Iron, Porphyrins and Chlorophyll

IRON is essential for chlorophyll formation[1]. A reasonable hypothesis is that this element acts in the conversion of coproporphyrinogen (COPROgen) to protoporphyrin (PROTO)[2,3]. We have tested this hypothesis and find the results contrary to expectation.

The plausibility of what may be called the COPROgen hypothesis stems originally from the finding of Pappenheimer and Johnson[4] that iron is essential for toxin production by *Corynebacterium diphtheriae*. Since then it has been found that these bacteria, when iron-deficient, apparently develop a metabolic lesion between COPRO and PROTO and consequently produce large quantities of COPRO; this response is shared by a wide variety of micro-organisms. As Granick and Mauzerall[5] have pointed out, all the available evidence points to the view that free porphyrins (or rather porphyrinogens) are precursors of chlorophyll; it naturally follows that a lesion in the porphyrin pathway would affect chlorophyll synthesis.

We have found that *Euglena gracilis* is an advantageous test object for studying chlorophyll synthesis. The alga may be grown heterotrophically on glutamate-ethanol[6] under low light intensity (25–50 ft.-candles) at 25°. After the desired growth has been achieved, the cells are washed and resuspended in 10^{-3} M potassium phosphate buffer, *p*H 6·8, and shaken under high light intensity (1,000–1,500 ft.-candles). Under these latter conditions the cells synthesize chlorophyll vigorously, but show no net increase in protein. Thus chlorophyll synthesis may be divorced from growth. Chlorophyll was estimated from the absorbancy at 663 mμ of aliquots extracted in 80 per cent acetone.

After the components of the medium were purified, we could demonstrate iron requirements for growth and for chlorophyll formation. At the lowest levels of iron, growth was inhibited (shaded part of Fig. 1). There was also a region of iron concentration throughout which growth was normal (unshaded part of Fig. 1); in part of this region of normal growth, capacity for chlorophyll formation was decreased. By confining the alga to the region of normal growth, we avoided complications due to consequences of growth inhibition.

From the COPROgen hypothesis one can deduce directly that the rate of chlorophyll synthesis in iron deficiency would be limited by the rate of PROTO formation. We expected specifically that the rate of PROTO formation would decrease with decreasing iron content, starting at some critical iron content which would be equal to, or greater than, the level that was critical for chlorophyll formation.

To test this hypothesis, we incubated suspensions of frozen and thawed *Euglena* of varying iron content with 2×10^{-4} M porphobilinogen in 0·06 M potassium phosphate, *p*H 6·8, and estimated the resulting free porphyrins. Separation was carried out by a modification of the extraction procedure of Dresel and Falk[7] and the chromatographic method recommended by Eriksen[8]. The several porphyrin components were eluted and the amounts determined spectrophotometrically at their Soret peaks. No unusual accumulations of COPRO were observed at any of the iron-levels reported here. On the contrary, the rates of COPRO formation decreased slightly with decreasing cell iron. At the same time, the iron-deficient cells, although defective in their capacity to form chlorophyll, produced PROTO at rates equal to or greater than the iron-sufficient controls (Fig. 1).

From the COPROgen hypothesis we expected that the rates of PROTO formation would decrease with decreasing iron concentration. Our results are clearly at variance with these expectations. While one can imagine a variety of *ad hoc* modifications to make the hypothesis fit the data, we believe that the COPROgen hypothesis is unlikely to prove correct.

