Control by Iron of Chlorophyll Formation and Growth in Euglena gracilis

C. A. Price and E. F. Carell

Plant Biology Department, Rutgers—The State University, New Brunswick, New Jersey

Iron has long been recognized as an essential element for growth and chlorophyll formation in higher plants (2) and, more recently, for photosynthetic bacteria (7, 8, 9).

Despite a wide variety of studies on iron deficiency chlorosis (1, 18), there is no specific information on the site of action of iron in chlorophyll formation. The purpose of our study was to identify this site or sites.

The use of higher plants for physiological and biochemical studies presents certain disadvantages. For reasons outlined elsewhere (15), the alga *Euglena gracilis* combines certain favorable characteristics of higher plants and microorganisms for investigations involving trace metals. Since, in addition, the physiology of chlorophyll production in *Euglena* at least superficially resembles that in higher plants (20) and an iron requirement for growth has been established (13), we thought that *Euglena* might be especially suitable for investigating the role of iron in chlorophyll formation.

In the present paper we report on some of the physiological variables concerned in the control of chlorophyll synthesis and growth by iron. We shall show that the iron-dependent chlorophyll-forming system of *Euglena* provides a remarkable test system for the study of the means by which an inorganic ion controls a metabolic process.

**Methods and Materials**

*Euglena gracilis* (Klebs), Z strain (Hutner), was originally obtained from Dr. S. Hutner.

**Growth Conditions.** The culture medium was as described previously (4, 15). The culture flasks were shaken at 25° in a Model G-25 Rotary Shaker (New Brunswick Scientific Company), modified so that the cultures could be illuminated through a large window in the top of the shaker. Two 22-w day-light fluorescent lamps were suspended to provide 25 or 50 ft-c illumination at the level of the culture media. Growth was monitored turbidimetrically in corrected Klett units (Kc) (15).

All subsequent incubations were carried out at 25°.

**Purification of Medium Components, Glassware, etc.** Potassium phosphate, magnesium sulfate, calcium nitrate, malic acid, and the surfaces of culture flasks and other containers were purified as described elsewhere (15). Deionized water, hydrochloric acid, and ammonium hydroxide were prepared according to Thiers (19). Glutamic acid was purified as follows: reagent grade glutamic acid was made m in metal-free hydrochloric acid and 1.5 m

---

1 Revised manuscript received Feb. 6, 1964.
2 From the doctoral thesis of E. F. Karali (now Carell). Supported in part by Grant G 9815 from the National Science Foundation to Dr. W. R. Robbins. This is a paper of the Journal Series of the New Jersey Agricultural Experiment Station, Rutgers—The State University, Plant Biology Department, New Brunswick.
3 Present address: Department of Botany and Bacteriology, North Carolina State College of the University of North Carolina, Raleigh, North Carolina.
in potassium thiocyanate. The solution was extracted with either ethyl acetate or amyl alcohol (16) several times until the organic phase was colorless. The aqueous solution was titrated to pH 3.0 with metal-free ammonium hydroxide and stored overnight in the refrigerator. The resulting crystals were collected on a large sintered-glass funnel previously soaked in 50% nitric acid, and washed with 95% ethanol until the filtrate was free of thiocyanate (negative to iron solution). The glutamic acid was air dried, weighed, suspended in water, dissolved and titrated to pH 6 with ammonium hydroxide, and diluted to a m concentration.

Iron Determination. A thiocyanate method (16) scaled down to 10 ml final volume, was used. The standard deviation of replicate samples was less than 0.02 μg with 0.2 μg amounts of iron. A sample in which iron was not detected was unlikely therefore to contain more than 0.03 μg. Solutions of the inorganic components of the medium were analyzed directly for iron. Organic samples or cell material were dry-ashed in platinum dishes (19) prior to iron determination. At the suggestion of Dr. R. E. Thiers, a small amount of organic matter (filter paper) was added to platinum dishes when standard solutions of inorganic iron were to be ashed; otherwise recovery was low.

Protein Nitrogen. Samples of cell suspension containing 0.2 to 1 mg of total nitrogen were diluted to about 5 ml volume with 6% trichloracetic acid, heated in a boiling water bath for 15 minutes, cooled, and centrifuged. The supernatant fractions were discarded and the sediments washed 3 times with 6% trichloracetic acid and transferred to Kjeldahl flasks calibrated to 50 ml. The sample was digested, cooled, and brought to 50 ml with distilled water. The acid digestion was then followed by nesslerization (10, 15).

