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Chlorophyll <latex>α</latex> Biosynthesis [and Discussion]

O. T. G. Jones and S. I. Beale

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Chlorophyll *a* biosynthesis

By O. T. G. Jones
Department of Biochemistry, Bristol University, Bristol BS8 1TD

Protoporphyrin IX is believed to be an intermediate common to both haem and chlorophyll biosynthesis. The pathway specific to chlorophyll starts with magnesium protoporphyrin and its monomethyl ester. Two routes have been proposed for conversion of the latter compound to protochlorophyllide: A, formation of the isocyclic ring followed by reduction of the 4-vinyl group, or B, reduction of the 4-vinyl group followed by formation of the isocyclic ring. Membranes prepared from isolated barley etioplasts are found to convert magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester to chlorophyllide *a* at a rate equal to that of chlorophyll synthesis in intact leaves: this result supports route A. NADPH is necessary to maintain the two successive reductive steps: reduction of the 4-vinyl group and then the photoreduction of ring IV to yield chlorophyllide.

The prohaem content of etiolated leaves does not increase during the phase of active chlorophyll synthesis although evidence is presented that suggests that the ALA synthesis reaction that regulates chlorophyll synthesis is common to both pathways. This and other regulatory aspects are discussed.

Protoporphyrin as an intermediate in chlorophyll *a* biosynthesis

Since the discovery by Granick (1948*a*) that protoporphyrin IX accumulated in considerable quantities in a mutant of Chlorella that was incapable of chlorophyll synthesis it has been widely accepted that protoporphyrin IX is an intermediate in the biosynthesis of chlorophyll *a*. Later Granick (1961) showed that the same porphyrin accumulated in higher plants; he grew barley in the dark, which prevents the light-dependent conversion of protochlorophyllide to chlorophyllide *a* (see figure 2) and supplied the cut shoots with δ-aminolaevulinic acid. This compound appears to bypass some regulatory step in tetrapyrrole biosynthesis and causes the formation of excessive amounts of protochlorophyllide and also of magnesium porphyrins and protoporphyrin. The same group of porphyrins have been found in leaves from chlorophyll-less mutants of barley (Gough 1972). Since magnesium protoporphyrin and magnesium protoporphyrin monomethyl ester were found in other chlorophyll-less mutants of Chlorella (Granick 1948*b*, 1961) it was thought likely that the following biosynthetic sequence was involved in chlorophyll formation:

precursors ⟶ protoporphyrin ⟶ magnesium protoporphyrin ⟶ magnesium protoporphyrin monomethyl ester ⟶ ⟶ ⟶ chlorophyll *a*.

Direct evidence that protoporphyrin may be converted to chlorophyll *a* is not available and since the recent questioning of such a fundamental belief as the absolute requirement for succinyl-CoA and glycine as substrates for ALA synthesis (see Beale, this volume) it may be necessary to maintain slight reservations about the central role of protoporphyrin in haem and chlorophyll synthesis. Such doubts would be quieted if the magnesium insertion reaction could be demonstrated to require protoporphyrin as its substrate.

Green plants contain all the enzymes required for protoporphyrin IX synthesis from ALA; ALA-dehydratase (Granick 1954) porphobilinogen deaminase, and uroporphyrinogen III cosynthetase (Bogorad & Granick 1953) and some form of coproporphyrinogen oxidative decarboxylase (e.g. Battersby, Staunton & Wightman 1972). These enzymes may not be involved exclusively (or even not at all!) in chlorophyll formation, since haem synthesis is also required by plant tissues.

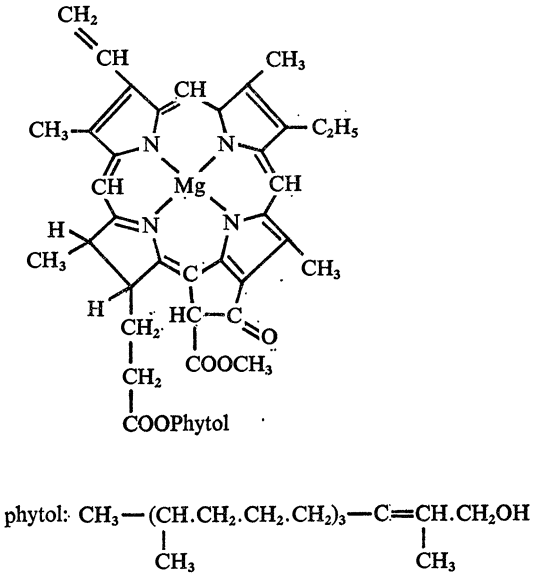


FIGURE 1. Chlorophyll *a*.

All photosynthetic organisms contain haem pigments in addition to their chlorophylls. The haems function as prosthetic groups of the cytochromes that are involved in photosynthetic electron flow and so the biosynthetic pathways to haem and porphyrins must be integrated to produce a balanced proportion of chlorophyll to cytochrome. It is not certain whether two completely independent pathways exist for haems and magnesium pigments or whether some portions of the biosynthetic routes are common and this problem is discussed in the final section of this article. However, the effect of added ALA in stimulating the synthesis of coproporphyrin(ogen), protoporphyrin(ogen) and magnesium protoporphyrin by etiolated seedlings makes it appear probable that the early steps in chlorophyll synthesis have the same intermediates as the early steps in protohaem biosynthesis:

precursors ⟶ ALA ⟶ uroporphyrinogen III ⟶ coproporphyrinogen III ⟶
protoporphyrinogen IX ⟶ protoporphyrin IX

Isolated etioplasts or chloroplasts will carry out these syntheses very effectively when supplied with ALA. Indeed, there have been a series of papers by Rebeiz and his co-workers (see a review by Rebeiz & Castelfranco 1973) in which the synthesis of magnesium tetrapyrroles, including protochlorophyll and chlorophyll *a*, was reported to take place when homogenates from greening cucumber cotyledons were incubated with [^{14}C]ALA. The enzymic reactions leading to protoporphyrin formation have been described elsewhere in this volume and will not be discussed in further detail here.