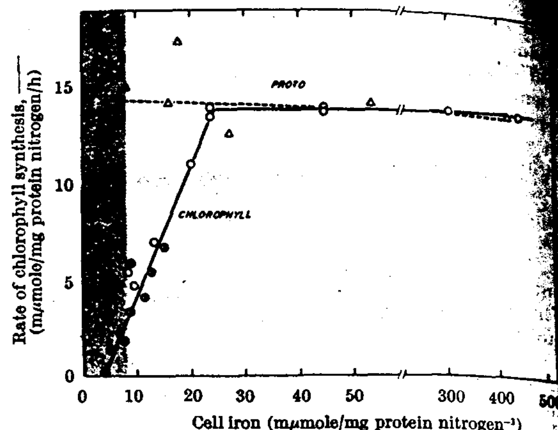

Fig. 1. Rates of chlorophyll and protoporphyrin formation at va[illegible] levels of cell iron. The different symbols represent data from se[illegible] experiments. The PROTO curve and the rising part of the chloro[illegible] curve are least mean square regressions

There are hypotheses assigning the action of ir[illegible] ALA formation directly or indirectly. Our experi[illegible] do not bear on these alternative suggestions.

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Age of the Baobab Tree

THE baobab tree *Adansonia digitata* L. (family Bombacaceae) has been variously estimated as being several hundred to several thousand years old. The French botanist Adanson, after whom the tree was named, contended that some specimens were as much as 5,000 years old[1].

It has, however, never been established that the baobab produces annual rings, and such estimates must therefore be regarded as largely guesswork[2]. Since many of these trees are more than 20 ft. in diameter and some even in excess of 30 ft.[1,2], the labour involved in obtaining a complete section and producing a sufficiently smooth surface to count the rings accurately would in any event be enormous, and it is doubtful whether this has ever been attempted. Moreover, many of the really larger baobabs have hollow centres due to natural causes or as a result of human intervention[1,2], thus rendering any attempt at ring-counting impossible.

As a result of bush-clearing operations at Lake Kariba we were fortunate enough to obtain a sample of wood from the heart of a 15-ft. diameter baobab tree (at a height of about 2 ft. above ground-level) which was kindly supplied to us by Mr. E. Swart. The tree was felled during 1960 near the confluence of the Sengwe and Zambesi Rivers (16° 55′ S.; 28° 05′ E.). Samples were also obtained from a point midway between the

centre and the outside of the tree and from a point directly adjacent to the bark.

We have measured the radiocarbon content of all three samples and deduced their ages using a carbon-14 half-life of 5,568 years. The heartwood gave an age of 1,010 ± 100 years B.P., and the sample midway between the centre and the outside of the tree gave an age of 740 ± 100 years B.P.

The sample adjacent to the bark which probably covered some 4 or 5 years of growth gave an activity of 1–2 per cent higher than that of age corrected 1,890 wood.

The indications are that the tree examined grew more slowly over the outer 7½ ft. of its diameter, and there thus appears to be no reason why some of the really large baobabs should not be several thousand years old. The average annual increase in radius over the last 3¾ ft. (1·14 m) of the total radius works out at 12 × 3·75 × 25·4/740 = 1·5 mm.

The actual measured average ring-width over the last 20 cm of growth was about 1·1 mm, which is not incompatible with the view that the rings are annual rings and that the tree did increase in radius more slowly during the latter half of its life. The baobab does have a very definite annual growth cycle, being completely bare of leaves during the dry season.

If it were not for the fact that so many of the really large baobabs are hollow they might well be ideal samples on which to determine the variation in the radiocarbon content of the atmosphere over the past few thousand years.

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Changes in Metabolism of Isolated Root Systems of Soy Bean

It is a familiar difficulty in work on nitrogen-fixation by excised root nodules that the capacity to fix nitrogen decreases rapidly. This may be due either to a shortage of some essential metabolite[1] or to an accumulation of some intermediary during the fixation process.

From the findings of Bond[2] and of Wieringa and Bakhuis[3] it was concluded that at least part of the fixed nitrogen is transported through the xylem. So it seemed worth while to examine nitrogen fixation in excised root systems by following the delivery of amino-acids by root nodules to the transpiration stream. So, after excision of the shoot, such a stream was induced artificially by applying either: (1) a high pressure (of 0·5–0·75 atm.) to the root system or (2) a lower pressure (of about 1 atm.) to the surface of the wound.

