MAGNESIUM PROTOPORPHYRIN AS A PRECURSOR OF CHLOROPHYLL IN CHLORELLA*

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(Received for publication, April 17, 1948)

In this paper, we wish to describe the separation and identification of another intermediate in chlorophyll synthesis from another Chlorella mutant.

The Chlorella vulgaris mutant 60 was isolated as an orange-colored colony after irradiation of normal green Chlorella cells with x-rays. Single cells from this colony showed constancy in their properties. The colonies which developed on the inorganic salts-glucose agar medium had a dull yellow color which turned orange-brown in 4 to 7 days when grown either in the light or dark at room temperature. At 36° growth was good but the colonies remained dull yellow. At 15° growth was very poor and colonies also remained dull yellow. The cells were grown in large flasks and harvested after 7 to 8 days. To minimize the chances of dealing with a mixed population due to spontaneous mutation, cultures for a large batch were always started from a typical colony derived from a single cell.

Extraction of Pigments from Supernatant Suspension of Cells---The cells were found to contain a complex mixture of pigments, the predominating ones being the carotenoids, with small amounts of protoporphyrin, magnesium protoporphyrin, and traces of a greenish pigment. By shaking the cells in distilled water, a reddish brown cloudy suspension was obtained which was relatively free of carotenoids and had absorption bands at 640, 590, 540 to 550, 470 to 480, and 420 to 425 mμ, as measured in a Beckman spectrophotometer (Fig. 1). Pigments from this cloudy suspension were readily isolated by treating the suspension with an equal volume of alcohol, saturating with NaCl, and extracting into ether. Preliminary tests showed that protoporphyrin was present in the ether solution. To get rid of the protoporphyrin the ether solution was washed with water several times and then rapidly extracted with an equal volume of ice-cold 1 N HCl.1 (The acid aqueous layer was shown spectrophotometrically to contain protoporphyrin.) The ether layer was immediately treated with an equal

* This is the fourth of a series of papers on porphyrins and related compounds. For the third paper, see Granick (1).

1 If no foaming occurs, then only a little Mg protoporphyrin will be decomposed by shaking the ether layer with cold 1 N HCl.
volume of ice-cold 1:1 solution of 0.02 M KOH and absolute alcohol. A pinkish fluorescent pigment entered the aqueous phase. (The residue in the ether now consisted of carotenoids and of a trace of a greenish pigment.)

The absorption spectrum of the aqueous alcohol solution was measured and the solution was found to contain two components (Fig. 2). The major component had two prominent bands in the visible with maxima at 550 and 588 m\(\mu\) and an intense ultraviolet maximum at 418 m\(\mu\); this comp-

ponent was later identified as magnesium protoporphyrin. The other, lesser component was found to be protoporphyrin which explained the bands at 630, 530, and 505 m\(\mu\). The bands seen in the cloudy aqueous suspension (Fig. 1) are best interpreted as representing colloidal aggregates of protoporphyrin with a small amount of magnesium protoporphyrin (1).

**Extraction of Pigments from Cells**—The isolation of the pigment, having bands at 550 and 588 m\(\mu\), was difficult because of the sensitivity of this pigment to acids, its low concentration, and the rather large amounts of contaminating yellow pigments. No simple procedure was found. Only
the general principles of the isolation will be described: it was necessary to control each step of the isolation by observations in the hand spectroscope.

To keep the cells slightly alkaline in order to avoid splitting out of the Mg, sodium bicarbonate was added to some 100 cc. of packed cells, and these were extracted until colorless with 80 per cent alcohol and 80 per cent acetone. To remove yellow pigments and fats and traces of green pigments, the alcoholic solutions were diluted with an equal volume of water, made alkaline with NH$_4$OH, and extracted with ether. The aqueous solution was brought to pH 5.5 to 6.0 with solid KH$_2$PO$_4$ and shaken with a small volume of n-amyl alcohol. Overnight in the ice box, the pigment was found to have collected in the amyl alcohol layer. The amyl alcohol layer was separated and evaporated to dryness under reduced pressure; the pink pigments were taken up in ether and shaken into a 50 per cent alcoholic layer containing dilute NH$_4$OH and again driven into ether by acidifying cautiously with acetate buffer and saturating the aqueous alcoholic layer with NaCl. This transfer between ether and aqueous alcohol was repeated twice more. The ether solution was then concentrated to 10 cc. The ether solution now contained as major components the two pigments, protoporphyrin and Mg protoporphyrin. By shaking

![Fig. 2. Absorption spectrum of alkaline alcoholic solution derived from supernatant. The three prominent bands are due to magnesium protoporphyrin. The extinction is 10 times higher on the left side than on the right side. The extinction signifies the observed densities.](image-url)
the ether solution with 0.05 cc. of 3 N NH₄OH, and then placing in the ice box overnight, the protoporphyrin was found to be precipitated out at the interface. (Under these conditions Mg protoporphyrin precipitated out only after 3 to 4 days.)

