Porphyrians and the Iron Requirement for Chlorophyll Formation in Euglena

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Although iron has been known to be indispensable for the formation of chlorophyll (11), the mechanism for the iron requirement has not been identified at the level of a chemical or enzymatic reaction. We report here experiments on the possible involvement of iron in porphyrin metabolism.

Pappenheimer (16) found that porphyrins were produced by iron-deficient Corynebacterium diphtheriae. Similarly, porphyrins accumulate in iron-deficient photosynthetic bacteria and the synthesis of bacteriochlorophyll is inhibited (15). In all cases of porphyrin formation with iron deficiency, coproporphyrin was the main porphyrin formed.

Townley and Neilands (20) found that lysed low-iron cells failed to metabolize COPROgen, while addition of iron to these lysates caused disappearance of COPROgen. They suggested a possible role of iron action in the conversion of COPROgen to PROTO. Sano and Granick (18) have found that COPROgen oxidase of beef liver mitochondria was inhibited by 3-phenanthroline and 2,2'-dipyridyl, but not by cyanide ion. The findings of Lascelles (15) are especially pertinent to the problem. Lascelles found that iron-deficient cultures of Rhodopseudomonas spheroides grown under conditions which stimulate chlorophyll synthesis formed predominantly COPRO III, but little or no PROTO, heme or bacteriochlorophyll. Addition of iron almost completely prevented the accumulation of COPRO and at the same time increased the amount of heme and bacteriochlorophyll. With 8-aminolevulinic acid as substrate for the cell suspension instead of glycine and succinate, the addition of iron does not suppress porphyrin synthesis, but it does influence the composition of the mixture of porphyrin formed; although COPRO remains the predominant component, considerable amounts of PROTO are also formed (15).

On the basis of COPRO accumulation in iron deficiency and the promotion of PROTO formation by iron, Lascelles (15) suggested that iron might participate catalytically in the oxidative decarboxylation of the propionic acid side chains of COPROgen III to the vinyl side chain of PROTO.

The aim of the studies reported here was to test the COPROgen hypothesis, that the requirement of iron in chlorophyll synthesis is due to a requirement for iron in the conversion of COPROgen to PROTO.

We found earlier (17) that the rate of chlorophyll formation in Euglena is linearly dependent on the iron content of the cells. We reasoned that the iron-sensitive reaction limiting chlorophyll synthesis should show the same dependency on cell iron content. We have tested several reactions of porphyrin metabolism for such a dependency.

Brief reports of this work have appeared elsewhere (13,14).

Materials and Methods

The organism used was Euglena gracilis (Klebs), Z strain (Hutner). Growth conditions, illumination, harvesting, purification procedures, and methods of iron and protein estimation were as described previously (17). The growth medium contained either 3.5 × 10⁻⁵ M iron (complete), 10⁻⁵ M iron (low iron), or no added iron.

A brief description of the test system (17) follows: Euglena are grown under low light intensity, washed free of growth medium, then resuspended in buffer.
For in vivo studies the cells are shaken under high light intensity whereupon chlorophyll is synthesized rapidly. The rate of chlorophyll synthesis is a strict function of the iron content of the cells. For in vitro studies the permeability of the cells is altered by freezing and thawing; the cells are then shaken with the appropriate substrate.

Cells of different iron contents were obtained by varying the concentration of iron in the medium and by harvesting at various stages during exponential growth.

**Chemicals.** δ-Aminolevulinic acid, URO octamethyl ester and COPRO tetramethyl ester, were purchased from K and K Laboratories, New York, N.Y. Stock solutions of 0.1 m δ-aminolevulinic acid in 0.1 m potassium phosphate buffer pH 7 were freshly prepared before use. The esters of URO and COPRO were hydrolyzed to the free porphyrin by Dr. S. F. MacDonald. Stock solutions of 5 × 10⁻³ m PBG in 0.01 n ammonium hydroxide (metal-free) were freshly prepared before use. PROTO was an gift from Dr. S. Granick. Stock solutions of COPRO, PROTO and URO of 3 × 10⁻³ m in 0.01 m ammonium hydroxide were prepared and stored at −20° in the dark for use as standards.

δ-Aminolevulinic acid was estimated by a modified method of Gibson et al. (7) and PBG as described by Gibson et al. (8).