Soy beans (*Glycine max* Merr.) were grown on Bond's medium[4]. The amount of amino-acids in the 'exudate' was determined according to the method of Moore and Stein[5]. The delivery of amino-acids to this artificial transpiration stream was found to decrease rapidly (up to 85 per cent within a few hours after excision of the shoot).

However, such a rapid decline appeared not to be restricted to nitrogen-fixing nodulated plants, because the same phenomenon was observed in non-nodulated plants in a medium containing NO_3^- or NH_4^+. With the latter plants, this decrease might be due either to a reduction in the capacity to take up the nitrogen needed (NO_3^-/NH_4^+) or to a reduction in the capacity to assimilate the inorganic nitrogen absorbed.

Therefore NO_3^--/NH_4^+-uptake, and respiration of the root system, of plants grown on the NO_3^--containing medium of Hoagland and Arnon[6] were determined before and after excision of the shoot. For the sake of comparison, the uptake of phosphate was determined as well.

To follow the respiration, both output of carbon dioxide and uptake of oxygen were determined. The two kinds of apparatus used for measuring carbon dioxide output were 'Ultragas-3' (Wösthoff, Bochum, Germany) and 'Uras' (Hartmann and Braun, Frankfurt am Main, Germany). The 'Magnos' apparatus of Hartmann and Braun was used to determine uptake of oxygen. The 'constant flow' technique[7] was used for measuring the rate of ion uptake. Concentrations of phosphate, nitrate and ammonium were determined according to the methods recommended by Allport[8].

The results obtained might be summarized as follows: (1) Within 1 h after excision there is a rapid decline in respiration (50 per cent or more), after which a new, 'pseudo' steady-state is reached in which the respiratory rate falls extremely slowly. (2) Changes in ion uptake vary with the nature of the ion: the uptake of nitrate and ammonium decreases rapidly, sometimes so rapidly that uptake comes to a standstill within a few hours; the uptake of phosphate is scarcely influenced.

The question arises whether this reduction in uptake of nitrate and ammonium is due to the elimination of a component in ion uptake which is linked to the transpiration stream[9,10]. Therefore experiments were performed in which the xylem was left intact and only the phloem was removed by cutting a ring, some inches high, from the base of the stem.

Plants treated in this way showed the same phenomena. It was concluded that it is the active uptake of nitrate and ammonium which decreases rapidly, presumably by lack of assimilates normally supplied through the phloem.

Looking for a relation between the phenomena mentioned in 1 and 2, I took into consideration that any decrease in respiration will be coupled with: (*a*) reduction in the energy supply needed for active uptake; (*b*) reduction in reducing power; (*c*) reduction in the amount of α-keto-acids needed for amination.

The question then arises as to which of those factors can be ascribed the decrease in uptake of ammonium and nitrate.

Point (*a*) must be rejected because it does not explain the absence of a similar reduction in phosphate uptake. On the other hand, point (*b*) is at variance with the equal reduction in uptake of both nitrate and ammonium. The cause of the decrease was therefore sought in a discontinuation in the supply of α-keto-acids (*c*). In more specific terms this means that this decrease was ascribed, not to the mechanism of uptake proper, but to the subsequent processes of amination. This is in accordance with the results of Lycklama[11].

The obvious inference from these considerations was to examine whether or not this reduction in uptake of nitrate and ammonium could be counteracted by adding these α-keto-acids or their precursors, that is, by adding acids of the Krebs cycle.

To start with it had to be established to what extent these acids entered the roots, any enhancing effect on respiration being considered as evidence for penetration of the acids.

For reasons of permeability, experiments were performed at low *p*H (3·5–4). Lowering the *p*H to these values did not reduce the respiration of the root systems; a finding which was in contrast with our experience with excised root tips in Warburg manometric experiments.

Of the acids of the Krebs cycle thus tested, succinic acid was especially effective. Addition of this weak acid in a concentration of 0·025 M, and excision of the shoot at the same time, resulted in an increase in respiration, remaining for hours at a level higher than before (as measured by uptake of oxygen and output of carbon dioxide).