![Graph](http://www.jbc.org/)

**Fig. 3.** Smooth curve, absorption spectrum of synthetic Mg protoporphyrin dimethyl ester in ether. X, absorption spectrum of synthetic Mg protoporphyrin in 0.02 N KOH containing 50 per cent ethanol. O, absorption spectrum of pigments isolated from *Chlorella 60*, measured in 0.02 N KOH containing 50 per cent ethanol; the extinction values were adjusted at 588 m\(\mu\) to the Mg protoporphyrin curve by a factor and the remaining points were multiplied by this factor.

An aliquot of this solution, evaporated to dryness, was taken up in a solution of 0.02 N KOH containing 50 per cent ethyl alcohol and measured spectrophotometrically. One point on this absorption curve (i.e. 588 m\(\mu\)) was adjusted to the curve for synthetic Mg protoporphyrin by a factor, and the remaining points were multiplied by this factor (Fig. 3). From
Fig. 3 it is seen that the predominating pigment in this solution corresponds to synthetic Mg protoporphyrin with respect to the positions of the absorption maxima (418, 551, and 589 m\(\mu\)) and the relative heights of the bands. The solution is still contaminated by small amounts of protoporphyrin, as seen in the bands at 505 and 630 m\(\mu\), possibly by carotenoids (i.e. band at 465 m\(\mu\)), and by a trace of a greenish pigment (640 to 670 m\(\mu\)). Since the solution was estimated to contain only about 0.5 mg. of Mg protoporphyrin, further purification by chromatographing was not attempted.

Identification of Porphyrin of Metal Complex As Protoporphyrin—Since only about 0.5 mg. of the magnesium protoporphyrin was isolated from Chlorella, and since the isolation in the crystalline state would have been too tedious, it was deemed necessary to obtain supporting evidence for the composition of this compound by identification of the kind of porphyrin and the kind of metal. An aliquot of the ether solution was extracted with 3 N HCl. At this acidity the metal was split off and all of the pink pigment entered the aqueous phase. The aqueous solution was neutralized and the porphyrin reextracted into ether. The ether solution was washed with water and then extracted successively with increasing concentrations of HCl. No porphyrins were extractable from ether with HCl solutions below 0.1 N. Two fractions were isolated by extraction between 0.1 and 0.4 N HCl and between 0.4 and 1.0 N HCl. The absorption spectra of both these fractions fell, within experimental error, on the curve of pure protoporphyrin (Fig. 4). This result indicates that neither a monovinyl nor any other porphyrin except protoporphyrin was present, and therefore the pigment originally isolated must be a derivative of protoporphyrin. Neither esters of magnesium protoporphyrin nor esters of protoporphyrin could be found in this preparation or in crude preparations that had been extracted from cells in which the use of alkaline fluids was avoided. Such esters would have been detected in the ether after extraction with 1 N HCl. (When Chlorella 60 was grown in a medium containing 1 mg. of Cu per liter, a small amount of pigment was observed which was stable in strong HCl; the positions of the band maxima were those of Cu protoporphyrin.)

Identification of Magnesium As Metal in Complex—The qualitative identification of magnesium was carried out with the quinalizarin reagent (2). The test is highly specific, only beryllium and lanthanum besides magnesium being reported to form a blue precipitate in strongly alkaline solution. However, the test is not particularly sensitive. A method was devised to remove the porphyrin, which interferes with observation of the blue precipitate, and at the same time to keep the magnesium as concentrated as possible.