**Separation and Identification of Porphyrins.** The analysis of porphyrins was carried out either by the differential extraction method of Dresel and Falk (4) or by a chromatographic method. The reaction was stopped by adding 0.1 volume of acetic acid to the incubation mixture. The mixture was frozen in a dry ice-acetone bath and then evaporated under vacuum to about 0.2 of its original volume. The slurry was extracted with 10 volumes of ethyl acetate-acetic acid (3:1) in a manner similar to that of Dresel and Falk (4) except that in a later stage of the extraction, the porphyrins were extracted with 15% HCl, the extract adjusted to pH 3.0 to 3.2, 3.5 and 3.7 to 3.9 and extracted with large volumes of ethyl acetate and ether (several times) until no fluorescence was detected in the last organic extract. The ethyl acetate and ether extracts were combined, washed with small volumes of water, and evaporated to dryness.

The dried porphyrins were then dissolved in a minimum volume of 0.5 n ammonium hydroxide and stored below 0° in the dark for separation and measurement.

The combined porphyrins were separated by the paper chromatographic method of Eriksen (5). Markers of URO, COPRO and PROTO were applied to every paper. The porphyrins were marked under a UV lamp and identified by comparison with the markers, by their RF and by their absorption maxima (6, 3, 10). The fluorescing spots were cut out and eluted in 10% HCl.

When 1 to 30 nmole amounts of porphyrins were applied to the paper, chromatographed, and eluted, recoveries were 96 to 99%, 90 to 96%, and 69 to 70% for URO, COPRO, and PROTO, respectively. When 20 to 50 nmole amounts were carried through the entire extraction and chromatographic procedure, with or without cell material, the recoveries were 34%, 75%, and 36% for URO, COPRO, and PROTO, respectively. The recoveries, although low, were repeatable.

**Estimation of Porphyrins.** By comparison of extinction of porphyrins when dissolved in concentrations of HCl, described in standard references, molar extinction coefficients in 10% HCl were found for PROTO, COPRO and URO to be E₉₅₄ (409 μμ) = 2.75 × 10⁴, E₉₅₄ (403 μμ) = 4.71 × 10⁵ and, E₉₅₄ (407 μμ) = 4.87 × 10⁶ respectively. The RF values of these porphyrins in our system were 0.8, 0.5, and 0.14 respectively.

Other unidentified porphyrins with RF values of 0.7, 0.6, 0.36, and 0.25 were always found as products in Euglena enzyme systems. Sano and Granick (18) assumed a molar extinction coefficient of 3.6 × 10⁶ for a 3-carboxyl porphyrin which probably corresponds to the porphyrin with RF of 0.7 in our system; for the rest of the unidentified porphyrins 4.71 × 10⁵ was assumed as suggested by Schwartz et al. (19).

**Enzyme Systems.** Washed cells were suspended in 0.1 m potassium phosphate buffer pH 7 and twice frozen in an acetone-dry ice bath and thawed.

**δ-Aminolevulinic Acid Dehydratase Activity.** The assay conditions were based on the method of Gibson et al (8). The final incubation mixture contained 0.3 to 0.4 mg protein nitrogen per ml, 0.005 m cysteine, and 0.07 m phosphate buffer pH 7.0. The mixture without substrate was shaken at 35°C under N₂ for 20 minutes, then δ-aminolevulinic acid was added to a final concentration of 0.01 m. The incubation continued anaerobically and samples were taken at short intervals to determine the rate of PBG formed. The activities of such preparations were found to be linear with time to at least 2 hours and with enzyme concentration between 0.1 and 2 mg protein nitrogen per ml.

**Porphyrin Formation from PBG.** The method of Heath and Hoare (12) was used. The final incubation mixture contained 0.5 to 0.6 mg protein nitrogen per ml, 0.1 m phosphate buffer pH 7.0, and 2 × 10⁻⁴ m PBG. The mixture was shaken at 37°C aerobically under laboratory light. Samples were taken at intervals to determine PBG disappearance and porphyrin formation. The rate of PBG disappearance was linear with time to at least 30 minutes, and with enzyme concentration between 0.2 and 1 mg protein nitrogen per ml. Activity was approximately optimal near pH 7.0.

**Results**

In order to test the COPROgen hypothesis, that iron is necessary for the conversion of COPROgen to PROTO, it was necessary to establish the conditions for measuring the activities of *Euglena* enzyme.
systems involved in porphyrin synthesis and then to
determine the dependence of these activities on the
iron concentrations in the cells.

