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An Electron Spin Resonance Study of Manganese in Wild-Type and Mutant Strains of Chlamydomonas reinhardi[1, 2]

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Summary. Changes in the intensity of the electron spin resonance signal of divalent manganese were found to occur in suspensions of wild-type *Chlamydomonas reinhardi*. The observed manganese signal decreased in the light and increased in the dark. Through the use of a continuous-flow system it was possible to determine that the manganous ions responsible for the observed signal were localized solely in the medium. Changes in the signal intensity associated with wild-type cells were independent of the ability of fragments prepared from these cells to perform the Hill reaction with 2,6-dichlorophenol-indophenol (DPIP) as the oxidant.

The manganese signal changes were still evident, though smaller, in cell suspensions of wild-type cells treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea, and in mutant strains unable to carry out the Hill reaction, *ac-115* and *ac-141*.

From these data it is concluded that the changes in intensity of the manganese resonance are not related to the function of manganese in photosynthesis but may reflect the capacity of cells for ion uptake in the light.

Two light induced electron spin resonance (ESR) signals, signals I and II, have been observed in studies of photosynthetic organisms. Some of the properties of these resonances have been determined in order to relate their occurrence to the operation of the photosynthetic electron transport chain (1). Signals I and II appear in the same portion of the magnetic field as does the six-peak ESR signal characteristic of divalent manganese, and frequently they are seen superimposed on the manganese spectrum. This superposition has been noted for *Chlorella pyrenoidosa* (15), *Chlamydomonas reinhardi* (7), *Scenedesmus sp.* (3), and *Anacystis nidulans* (5). Manganese has long been established as a requirement for plant growth, and it has been strongly implicated in photosynthetic oxygen evolution. However, no mechanism for its role in photosynthetic oxygen evolution is known.

Treharne and co-workers (15) have reported that the manganese signal observed with preparations of *C. pyrenoidosa* decreased in magnitude in the light and increased in magnitude in the dark. They suggested that the changes in signal intensity were related to some function of manganese in photosynthesis (13). Allen and her coworkers (1) were unable to detect any light-induced change in the intensity of the manganese signal that they observed in suspensions of cells of *C. pyrenoidosa*. Also, no light-induced change in the manganese signal has been reported for the other algae.

The study of the ESR manganese signal in cells of *C. reinhardi* reported here was undertaken in order to determine whether the magnitude of the manganese signal was affected by incubation in the light or in the dark and, if so, whether such changes in magnitude could be related to photosynthesis. From our evidence it appears that changes in manganese signal intensity in light and dark do occur but that these changes are not related to the role of manganese in photosynthetic oxygen evolution.

Materials and Methods

Cell Cultures. Wild-type and mutant strains *ac-115*, *ac-141*, of *C. reinhardi*, (strain 137c), were used in this study. The mutant strains examined are unable to carry out normal photosynthesis and require acetate for growth. In *ac-115* and *ac-141* mutant strains cytochrome 559 is either missing or nonfunctional (6), and chloroplast fragments prepared from these strains cannot carry out a Hill reaction though they are able to photoreduce pyridine nucleotide with reduced indophenol dye as the electron donor (8).

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Cells were cultured on minimal medium (12) supplemented with 0.2 % sodium acetate. Manganese-deficient cells were prepared by growth on medium which was treated to remove manganese contamination. The cultures were illuminated continuously at an intensity of 4000 lux from daylight fluorescent lamps and agitated on a Gyrotory shaker. The temperature was controlled at 25°. The cells were harvested in late logarithmic phase of growth. The medium in which they were grown was employed as the suspending medium in ESR studies.

Direct cell counts were made with the aid of a hemacytometer. Chlorophyll concentration was carried out according to a modification (2) of the method of MacKinney (9).

