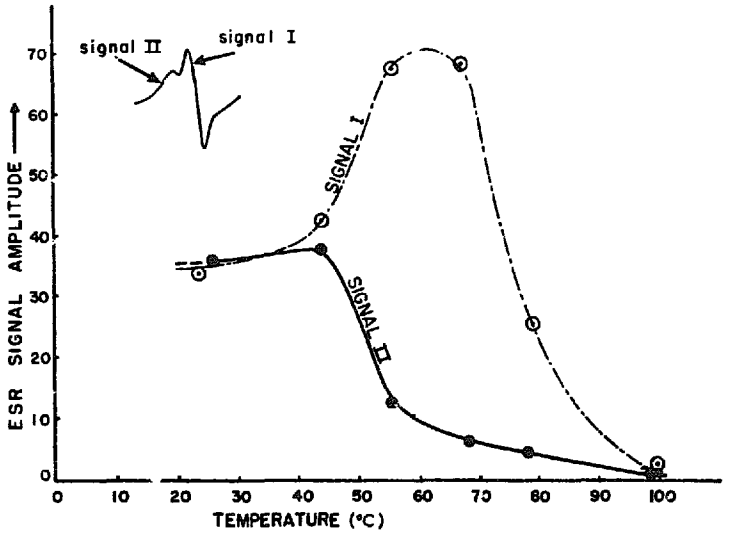

Fig. 2. Amplitude of ESR signals from *Chlorella pyrenoidosa* as a function of temperature of pretreatment. Signal I and Signal II designations are described in text. Samples were heated for 5 min at the temperature indicated prior to making the ESR measurements.

Sonication results

Chloroplast fragments prepared by sonic oscillation also showed enhancement of Signal I. Fig. 3a again shows the ESR spectrum of normal cells for reference. Fig. 3b is the ESR spectrum obtained from chloroplast fragments prepared with sucrose under refrigeration in order to preserve some Hill-reaction activity. An enhanced Signal I is evident along with a nearly normal Signal II. If the chloroplast fragments are left standing overnight at room temperature, they lose Hill-reaction activity. The ESR signal from these fragments then exhibits only an enhanced Signal I and no Signal II (Fig. 3c). Thus, here again, there appears to be a correlation between the loss of Signal II and the loss of Hill-reaction activity.

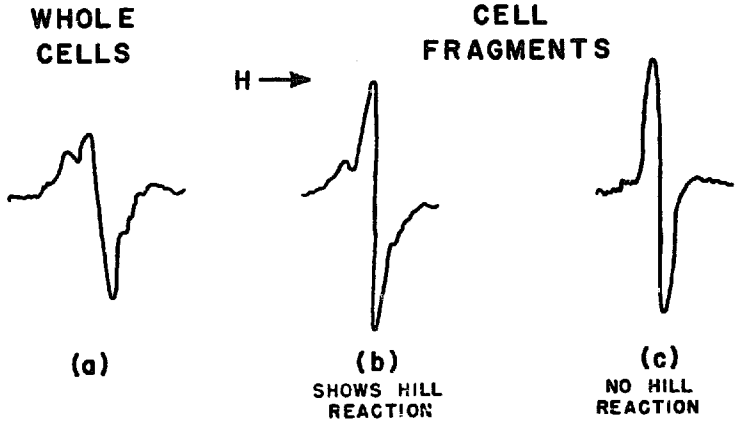

Fig. 3. Effect of cell fragmentation on light-induced ESR signals from *Chlorella pyrenoidosa*. Signal II is shown only by fragments able to perform a Hill reaction. Those fragments showing Hill-reaction activity were prepared by sonic oscillation in sucrose, as described in the METHODS section.