Chlorophyll. One ml of cell suspension was mixed with 4 ml of acetic in centrifuge tubes cooled in ice water. After one hour in the dark the samples were remixed and centrifuged, and the extinction of the clear supernatant solution determined at 663 μm in a Beckman DU spectrophotometer. (The residue from a single extraction was colorless and further extractions did not increase the yield.) The concentration and amounts of chlorophyll were then calculated from the extinction coefficient for chlorophyll, as reported by MacKinney (11).

Chromatography of chlorophyll was carried out by a slight modification of the glass fiber method of Hager (3). Cells extracted in boiling aceticene were mixed with an equal volume of ethyl acetate and washed twice with 10% (w/v) sodium chloride and once with water (6). The organic phase was dried with a minimum amount of sodium sulfate, evaporated to dryness at reduced temperature and pressure, and dissolved in a minimum volume of acetone. The solution of pigments was applied to sheets of glass fiber (S & S No. 25) prepared as described by Hager (3). The amount of pigment applied was less than 30 (E660 optical density units) μl cm⁻¹. The chromatograms were run descending in petroleum ether (30° to 60°): kerosene: absolute ethanol (50: 48: 2) under pressure for about 1 hour.

Hager's glass fiber method has the advantage over chromatography on cellulose paper of many-fold greater capacity and superior resolution. Under our conditions chlorophyll a and b ran at Rf of about 0.8. The exact values varied from one run to another. Chlorophyllides, obtained by extracting spinach or light-grown *Euglena* with cold acetone, ran at Rf values of about 0.2.

Results

*Chlorophyll as a Function of Iron Level.* The components of the growth medium were analyzed for iron and those that contributed more than 0.2 μg per liter of final medium were purified. Unpurified glutamic acid from several sources contained sufficient iron to satisfy completely the requirements of the organism. By means of our extraction method, we succeeded in purifying glutamate sufficiently to limit growth to about 10% of the iron-supplemented controls (table 1).

In order to establish the iron requirement for growth we studied the relationship between iron concentration in the growth medium and the kinetics of growth of the organism. Ferrous sulfate was added to the purified medium to make a final concentration of added iron between 0 and 3.6 × 10⁻⁵ M; 50 μl volumes were inoculated in 250 ml naphtho-culture flasks and the cultures shaken at 25° under laboratory light. Growth was measured continuously until the cells ceased to grow. The time course of growth of some cultures are shown in figure 1. Over most of the growth period the cultures containing 5 × 10⁻⁵ M iron or more showed exponential kinetics; at lower concentrations of iron growth kinetics were at no point exponential.

Preliminary Chlorophyll Studies. *Euglena* were grown under high light intensity with and without 3 × 10⁻⁵ M added iron and the growth and chlorophyll contents determined. The culture with added iron reached 740 Kc and without added iron, 78 Kc. In addition to the limitations of growth in the absence of added iron, the chlorophyll content was disproportionately low. The chlorophyll content with added iron was 38 μg per mg protein nitrogen. Without added iron the concentration of chlorophyll was 14 μg per mg protein nitrogen or 37% of the controls. In different experiments the iron contents of the chlorotic cells were 0.23 to 0.5 μg iron per mg protein nitrogen compared to more than 20 μg per mg protein nitrogen in control cells. Thus, *Euglena* reacted to low nutrient iron levels in a manner characteristic of plants generally.

There were 2 possible reasons for the low level of chlorophyll in the iron-deficient cells: decreased synthesis of chlorophyll or increased destruction. Clearly, if we were to study the role of iron in...
chlorophyll synthesis, we had to exclude interference due to destruction. Iron-sufficient and iron-deficient cells were grown under 500 ft-c, harvested, and made up in concentrated cell suspensions containing about equal concentrations of chlorophyll. The cells were then shaken in a water bath shaker at 1500 ft-c and samples removed at intervals for chlorophyll determination. We expected that if differential bleaching of chlorophyll was occurring, the iron-deficient cells would lose chlorophyll under the high light intensity, while the iron-sufficient cells would lose less or not be affected at all. As shown in Table II this did not occur. Both suspensions increased their chlorophyll concentration, the iron-sufficient controls more rapidly than the deficient cells. While still higher light intensities might have caused bleaching, the low levels of chlorophyll observed in iron-deficient cultures occurred at light intensities of less than 500 ft-c. Thus, simple bleaching could not account for the earlier results.