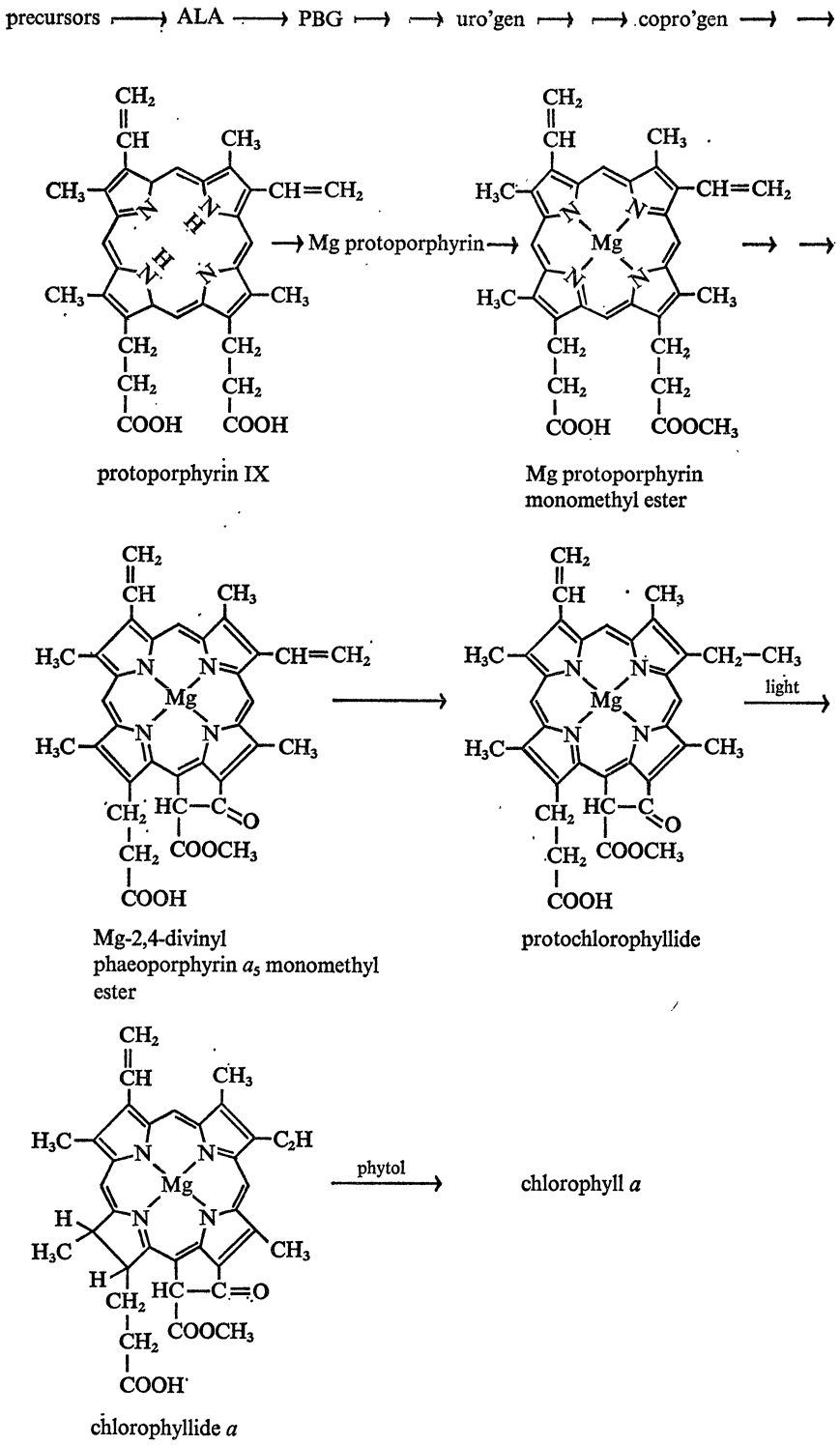


FIGURE 2. A suggested pathway for the biosynthesis of chlorophyll *a*.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

A scheme for the biosynthesis of chlorophyll *a* from protoporphyrin is given in figure 2 and the evidence for some of the suggested intermediate reactions will be discussed below.

Protoporphyrin to magnesium protoporphyrin monomethyl ester

Since three different chlorophyll-less mutants of Chlorella have been found that accumulated protoporphyrin, magnesium protoporphyrin or magnesium protoporphyrin monomethyl ester (Granick 1948*a*, *b*, 1961), it has been widely accepted that these tetrapyrroles are intermediates in chlorophyll synthesis, following each other in this order. It has not yet been possible to prepare any form of extract from photosynthetic organisms in which the insertion of Mg^{2+} into protoporphyrin can be unequivocally demonstrated, and this remains one of the problem areas of chlorophyll biosynthesis. Barley plants contain an enzyme that actively inserts Zn^{2+} into protoporphyrin (Goldin & Little 1969) and Jones (1968) and Porra & Lascelles (1969) showed that metal chelatase activity was localized in the plastid fraction of higher plants. This chelatase did not insert Mg^{2+} into porphyrins and is possibly associated with enzymic haem synthesis, since like the ferrochelatase of rat liver mitochondria, it will insert Zn^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} or Ni^{2+} as well as Fe^{2+} into protoporphyrin. Since Rebeiz & Castelfranco (1971*a*, *b*, 1973) have evidence that protochlorophyll and chlorophyll are formed by cell-free homogenates of greening cucumber plants when they are supplied with [^{14}C]ALA it is possible that the magnesium insertion reaction may proceed *in vitro* and further development and exploitation of this system is awaited.

By using the photosynthetic bacterium *Rhodopseudomonas spheroides*, some progress in the study of magnesium chelation has been made by Gorchein (1972, 1973). He found that whole cells incorporated magnesium into exogenous protoporphyrin, but the product was magnesium protoporphyrin monomethyl ester, not magnesium protoporphyrin. Gorchein proposes that in *R. spheroides* insertion of magnesium is obligatorily coupled with methylation of the magnesium porphyrin and that the overall reaction is dependent upon a readily disrupted multienzyme complex. Some such fragile complex may be required in green plants and its destruction explains the failure by many workers to measure magnesium chelatase *in vitro*, but the isolation of chlorophyll-less mutants of barley and Chlorella that accumulate free magnesium protoporphyrin suggests that magnesium insertion is not necessarily coupled to methylation in higher organisms.

The enzyme that forms the monomethyl ester of magnesium protoporphyrin was first detected in extracts from *R. spheroides* (Gibson, Neuberger & Tait 1963). It was shown that the methyl donor was *S*-adenosylmethionine and the enzyme was given the name *S*-adenosylmethionine-magnesium protoporphyrin methyl transferase. Later an enzyme with the same properties was found in maize chloroplasts (Radmer & Bogorad 1967) and in chlorplasts of light-grown *Euglena gracilis* or in the proplastids of dark-grown *Euglena* (Ebbon & Tait 1969). The activity increased two or threefold after cells were transferred from dark to light. In all the cases that have been studied the specificity of the enzyme is such that magnesium protoporphyrin is a much better substrate than protoporphyrin, in agreement with the suggested sequence:

protoporphyrin ⟶ magnesium protoporphyrin ⟶ magnesium protoporphyrin monomethyl ester.