Spectrometer System. ESR measurements were made on a Varian 4502 EPR spectrometer operating at a microwave frequency of 9.5 kilomegacycles ($\nu = 9.5 \times 10^9$ sec^{-1}) with field modulation at 100 kilocycles. The signal was displayed on a Varian recorder. Two general methods, the static method and the continuous flow method, were used for examining the cells. In the static method the cells were concentrated, resuspended in their original growth medium (30 % cell volume/total volume), and placed in the Varian quartz aqueous solution sample cell having an effective volume of 0.05 ml. The sample cell was placed in a slotted cavity and illuminated with water-filtered red light from an air-cooled 500 watt tungsten lamp 0.6 meters distant from the cavity. The red filter used was a Corning 2-64 glass filter, transmitting wavelengths above 650 mμ. All the measurements were made at room temperature. The average chlorophyll concentration in the sample was 1 mg/ml and the average cell number 2.0×10^8/ml.

There are many difficulties inherent in studies of cells using the static method. The high volume of cells to total volume loaded in the sample holder and the limited availability of air make it unlikely that cells are being studied under physiological conditions. The geometry of the sample holder is such that only a fraction of the cells loaded is examined, and the cells that do not fall within the walls of the cavity can never be illuminated. Furthermore, in the course of a single experiment, the cells settle out so that the number of cells actually within the walls of the cavity is constantly diminished. To overcome these difficulties and to permit long term studies of manganese signal changes under different conditions, a continuous flow system was developed.

The flow system was constructed so that the cells, suspended in their original medium, moved from an open glass reservoir and through transparent Tygon tubing coiled in a plane. The tubing was illuminated when desired with a bank of five 30-watt fluorescent lights providing 10,000 lux of illumination. The suspension was circulated through the tubing to the sample cell in the cavity and back to the reservoir in a continuous cycle with a Manostat 72-590 Varistaltic pump. The total volume of the system was 50 ml, and the time required for a single circulation of this volume around the flow circuit was about 30 seconds under typical conditions. The concentration of cells was kept sufficiently low to allow the light to penetrate to a depth that was greater than or comparable to the thickness of the suspension used. In the system used here the thickness of the suspension was equivalent to the inner diameter of the Tygon tubing (about 3 mm). The cell concentration ranged from 2.5 to 5.0×10^7 cells/ml. The corresponding range of chlorophyll was 0.05 to 0.1 mg/ml.

Hill Reaction Studies. The rate of the Hill reaction with DPIP as the oxidant was measured to determine if a correlation existed between changes in the magnitude of the ESR manganese signal and changes in the ability to perform this partial reaction of photosynthesis. Wild-type cells were spun down and resuspended in their own medium at a concentration equivalent to that used in static ESR studies. Aliquots were placed in each of several Thunberg tubes, and the air in these tubes was replaced with nitrogen. The contents of some tubes were removed immediately, and the cells collected by centrifugation. The other tubes remained in the dark at room temperature for 2 hours at which time these cells were

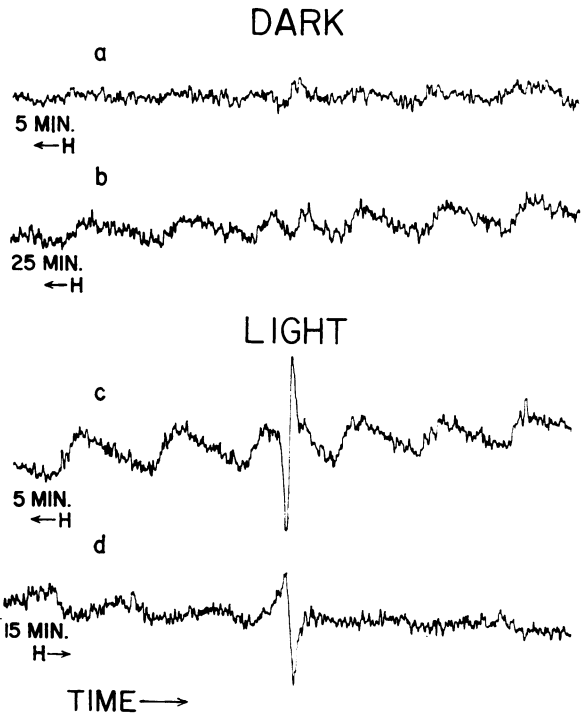

FIG. 1. Time-course study of manganese ESR signal changes of wild-type *C. reinhardi* cells examined in the static system. The time of initiation of the scan in the dark and after the light is turned on is given at the left; H→ indicates the direction of change of magnetic field during each scan. Time for a single scan was about 5 minutes. The light was turned on after the cells had been in the dark for approximately 40 minutes. The 6 lines of the manganese signal are centered at a magnetic field of 3200 gauss.

collected by centrifugation. In both cases chloroplast fragments were prepared as described (11) using 5 mM phosphate buffer (pH 7.4) as the suspending medium, and the Hill reaction was measured according to the method described by Levine and Smillie (8).