Chlorophyll Formation in Resting Cells. The rates of chlorophyll synthesis of cells exposed to high light intensity and also the experiments of Huzisige et al. (5) suggested to us that we might be able to induce *Euglena* to synthesize large amounts of chlorophyll by growing the cells under low light and then incubating a washed cell suspension under higher light intensities.

We tested this by growing cells at 25 ft-c, washing and suspending the cells in buffer, and then shaking them under 1500 ft-c (fig 2). No, or in this case, little chlorophyll synthesis occurred in the iron-deficient cells, whereas in the iron-sufficient controls, synthesis occurred rapidly after an initial lag period and was, for an interval, nearly linear with time.

This experiment provided a convenient means for comparison of the rates of chlorophyll synthesis; the cells could grow at normal rates under low light, but the chlorophyll content would be low. By then subjecting a suspension to high light, the cells would be induced to form easily measurable amounts of chlorophyll within a few hours. Since this procedure appeared to constitute a general test system, we sought to characterize it further.

Iron-deficient cells were suspended in different medium components and the rates of chlorophyll formation measured. The rate of chlorophyll synthesis was promoted by iron: the effect of iron was not increased by ethanol, glutamate or other components of the medium (table III and fig 2).

The promotion by iron of chlorophyll synthesis in iron-deficient cell suspensions indicated to us that the iron lesion was partially reversible. While the addition of iron to the deficient cells increased the rate of chlorophyll synthesis, it did not alter the increased length of the lag period that occurs in iron deficiency. Under severe iron-deficiency conditions, no chlorophyll was detected over a 20-hour interval, even with addition of iron.

An optimum concentration for the promotion of chlorophyll formation by iron was found at about 10^{-5} M added iron (fig 3); significant promotion of chlorophyll synthesis was obtained with as little as 3 \times 10^{-8} M iron added to the deficient suspension.

Promotion of chlorophyll synthesis by added iron was found to be independent of the pH of the induction medium from pH 3.5 to 8.

Identity of Newly Formed Chlorophyll. We observed that 80% acetone extracts of cells exposed to light under the standard conditions absorbed with
Price and Carell—Iron, Chlorophyll, and Growth in Euglena

**Fig. 1 (upper left).** Time course of growth of *Euglena gracilis* with different amounts of added iron. Growth monitored in nephelo-culture flasks turbidimetrically in the light at 25°; iron (as ferrous sulfate) added to final concentrations as follows: none, △; 5 × 10⁻⁸, X; 5 × 10⁻⁷, □; 10⁻⁵, ○; 3.6 × 10⁻³, ●. Ordinate units (K₇) are corrected Klett units (15).

**Fig. 2 (upper right).** Time course of chlorophyll synthesis. Cells grown under low (50 ft-c) light intensity with 3 × 10⁻³ M iron (□) or low iron (1.8 × 10⁻⁷ M) (○, △). After harvest the cells were resuspended in 10⁻³ M phosphate buffer pH 6, with (△) or without (○) 3 × 10⁻⁵ M added iron and incubated under high light intensity; samples taken at various intervals for chlorophyll analysis.

**Fig. 3 (lower left).** Rates of chlorophyll synthesis with addition to the cell suspension of various concentrations of iron. Cells grown with 7 × 10⁻⁸ M added ferrous sulfate under 50 ft-c light intensity, harvested and resuspended in 10⁻³ M phosphate buffer, pH 6, at various concentrations of ferrous sulfate as shown; rate of chlorophyll synthesis estimated over following intervals: 8 to 11 hours (○), 11 to 13 hours (△), and 13 to 15 hours (●); suspensions contained 0.47 mg protein nitrogen and 0.14 μg (0.0025 μmole) cellular iron per ml of incubation mixture.

**Fig. 4 (lower right).** Rates of chlorophyll synthesis in *Euglena* as a function of the iron content of the cells. The details of the calculations are in the text; the shaded area represents a region where growth is inhibited. The different symbols represent results from different experiments. In one experiment the cells had been grown at 20° (○), in the remainder at 25°. All rates of chlorophyll formation were determined during incubation at 25°.
Table II.  Time Course of Chlorophyll Levels in Cells Exposed to High Light Intensity

*Euglena* was grown under 450 ft-c, harvested, washed and resuspended in water at concentrations such that suspensions had nearly equal concentrations of chlorophyll. Then the cells were exposed to 1500 ft-c and samples were taken for chlorophyll analysis at various times. The iron-sufficient cells had 0.87 mg protein-N/ml; iron-deficient cells had 1.8 mg protein-N/ml.