The test was carried out in the following way: 5 cc. of the ether solution, estimated to contain approximately 250 \(\gamma\) of magnesium protopor-
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phyrin, were placed in a 15 cc. conical centrifuge tube. Then 0.10 cc. of 4.6 N HCl was added, and air was bubbled through to stir the HCl into the ether layer. After several minutes the ether layer became completely colorless and all of the protoporphyrin was now collected into the drop of aqueous HCl at the bottom of the tube. The HCl was neutralized by adding 0.12 cc. of 4.00 N NaOH plus 0.01 cc. of glacial acetic acid to the tube. Air was again bubbled through until all of the protoporphyrin had passed back into the ether layer. The tube was then centrifuged. The clear colorless aqueous droplet at the bottom of the tube was now removed with a capillary pipette and placed on a drop plate, and 2 drops of alcoholic quinalizarin (10 mg. per cent) and 2 drops of 2 N NaOH were added. Blue granules appeared within 1 minute. At the same time and on the same drop plate a series of known concentrations of Mg++ was run, including controls of the reagents. From the rate at which the blue granules appeared and their volume it was estimated that the quantity of Mg++ was about 10 γ, which was in the predicted range if the compound was Mg++ protoporphyrin. (At this concentration Ca++ does not give a characteristic blue precipitate.)

Neither magnesium protoporphyrin nor its ester has ever been prepared

Fig. 4. Smooth curve, absorption spectrum of synthetic protoporphyrin IX in ether. Absorption of porphyrin derived from magnesium protoporphyrin isolated from mutant Chlorella; X, porphyrin extracted between 0.1 and 0.4 N HCl; O, porphyrin extracted between 0.4 and 1.0 N HCl. The extinction is 10 times higher on the left side than on the right side.
in the crystalline form. It was deemed necessary to prepare the magnesium protoporphyrin and to study its properties, in order to compare it with the pigment derived from the Chondra mutant. The dipotassium salt of magnesium protoporphyrin was made by way of the dimethyl ester.

Preparation of Magnesium Protoporphyrin Dimethyl Ester—The method used here, of inserting the magnesium into protoporphyrin by means of a decomposed Grignard reagent, is a modification of that used by Fischer and Dürr (3). To obtain this compound in its crystalline form, it was found necessary to use highly purified protoporphyrin ester, to avoid temperatures above 80°, and to run the reaction preferably in the absence of O2.

In a 500 cc. round bottom triple necked flask was placed 1.0 gm. of Mg ribbon. This was washed by decantation with anhydrous ether. Then 30 cc. of anhydrous ether and 15 cc. of ethyl bromide were added. The flask was connected to a reflux condenser with a drying tube attached, and the contents warmed gently. After some 20 minutes the reaction was ended. To distil off the ether and excess ethyl bromide most easily, the water in the reflux condenser was emptied, and the flask, still attached to the condenser, was placed in hot water. Toward the end of the evaporation, suction was applied through the drying tube to aid in the distillation and drying.

The flask containing the dry ethyl magnesium bromide was cooled in ice water, and 75 cc. of dry n-propyl alcohol (distilled over CaO) were added in small portions through the top of the reflux condenser. The residue dissolved completely. The solution was then heated and refluxed for 10 minutes to decompose the last traces of the Grignard reagent. After cooling to 50°, 280 mg. of twice crystallized protoporphyrin dimethyl ester were added to the flask, arrangement being made to pass dry N2 slowly into one arm of the flask. The flask was placed on a water bath and kept at 70-75° for 5 to 7 hours or until the protoporphyrin band at 630 µm had disappeared. Higher temperatures or prolonged heating led to yellow decomposition products.

The material in the flask was now transferred, with the aid of a small amount of ether, to a Claisen distilling flask and the propyl alcohol distilled almost to dryness under diminished pressure in the presence of N2. The dry material was now transferred with the aid of 750 cc. of water and 750 cc. of ether to a 2 liter separatory funnel. The solution was shaken to extract most of the Mg protoporphyrin ester into the ether. Then 100 cc. of a solution containing 10 gm. of ammonium acetate and 10 gm. of Na2HPO4 were added. A flocculent precipitate of MgNH4PO4 was produced. This was drawn off and the ether layer washed several times with water. A small amount of impurity went to the interphase and was drawn off. The ether solution was dried with anhydrous sodium sulfate and evaporated.
to dryness under diminished pressure in the presence of N₂. The dark red powdery residue was dissolved in some 30 cc. of wet ether and filtered. A small brownish residue with a band at 470 to 480 mμ collected on the filter and was discarded.

Even when pure, the Mg protoporphyrin ester is difficult to crystallize from solution, although crystals will be found to form on a glass slide under the microscope. Crystallization was accomplished in the following manner: To the concentrated ether solution, 3 cc. of xylene were added, and the solution further evaporated down to about 7 to 10 cc. Then 5 cc. of low boiling petroleum ether were added (b.p. 30–60°) and a crystalline precipitate rapidly formed. After cooling for several hours the precipitate was centrifuged, washed with low boiling petroleum ether by centrifuging, and then filtered off. The yield of this crystalline pinkish powder was 220 mg. or about 75 per cent of theory.