Results

Static System Studies. In order to ascertain the existence of the light-induced changes in the magnitude of the manganese signal, aliquots of cells were examined under conditions of light and dark. A typical result is shown in figure 1. The manganese signal, barely visible at first, increases in intensity during the dark period (scans a and b). On illumination the signal decreases in intensity (scans c and d). The six peaks of the manganese signal appear asymmetrical indicating that changes affecting the signal strength have occurred continuously during the 5 minute scan. This asymmetry can be noted particularly in the light period. Signal I which appears in the middle of the manganese signal during scans in the light is smaller in scan d than in scan c in agreement with the time-course studies of signal I in *C. reinhardi* (16). Signal II which appears in the dark and along with signal I in the light is present but not well resolved in these studies. The observations of changes in manganese signal intensity in the light and in the dark have been verified on numerous preparations of cells. The maximum manganese signal heights observed have corresponded to divalent manganese concentrations of 10^{-5} to 10^{-6} M.

Continuous-Flow System Studies. The sequence of changes associated with the wild-type cells examined in the continuous-flow system under conditions of light and dark is presented in figure 2. The rate of signal increase in the dark and the rate of decrease in the light remained approximately constant throughout each cycle of light and dark during the 10 hours

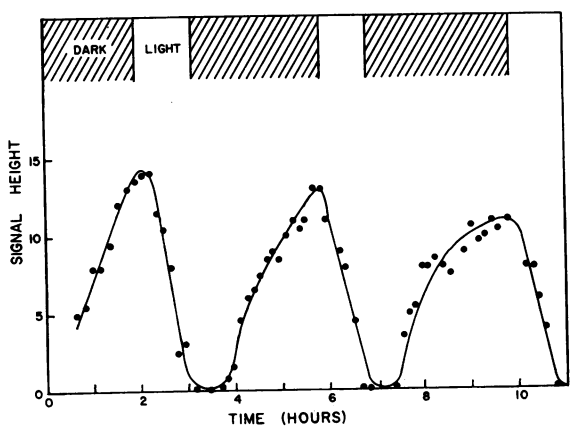

FIG. 2. Changes in manganese signal intensity of wild-type *C. reinhardi* cells examined in the continuous-flow system. Signal height is given as the peak-to-peak height of the signal averaged over the 6 peaks of the signal and expressed in arbitrary units.

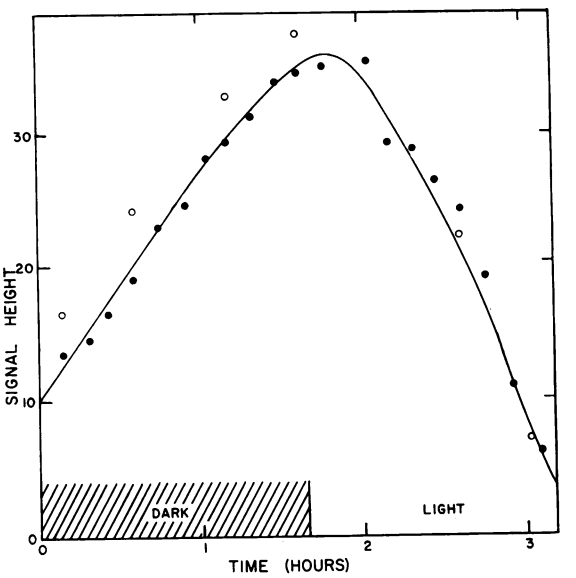

FIG. 3. Manganese signal intensity of a wild-type *C. reinhardi* suspension examined in the continuous-flow system. Closed circles: manganese signal intensity as observed with the circulating cell suspension; open circles: manganese signal associated only with suspending medium.