<table>
<thead>
<tr>
<th>Concentration of iron added to medium</th>
<th>Chlorophyll concentration (µg chlorophyll/ml cell suspension)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero time</td>
</tr>
<tr>
<td>3 X 10-8 M</td>
<td>34</td>
</tr>
<tr>
<td>None added</td>
<td>26</td>
</tr>
</tbody>
</table>

a peak in the red at 663 mλ characteristic of chlorophyll a; this was true for iron-sufficient cells, iron-deficient cells, and iron-deficient cells treated with iron. We also examined such extracts by glass fiber chromatography. Judging from the Rp and absorption spectrum, the principal green pigment formed was probably the chlorophyllide of chlorophyll a. Chlorophyll a, the chlorophyllide of chlorophyll b, phenophytin a and a fourth unidentified green pigment were also formed in a 10-hour incubation, but in much smaller amounts (about 0.1 as much as the principal pigment). Increases in β-carotene were also observed.

**Protein Synthesis.** We wished to determine if the observed increases in chlorophyll were accompanied by any net increase in cell protein. Samples of different treatments were taken at zero time and after 9 hours, and analyzed for cell protein. No significant change in cell protein was detected under these conditions (table IV). Thus, chlorophyll formation was disengaged from net protein synthesis.

The observation that no net synthesis of protein occurs when chlorophyll is synthesized in our system does not exclude the possibility that protein turnover occurs. However, we find chlorophyll synthesis is also insensitive to dinitrophenol; in fact, the rate is actually increased about 30% (table V).

**Chlorophyll Formation as a Function of Iron Content.** We reasoned that if iron controls a single step in the formation of chlorophyll, we might find a simple relation between the concentration of iron in the cells and the rate of chlorophyll synthesis. We examined, therefore, the rate of chlorophyll formation in cells grown with 10-3 M and 10-6 M added iron. The choice of the lower concentration of added iron was based on the finding that at 10-6 M there was normal growth (cf fig 1) but decreased chlorophyll formation, and that reversal of chlorosis and maximum rates of chlorophyll synthesis were obtained with about 10-6 M iron added to the cell suspension.

Cells grown with 10-5 M and 10-6 M added iron were harvested at different stages of growth in order to obtain cells with varying levels of cell iron. The cells were then suspended in buffer and induced to grow in the presence of dinitrophenol.

Table III. Rates of Chlorophyll Synthesis with Various Additions to *Euglena* Cell Suspensions

*Euglena* was grown under low light intensity either with (iron-sufficient) or without (iron-deficient) the addition of 3 X 10-8 M iron, then harvested, washed, resuspended in water, and shaken at 25° under 1500 ft-c of light. Chlorophyll synthesis was measured as µg chlorophyll.hour⁻1. mg protein-N⁻¹.

<table>
<thead>
<tr>
<th>Composition of suspending medium</th>
<th>Maximum rate of chlorophyll synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iron-sufficient</td>
</tr>
<tr>
<td>Water</td>
<td>4.83</td>
</tr>
<tr>
<td>Water</td>
<td>6.5</td>
</tr>
<tr>
<td>Water</td>
<td>6.25</td>
</tr>
<tr>
<td>Water</td>
<td>6.0</td>
</tr>
<tr>
<td>Complete growth medium less iron</td>
<td>...</td>
</tr>
<tr>
<td>Complete growth medium + iron</td>
<td>...</td>
</tr>
<tr>
<td>2 X 10⁻³ M glutamate + iron</td>
<td>...</td>
</tr>
<tr>
<td>2 X 10⁻³ M glutamate + 0.05 M</td>
<td>...</td>
</tr>
<tr>
<td>ethanol + iron</td>
<td>...</td>
</tr>
</tbody>
</table>

Table IV. Protein Content During Interval of Induced Chlorophyll Synthesis

<table>
<thead>
<tr>
<th>Cell iron content</th>
<th>Protein nitrogen (mg/ml cell suspension)</th>
<th>Protein nitrogen % increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg·mg protein-N⁻¹</td>
<td>Time</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 hours</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>9</td>
</tr>
</tbody>
</table>

* Incubated with 3 X 10⁻⁵ M iron; analyzed for iron prior to incubation.

