Phytochemistry, Vol. 20, No. 5, pp. 1005–1009, 1981.
Printed in Great Britain.

0031-9422/81/051005-05 $02.00/0

TETRAPYRROLE BIOSYNTHESIS IN GREENING ETIOLATED BARLEY SEEDLINGS

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(*Received* 22 *August* 1980)

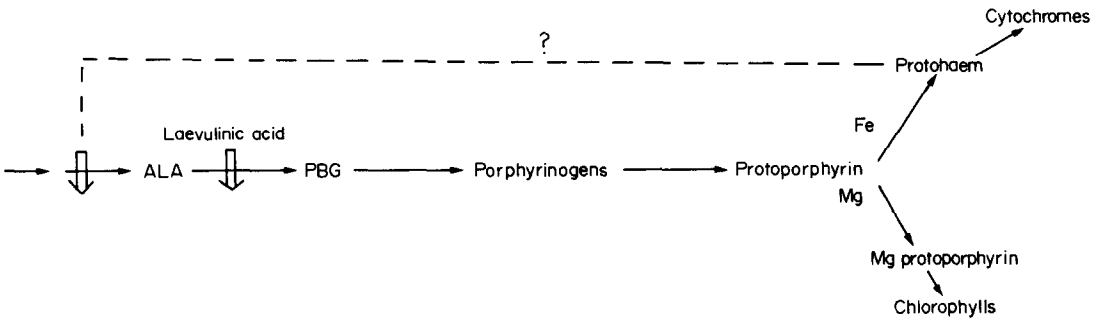
Key Word Index—*Hordeum vulgare*; Gramineae; greening barley; tetrapyrrole biosynthesis; haem biosynthesis; laevulinic acid inhibition.

Abstract—Allyl isopropylacetamide (AIA) does not stimulate porphyrin biosynthesis in greening barley; AIA inhibits the synthesis of 5-aminolaevulinate (ALA) in plants and does not overcome the repression of ALA-synthetase. This indicates that the ALA synthesis system of green plants is regulated differently from ALA synthetase of mammalian systems. Laevulinic acid (LA) inhibited the biosynthesis of tetrapyrrole pigments in greening barley and diminished the insertion of ^{55}Fe into extractable protohaem, confirming that haem was synthesized at a time of little net increase in protohaem. ALA feeding increased iron incorporation into protohaem without increasing either extractable protohaem or cytochromes *b* and *f*. Since ALA feeding greatly increased the protochlorophyllide content of dark-grown plants and subsequent chlorophyll levels in the light, the regulation of haem pigment synthesis in plants occurs after protoporphyrin and protohaem synthesis and is likely to involve the turnover of protohaem produced in excess of haem protein requirements.

INTRODUCTION

When seeds of higher plants are allowed to germinate in the dark the shoots form no chlorophyll but contain small amounts of its immediate precursor, protochlorophyllide. The etiolated leaves do not contain chloroplasts but instead contain etioplasts, which are converted to normal chloroplasts on steady illumination. Etioplasts contain nearly all the enzymes necessary for carbon dioxide fixation and most of the cytochromes believed to be involved in photosynthetic electron flow [1, 2]. Illumination of etiolated barley shoots causes a dramatic rearrangement of the membranes of the etioplasts, the conversion of protochlorophyllide to chlorophyllide *a* and then to chlorophyll *a*. After a lag period of about 2–3 hr, a rapid and increasing synthesis of chlorophylls *a* and *b* commences. During this period of continuing chlorophyll synthesis there is little change in the concentration of protohaem-containing pigments within the greening tissue [3].

There is evidence that 5-aminolaevulinate is a precursor of both haem and chlorophyll in greening barley [3] since both are labelled when cut shoots are treated with labelled 5-aminolaevulinate (ALA). In animals and bacteria ALA is formed from succinyl CoA and glycine by the action of the enzyme 5-aminolaevulinate synthetase (ALAS), a regulatory enzyme in the biosynthesis of haem pigment; in green plants, however, it is formed by a different reaction which is as yet incompletely characterized but which is well known to utilize all the five carbons of an intermediate such as glutamate [3–5]. In green plants, as



Scheme 1. Biosynthetic route from ALA to haem pigments and chlorophylls. PBG is porphobilinogen; ALA is 5-aminolaevulinate.

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in mammals and bacteria, the synthesis of ALA is the rate-limiting step in the synthesis of tetrapyrrole pigments.

