MANGANESE REQUIREMENT WITH RESPECT TO GROWTH, HILL REACTION AND PHOTOSYNTHESIS

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The necessity of manganese for the growth of both autotrophic and heterotrophic plants was reported first by Bertrand (5, 6) more than 50 years ago, and the essentiality of manganese for the growth of Chlorella was shown originally by Hopkins (20) of Cornell University in 1930.

The relation of manganese to photosynthesis was suspected by McHargue (24) and Bishop (7). However, it was not until 1937 when Pirson (25) showed that the photosynthesis of manganese-deficient Chlorella cells was partially inhibited and that the inhibition could be relieved instantaneously by the addition of manganese to the medium. Emerson and Lewis (12, 13) observed that the absence of micronutrient elements in the culture medium caused a decrease in the quantum yield of photosynthesis, and that the efficiency of photosynthesis was improved more with the addition of manganese than with the addition of any other micronutrient element. The literature on this subject has been reviewed by Pirson (26). More recently, there has been the work of Kessler (21), Habermann (18), Pirson and Bergmann (27) and Reisner and Thompson (31, 32, 33).

This paper reports a study of the growth, Hill reaction, and photosynthesis of Chlorella pyrenoidosa cultured autotrophically and heterotrophically at various levels of manganese, and similar studies with autotrophically cultured Nostoc muscorum, Scenedesmus quadricauda, Porphyridium cruentum and Lemna minor. Measurements pertaining to the chlorophyll-manganese ratio and the relation of manganese both to the chlorophyll content and to the destruction of chlorophyll by light are also included. Preliminary results have already been published (14), and some of the present results were reported at the 1956 meeting of the American Institute of Biological Sciences (15).

Methods

Chlorella pyrenoidosa (Emerson strain) was obtained from Dr. Jack Myers of the University of Texas. This alga was cultured in Warburg and Burk medium (34) supplemented with 1 ppm Fe as FeSO₄·7H₂O, 0.02 ppm Cu as CuSO₄·5H₂O, 0.5 ppm B as H₃BO₃, 0.05 ppm Zn as ZnSO₄·7H₂O, and 0.01 ppm Mo as (NH₄)₆Mo₇O₂₄·4H₂O. The manganese was added separately as MnSO₄·7H₂O from a dilution series. Fisher Certified and Baker Analyzed reagents supplied the macronutrients, and Matthey Specpure compounds furnished the micronutrients. Scenedesmus quadricauda and Porphyridium cruentum were obtained from the University of Indiana Algae Culture Collection. The former was cultured on a modified Chicago medium containing 1 liter H₂O, 500 mg MgSO₄·7H₂O, 250 mg KNO₃, 250 mg K₂HPO₄, 200 mg NaCl, 27 mg Specpure CaCl₂, 1.88 millimoles NaOH prepared from C. P. Baker's Analyzed metallic sodium, and supplemented with iron and trace elements as for Chlorella. Porphyridium cruentum was cultured in a medium formulated as follows: 1 liter H₂O, 2.5 g NH₄NO₃, 3.75 g Mg(NO₃)₂·6H₂O, 6.7 g NaCl, 625 mg KCl, 300 mg CaCl₂·2H₂O, 24 mg K₂SO₄, 25 mg KH₂PO₄, 649 mg NaHCO₃, and supplemented with iron and trace elements as for Chlorella, with molybdenum increased from 0.01 ppm to 0.1 ppm. Nostoc muscorum, obtained from Dr. G. C. Gerloff, University of Wisconsin, was cultured on a modified Chu medium containing 1 liter H₂O, 125 mg MgSO₄·7H₂O, 250 mg KNO₃, 30 mg K₂HPO₄, 27 mg Specpure CaCl₂, 3.76 millimoles NaOH specially prepared as described above, and supplemented with iron and trace elements as for Porphyridium. Lemna minor, from the Carolina Biological Supply Co. was cultured in Hoagland's medium (17).