Table V. Rates of Chlorophyll Synthesis in the Presence of Varying Concentration of Dinitrophenol

Cells suspended in 10⁻³ M phosphate buffer, pH 6.8, and under conditions as described in table III.

<table>
<thead>
<tr>
<th>Concentration of dinitrophenol (m)</th>
<th>Rate of chlorophyll synthesis (µg·(ml of incubation mixture)⁻¹·hour⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.44</td>
</tr>
<tr>
<td>0</td>
<td>2.42</td>
</tr>
<tr>
<td>3 X 10⁻⁶ M</td>
<td>2.44</td>
</tr>
<tr>
<td>3 X 10⁻⁵ M</td>
<td>2.71</td>
</tr>
<tr>
<td>3 X 10⁻⁵ M</td>
<td>2.76</td>
</tr>
<tr>
<td>3 X 10⁻⁵ M</td>
<td>3.21</td>
</tr>
</tbody>
</table>
form chlorophyll. Cells harvested early during growth, contained enough iron to form chlorophyll at normal rates. Cells harvested at more advanced stages of growth, contained less cell iron and synthesized chlorophyll at reduced rates.

Figure 4 contains combined data from different experiments of this sort. The rising part of the curve is the least mean square regression. A clear linear relationship was found between low concentrations of cell iron and the rates of chlorophyll synthesis. Beyond a critical value of cell iron there was no increase in rates of chlorophyll formation as cell iron increased.

The linear part of the curve passes through 2 regions, one (fig 4, shaded area) where growth is limited by iron and the other where growth is normal.

Discussion

Euglena gracilis has a number of promising characteristics as a test system for the study of the means by which iron controls chlorophyll synthesis and growth: under our specified conditions chlorophyll synthesis is rapid, highly repeatable and absolutely dependent on the iron content of the cells. Growth is also dependent on iron, but chlorophyll synthesis can occur independent of growth. Thus Euglena appears to represent the kind of organism recommended by Pirson (15) for the solution of problems in mineral nutrition.

While we have not obtained direct information by which the site or sites of iron action can be identified, the data does permit certain generalities about iron in Euglena.

From the observation that decreased chlorophyll synthesis occurred in cells which had been growing at normal rates (fig 1,3), it is clear that the iron required for growth is associated with sites that are distinct from the iron required for growth. Another observation, that the x-intercept is positive in the chlorophyll-iron curve, shows that there exists a pool of iron in the cell which is absolutely unavailable for chlorophyll synthesis. Oertti and Jacobson (12) published a composite graph for a variety of higher plants, qualitatively similar to our figure 4, and also showing iron unavailable for chlorophyll synthesis.

In view of our finding that 2,4-dinitrophenol fails to inhibit chlorophyll formation (table V), it seems unlikely that oxidative phosphorylation could be the energy source for this synthetic process. It may be that chlorophyll formation is dependent on photophosphorylation. This possibility is supported by Carell's (unpublished) finding that inhibitors of photophosphorylation do inhibit chlorophyll formation.

We shall report later (Carell and Price, in press) on the employment of this Euglena system in studying the relation of porphyrin metabolism to chlorophyll synthesis.

Summary

When Euglena gracilis are grown first under low light intensity and then shaken under high light intensity in a nonnutrient buffer, chlorophyll is synthesized rapidly with high repeatability, and in absolute dependence on the iron content of the cells. Chlorophyll formation in iron-deficient cells is promoted by iron but not by other components of the growth medium. The iron deficiency "lesion" is partially and specifically reversible.

A graph of chlorophyll synthesis as a function of the iron content of the cells shows the rate of chlorophyll synthesis rising linearly from zero to a saturating value of iron, beyond which the rate remains constant over a 20-fold range of iron contents.

Chlorophyll synthesis may be as little as one-third of control rates at iron contents where growth remains normal.

Chlorophyll synthesis may occur without net synthesis of protein and is not inhibited by 2,4-dinitrophenol.

The form of the newly found chlorophyll appears to be principally chlorophyllide a.

Acknowledgments

We thank Dr. W. R. Robbins and Dr. H. E. Clark for their criticisms and suggestions.

Literature Cited