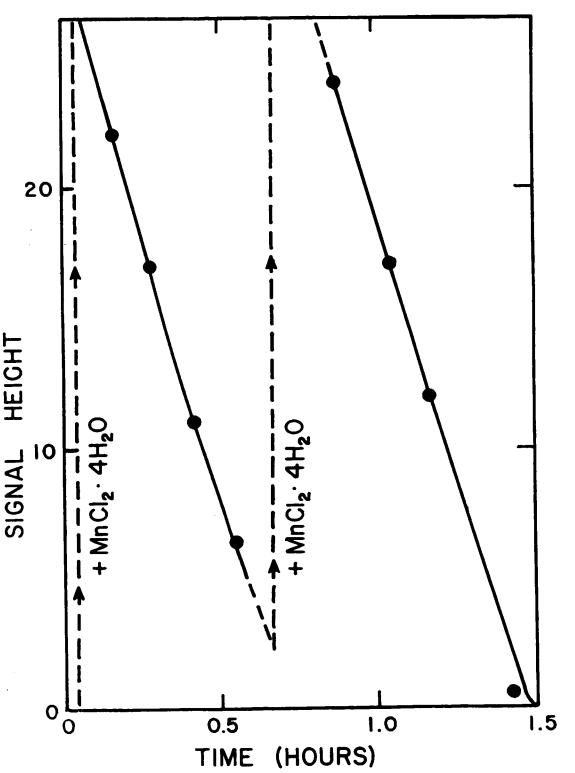

FIG. 4. Manganese signal changes of a wild-type cell suspension in the light following addition of $MnCl_2 \cdot 4H_2O$ to the continuous-flow system.

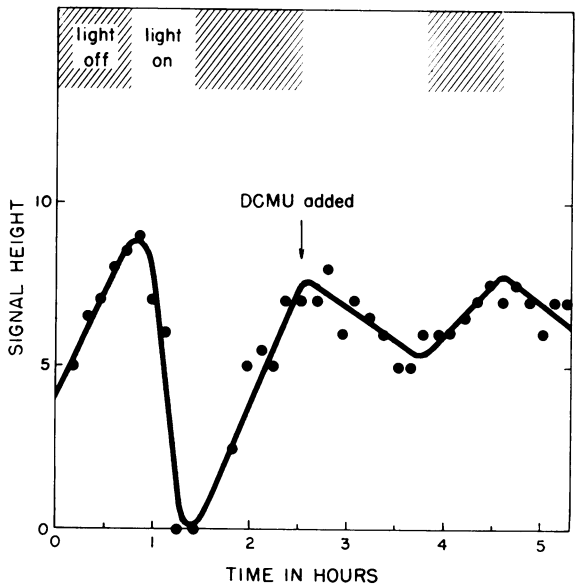

FIG. 5. Effect of addition of 10 μM DCMU in the light on manganese signal changes of wild-type *C. reinhardi* cells.

of the experiment shown. When small aliquots of the circulating suspension were removed in the course of an experiment and the cells removed by centrifugation, all the manganese signal observed with the cell suspension was recovered in the medium (fig 3). The slight differences between manganese signal intensity of the cell suspension and signal intensity in the medium alone probably reflect physiological changes which took place during the centrifugation of the aliquot in the dark. Thus, when the ESR manganese signal is detected it is not associated with the cells but with their surrounding medium.

When divalent manganese was added to a circulating cell suspension the manganese signal generated by the added ions diminished in the light (fig 4). Since bound manganese does not show a spin-resonance signal, the loss of the manganese signal must be due to a complexing or binding of the ion with a cell component.

When 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to completely block the ability of the cells to carry out the Hill reaction, the changes in the manganese signal in the light and the dark were still evident though reduced in rate to from 50 to 90 % of untreated cells (fig 5). This result prevailed whether DCMU was added in the light or in the dark.

Preparations of *ac-115* and *ac-141* were also examined in the continuous-flow apparatus. The decrease in the manganese signal in the light and its increase in the dark occurred in cell suspensions of these mutant strains at rates similar to that of DCMU-treated wild-type cells.