Free Porphyrin Formation from PBG. When
PBG was incubated with frozen and thawed Euglena
which had been harvested from complete nutrient
media, URO, COPRO, and PROTO were formed
(fig 1). In addition unidentified porphyrins, prob-
ably with 3, 5 or 6, and 7 carboxyls were found on the
chromatograms. There was also a component with
an $R_F$ of about 0.6 and a COPRO-like Soret band
which was invariably found when commercial COPRO
was run through the extraction procedure. We des-
ignated it “altered COPRO.”

The disappearance of PBG was rapid and linear
with time during the first 30 to 60 minutes of incuba-
tion; more than 80% disappeared within 1 hour (fig
2). Since 4 molecules of PBG are necessary to syn-
thesize 1 molecule of porphyrin, the yield as measured
by chromatography accounted for only about 25% of
the PBG consumed.

![Fig. 1. Time course of porphyrin formation from PBG by frozen and thawed Euglena preparation. Total porphyrin (●), PROTO (○), COPRO (□), and URO (△).](image)

![Fig. 2. Time course of PBG equivalent disappearance and porphyrin formation by frozen and thawed Euglena preparation. The actual amount of PBG disappearing is divided by 4 to be equivalent to porphyrin formed; equivalent PBG disappearance (○), porphyrin formed (□).](image)

Tests of the COPROgen Hypothesis. Frozen and
thawed cell suspensions of Euglena containing differ-
ent concentrations of cell iron were incubated with
PBG under standard conditions. The different sam-
plies of cells had widely varying capacities to form
chlorophyll but all cultures in the series had shown
normal growth rates; thus, iron was limiting to chlo-
rophyll formation but not to growth. Porphyrin
formation was measured by the chromatographic
method.

The time course of porphyrins formed is shown in
table I. PROTO was in all cases the largest com-
ponent. None of the samples showed any unusual ac-
cumulation of COPRO. The kinetics of individual
porphyrin formation from PBG were not simple under
these conditions but the rates in the different prepara-
tions were similar. We elected to compare the prepa-
trations on the basis of the maximum observed rates
in any 1-hour interval. The rates of PBG disappear-
ance were measured in the same experiment. δ-
Aminolevulinate dehydratase activities were measured
in a different set of cell suspensions. The foregoing
enzyme activities in the different cell suspensions were
Table 1. Time Course of Porphyrins Formed by Frozen and Thawed Euglena
Having Different Concentrations of Cell Iron

<table>
<thead>
<tr>
<th>Identified porphyrins</th>
<th>23.5</th>
<th>2.99</th>
<th>1.52</th>
<th>1.0</th>
<th>0.79</th>
<th>0.46</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROTO</td>
<td>1 hr</td>
<td>0.25</td>
<td>0.50</td>
<td>0.27</td>
<td>0.62</td>
<td>1.63</td>
</tr>
<tr>
<td>COPRO</td>
<td>2 hr</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>URO</td>
<td>3 hr</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>Total identified</td>
<td>1 hr</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>porphyrins</td>
<td>2 hr</td>
<td>4.04</td>
<td>4.04</td>
<td>4.04</td>
<td>4.04</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td>3 hr</td>
<td>4.73</td>
<td>4.73</td>
<td>4.73</td>
<td>4.73</td>
<td>4.73</td>
</tr>
</tbody>
</table>

Unidentified porphyrins
(listed by $R_p$ and $E_{max}$ in Soret region)

| $R_p$ 0.7 | 1 hr | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
|           | 2 hr | 0.27 | 0.27 | 0.27 | 0.27 | 0.27 | 0.27 |
|           | 3 hr | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 |
| $R_p$ 0.6 (altered COPRO) | 1 hr | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |
|           | 2 hr | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
|           | 3 hr | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 |
| $R_p$ 0.4 | 1 hr | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
|           | 2 hr | 0.27 | 0.27 | 0.27 | 0.27 | 0.27 | 0.27 |
|           | 3 hr | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 |
| $R_p$ 0.25 | 1 hr | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
|           | 2 hr | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 |
|           | 3 hr | 0.51 | 0.51 | 0.51 | 0.51 | 0.51 | 0.51 |

N.D. = not detected.

then plotted against the iron contents of the cells (fig 3). The rates of chlorophyll formation were included for comparison. Clearly none of the activities paralleled chlorophyll synthesis.

We recognized that the comparison of chlorophyll formation and enzymic activities was somewhat inexact, since chlorophyll formation was measured in cells strongly illuminated for several hours whereas the measurements of the enzyme activities of porphyrin metabolism were performed at what would correspond to zero time in the chlorophyll formation assay. We therefore repeated the tests with cells which had been shaken under 800 ft-c for 6 hours before being frozen and thawed. In this experiment porphyrin formation was determined by the method of differential extraction. The results (fig 4) were essentially the same as with cells that had not been induced to form chlorophyll.