In higher plants there is a requirement for the close regulation of tetrapyrrole pigment and apoprotein synthesis so that haem pigments, for mitochondria and etioplasts, are produced in dark growth at about the same rate as in the light, although very rapid synthesis of chlorophylls takes place on illumination, necessitating a huge increase in the flow along the tetrapyrrole biosynthetic path. The intermediates between ALA and protoporphyrin are likely to be common to the haem and chlorophyll pathways [6] although compartmentation of enzymes in mitochondria, chloroplasts and cytosol may contribute to regulation. A simple representation of these pathways is presented in Scheme 1.

We have examined changes in haem and chlorophyll levels during the greening of excised, etiolated barley leaves and have made use of two inhibitors in order to examine the interactions of chlorophyll and haem pigment synthesis. Laevulinic acid inhibits the formation of porphobilinogen by competing with the natural substrate aminolaevulinate and has been shown to inhibit the induction of the haem enzyme nitrate reductase in barley [7]. Allylisopropylacetamide, which is used in mammalian systems to induce aminolaevulinate synthetase, probably by catalysing the breakdown of the repressor protohaem [8], has been reported to inhibit haem synthesis in maize [9].

RESULTS

The total extractable protohaem from whole excised shoots of barley increased slightly on greening [3]. Both allylisopropylacetamide (AIA) and laevulinic acid caused a small fall in this protohaem level (Table 1) in the etiolated and greening shoots. AIA also inhibits the induction of nitrate reductase, a haem-containing enzyme induced by light [7], and the formation of chlorophyll (Table 2). These results suggest that there is some *de novo* synthesis of protohaem which can be blocked by inhibitors.

Table 1. The effects of laevulinic acid and allylisopropylacetamide on total protohaem levels in etiolated and greening barley

Treatment	Protohaem (nmol/g fr. wt)
Experiment 1: Light	
Control	6.89
+ Laevulinic acid (40 mM)	5.68
+ Allylisopropylacetamide (40 mM)	5.40
+ Laevulinic acid (40 mM) + Allylisopropylacetamide (40 mM)	5.35
Experiment 2: Dark	
Control	4.65
+ Laevulinic acid (40 mM)	3.02
Experiment 3: Dark	
Control	5.52
+ Allylisopropylacetamide (40 mM)	4.87

In the greening experiment (1) cut shoots were illuminated in the presence and absence of the inhibitor for 18 hr. Incubation in the dark experiment (2 and 3) was for 24 hr.

Table 2. Effects of allylisopropylacetamide on nitrate reductase and chlorophyll levels in greening barley

Treatment	Nitrate reductase (μmol NO_2^- prod. g fr. wt. hr)	Chlorophyll (nmol/g fr. wt hr)
Control	4.2	448.4
+ Allylisopropylacetamide (40 mM)	2.3	308.1

Excised shoots were illuminated in the presence of 100 mM KNO_3 for 16 hr.

In mammals, AIA is a potent inducer of aminolaevulinate synthetase [10]. In green plants we found no such effect. Qualitative observations of porphyrins extracted from both the acetone–NH_4OH and acetone–HCl extracts of the protohaem extraction procedure [3] under a 365 nm UV lamp showed no increase in the porphyrin concentrations in AIA-treated shoots. This result suggested that there was no induction of ALA-synthetase by the porphyrogenic drug. ALA production can only be measured in the presence of laevulinic acid [4, 5]. Exogenously fed AIA was also found to be without effect on the dark production of protochlorophyllide by tissue fed with ALA (Fig. 1), nor did it affect protochlorophyllide reductase or phytolation (results not shown). This was surprising since AIA inhibited the synthesis of protohaem and chlorophyll (Tables 1 and 2). However, it was observed that in greening, excised shoots fed with 40 mM AIA and 40 mM laevulinic acid less ALA accumulated than in shoots incubated in laevulinic acid alone (Table 3). Thus it appears that AIA exerted its effects on tetrapyrrole biosynthesis by inhibiting the formation of ALA from its precursors. This is in complete contrast to its effect in mammalian systems, where it acts

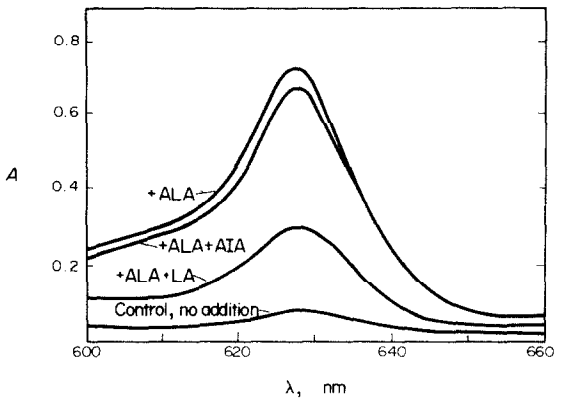

Fig. 1. Effect of 20 mM aminolaevulinic acid on the induction of protochlorophyllide in excised, etiolated shoots in the dark; in the presence of 40 mM laevulinic acid and 40 mM allylisopropylacetamide. Protochlorophyllide was extracted from 1 g of shoots in 7 ml of 80% acetone, the extract centrifuged and spectra were recorded using 1 cm light path.