The glucose for heterotrophic growth was dissolved double-strength in water, autoclaved, and when cool added to autoclaved double-strength medium. The final concentration in the medium was 1%. For preparing the low-manganese medium, glucose was purified by passing a 10% solution in redistilled water successively through two 1.1 x 25 cm Pyrex columns containing 50-100 mesh Dowex 50—X₂ cation exchange resin which was operated in the hydrogen cycle.

The water was redistilled in a Pyrex-glass distillation apparatus and the glassware was supercleaned by the method of Waring and Werkman (35). The culture flasks were 300-ml Pyrex flasks with side arms, containing 100 ml of medium, plugged with cotton and covered with rubber caps. The cultures
Fig. 1. Autotrophic and heterotrophic growth of *Chlorella pyrenoidosa* without added manganese and with 0.5 ppm added manganese in the culture medium.

Fig. 2. Heterotrophic growth of *Chlorella pyrenoidosa* at very low concentrations of added manganese.
were bubbled with 5% CO₂ in air at the rate of about 35 ml/min, agitated continuously on a shaker in a room maintained at 25°C, and illuminated with approximately 1000 ft-c of daylight fluorescent light.

The heterotrophic Chlorella cultures were started with one loopful of an autotrophic bacteria-free stock culture which contained 0.5 ppm manganese. The manganese series of autotrophic cultures were begun from a minus-manganese heterotrophic culture that had been washed to remove the glucose. Fifty to 100 µl of washed cells per 100 ml of culture were used.

The procedure for the determination of chlorophyll in Chlorella and Scenedesmus was based on the work of MacKinney (23), using methanol extraction. Chlorophyll determinations for Nostoc were obtained by Arnon’s method for chloroplasts (3) using acetone since methanol did not satisfactorily remove the chlorophyll. Standard procedures were used for the isolation of chloroplasts from Lemna minor (9) and for the determination of chlorophyll in these chloroplasts (9).

Manometric measurements of photosynthesis and of the Hill reaction have been described by Brown (8), and Clendenning and Erhmantraut (10), respectively. For measurements of photosynthesis, manganese-free 0.1 M sodium carbonate-bicarbonate buffer at pH 9.4 to 9.5 was prepared from the specially synthesized NaOH which had been used in the culture media. All of the results reported are based on the direct method of Warburg, although some photosynthetic measurements have been obtained with the indirect method.

RESULTS AND DISCUSSION

Figure 1 compares the growth rates of Chlorella grown autotrophically and heterotrophically without added manganese and with 0.5 ppm added manganese. Heterotrophic growth showed a lag period of about 10 days. The most rapid growth occurred in plus-manganese cultures grown autotrophically. Minus-manganese autotrophic cultures did not produce visible growth. Pirson and Bergmann (27) and Bergmann (4), reported a reduction in growth of the minus-manganese autotrophic cultures to about 50% of that of normal, manganese-free cultures grown by Reisner and Thompson (33) in a medium prepared with purified nutrients. As early as 1930 Hopkins (20) reported that he was able to reduce autotrophic growth 10- to 600-fold in culture solutions from which the contaminating manganese had been removed by adsorption on calcium phosphate.

Growth in the minus-manganese heterotrophic cultures was 50 to 70% of that in plus-manganese heterotrophic control cultures. The manganese contamination in the minus-manganese medium was further reduced by raising a heterotrophic crop of Chlorella in it. Heterotrophic growth of the second crop in this used medium was 34% of the growth in the same used medium to which 0.5 ppm manganese had been added.

The complete elimination of heterotrophic growth was achieved by reducing the concentration of the nutrients 100-fold for the macronutrients and 10-fold for the iron and micronutrients, thus minimizing the contaminating manganese. There was no growth in this medium with 5 x 10⁻⁷ ppm Mn (10⁻¹¹ M) added, but growth did occur with 5 x 10⁻⁶ ppm Mn (10⁻¹⁰ M) or more added (fig 2). The culture with 5 x 10⁻⁶ ppm Mn was appreciably slower in getting started than the cultures with higher concentrations of manganese. Pirson and Bergmann (27) obtained no reduction in growth in minus-manganese heterotrophic cultures compared with cultures containing 0.056 ppm Mn. However, Reisner and Thompson (33) were able to show reduction in the growth of heterotrophic manganese-deficient Chlorella cultures down to 75% of the manganese-sufficient cultures.