The data were contrary to the expectations from the COPROgen hypothesis. We concluded therefore that iron does not limit chlorophyll production in Euglena via a role in the transformation of COPROgen to PROTO. But the data were also contrary to
experience with nearly a score of other microorganisms. We faced the disturbing possibility that conclusions about iron metabolism in *Euglena* might not be generalized to other organisms. Close examination of the literature for which data was available showed that the iron lesion between COPRO and PROTO appeared only in microorganisms made so deficient in iron that growth was impaired. For the tests of the COPROgen hypothesis we had specifically avoided growth-limiting iron deficiencies in order to avoid secondary effects. We therefore tested the possibility that *Euglena* would behave like other microorganisms in extreme iron deficiency.

Cells were grown to plateau stage with either no added iron (approx $5 \times 10^{-8}$ M of residual iron) or $2 \times 10^{-8}$ M added iron (complete medium). The cells were harvested, frozen and thawed, and incubated with PBG in the usual way. The results (fig 5) showed substantially decreased production of PROTO and an enhanced accumulation of COPRO relative to the controls in the severely deficient cells. Moreover the relative increase in COPRO formation is enhanced with time over the course of the incubation (table II).

![Graph showing enzyme activities of preinduced cells as functions of iron contents.](image)

**Fig. 4.** Enzyme activities of preinduced cells as functions of iron contents. Rates of equivalent PBG disappearance (□), COPRO (●), chlorophyll (○), and PROTO (△) formation were determined with frozen and thawed *Euglena* which had been incubated for 6 hours under high light intensity.

![Graph showing porphyrin accumulation by *Euglena* grown with sufficient and with extremely deficient iron.](image)

**Fig. 5.** Porphyrin accumulation by *Euglena* grown with sufficient and with extremely deficient iron. *Euglena* were grown with sufficient iron (solid symbols, ● □) or extremely deficient iron (open symbols, ○ □); frozen and thawed cells were incubated with PBG and analyzed at intervals for PROTO (□, ■) and COPRO (○, ●).

**Discussion**

The COPROgen hypothesis, that the COPROgen-toPROTO reaction is the site of action of iron in chlorophyll formation, has been considered in one

<table>
<thead>
<tr>
<th>Table II. Time Course of COPRO, PROTO and Percent COPRO to PROTO Formed by Frozen and Thawed <em>Euglena</em> Having Different Concentrations of Cell Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells were grown in either no added iron or complete medium. The conditions and detail are the same as in figure 5.</td>
</tr>
<tr>
<td>Cell iron</td>
</tr>
<tr>
<td>0.29</td>
</tr>
<tr>
<td>mmoles</td>
</tr>
<tr>
<td>Time hours</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

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form or another by several writers (15). We have tested a deduction from the hypothesis, that with increasing cellular iron concentrations over the range where iron limits chlorophyll formation, the rates of chlorophyll formation should increase at the same pace as increases in the rate of the COPROgen-to-PROTO reaction.

If this expectation had been realized, the data would have comprised evidence in support of the hypothesis. Since the expectation was not realized, we must inquire whether the evidence requires that the hypothesis be rejected.

The validity of the physiological test system was established previously (17): chlorophyll a was the principal pigment formed by the cells and the rates of formation were substantially linear with time over the range tested, etc.

Did the enzymological test system correctly measure the capacity of the cells to transform COPROgen to PROTO? The frozen and thawed cells were fed PBG rather than COPROgen. The time course of porphyrin accumulation determined chromatographically (fig 1,2) was consistent with the sequence PBG → UROgen → COPROgen → PROTO. We need assume only that the porphyrinogens were oxidized to porphyrins during the separation procedure. The conditions, if not optimal for measuring COPROgen oxidase (18), were at least comparable to those under which the iron lesion had been observed with other microorganisms.

The rates of PROTO formation were small relative to chlorophyll formation in the experiments where porphyrins were separated chromatographically. We knew, however, that recovery of PROTO in the extraction procedure was low (Methods). When the porphyrins were measured by differential solvent extraction (fig 4) the rates of PROTO formation were of the same order of magnitude as those of chlorophyll formation. It thus appears that we were measuring a metabolically significant fraction of porphyrin metabolism. The conclusion appears inescapable that iron-deficient cells can have an impaired capacity to form chlorophyll and have at the same time normal COPROgen oxidase activities.