Since the enzyme will methylate zinc protoporphyrin, zinc protoporphyrin monomethyl ester may arise as an artefact *in vitro*.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Protochlorophyllide

Protochlorophyllide is perhaps the best authenticated and accepted intermediate in chlorophyll biosynthesis. When seeds of angiosperms are germinated in the dark they do not form chlorophyll. Instead, small amounts of protochlorophyllide are formed in the abnormal plastids (the etioplasts) and this compound is rapidly converted to chlorophyllide when the plants are transferred to light. Protochlorophyll differs from chlorophyllide only in the state of reduction of ring IV of the tetrapyrrole (see figure 2). The reduction of this ring is a light-dependent reaction. Protochlorophyllide in most dark grown plants appears to be present in the etiolated leaf in more than one form. These may be recognized by their characteristic absorption maxima in the red region of the spectrum. The bulk of the protochlorophyllide absorbs with a maximum near 650 nm, another form has a maximum near 636 nm and a third form, more often found in older tissues, absorbs with a maximum at about 628 nm. If etiolated leaves containing these three protochlorophyllide complexes are extracted with a polar water-miscible solvent such as acetone, then only one form of protochlorophyllide is found in solution, with an absorption maximum in ether at 623 nm. It is likely that the different spectroscopic forms found *in vivo* are due to differences in the physical environment of the protochlorophyllide molecules. They may be aggregated or complexed to specific proteins or membranes. Protochlorophyllide *in vitro*, when detached from protein, is not light-transformable.

Not all of the forms of protochlorophyllide present in etiolated barley or bean leaves are converted to chlorophyllide on brief illumination. Kahn, Boardman & Thorne (1970) found that the 650 and 637 nm forms were phototransformed but not the 628 nm form. There appeared to be energy transfer between the 637 nm (P_{637}) form and the 650 nm (P_{650}) form, suggesting that these two are in close physical proximity. In a later paper it has even been suggested the P_{635} and P_{650} may be two absorption bands of a single species of protochlorophyll complex or perhaps the absorption bands of two species in dynamic equilibrium (Kahn & Nielsen 1974). This interpretation appears too simple since there are many examples of purified protochlorophyllide complexes that are completely light-transformable and yet have only one absorption band in the red region: either near 650 or near 636 nm.

When etiolated leaves are supplied, in the dark, with added ALA these leaves produce unusually large amounts of protochlorophyllide (Granick 1959, 1961), apparently because even in dark grown plants the enzymes for the reactions between ALA and protochlorophyllide are present in non-limiting amounts. The protochlorophyllide that is formed absorbs around 636 nm, is not light-transformable when the leaves are exposed to steady illumination (see figure 3) and it was suggested that this inactivity results from the lack of sufficient catalytic protein to bind to the excess protochlorophyllide and convert it to the light transformable complex called the protochlorophyllide holochrome. There is considerable experimental support for this suggestion since it has been found that exposure of ALA-fed leaves to alternating flashes of light and dark intervals results in the utilization of the ALA-induced protochlorophyllide (Gassman & Bogorad 1967; Sundqvist 1969), possibly because the catalytic protein has time to recombine with protochlorophyllide and form an active complex during the dark intervals.

It will become apparent from any study of the literature that there is a lack of agreement about the wavelength absorption maximum of the transformable and non-transformable forms of protochlorophyllide. In general it is accepted that P_{650} is transformable on illumination and

P_{628} (where observed) is not; P_{635} is reported by some to be immediately transformable, by others to be transformed only in a 'flashing' illumination with dark intervals. The alternative possibility is that P_{635} is not a single species but has two components, one transformable the other not.

Sundqvist (1970) showed that in ALA-treated leaves a flash caused the disappearance of P_{650}, with formation of chlorophyllide, and that during the subsequent dark period the P_{650} was reformed, presumably from the pool of P_{636}. The two reactions could be clearly separated by their temperature dependence, since the light reaction proceeded at 5 °C but the dark replacement of the photoactive form was slow at 5 °C. Distinction between these two protochlorophyllide forms in sensitivity to hydrogen sulphide was observed by Gassman (1973). Hydrogen sulphide caused P_{650} to be converted to non-photoconvertible P_{633} in a reaction that was reversible when the gas was removed by flushing with nitrogen.

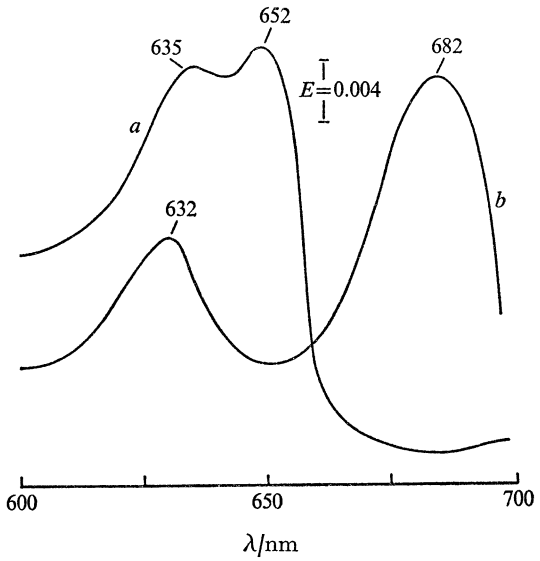


FIGURE 3. Spectra of protochlorophyll(ide)-enriched etioplasts from 7-day-old dark-grown barley (after Griffiths 1975). Etioplasts containing a high concentration of protochlorophyll(ide) were isolated by immersing the cut-ends of the etiolated shoots in 10 mM δ-aminolaevulinate for 6–8 h in darkness. Etioplasts were prepared from this material as described by Griffiths (1975) and suspended in buffer at pH 7.2 at a concentration of 6 mg protein/ml. (*a*) Spectrum before illumination. (*b*) Spectrum after illumination for 90 s with an unfiltered 100 W tungsten lamp.

The first product of photoconversion of the protochlorophyllide holochrome *in vivo* is a form of chlorophyllide having an absorption maximum at 678 nm. This is rapidly converted, in the dark, to a form with an absorption maximum at 683 nm (Schopfer & Siegelman 1968; Gassman, Granick & Mauzerall 1968). A slower dark reaction (the Shibata shift, Shibata 1957) then occurs, leading to the formation of a form of chlorophyll with an absorption maximum at 673 nm. This slow spectroscopic change may coincide with the phytylation of chlorophyllide to chlorophyll. Granick & Gassman (1970) proposed that the sequence of events in the photoconversion of protochlorophyllide may be represented as shown in figure 4. P_{635}, formed in the presence of added ALA, is converted to P_{650} by combination with the specific holochrome protein and a reductant. Chlorophyllide 678 is still attached to the protein; detachment from the specific protein results in the spectroscopic shift to 683 nm. The final spectroscopic shift to 673 nm coincides with

esterification with phytol. Other spectroscopically distinct intermediates in chlorophyllide and chlorophyll formation have been reported and alternative mechanisms have been proposed. By making measurements of the circular dichroism of protochlorophyllide of etiolated and greening tissue Schultz & Sauer (1972) and Mathis & Sauer (1972) have found evidence of pronounced pigment–pigment interaction and propose that some of the spectroscopic forms of protochlorophyllide and chlorophyllide are due to dimers of protochlorophyllide and chlorophyllide, and to mixed forms, as suggested below.