Growth, Hill reaction and photosynthesis of autotrophic Chlorella cultures containing various concentrations of manganese were found to closely parallel each other (fig 3). Those with zero or 5 x 10⁻⁵ ppm added manganese usually showed no growth, no Hill reaction activity, and low photosynthesis. As the manganese was increased to 5 x 10⁻⁴ ppm there was very slow growth, and a small corresponding increase in both Hill reaction and photosynthesis. Most of the increase consistently occurred between 5 x 10⁻⁴ ppm and 5 x 10⁻³ ppm manganese, and fairly uniform values resulted at the higher manganese concentration levels. Furthermore, there was consistently somewhat less growth at 5 x 10⁻¹ ppm than at 5 x 10⁻² ppm, and growth declined progressively at still higher levels of manganese. Hill reaction values for Chlorella cells grown with 100 ppm manganese showed a marked decline from the values for Chlorella cells grown with 10 ppm manganese.

In order to show the low photosynthetic rate, 6% of normal, at the lowest manganese concentrations in
figure 3, it was necessary to use specially purified manganese-free sodium carbonate-bicarbonate buffer in the Warburg vessels.

Figure 4 shows that although heterotrophic growth of Chlorella was the same at all of the levels of manganese concentration tried, there was Hill reaction activity and capacity for photosynthesis only if manganese exceeded a minimum value in the cultures. It has been shown repeatedly that "zero-manganese" heterotrophic cultures have no Hill reaction activity and no capacity for photosynthesis. The pronounced increase in Hill reaction values and photosynthesis occurred between $5 \times 10^{-6}$ ppm and $5 \times 10^{-3}$ ppm manganese.

Figure 5 gives the results of an experiment which was designed to yield more detailed information on the portion of the manganese concentration growth curve where autotrophic growth and Hill reaction values showed a pronounced increase. Both one-day- and three-day-growth curves and Hill reaction measurements on the three-day cultures are shown. Autotrophic growth began its pronounced increase at a point slightly above $1 \times 10^{-3}$ ppm manganese. There was a straight line relationship of Hill reaction values for cultures grown with manganese concentrations between $5 \times 10^{-4}$ ppm and $1 \times 10^{-2}$ ppm.

The very small but definite manganese requirement for heterotrophic growth of Chlorella suggests that it is a necessary co-factor of an enzymatic system. A requirement for manganese has been shown by Anderson and Evans (2) for iso-citric dehydrogenase and the "malic enzyme," and by Kornberg et al (22) for oxaloacetic decarboxylase.

The 1000-fold larger manganese requirement for autotrophic growth, and the parallel responses of autotrophic growth, Hill reaction and photosynthesis with respect to manganese content, strongly indicate that manganese is an essential component of that part of the photosynthetic apparatus which is common to these three processes. This conclusion is compatible with the suggestion of Pirson et al (28) and with the further evidence of Kessler (21) that manganese is involved in the photosynthetic liberation of oxygen.

*Scenedesmus quadricauda, Nostoc muscorum, Porphyridium cruentum,* and *Lemna minor* were made manganese deficient by repeated autotrophic sub-culturing until zero Hill reaction activity was attained. The response to the addition of various concentrations of manganese was observed to be generally the same as that shown by Chlorella (figs 6 to 9). *Scenedesmus, Porphyridium,* and *Lemna,* however, required higher levels of manganese, whereas *Nostoc* responded to lower levels of manganese than Chlorella. There was a small but significant increase in growth and Hill reaction of *Nostoc* at $5 \times 10^{-5}$ ppm manganese, a concentration which has consistently been insufficient to cause Chlorella to respond. The possibility that this variation shown by *Nostoc* may have been due to the medium was ruled out by an experiment in which Chlorella was grown in modi-
fied Chu medium. It was found that the same concentration of manganese was required to support autotrophic growth, Hill reaction and photosynthesis as was required in the modified Warburg and Burk medium.