Table 3. Accumulation of aminolaevulinic acid in the presence and absence of allylisopropylacetamide and laevulinic acid in the dark and in the light

Treatment	Aminolaevulinic acid (nmol/g/fr. wt) Dark	Light
Control	0	0
+ Laevulinic acid (40 mM)	22.0	700
+ Allylisopropylacetamide (40 mM)	0	0
+ Laevulinic acid (40 mM) + Allylisopropylacetamide (40 mM)	23.1	523

The excised shoots were illuminated for 7 hr after a preincubation period of 4 hr.

as an inducer. It was not possible to measure ALA accumulation in the dark because the very small accumulation was not within the limits of accuracy of the assay method.

It has been shown that laevulinic acid causes a drop in protohaem concentrations in the etiolated and greening barley leaf (Table 1), [7]. We have further confirmed this effect of laevulinic acid on haem concentration in a separate series of experiments. Excised barley shoots were incubated with labelled $FeCl_3$, in the presence of laevulinic acid or ALA. After incubation, the total protohaem was extracted and crystallized following the addition of carrier. Laevulinic acid caused over 50% inhibition of the incorporation of iron into haem, in both the dark and in the light (Table 4) confirming that haem was synthesized even when there was no net increase in tissue haem and that this process was sensitive to laevulinic acid. The addition of ALA to the excised shoots increased slightly the incorporation of labelled iron into protohaem (Table 5). Furthermore, no increase in the total protohaem content, or the cytochrome (haemproteins) levels was observed in the excised shoots fed with exogenous ALA. Cytochrome *f* was unchanged at 0.18 nmol/mg protein and total cytochrome *b* unchanged at 0.44 nmol/mg protein.

Greening in the presence of either laevulinic acid or AIA had some slight effect on the cytochrome content of the plastids from greening barley. The development of cytochrome b_{559HP} was strongly inhibited and cytochrome b_{563} was also affected. This supports the view that cytochrome b_{559HP} is formed *de novo* and that there is a limited supply of haem available for synthetic purposes

Table 4. Effects of laevulinic acid on $^{55}Fe^{2+}$ incorporation into haem in the dark and in the light

Treatment	Specific activity (dpm/nmol) Dark	Light
Control	2988	1415
Laevulinic acid (40 mM)	1300	493

The period of incubation was 17 hr and 10 hr respectively after a period of 4 hr preincubation. 10μC of labelled iron was used. Tissues contained 117 nmol iron/g wet wt.

Table 5. Incorporation of $^{55}Fe^{2+}$ into haem in the presence of 20 mM aminolaevulinic acid

Treatment	Specific activity (dpm/nmol)
Control	1420
+ Aminolaevulinic acid	1804

Excised shoots were fed with 10μCi $^{55}Fe^{2+}$ for 16 hr in the dark.

which is seriously depleted by addition of laevulinic acid or AIA. The small fall in content of other cytochromes may indicate that these turn over during the greening period.

Since the amount of labelled iron taken up by the leaves was known and the tissue content of the total iron was determined to be 117 nmol/gram of tissue, it was possible to calculate an approximate turnover of the protohaem, assuming that the total iron diluted out the labelled iron. The turnover thus calculated was about 1.2% of the total haem per hr.

Since haem concentrations did not increase under conditions where protochlorophyllide concentrations were increased by feeding exogenous ALA (Fig. 1), it was possible that the activity of ferrochelatase may be limiting haem synthesis. It is also possible that added ALA did not cause an increase in haem because ferrochelatase may be inhibited by any excess haem produced. Measurements of ferrochelatase activity in the plastid fractions of etiolated or partially greened barley showed no difference in activities following ALA or laevulinic acid addition. The specific activities were unchanged at 0.22 nmol/min/mg protein.