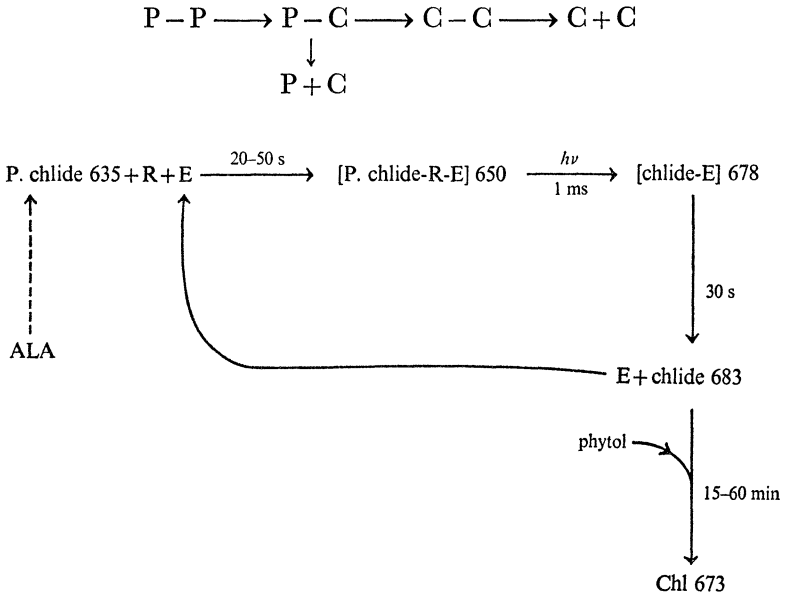


FIGURE 4. Representation of some of the early events in the formation of chlorophyll *a*, as suggested by Granick & Gassman (1970). R is a reductant (possibly NADPH, see Griffiths 1974), E is a photoenzyme, P. chlide 635 is a form of protochlorophyllide with an absorption maximum at 635 nm, chlide is chlorophyllide, Chl is chlorophyll. Numbers after pigments refer to their absorption maxima in the red region.

This postulate is not necessarily incompatible with the scheme shown in figure 4. The aggregation of the molecules may arise as a consequence of their binding to a specific protein; phytylation may also catalyse the disaggregation of a dimer.

It is possible to extract and purify a complex between protochlorophyllide and an associated protein, the protochlorophyllide holochrome. There are varying reports of the molecular mass of the bean leaf holochrome ranging from 960 000 (Smith 1960) to 600 000 (Boardman 1962) and of the separation of two forms with molecular masses of 300 000 and 550 000 (Schopfer & Siegelman 1968). Although early work suggested that there was an average of one protochlorophyllide molecule per protein, later work indicates that there are several chromophores, with up to twenty proposed by Thorne (1971). Since photoconvertible subunits with a molecular mass of 63 000 can be prepared by extracting leaves with saponin (Heningsen & Kahn 1971) there is little doubt that the original complex must have more than one chromophore.

The quantum yield of the photoconversion of protochlorophyllide to chlorophyllide was calculated by Smith (1958) to be near 0.6 and his data suggest that the transformation is a one quantum process. The rate of reaction is independent of holochrome concentration and of the viscosity of the medium, suggesting that phototransformation does not involve a collision process between photoactivated protochlorophyll and some reductant molecule. The nature of the

reductant remains unknown, although the action spectrum for the photoconversion is that of protochlorophyllide.

Etioplasts from dark-grown seedlings seem to lose fairly rapidly the capacity to phototransform their endogenous protochlorophyll to chlorophyllide and to carry out the morphological changes that, *in vivo*, are associated with the transition from etioplast to chloroplast structure. Horton & Leech (1972) found that etioplasts from *Zea mays* retained for 5 h the capacity to phototransform their endogenous protochlorophyllide provided that 1.5 mM ATP was present in the incubation and isolation medium. In the absence of ATP the ratio of P_{650} to P_{630} declined to 1.0 from 1.6 and only 33 % of the protochlorophyllide was phototransformable. Addition of ATP to such etioplasts caused an increase in phototransformability but without evidence for prior conversion of P_{630} to P_{650}. This system has been very considerably developed by Griffiths.

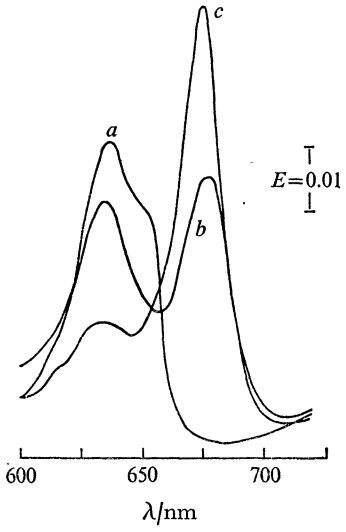


FIGURE 5. A comparison of the phototransformation of protochlorophyllide in isolated barley etioplasts by repetitive flashing illumination or by one saturating flash (after Griffiths 1975). Protochlorophyllide enriched etioplasts were prepared from etiolated barley as described in figure 3. (*a*) Spectrum before illumination. (*b*) Spectrum after illumination for 90 s with a 100 W lamp. (*c*) Spectrum following exposure to a 1 ms flash every 15 min for 10 h.

He prepared etioplasts from barley leaves which had been exposed to exogeneous ALA in darkness for 6 to 8 h, to cause a rise in P_{634}. Unlike the endogenous P_{652} this was not immediately converted to chlorophyllide on illumination (see figure 5) but exposure of the etioplasts to a régime of flash illumination, with dark intervals, resulted in utilization of much of the P_{634}, although some residual unchanged material was found (Griffiths 1975). It was found that if P_{634}-enriched etioplasts were carefully prepared they could subsequently be lysed in water and that the resulting etioplast membranes retained the capacity to transform not only their P_{650} but also, in a 'flashed' incubation, their capacity to use P_{634} (Griffiths 1974). In order that P_{634} could be used it was necessary to supply a reductant, and much the most effective reductant was NADPH (see table 1). NADH was a relatively poor activator of this reaction. Since the quantity of chlorophyll produced by these lysed etioplast membranes supplemented with NADPH was two or threefold greater than that of the control, intact, unsupplemented

etioplasts it is reasonable to deduce that the supply of NADPH is limiting in the conversion of P_{634} to chlorophyllide and that NADPH is the reductant, direct or indirect, for reduction of ring IV of protochlorophyllide.

When difference spectra were recorded to show the effect of adding NADPH to lysed P_{634}-enriched etioplasts, in the dark, it was apparent that there was a disappearance of P_{634} linked to the formation of P_{652}. The $t_{\frac{1}{2}}$ for the restoration of P_{652}, after a flash illumination, was less than 10 s and this process was relatively temperature-insensitive. There seems an excellent prospect that this experimental system will be useful in determining precisely the nature of the P_{634}–P_{652} transformation and the reductant for ring IV of protochlorophyllide.