A portion of the pink powder was washed on the filter with anhydrous ether. The filtrate consisted of a colloidal solution; a slight residue remained on the filter paper. To the filtrate was added low boiling petroleum ether, and a precipitate of plates and highly twinned crystals resulted. This was centrifuged, washed with low boiling petroleum ether, and dried in vacuo. Analyses of this material showed the following percentage composition.

$$C_{36}H_{26}O_{4}N_{4}Mg.$$  
Calculated:  C 70.5, H 5.89, N 9.15, Mg 3.98

Found:  C 70.36, H 6.01, N 9.04, Mg 4.00

An aliquot of this crystalline material was dissolved in moist ether and the absorption spectrum of the red fluorescent pigment was determined (smooth curve, Fig. 3). The absorption bands are very sharp and high. The molar extinction per cm. of light path for a given wave-length is given by

$$E_{\text{mole per liter}} = \log_{10} \frac{I_0}{I} \times \frac{1}{\text{cm.} \times \text{mole per liter}}$$

$E$ at 419 mμ = 308,000, at 340 mμ = 20,550, at 511 mμ = 18,200, at 589 mμ = 18,200, and at 510 mμ = 2450.

A comparison of the position of the visible absorption spectra of various divalent metal mesoporphyrins which have been studied shows that the Mg compound has its bands furthest displaced toward the red end of the spectrum (4).

Preparation of Dipotassium Salt of Mg Protoporphyrin—30 mg. of the Mg protoporphyrin ester were treated in a 50 cc. centrifuge tube with 5 cc. of 30 per cent methyl alcoholic KOH for 15 minutes at 40°. Then 10 to 15 cc. of water were added, resulting in a flocculent precipitate. The precipitate was centrifuged down and the supernatant liquid discarded. The
precipitate was dissolved in 5 cc. of hot methanol and placed in the ice box. Crystals arose, consisting of rhomboid plates, often highly twinned, especially if rapidly formed (Fig. 5). If crystallization did not occur under these conditions, then crystallization could be induced by adding small portions of a solution made up by diluting the methyl alcoholic KOH 1:10 with water. The plates were dichroic, dark red and pale yellow. On the basis of \( \text{K}_2\text{C}_{34}\text{H}_{30}\text{O}_4\text{Mg} \), calculated, \( N = 8.4 \) per cent; found, 8.2 per cent. The absorption spectrum of this compound was measured in 0.02 n KOH containing 50 per cent ethanol. The extinction values in this solution are lower than for the ester in the ether solution (Fig. 3).

Biological Activity—\textit{Hemophilus influenzae} Turner requires heme or protoporphyrin for growth and for the reduction of nitrate to nitrite (4).

![Fig. 5. Crystals of the dipotassium salt of magnesium protoporphyrin. Left-hand, formed rapidly from aqueous methanol, \( \times 400 \); right-hand, formed slowly from methanol, \( \times 100 \).](image)

It was found that Mg protoporphyrin would support the growth of this organism at a concentration one-fifth of that of protoporphyrin. As with protoporphyrin, the nitrate reducing activity of organisms grown on Mg protoporphyrin was proportional to the growth of the organisms. A tentative explanation for the fact that the growth-promoting effect of magnesium protoporphyrin is greater than that of the protoporphyrin itself may be found in the fact that the magnesium porphyrin has a smaller tendency to form colloidal solutions and will therefore be more readily available to the cell than porphyrin itself. Once in the cell, it is probable that the magnesium is split out and then the iron inserted (4).

We desire to express our thanks to Dr. L. Michaelis for his constant stimulation and advice.
SUMMARY

From 100 cc. of cells of *Chorella* mutant 60, about 0.5 mg. of a pinkish fluorescent pigment was isolated. This was identified as Mg protoporphyrin in several ways. Its absorption spectrum agreed with the spectrum of synthetic magnesium protoporphyrin in position and relative heights of the bands. The metal was split out of the complex with acid and identified as Mg$^{++}$ by the quinalizarin lake method. The porphyrin was identified as protoporphyrin by its HCl number and by its absorption spectrum.

Methods for the synthesis of magnesium protoporphyrin dimethyl ester and of the dipotassium salt of magnesium protoporphyrin are described.

The isolation of magnesium protoporphyrin suggests that, after the synthesis of protoporphyrin, insertion of magnesium is the next step in the biological synthesis of chlorophyll by *Chlorella*.

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