It has been shown for barley that ferrochelatase can use Co^{2+} in preference to Fe^{2+} as the metal substrate *in vitro* [11] and it is known that injected Co^{2+} inhibits haem synthesis in the rat by affecting both ALA synthetase and ferrochelatase [8]. It was thus interesting to see whether addition of Co^{2+} might yield information about regulation of haem synthesis in barley. However, we observed that $CoCl_2$ (1 mM) had no effect on the total protohaem levels in these tissues, nor did it affect cytochrome *f* concentrations in isolated plastids. Cytochrome *f* is a *c*-type cytochrome and thus the haem moiety of the haemprotein is not extractable with acetone–HCl (see Experimental). The total chlorophyll formed after 10 hr illumination was inhibited by 23% by the Co^{2+} treatment.

DISCUSSION

Studies in this laboratory have shown that the effects of allyl isopropylacetamide (AIA) in plant tissues are totally different from its reported effects on the mammalian system. This is not surprising since plants synthesize chlorophyll in addition to protohaem and the regulation of the tetrapyrrole biosynthetic path may be completely different. In mammals, protohaem, the end product of the pathway, is a feedback inhibitor of the synthesis and possibly of the activity of the enzyme ALA-synthetase which catalyses the first and rate-limiting step [10]. AIA causes a drop in total liver haem concentration probably by inducing the breakdown of P-450 in the liver. This removal of repressor haem causes an enormous increase in the activity of ALA-synthetase and as a result an

increase in the intermediates of the pathway, especially protoporphyrin [8, 10]. However, we found that in barley shoots AIA feeding caused no increase in protoporphyrin or other porphyrins (qualitative observations) although it did reduce the total protohaem and chlorophyll in these tissues. It thus appears that ALA formation was not induced. It has been suggested that protochlorophyllide may act as a feedback inhibitor of the enzyme(s) responsible for ALA formation [12, 13]. Since AIA did not affect the conversion of protochlorophyllide to chlorophyll it is unlikely that it would have caused protochlorophyllide to accumulate to concentrations inhibitory to ALA formation.

Measurements of the effect of AIA on the laevulinic acid-induced accumulation of ALA indicated that its site of action is probably more directly on the formation of ALA. It is possible that AIA may affect P-450 concentrations in plant microsomes in a similar fashion to that reported in animals. Little is known of the function of P-450 in higher plants and P-450 concentrations were not measured in the course of experiments described here but have been reported to be very low in barley [14]. Thus P-450 would not contribute significantly to total protohaem concentrations in these tissues.

Incubating excised shoots in high concentrations of ALA led to a large increase in protochlorophyllide concentration in the etiolated tissues (Fig. 1). Similar findings have also been reported elsewhere [3, 15]. However, under these conditions, although ALA is taken up by the tissues, little increase in protohaem concentration was detectable. This is in agreement with the results of Castelfranco and Jones [3] and is surprising since both protohaem and chlorophyll synthesis from ALA are equally rapid [3]. It is not possible that excess ALA present inhibited ferrochelatase activity since tissues fed with the precursor incorporated labelled iron into haem as efficiently as the untreated times (Table 5). Nor did it increase the concentration of *b*-type cytochromes in the plastids or the concentration of cytochrome *f*, a *c*-type cytochrome, the haem group of which is not present in protohaem extracts. Castelfranco and Jones [3] have shown that synthesis of haem is as rapid as that of chlorophyll from labelled ALA during the early hours of greening. Since no increase in total protohaem was detectable during greening [3], it was suggested that there is a rapid turnover of a 'pool of haem'. An approximate calculation of protohaem turnover, to determine the size of a free protohaem pool, revealed that only about 1.2% of the total plant protohaem is synthesized per hr; this is within limits of experimental error for the measurements of changes in total haem contents of shoots.

The failure to observe an increase in protohaem concentration in the presence of ALA cannot be due to a control at the ferrochelatase step since ferrochelatase activity was not limiting. In addition, there is a rapid synthesis of haem during greening which also suggests that ferrochelatase activity is not limiting [3]. Although metal porphyrins have been found to be inhibitory to ferrochelatase activity [11], we observed that protochlorophyllide (18 nmol/2.5 ml incubation mixture) had no effect on its activity.