TABLE 1. EFFECT OF DIFFERENT REDUCTANTS UPON THE UTILIZATION OF PROTOCHLOROPHYLLIDE BY WATER LYSED ETIOPLASTS PREPARED FROM PROTOCHLOROPHYLLIDE ENRICHED ETIOLATED BARLEY (AFTER GRIFFITHS 1974)

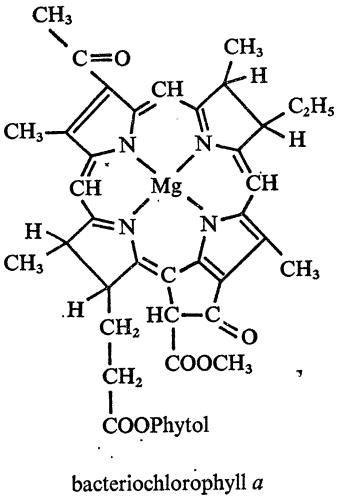
Water-washed etioplast membranes (free of soluble enzymes) were resuspended in isolation medium and incubated overnight under flashing illumination (see figure 5) with the supplements shown. Yields of chlorophyll are compared with that obtained by the flashing illumination of intact un-lysed etioplasts.

medium supplement	chlorophyll yield (% of that from intact etioplasts)
none	36
1.5 mM ATP	38
5 mM Cysteine	98
ATP + NADH generating system	95
ATP + NADPH generating system	274
NADPH generating system	269

THE FORMATION OF PROTOCHLOROPHYLLIDE FROM MAGNESIUM PROTOPORPHYRIN MONOMETHYL ESTER

In order to convert magnesium protoporphyrin monomethyl ester to protochlorophyllide two separate operations are necessary. The vinyl group at position 4 must be reduced to ethyl and the isocyclic ring must be formed from the propionyl methyl ester substituent at position 6 of the porphyrin ring. The order of these events is subject to some discussion. The earliest evidence was obtained when cultures of *Rhodopseudomonas spheroides* were treated with 8-OH-quinoline to inhibit the synthesis of bacteriochlorophyll *a* (figure 6). A pigment accumulated that was spectroscopically very similar to protochlorophyllide (Jones 1963*a*) and was identified as magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester (Jones 1963*b*). Since there is now reasonable evidence from mutant work that chlorophyllide *a* is an intermediate in bacteriochlorophyll biosynthesis (see, for example, Richards & Lascelles 1969) it appeared very possible that magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester was the intermediate directly preceding protochlorophyllide, even in green plants. Subsequently this phaeoporphyrin was identified in higher plants: it accumulates in considerable quantities in the inner seed coats of the Cucurbitaceae (Jones 1966; Houssier & Sauer 1969), where it appears to have no physiological function. The evidence that this phaeoporphyrin is an intermediate in chlorophyll synthesis has been circumstantial, but recently more direct evidence have been obtained (Griffiths & Jones 1975). Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester was obtained in fairly considerable quantity from a mutant, V_3, of *R. spheroides* that is unable to synthesize bacteriochlorophyll. The

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



bacteriochlorophyll *a*

FIGURE 6. Bacteriochlorophyll *a*.

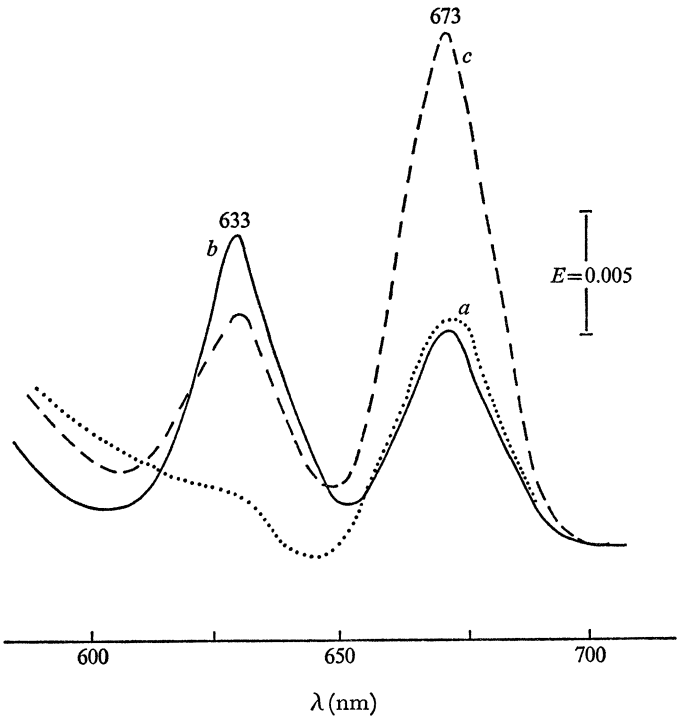


FIGURE 7. The formation of chlorophyllide *a* from added magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester+NADPH, by water-lysed barley etioplasts. Etioplast membranes were prepared normal etiolated barley as described in table 1 and suspended in isolation medium. Three aliquots were taken and incubated at 18 °C for 0.5 h in flashing illumination (1 ms xenon flash every 80 s) to convert all endogenous protochlorophyllide to chlorophyllide. Various additions were then made and the flashing illumination continued for 2 h. Spectra were recorded as shown. (*a*) Medium supplemented with NADPH generating system. (*b*) Medium supplemented with magnesium 2,4-divinylphaeoporphyrin a_5 ester in 0.1% sodium cholate. (*c*) Medium supplemented with magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester and NADPH generating system.

phaeoporphyrin was dissolved in sodium cholate solution, added to lysed etioplast membranes prepared from dark-grown barley and incubated under flashing illumination (1 ms xenon flash every 80 s, for 2 h). Little change took place until an NADPH-generating system was added to the incubation mixture, when a fairly rapid production of chlorophyllide was found (figure 7) in quantities that are easily measured spectroscopically. The rate of magnesium 2,4-divinyl-phaeoporphyrin consumption was close to that of protochlorophyllided used under the same incubation conditions (figure 8), and both compounds gave rise to the same pigment, which is spectroscopically and chromatographically indistinguishable from chlorophyllide *a*. NADH was ineffective in promoting this reaction, which is not surprising since NADPH is specifically required in the protochlorophyllide reduction sequence (see p. 214). Since the rates of chloro-phyllide synthesis from magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester by etioplast lysates are as great as those obtained in intact leaf tissue it is reasonable to suggest that it is a 'normal' intermediate in chlorophyll synthesis, i.e. formation of the isocyclic ring precedes reduction of the vinyl group at position 4.