Measurements of Hill reaction and photosynthesis on Porphyridium cruentum were not attempted. In the case of Lemma minor, the Hill reaction was determined on isolated chloroplasts.

These results indicate that the photosynthetic role of manganese is quite general, extending to at least several species of algae with different pigment systems and to higher plants.

Table I presents the chlorophyll content of one-day-old autotrophic cultures of three different algae, and that of an 18-day-old heterotrophic culture of Chlorella, for various concentrations of manganese in the medium.

Prolonged continuous illumination of manganese-deficient autotrophic cultures of Chlorella caused a bleaching effect and resulted in marked chlorosis. Two Chlorella cultures, one with 0.5 ppm Mn added and another with no Mn added, which were exposed to about 1000 ft-c of light autotrophically for three days, were found to contain 7.5 mg and 0.9 mg chlorophyll per ml of packed cells, respectively. When the experiment was repeated, values of 11.3 mg and 2.3 mg chlorophyll per ml of packed cells were obtained.

There are conflicting reports on the chlorophyll content of manganese-deficient plants. Bishop (7) reported that manganese deficiency interfered with the synthesis of chlorophyll thereby affecting carbon assimilation. Portsmouth (29) found that manganese-treated potato leaves became much greener and healthier looking than the manganese-deficient controls. However, Piron et al. (28) as well as others generally agree that manganese deficiency does not directly produce chlorosis. Rao and Lal (30) studied the effect of manganese deficiency on barley plants and reported that the chlorophyll content of the manganese-deficient plants was more than double that of the controls.

The pronounced protective action of manganese against photobleaching of the chlorophyll in Chlorella may also indicate that it is a component of the oxygen disposal system. Photobleaching can result from the oxidizing action of peroxides which are formed by the reduction of oxygen. Thus, the efficient removal of oxygen would decrease photobleaching.

Radioactive manganese was used to determine the manganese content of one-day-old autotrophic Chlorella cells which were cultured in a medium containing the concentration of manganese (5.0 × 10⁻⁵ ppm) just sufficient for maximum growth. The manganese was found to be 114 times more concentrated in the cells than in the medium. Hence, every μl packed cells contained 1.0 × 10⁻¹¹ moles of manganese. Since there were 50 × 10⁶ cells per μl packed cell volume each cell had an average of 120,000 atoms of manganese. Identically grown cells had a chlorophyll content of 5.3 mg per ml packed cell volume (table I) or an average of 7 × 10⁷ chlorophyll molecules per cell. Thus, there is a ratio of about 600 chlorophyll molecules per atom of manganese at the concentration where manganese ceased to be a rate limiting factor. It is interesting to compare this ratio with the calculations of Emerson and Arnold (11) who measured the maximum oxygen yield per flash of light. They found roughly one mole of oxygen for every 2500 moles of chlorophyll for a Chlorella culture comparable to ours. There are four electrons required to form one molecule of oxygen from water, so there has been one electron transferred for every 625 chlorophyll molecules. The general agreement with the manganese-chlorophyll ratio suggests that, in some cases, the limiting factor for photochemical oxygen evolution may be the manganese component. Clendenning and Ehrmantraut (10) reported that there was a rate-limiting catalyst which controlled maxi-

### Table I

**Chlorophyll Content of Algae Cultured at Different Levels of Manganese**

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>TYPE OF CULTURE</th>
<th>CHLOROPHYLL CONTENT AT INDICATED Mn CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml p.c.</td>
<td>mg/ml p.c.</td>
</tr>
<tr>
<td>Chlorella</td>
<td>Autotrophic</td>
<td>6.00</td>
</tr>
<tr>
<td>Chlorella</td>
<td>Het.</td>
<td>6.25</td>
</tr>
<tr>
<td>Scenedesmus</td>
<td>Autotrophic</td>
<td>3.52</td>
</tr>
<tr>
<td>Nostoc</td>
<td>Autotrophic</td>
<td>1.22</td>
</tr>
</tbody>
</table>

* Incomplete extraction.