Our results would not exclude the hypothesis only if there were a separate system for synthesizing porphyrins leading to chlorophyll formation, if this system were peculiarly sensitive to iron, and if this system comprised a negligible fraction of the total porphyrin production. Carell and Kahn (2) have in fact found that isolated Euglena chloroplasts can synthesize porphyrins. A separate system therefore does exist, but the activity of these organelles appears to comprise a sufficiently large fraction of the total porphyrin production that we should have detected any sensitivity to iron deficiency.

It thus seems that the iron lesion in chlorophyll synthesis cannot be at the COPROgen oxidase step nor, it would appear, at any step between δ-aminolevulinate and PROTO. In Euglena as in other microorganisms iron ap- pears to be required for the conversion of COPROgen to PROTO. This iron requirement is apparently satisfied before that for chlorophyll synthesis. Euglena (and green plants generally) may be quantitatively different from bacteria, fungi, and animal cells in their response to iron deficiency. Free porphyrins do not accumulate even in severely iron-deficient green plants unless a porphyrin precursor is fed.

The site of the iron lesion in chlorophyll deficiency remains unidentified. The suggestions of a feedback mechanism are attractive but unproved (9, 2).

A few other plant enzyme systems capable of transforming δ-aminolevulinate and PBG into porphyrins have been described (10).

Note added in proof: we have unaccountably neglected to note the excellent work of H. V. Marsh, H. J. Evans, and G. Matrone (Plant Physiol. 38: 638, 1963), in which they found normal rates of δ-aminolevulinate metabolism in iron deficient cow peas.

Summary

We have tested the hypothesis that the requirement of iron in chlorophyll synthesis is due to a requirement for iron in the conversion of coproporphyrinogen (COPROgen) to protoporphyrin (PROTO).

The rate of synthesis of chlorophyll in Euglena gracilis can be made linearly dependent on the iron content of the cells. Over that part of the range of iron contents where growth is not limited, the activities of several enzyme systems presumably required for porphyrin formation are also independent of iron. These include δ-aminolevulinate dehydratase, porphobilinogen deaminase, and the enzymatic synthesis of COPRO and PROTO from porphobilinogen.

Only at extreme iron deficiency where growth is limited did we observe accumulation of COPRO and decreased rates of PROTO formation.

We conclude that while iron may be required for the conversion of COPROgen to PROTO in Euglena as it is in many other organisms, this requirement has nothing to do with the iron requirement in chlorophyll synthesis. We conclude that the COPROgen hypothesis seems not to be correct.

Literature Cited


2. Carell, E. F. and J. S. Kahn. 1964. Arch Bio-


Oxygen Uptake and Evolution Following Monochromatic Flashes in Ulva and an Action Spectrum for System I.1

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Carnegie Institution of Washington, Department of Plant Biology, Stanford, California

The time courses of O2 exchange induced in Ulva by monochromatic light flashes lasting 1 second or less is reported here for a variety of conditions. The resulting O2 exchange may be entirely positive corresponding to O2 evolution, entirely negative due to O2 uptake, or a complex mixture of both effects. Following up studies on the nature of the pressure-insensitive initial O2 outburst, Vidaver (12) has found Ulva lobata (sea lettuce) to show a particularly strong initial O2 uptake, negative spike, from flashes of appropriate wavelength, intensity and duration. The O2 uptake in Ulva may be so large as to completely obscure photosynthetic O2 evolution. The O2 uptake is presumably caused by the formation of a rapidly oxidizable photoproduct by the long wavelength chlorophyll a (system I) as previously described by French and Fork (6,7). They observed O2 uptake transients in Porphyridium. There was an O2 uptake lasting several minutes after turning off the light. This uptake was partially obscured by what we consider to be delayed O2 release in the dark. Furthermore, O2 uptake was seen also as a small but initial negative spike. In the present experiments the initial negative spikes are larger than in Porphyridium.

Variations in wavelength, intensity, flash duration, and dark time between flashes were studied. The time course of gas exchange induced by these flashes is complex. The following recognizable components of the time course may overlap each other but their individual contributions to the curves is clear. The events start with a rapid O2 evolution followed by an O2 uptake which is nearly as fast. Either of these initial events may completely or partially obscure the other. After these positive or negative spikes there is a slow and complex return to the previous rate of dark respiration which lasts for 1 or 2 minutes. After an O2 uptake spike there is an overshoot which

1 Received May 11, 1964.