Hill Reaction Studies. Cells were incubated, as described in the Methods section, under conditions of prolonged darkness to produce a high manganese signal. The Hill reaction ability of such preparations (high signal) was compared to that of cell preparations which had not been incubated and which had not generated a manganese signal (low signal) (table I). The Hill reaction rate obtained from chloroplast fragments was unchanged even when the external manganese signal was high. In contrast, chloroplast fragments prepared from cells that had been made manganese deficient by continuous growth in manganese-free medium exhibited a markedly reduced rate of Hill reaction.

Table I. *The Rate of the Hill Reaction by Chloroplast Fragments having a Low or High Mn^{2+} ESR Signal Compared with the Rate Obtained with those Isolated from Manganese-Deficient Cells*

The reaction mixture (0.8 ml) contained chloroplast fragments equivalent to 3 to 4 μg chlorophyll and the following in μmoles: potassium phosphate buffer, pH 7.0, 10; KCl, 5; and 2,6-dichlorophenol-indophenol (DPIP), 5. The reaction was run in a cuvette placed in the sample compartment of a Cary model 14 spectrophotometer. The cuvette in the reference compartment contained a control reaction mixture from which DPIP was omitted. The reactions were run at 25 °.

Sample	μmoles DPIP reduced hr^{-1} mg^{-1} chlorophyll
Low signal	102
High signal	99
Manganese-deficient	7

Discussion

Relation of the Manganese Signal to Photosynthesis. Cells of the wild-type *C. reinhardi*, when examined for spin resonance signals in the static system and under more physiological conditions in the continuous-flow system, showed the well-defined, six peak spectrum of divalent manganese in addition to the resonances I and II typical of photosynthetic systems. Under both sets of experimental conditions the manganese signal was found to increase in intensity in the dark and decrease in the light. The divalent manganese when detected in ESR experiments was not located within the cells but in their surrounding medium. It is unlikely that the changes in the intensity of the manganese signal are in any way related to the functioning of manganese in photosynthesis for the following reasons: 1) When electron flow from system II is blocked either as a result of inhibition with DCMU or by mutation (*ac-115, ac-141*) the changes in the manganese signal in the light and dark are still evident; 2) Under conditions in which a large external manganese signal is produced, Hill reaction activity is unaffected. The Hill reaction is diminished only in cells made manganese deficient through growth in medium lacking manganese.

Thus, the loss of manganese from cells in the dark

as detected by the increased ESR signal and the removal of manganese from the medium by cells in the light as indicated by the reduction of the ESR signal do not appear to be changes in the localization of manganese which is active in photosynthesis.

Interpretation of the Manganese Signal Changes. The changes in intensity of the divalent manganese signal may be related to the ability of cells to support an energy-dependent ion uptake. In the light the uptake of different ions, including manganese, from the external medium into the cell may be facilitated by ATP provided by photosynthetic phosphorylation. The major site of accumulation of the manganese is probably the mitochondrion since this organelle is able to sequester large quantities of this ion (4). In vitro, at least, there does not appear to be a net uptake of manganese by chloroplasts in the light (10). The uptake and subsequent binding of manganese within the cell could result in the loss of divalent manganese from the medium and a concomitant reduction in the ESR manganese signal observed. In the dark, a net efflux of the stored or excess manganese from the cells into the medium, where it is once again detected as divalent manganese, would yield the observed increase in ESR signal magnitude. The reason for this loss of manganese in the dark is not clear, but it may be related to conditions of anaerobiasis which obtain in the dark in both the static and continuous-flow systems.

Wild-type cells treated with DCMU, and the photosynthetic mutants *ac-115* and *ac-141*, also exhibit the manganese signal changes in the light and dark. The explanation given here would require that such cells are capable of making ATP in the light even though they cannot carry out complete photosynthesis. Chloroplast fragments isolated from wild-type and mutant strains can carry out cyclic photosynthetic phosphorylation with phenazine methosulfate (PMS) as the electron carrier (R. P. Levine, unpublished observations) and recently, in vivo cyclic photosynthetic electron flow which persists in the presence of DCMU has been found in wild-type *C. reinhardi* cells (14) and in the mutant strains *ac-115* and *ac-141* (D. Teichler-Zallen, T. T. Bannister, and G. Hoch, in preparation). In these cells, ATP available for ion uptake is probably less than under conditions of normal photosynthesis since the rate of uptake observed is less than that observed in untreated wild-type cells.

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