The alternative pathway from magnesium protoporphyrin monomethyl ester to chlorophyll-lide *a* has been supported by Ellsworth & Aronoff (1968*a*, *b*, 1969), who found that in two mutants of Chlorella a number of magnesium tetrapyrroles accumulated, which they purified and

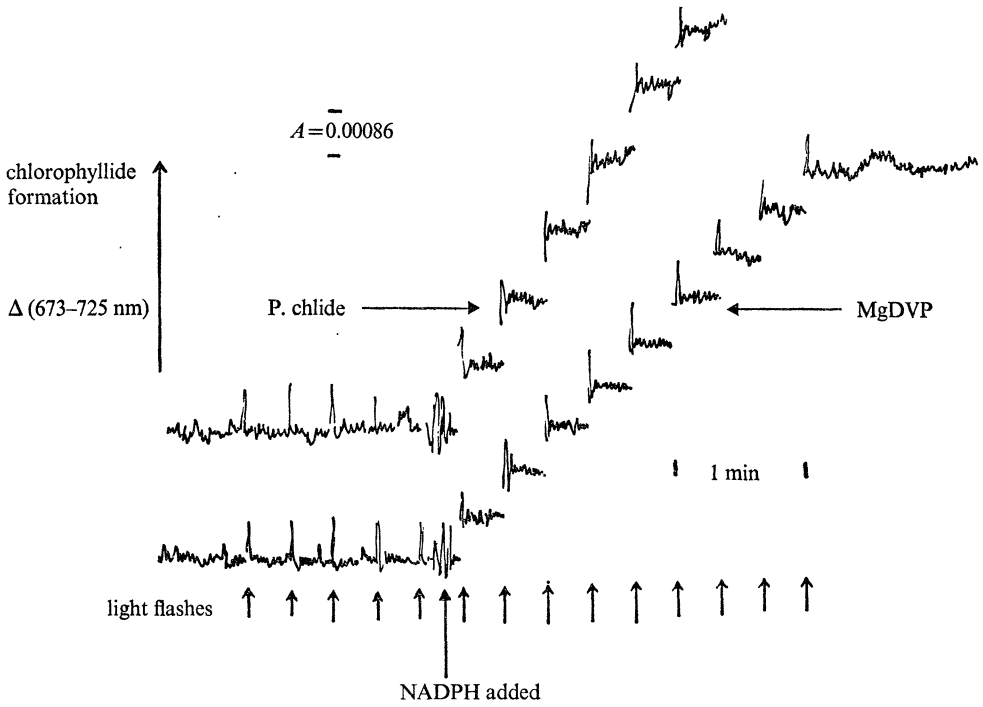


FIGURE 8. Flash-dependent synthesis of chlorophyllide by lysed barley etioplast membranes, using added proto-chlorophyllide or magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester as substrates. Lysed etioplasts (see figure 6) were suspended in isolation medium in the cuvette of a dual wavelength spectrophotometer and subjected to flashing illumination (1 ms xenon flash every 20 s), with flashes indicated thus ↑. After several flashes an NADPH generating system was added and the flashing regime continued. The upper trace records the synthesis of chlorophyllide when etioplasts suspensions were supplemented with protochlorophyllide (added in 0·1 % sodium cholate). The lower trace records chlorophyllide synthesis when the supplement was magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester. No chlorophyllide was synthesized in the absence of these supplements. Chlorophyllide synthesis was measured as the increase in extinction at 673 nm, with 725 nm as an isosbestic wavelength.

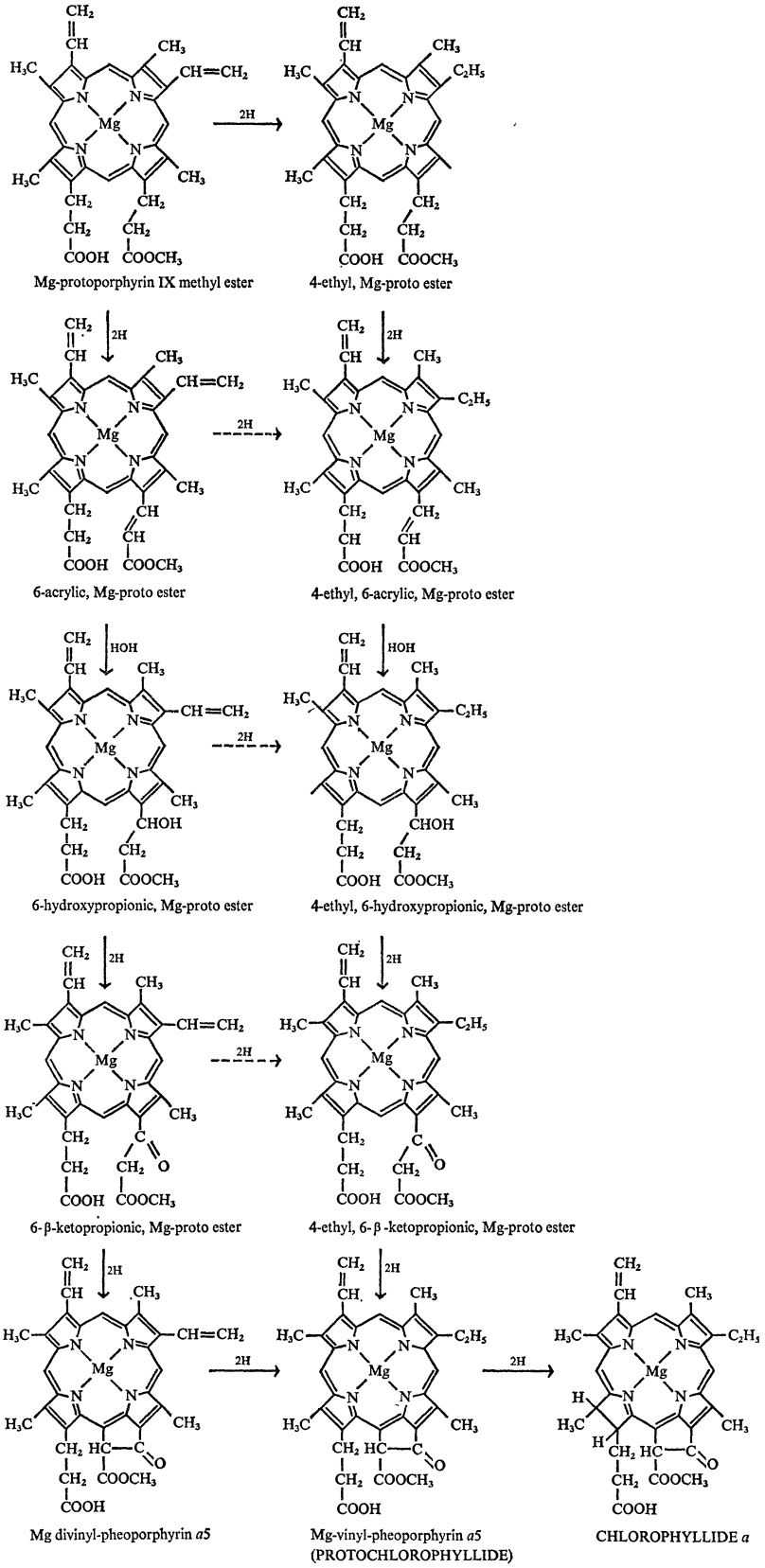


FIGURE 9. A scheme for the biosynthesis of chlorophyll *a* postulated by Ellsworth & Aronoff, based upon pigments found to accumulate in chlorophyll-less mutants of Chlorella.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