### Table II

**Cell Numbers, Cell Volumes, and Cell Diameters of Heterotrophically Grown Normal and Manganese Deficient Chlorella**

<table>
<thead>
<tr>
<th></th>
<th>NORMAL + Mn</th>
<th>ZERO Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average cell number</td>
<td>43.5 x 10⁶ cells</td>
<td>21.4 x 10⁶ cells</td>
</tr>
<tr>
<td>Average cell volume</td>
<td>23.0 μ³</td>
<td>46.7 μ³</td>
</tr>
<tr>
<td>Average cell diameter</td>
<td>3.5 μ</td>
<td>4.5 μ</td>
</tr>
</tbody>
</table>
mum oxygen evolution from intact cells of *Chlorella pyrenoidosa* during both photosynthesis and quinone Hill reactions.

There was a marked difference in size, growth habit, and morphology between normal and manganese-deficient heterotrophic Chlorella cells. Normal heterotrophic cells were slightly larger than normal autotrophic cells, and deficient heterotrophic cells had twice the volume of normal heterotrophic cells (table II). Deficient cells had a pronounced tendency to clump and the chloroplasts were poorly defined. Alberts-Dietert (1) also observed the relation of Chlorella cell size to manganese supply.

A good Hill reaction rate was readily obtained by adding manganese to deficient cells. The deficient cells were suspended in the culture medium containing the manganese compound and were incubated in the light (approx. 1000 ft-c) at room temperature for one hour. Some of the results are reported in table III. These results indicate that there were no differences in recovery due to using manganese of different valence states. The importance of incubation time and substrate are also shown. The comparatively rapid response of photosynthesis and Hill reaction when manganese was added to a deficient culture indicates direct participation rather than a secondary action.

In order to rule out the possibility that the manganese effect on the Hill reaction could be unique to the presence of quinone as a Hill reaction oxidant, other oxidants were tested. Hill’s reagent (19), and benzaldehyde (16) were used in comparison with quinone. Similar results were found in all cases, with both manganese-deficient and normal Chlorella. Activity differences between “minus” Mn and “plus” Mn cells with the different oxidants were as follows: benzaldehyde > quinone > Hill’s reagent.

**Summary**

*Chlorella pyrenoidosa* required a minimum of 10⁻⁷ ppm manganese in the culture medium for heterotrophic growth and a 1000-fold larger amount for autotrophic growth. Autotrophic growth, Hill reaction, and photosynthesis responded equally when increments of manganese were added to manganese-deficient cultures. Similar results were obtained with *Scenedesmus quadricauda*, *Nostoc muscorum*, *Porphyridium cruentum*, and *Leucaena minor*. The chlorophyll content of one-day-old autotrophic cultures of *Chlorella* and *Scenedesmus* was not affected adversely by manganese deficiency, but after being illuminated three days, manganese deficient cultures of *Chlorella* had much less chlorophyll than similarly treated non-deficient cultures. When manganese was added to deficient cultures, their capacities for photosynthesis and Hill reaction were quickly restored. *Chlorella* cultured in a medium containing manganese just sufficient for full photosynthesis and Hill reaction had a manganese-chlorophyll molar ratio of 1 to 600. The results indicate that a very small amount of manganese suffices as an essential enzymatic co-factor for heterotrophic growth, and that a much larger amount forms an essential part of the photosynthetic oxygen-evolving apparatus as previously suggested by others.

NaOH was prepared from C. P. Baker’s Analyzed reagent grade sodium by J. Eichel, Chemist. Modifications in chlorophyll determinations for *Nostoc* and *Scenedesmus* were developed by Miss Joan Groulx. Cell counts were done by Miss Grace Norris, and the drawings were prepared by Richard Miller.

**Literature Cited**