Results from this laboratory indicate that there is a rapid turnover of a fraction of the total protohaem, as suggested earlier [3]. It now appears that there are at least two pathways leading to the formation of both protohaem and chlorophyll. Two ferrochelatases have been separated in different membrane fractions [11] and it appears that some ALA can be synthesized from succinyl CoA and glycine as well as from the intact carbon skeleton of glutamate ([16, 17] and S. Beale, private communication). It is possible that the conventional ALA-synthetase pathway operates in mitochondria in the dark and that the glutamate pathway is in the plastids and is induced in the light. As a result of this, two independent regulatory mechanisms may be operating for the two pathways. Our results tend to suggest that the supply of ALA to either haem or chlorophyll is influenced similarly by inhibitors and that the level of protohaem is regulated by a turnover process. The level of chlorophyll is regulated by light.

EXPERIMENTAL

Plant materials and application of inhibitors. Dark-grown barley (*Hordeum vulgare* L. cv Proctor) seedlings, 7–8 days old, were cut under water and placed in beakers containing 10 mM K-Pi at pH 7.0. A preincubation period of 4 hr was allowed before the cut shoots were illuminated. Inhibitors were present in the buffer during this preincubation when necessary. A fan was placed in front of the beakers to facilitate uptake of the solutes.

Plastid preparation. Plastids were isolated from the barley shoots using the method of ref. [18].

Enzyme assays. Nitrate reductase was assayed as previously described [7]. Protochlorophyllide reductase was assayed by the method of ref. [19]. Phytolation of chlorophyllide was measured by the method of [20] with the following modifications. Excised shoots were preincubated in the dark for 4 hr in a solution of allylisopropylacetamide, as described above. The shoots were illuminated for 1 min then returned to the dark. At intervals of 0, 3, 6, 12 and 20 min, samples (1 g) were removed and stored in liquid N_2. They were transferred to boiling distilled water for 1 min and then homogenized in a mixture of 35% (w/v) NH_4OH–Me_2CO–H_2O (1:44:5). Chlorophyll and chlorophyllide were then separated by the method of ref. [20]. Chlorophyll and chlorophyllide concentrations were determined from absorbance spectroscopy in the region 710–600 nm. Ferrochelatase was assayed with Co^{2+} and deuteroporphyrin as substrates by the method of ref. [21].

Protohaem measurements. Protohaem levels in barley shoots were measured as described in ref. [3].

Cytochrome measurements. Cytochrome concentrations in plastids were measured by difference spectroscopy as described in ref. [22].

Determination of soluble iron. This was performed following the method of ref. [23].

Aminolaevulinic acid measurements. ALA was measured by the method of ref. [24].

Incorporation of ^{55}Fe into protohaem. Cut shoots (1 g) were fed with 10 μC of carrier free $^{55}FeCl_3$ (442 μCi/μmol) in 0.1 M HCl. At the end of the incubation period the shoots were washed in large amounts of distilled H_2O. Haem was extracted, carrier-free protohaem was added and crystallized as described in ref. [3]. The crystals were dissolved in alkaline C_5H_5N and spotted onto glass fibre discs and bleach applied to prevent colour quenching. The discs were assayed for radioactivity as described below.

Measurement of protein synthesis by incorporation of ^{35}S from sodium [^{35}S] sulphate. Excised shoots (1 g) were fed with 10 Ci of labelled aq. sulphate carrier-free (pH 6–8) for the times given in the Results. After washing in copius amounts of distilled H_2O the shoots were ground in 7 ml 0.1 M K-Pi, pH 7.0 and the homogenate centrifuged for 10 min in a bench centrifuge. To 1 ml of the supernatant was added 5.0 ml of hot (80°) 5% (w/v) TCA. The ppt. was filtered on to a Whatman glass microfibre paper (2.5 cm GF/C). The ppt. was washed twice with mixtures of H_2O–MeOH (4:1), H_2O–MeOH (2:1) and finally

MeOH–Me_2CO (1:1) and placed in a vial containing 4.0 ml of scintillant, (5.0 g PPO, 0.25 g POPOP in 1 l. sulphur-free C_6H_5.Me). Radioactivity was determined in an Isocap liquid scintillation counter. Quench corrections were made by the Channels ratio method.

Chlorophyll estimation. Chlorophyll was extracted and determined by the method of ref. [25].

Protein. Protein was determined by the method of ref. [26].

Replication of experiments. The results given in tables and figures are from experiments repeated three times. The haem, protochlorophyll and chlorophyll content of control cut barley shoots varies from experiment to experiment by up to 30%. The effects of treatments are, however, consistently within the range ±10% of the quoted values.

Acknowledgements—This work has been supported in part by a grant from the Science Research Council. We are also grateful to the Government of Malaysia and the University of Malaya for financial support, and to Mrs. E. Burd for her skilled assistance.

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