indentified by techniques based on mass spectrometry. These compounds had reduced vinyl groups as well as modifications to the propionic acid ester chain (see figure 9), which suggest that the isocyclic ring is formed following successive dehydrogenation, hydration and dehydrogenation of this side chain. Since these authors detected magnesium mesoporphyrin and magnesium 2,4-divinylphaeoporphyrin a_5 as products of their mutants they were led to propose that there may be several routes to protochlorophyllide and also some 'abortive' sequences. More recently Ellsworth & Hsing (1973, 1974) have found that tritiated NADH acted as a source of label for the porphyrin fraction produced when magnesium protoporphyrin was incubated with a homogenate of etiolated wheat seedlings. ^3H was incorporated into the fraction that, on chromatography, was found to contain reduced vinyl groups, apparently largely in a Mg-monoethyl, monovinyl deuteroporphyrin fraction. Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester was not a substrate in this reduction.

The formation of chlorophyll *a* from chlorophyllide *a*

All green leaves appear to contain an enzyme that will catalyse the hydrolysis of phytol from the propionic ester side chain of chlorophyll *a*. This enzyme, chlorophyllase, is active in high concentrations of organic solvents (40 % acteone is often present during assay). In ethanolic or methanolic solutions chlorophyllase catalyses the exchange of ethanol or methanol for the phytol. It acts only on chlorophylls and phaeophytins in which ring IV is reduced, including bacteriochlorophyll and chlorobium chlorophyll; this specificity is what might be expected if the enzyme were normally work required to work in the forward direction, to catalyse the synthesis of chlorophyll *a*, but purified preparations from barley obtained by Klein & Vishniac (1961) did not catalyse the esterification of chlorophyllide by phytol. Chlorophyllase activity increases in tissues that are greening (Holden 1961; Sudyina 1963), which does suggest that it functions in synthetic reactions, but in general it has been found that activity is much greater in transesterification reactions, catalysing the phytylation of methyl chlorophyllide *a* rather than the phytylation of chlorophyllide *a* or phaeophorbide *a* (e.g. Chiba *et al.* 1967; Wellburn 1970; Ellsworth 1972*a*, *b*). Inconsistent results may be the result of the wide variety of solvents used in the assay of this enzyme. An alternative explanation may be that *in vivo* the natural substrate is not phytol. It is noteworthy that in the photosynthetic bacteria the esterifying alchol of bacteriochlorophyll is sometimes *all-trans*-geranylgeraniol (Katz *et al.* 1972). It may be that late stages in phytol biosynthesis occur after some precursor has become esterified to chlorophyllide *a*.

Relation between chlorophyll synthesis and haem synthesis

Etiolated plants, which have no chlorophyll, synthesize mitochondria with a normal complement of cytochromes, and etioplasts also contain cytochromes that will be integrated into a functioning photosynthetic electron transport system during greening (cf. Plesnicar & Bendall 1973). Since it has been found that isolated etioplasts are capable of carrying out the series of reactions converting ALA to protoporphyrin it has been attractive to propose that the problem of permitting haem synthesis in dark conditions where chlorophyll synthesis is not possible could be resolved by having two separately regulated sites for ALA synthesis, in mitochondria and etioplasts. The discovery by Beale & Castelfranco (1973, 1974*a*, *b*) that the ALA synthetase that appears to be regulatory in chlorophyll synthesis is quite different in character from

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

previously described ALA-synthetases of haem-synthesizing systems made such a suggestion reasonable. This hypothesis was tested by Castelfranco & Jones (1975) who incubated greening etiolated barley leaves in solutions of ^{14}C-labelled substrates and then isolated and counted the chlorophyll *a* and protohaem from the greened tissues. The results, shown in table 2, were surprising. The incorporation of counts into haem was as efficient as into chlorophyll, although during greening there was no net synthesis of protohaem (figure 10). This suggests that there is a rapid turnover of protohaem during greening. Further, the labelling pattern of haem and chlorophyll was very similar and neither appeared to be formed via the well-characterized ALA-synthetase reaction:

$$\underset{\text{(glycine)}}{\text{succinyl CoA} + \text{NH}_2\text{-CH}_2\text{-*COOH}} \longrightarrow \text{ALA} + *\text{CO}_2.$$

TABLE 2. INCORPORATION OF ISOTOPICALLY LABELLED COMPOUNDS INTO PROTOHAEM AND PHAEOPHORBIDE *a* BY BARLEY SHOOTS (AFTER CASTELFRANCO & JONES 1975)

Samples (5 g) of etiolated barley shoots were incubated for 5 h in light with 5–10 μCi of labelled precursor dissolved in 5 ml water. When the uptake of $^{14}CO_2$ was measured the incubation was carried out in a closed glass tank. At the end of the incubation chlorophyll was extracted in ammoniacal acetone, purified by thin-layer chromatography, converted to phaeophytin *a* and purified, finally converted to phaeophoride *a* and purified. Protohaem was extracted into acetone-HCl and purified by crystallization following the addition of carrier. Samples were counted in a gas flow counter.

precursor added	10^{-5} × uptake (count/min)/g fresh mass	molar activity (count/min)/nmol	
		protohaem	phaeophorbide *a*
$^{14}CO_2$	—	62.9	71.5
[1-^{14}C]glycine	7.1	17.0	6.2
[2-^{14}C]glycine	11.9	17.8	21.7
[1-^{14}C]DL-glutamate	8.2	97	174
[U-^{14}C]L-glutamate	11.5	79.5	67.4
[4-^{14}C]ALA	6.8	1240	1460
$^{59}FeCl_3$	6.1	1090	0

In this reaction C_1 of glycine is lost and only C_2 is incorporated into the tetrapyrrole. In table 2 it is seen that neither carbon of glycine is a particularly effective precursor of haem or chlorophyll. On the other hand [1-^{14}C]glutamate is well incorporated into both tetrapyrroles. This would not be expected if glutamate were effective only as a precursor of succinyl-CoA, which would follow the pathway, glutamate ⟶ oxoglutarate ⟶ succinyl-CoA. In the oxoglutarate dehydrogenase reaction C_1 is lost. So it is apparent that in barley both haem and chlorophyll are formed by similar pathways, neither of which utilizes the usual ALA synthetase enzyme.