Light-intensity effects

Light-saturation studies have been made on both whole cells and chloroplast fragments. Fig. 4 shows the ESR-signal amplitude *versus* light-intensity studies for whole cells of *Scenedesmus obliquus*, *Chlamydomonas reinhardi*, *Plectonema boryanum*, and *Anacystis nidulans*. Similar studies have been reported previously for *Chlorella pyrenoidosa*[8]. These data are not to be considered quantitative, since no attempt was made to have the same number of cells in the sample tube in each case. Even the ratio of Signal I to Signal II has been observed to change from sample to sample of the same algal species—probably due to different physiological states of the specimens. However, we do believe it significant that in every species tried to date,

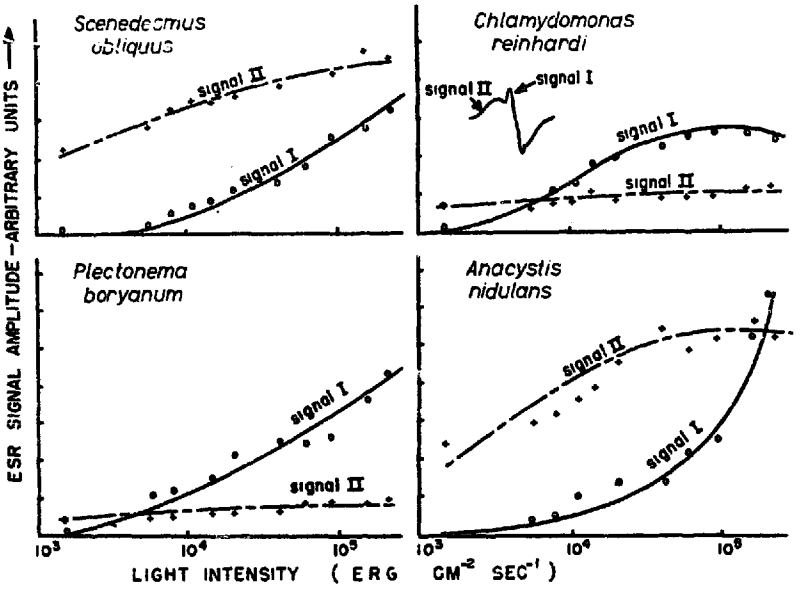

Fig. 4. The effect of light intensity on the ESR signals from cells of several algal species. Light intensity is plotted on a logarithmic scale. A tungsten lamp was used, as described in the METHODS section.

Signal II appears first in the low to normal light-intensity range best suited for cell growth, followed by Signal I in the higher light-intensity range. Moreover, Signal II consistently shows light-saturation tendency whereas Signal I does not.

Light-intensity studies on cell fragments obtained from the same set of algal species are shown in Fig. 5. Since these cells were broken by sonication in phosphate buffer without sucrose, the cell fragments were not active in the Hill reaction and only Signal I was observed. The nearly straight-line response which exists between the ESR-signal amplitude and the logarithm of light intensity is reminiscent of a purely photophysical-type response.

Ochromonas danica

To date we have found only one algal species which failed to yield an enhancement of Signal I by either the sonication or heat-treatment procedure outlined above. *Ochromonas danica* exhibits Signal II and a very weak Signal I in the whole cells but shows neither signal in the heated cells or chloroplast fragments (see Fig. 6). It may

be significant that *Ochromonas danica* also is the only one of the species studied which requires fixed carbon in the form of amino acids for growth.

Inhibitor effects

The effect of photosynthesis inhibitors on the two light-induced ESR signals in *Chlorella pyrenoidosa* is shown in Table I. *o*-Phenanthroline and DCMU were used as inhibitors in these experiments. The ratio of Signal I to Signal II is considered more significant than either signal measured alone, since this tends to compensate for the uncertainty in the number of cells in the sample tube during each run. This

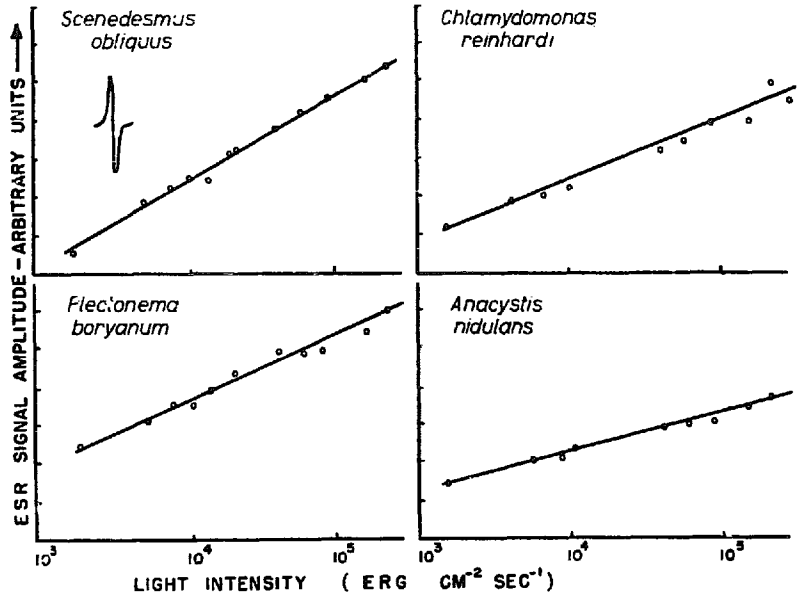

Fig. 5. The effect of light intensity on cell fragments from several algal species. Light intensity is plotted on a logarithmic scale. See METHODS section for details.

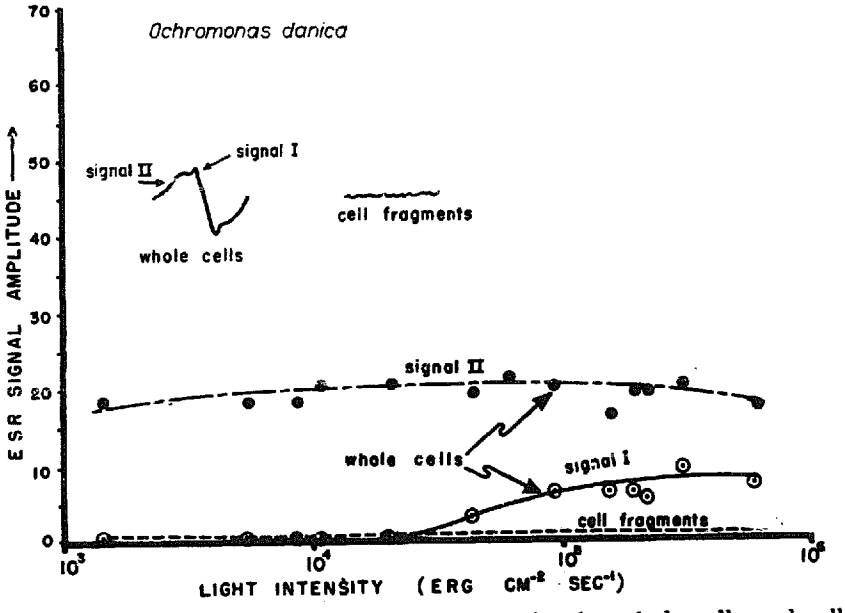

Fig. 6. ESR-signal amplitude as a function of light intensity for whole cells and cell fragments of *Ochromonas danica*. Enhancement of Signal I by cell fragmentation is not observed in this species.

table shows that in the presence of these compounds which inhibit O_2 evolution, the ratio of Signal I to Signal II increases. This is consistent with our findings using heat-treated and sonicated cells. WEAVER has previously reported the same enhancement effect for *Chlamydomonas reinhardi* using DCMU[9]. HEISE also has reported enhancement of Signal I and reduction of Signal II using *o*-phenanthroline with spinach chloroplasts[10].

TABLE I

EFFECT OF PHOTOSYNTHESIS INHIBITORS ON THE RATIO ESR SIGNAL I TO SIGNAL II IN *Chlorella pyrenoidosa*

Enhancement of the ratio was observed within 10 min after addition of the inhibitor. Expt. No. 4 was performed after an exposure of several hours to the inhibitor.

Expt. No.	*Inhibitor*	*Ratio Signal I/Signal II*	
		Control	*+ Inhibitor*
1	0.1 mM DCMU	63/48 = 1.31	100/39 = 2.57
2	0.1 mM DCMU	60/43 = 1.39	102/30 = 3.4
3	0.1 mM DCMU	56/41 = 1.36	83/23 = 3.6
4	1 mM o-phenanthroline	60/43 = 1.39	47/5 = 9.4
5	1 mM o-phenanthroline	60/43 = 1.39	100/45 = 2.23
6	1 mM o-phenanthroline	56/41 = 1.36	88/39 = 2.26

Fluorescence

Fluorescence enhancement has been reported in algal cells when normal physiological processes have been impaired. For example, ZWEIG *et al.*[11] report fluorescence enhancement in algal cells with DCMU. Using *Chlorella pyrenoidosa* with 0.1 mM DCMU we were able to demonstrate a correlation between the enhancement of Signal I and the enhancement of fluorescence. Carrying the analogy one step further, we found that enhancement of both fluorescence and Signal I had a very similar dependence upon temperature of pretreatment of the cells (Fig. 7). Both showed maximum

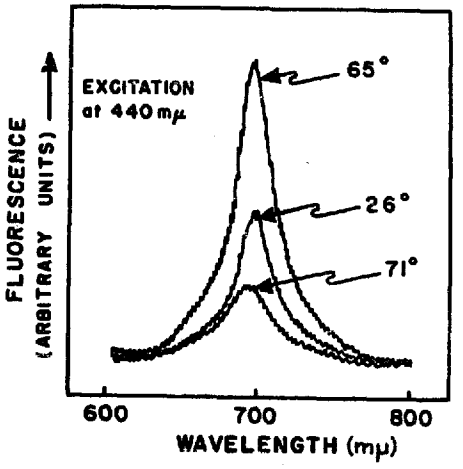

Fig. 7. Effect of temperature on fluorescence of *Chlorella pyrenoidosa*. Excitation was at 440 mμ and fluorescence was observed at 689 mμ. Cells were pretreated for 5 min at the temperature shown prior to making measurements on a spectrophotofluorometer.

enhancement at about 65° and fell off sharply above that temperature. Thus, there appears to be a correlation between chlorophyll fluorescence enhancement and enhancement of Signal I.

DISCUSSION

A previous communication from this laboratory[12] reported that *Chlorella pyrenoidosa* cells deficient in manganese show only a weak light-induced signal. WEAVER[3] repeated this experiment and determined that Signal II was missing in manganese-deficient cells. She further postulated that Signal II may be due to the semiquinone form of plastoquinone (Q255). Although both Mn^{2+} and plastoquinone are essential components of a functional unit for the Hill reaction, they appear to act in different places. Mn^{2+} most likely acts in the system involved in the oxidation of water leading to O_2 production, while the plastoquinone most likely acts in a coupled manner as the electron acceptor. Since both of these systems must be functional to observe O_2 evolution, it is difficult to determine which part is responsible for Signal II. The experiments of LEVINE AND PIETTE[4] strongly indicate that Signal II is due to the water oxidation system, since two mutants of *Chlamydomonas reinhardi* which can perform NADP photoreduction only when supplied with alternate electron donors do not show the signal, yet one mutant strain which can evolve O_2 (yet can't reduce NADP unless an alternate electron donor is added) does show Signal II.

There is further evidence in the literature that the broad signal is due to the O_2 evolution system. ALLEN *et al.*[5] observed that "water-treated" cells with low O_2-evolving ability exhibit a weak Signal II. This treatment should not remove plastoquinone. (These authors and LEVINE AND PIETTE use a reverse notation for the signals from that originally established by COMMONER. For this paper we have changed the differing notation used at several laboratories to conform with COMMONER's and our notation.) In addition, ANDROES *et al.*[13] found that washed spinach "quantasomes" with weak Hill-reaction activity also have a small Signal II. A final bit of indirect evidence linking Signal II with O_2 evolution is that it has not been observed in photosynthetic bacterial systems which, of course, do not evolve O_2.

In summary, Signal II appears to be a physiological-type response because it apparently exists only in intact cells and/or systems exhibiting Hill-reaction activity. It is most prominent in the normal temperature range conducive to biological activity. It appears at very low light intensity and shows light-saturation tendency at light intensities higher than normal for best cell growth. The evidence presented above indicates that Signal II is due to free radicals which are probably involved in the O_2 evolution pathway.

Signal I, on the other hand, appears to be a photophysical-type response. We have confirmed earlier work[14,6] that it can be observed at temperatures as low as −135°, and also have detected it at temperatures as high as 80°. Therefore, the production of Signal I does not involve ordinary enzymic processes. Moreover, Signal I does not require biologically active chloroplast fragments or systems, being apparent in systems which exhibit no Hill-reaction activity. With whole cells at room temperature, Signal I is most prominent only in the high light-intensity range. In chloroplast fragments it shows a logarithmic response to light intensity over a wide range from $1 \cdot 10^3$ to $6 \cdot 10^5$ ergs·cm^{-2}·sec^{-1}. In addition, Signal I appears to involve the same electronic transistions which produce chlorophyll fluorescence.

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