Since exogenous [^{14}C]ALA is efficiently incorporated into both protohaem and chlorophyll *a* it is very unlikely that there can be separate pools of ALA in mitochondria and etioplasts, each under separate regulation. Such separate regulation could not be effective if there is free diffusion of ALA to both synthetic systems. Regulation of these two pathways does not appear to be through the synthesis of separate pools of ALA. This is interesting in the light of the results shown in table 3. The addition of ALA to dark-incubated etiolated barley tissue caused a very large increase in the tissue content of protochlorophyllide (from 10.6 to 113 nmol per gram fresh mass), without any increase in the tissue haem. Other porphyrins in the tissue, coproporphyrin

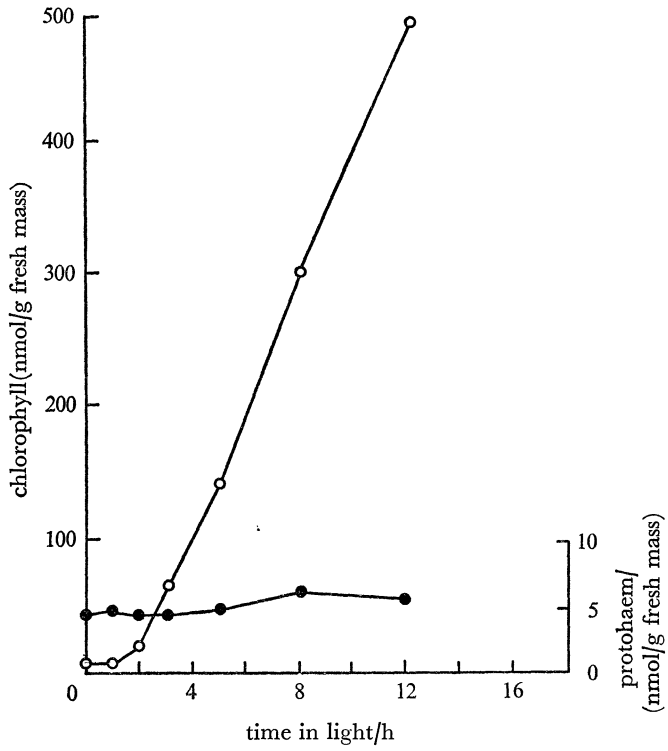


FIGURE 10. Changes in the content of chlorophyll *a* and protohaem during the greening of etiolated barley leaves. Dark-grown barley shoots were cut and placed in constant illumination with the cut ends dipping beneath water. Samples were removed at the intervals shown and the tissue level of chlorophyll *a* and protohaem determined using standard extraction methods (from Castelfranco & Jones 1975).

TABLE 3. EFFECT OF EXOGENOUS ALA ON THE ACCUMULATION OF VARIOUS TETRAPYRROLES

5 g of etiolated barley leaves were incubated for 5 h, the cut end dipping in either 20 mM ALA or H_2O. The light and dark incubations were carried out in two separate experiments. The samples were extracted and analysed for tetrapyrroles by standard methods.

incubation conditions		chlorophyll *a*	protochloro-phyllide	protoporphyrin	coproporphyrin	protohaem
		nmol/g fresh mass for each tetrapyrrole				
light	ALA	182.6	17.4	9.6	0.4	6.8
	H_2O	150	0	0	0	6.6
dark	ALA	0	113	11.2	trace	3.9
	H_2O	0	10.6	0	0	4.2

TABLE 4. INCORPORATION OF ^{59}Fe INTO PROTOHAEM UNDER LIGHT AND DARK CONDITIONS

Two 5.0 g samples of barley tissue were incubated with 4.2×10^6 count/min of $^{59}FeCl_3$ in 5.0 ml of 10 mM ascorbic acid, respectively in the light and in the dark. After 5.0 h the protohaem was extracted and the molar radioactivity determined.

	$10^{-5} \times$ uptake (count/min)/g fresh mass	molar radioactivity protohaem (count/min)/nmol
light	7.6	578
dark	6.1	295

and protoporphyrin increased slightly in concentration. There must, then, be some way of maintaining the haem level unchanged while protochlorophyllide synthesis is proceeding. The high apparent rate of synthesis and turnover of protohaem may yield a clue to this regulatory mechanism. Castelfranco & Jones (1975) have proposed a tentative scheme in which free protohaem is seen to act as direct or indirect inhibitor of the one ALA synthesis system of higher plants (see Granick & Sassa (1971) for a review of the effect of haem in regulating ALA-synthetase). In the dark, ALA synthesis leads to excess haem production; this haem inhibits ALA synthesis. In the light, haem breakdown is stimulated (see table 4) and ALA synthesis for chlorophyll production is permitted. This scheme described in figure 11 is supported by the experiments of Duggan & Gassman (1974), who showed that the treatment of etiolated leaves, in the dark, with iron chelators caused effects very similar to the addition of ALA, i.e. porphyrins and magnesium porphyrins increased in concentration at the same time that the tissue concentration of protohaem diminished, presumably because of the turnover of protohaem and the scavenging of the liberated iron by the iron chelator.

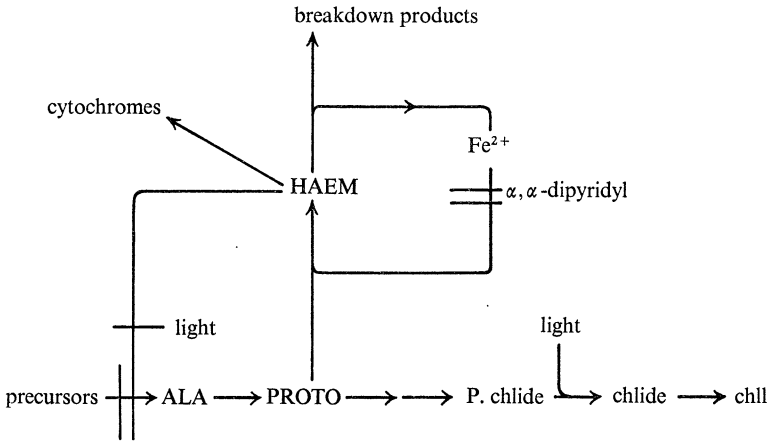


FIGURE 11. A scheme showing the possible mechanism for the regulation of haem and chlorophyll synthesis. It is proposed that protohaem inhibits the conversion of precursors to ALA, either as a direct enzyme inhibitor or by inhibiting the synthesis of some rate limiting enzyme; light overcomes this inhibitory effect.

The effect of light in activating ALA synthesis has been discussed elsewhere in this volume by Beale and the proposal that the delay in the production of ALA synthesizing enzymes is responsible for the lag phase in initiating chlorophyll synthesis (see figure 10) have been discussed in the interesting papers by Nadler & Granick (1970) and Suzer & Sauer (1971). In addition it is now apparent (see Griffiths 1974) that the supply of NADPH may be important in controlling the rate of chlorophyll synthesis.

It is a pleasure to acknowledge my debt to Dr W. T. Griffiths of the Biochemistry Department of Bristol University for interesting discussion, in many cases involving his unpublished work.

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Discussion

S. I. Beale (*The Rockefeller University, New York, N.Y.* 10021, *U.S.A.*). Have you detected haem degradation products in greening barley tissue or found any other direct evidence for the ability of the tissue to metabolize haem?

O. T. G. Jones. We have not carried out a detailed search but have not observed any likely compounds in our extracts.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES