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HAEM AND CHLOROPHYLL FORMATION IN ETIOLATED AND GREENING LEAVES OF BARLEY

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; chlorophyll; haem; ageing leaves; etiolation; greening.

Abstract—The amounts of protochlorophyllide (P650) and protohaem were measured in ageing dark-grown barley leaves. Maximum amounts of P650 and protohaem were found in 6- to 8-day-old material after which P650 declined rapidly and protohaem more slowly. In leaves exposed to light maximum chlorophyll was produced in 6-day-old material with progressively less the older the leaves. Haem concentrations increased in seedlings of all ages exposed to light. A lag phase was observed for both chlorophyll and haem formation in leaves given a light treatment. Haem, however, showed a slight yet significant decline as chlorophyll production commenced. The results indicate that chlorophyll and haem synthesis share a common pool of δ-aminolaevulinic acid (ALA). At a certain stage of development, the magnesium porphyrin pathway diverts precursors away from haem synthesis. It is only when the ALA synthesising system is well developed that the production of ALA can satisfy pathways to both haem and chlorophyll. The observed changes in haem under certain conditions suggest that, as in animal systems, haem levels may regulate porphyrin formation (chlorophylls) by controlling the supply of ALA.

INTRODUCTION

Leaves of barley seedings grown in the dark accumulate protochlorophyll(ide), of which the P650 form (and perhaps others) is converted to chlorophyllide and chlorophylls on illumination [1, 2]. During the early stages of continuous illumination more chlorophyll is formed, but only after an appreciable time lag [3]. The commencement of the linear phase of chlorophyll synthesis is associated with the light-induced formation of δ-aminolaevulinic acid (ALA) [4, 5]. The amount of dark-formed P650 and the net rate of chlorophyll synthesis decline with the age of the etiolated seedling [6, 7]. Stobart *et al.* [8] demonstrated that the decline in P650 formation in ageing barley leaves was due to a decrease in the rate of ALA formation rather than to the conversion of ALA to protochlorophyll (P650).

It is now well documented that haem is important in the control of porphyrin synthesis in bacterial and animal systems [9–14] and the existence of 'free' haem (protohaem) pools in higher plants was recently described [15]. A tentative scheme was proposed in which protohaem may function to suppress ALA synthesis in dark-grown leaves. On illumination, haem breakdown may be stimulated and ALA synthesis for chlorophyll formation permitted. In etiolated bean leaves haem increased on illumination [17] whereas no change in haem was found in illuminated barley seedlings [16].

The present report is an attempt to correlate endogenous haem with the capacity of ageing etiolated barley leaves to synthesise chlorophyll. Such an approach should provide information on the suggested regulatory role of haem under physiological conditions.

RESULTS

Protochlorophyllide (P650) and protohaem in dark-grown leaves

Seedlings were grown in the dark for 6–16 days. At various intervals primary leaves were removed and assayed for P650 and protohaem. The amount of P650 in 6-day-old dark-grown leaves was 0.35 nmol/g fr. wt and this increased to 3.55 nmol/g fr.wt by day 8: a rate of net synthesis of 1.60 nmol/day. After day 8, P650 was lost rapidly and by day 10 the amount was 1.65 nmol/g fr. wt, giving a net rate of decline of 0.95 nmol/day between days 8 and 10. In the following 6 days there was little further change.

The amounts of protohaem in dark-grown leaves of different ages were determined. From 4.8 nmol/g fr. wt at day 6 there was a slow decline to 3.3 nmol at day 17. This represents a net rate of decline of 0.14 nmol/day.

The decline in protohaem in dark-grown leaves is therefore relatively slow with no indication of a rapid decrease at or around the 8th day of growth as found for P650.

Effect of light on chlorophyll and protohaem in ageing leaves

Seedlings grown in the dark for 6, 10 and 14 days were transferred to the light for a further 6 days. The amounts of chlorophyll (Fig. 1) and protohaem (Table 2) in the primary leaves were measured daily. In 6-day-old dark-grown leaves, illumination caused a linear increase in

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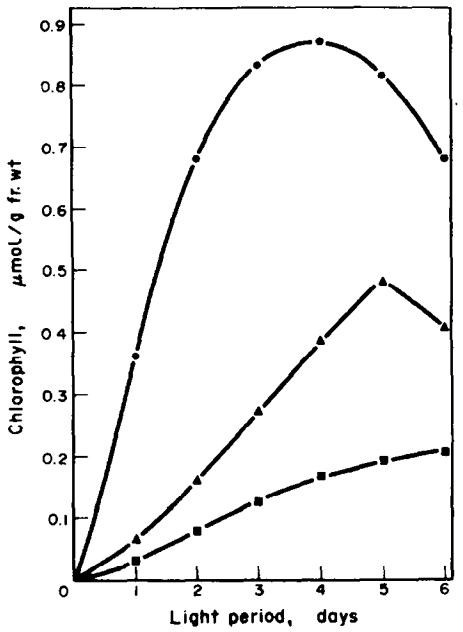

Fig. 1. Chlorophyll formation in dark-grown leaves exposed to light. Seedlings grown in the dark for 6, 10 and 14 days were transferred to the light for a further 6 days. Chlorophyll was measured daily in 80% acetone extracts of the primary leaf. ●——● 6 day; ▲——▲ 10 day; ■——■ 14 day.

chlorophyll lasting for *ca* 48 hr, reaching a maximum after 4 days of light of 876 nmol/g fr. wt. Ten-day old dark-grown leaves had a longer linear phase of chlorophyll formation, lasting *ca* 5 days. Dark-grown 14-day-old leaves did not show a clear linear phase of chlorophyll synthesis on illumination and the rate declined from the 2nd day of light treatment. In 6-day-old leaves the mean rate of chlorophyll formation was 219 nmol/day. There was a decrease to 97 nmol/day in 10-day-old material. Extending the dark growth to 14 days diminished the rate of chlorophyll formation to 35 nmol/day.

Protohaem in dark-grown leaves of various age was measured after 24 hr light together with that in dark controls (retained in the dark for an additional 24 hr). The results (Table 1) show that light induced an increase in the amount of protohaem at all ages. Seven-day-old dark-grown leaves showed an increase in protohaem of 2.68 nmol/g fr. wt after 24 hr light. As the leaves aged in the dark the amount of protohaem produced on illumination diminished. In 16-day-old leaves light treatment increased protohaem by only 1.26 nmol/g fr. wt.

Table 1. Protohaem concentrations in ageing dark-grown barley leaves exposed to light

Leaf age	Protohaem (nmol/g fr.wt)			% Increase in the light
	Dark	Light	Difference	
7	4.47	7.15	2.68	59.9
9	3.13	4.84	1.71	54.6
13	3.69	5.62	1.93	52.3
16	3.32	4.58	1.26	38

Seedlings were grown in the dark for 7 to 16 days at 25°. Half the samples were transferred to the light for 24 hr and the remainder kept in the dark as controls.

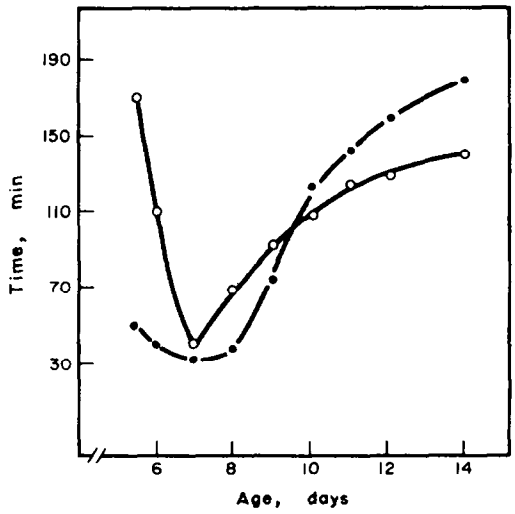

Fig. 2. The lag-phase and doubling-time of chlorophyll synthesis in barley leaves. Dark-grown seedlings of different ages were illuminated and chlorophyll measured at 15 min intervals for up to 3 hr. The lag phase is the time elapsed from the start of illumination to the first detectable increase in chlorophyll above that formed from pre-formed P650. The doubling period is the time required from the end of the lag phase to the point at which the chlorophyll was double the amount derived from preformed P650. O——O doubling time ●——● lag phase.

Kinetics of chlorophyll and protohaem synthesis

The lag phase in chlorophyll synthesis in several experiments was measured to establish any relationship with protohaem over this period.

Dark-grown seedlings of various ages were illuminated for 2–3 hr; at 15 min intervals chlorophyll was determined (Fig. 2). The length of the lag phase was taken as the time that elapsed from the start of illumination to the first indication of an increase in the chlorophyll content. In order to establish whether the length of the lag phase was related to the subsequent rate of chlorophyll formation further chlorophyll measurements were made. The point in time was established at which the chlorophyll level doubled (from the amount of preformed P650). This second period, referred to as the 'doubling time', gives a measure of the rate of ALA synthesis after the end of the lag phase. The lag phase in 5.5-day-old leaves was *ca* 50 min. It was shorter in 7- and 8-day-old leaves but thereafter continued to lengthen as the leaves aged. The doubling period, however, showed a similar pattern of change but with several important differences. The doubling period was 170 min in 5.5-day-old leaves, nearly 3.5 times longer than that of the lag phase. By day 7, however, the doubling time had fallen to its minimum value of 40 min, nearly the same as the lag phase. It then increased and after 8 days became increasingly shorter than the lag phase.

In other experiments protohaem formation was measured to see whether it followed a similar pattern. Seven-day-old dark-grown seedlings were illuminated for 105 min. At 15 min intervals during this period protohaem and chlorophyll were measured in the primary leaves (Fig. 3). Chlorophyll increased after 30 min light whereas protohaem did not rise until after 1 hr and then only after there had been a brief period of decline.

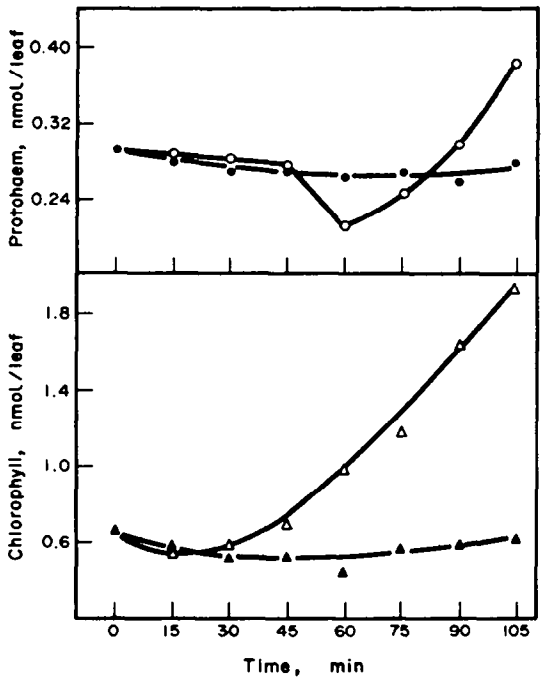

Fig. 3. Chlorophyll and protohaem in barley leaves exposed to light. Dark-grown 7-day-old seedlings were illuminated for 105 min. At 15 min intervals, the primary leaves were removed and assayed for chlorophyll and protohaem. ○——○ and △——△, light treated leaves; ●——● and ▲——▲, dark controls.

This 'dip' in protohaem level was observed on numerous occasions and tends to coincide with the start of the linear phase in chlorophyll production.

Distribution of protohaem and chlorophyll in the primary leaf

The distribution of protohaem in 7- and 10-day-old dark-grown leaves given a 7 hr light treatment is given in Table 2. In 7-day-old dark-grown leaves, 51% of the total protohaem was present in the top third of the leaf blade. Light treatment increased this slightly. The basal parts (bottom third of the blade and coleoptile region) contained *ca* 35% of the total protohaem in the dark-grown controls, and rather less after 7 hr light. In absolute terms (pmol/g fr. wt) there was a small increase in the basal sections of 406 pmol on illumination. In 10-day-old dark-grown leaves the protohaem was more equally distributed throughout the shoot. The effect of 7 hr light was to increase the absolute amounts of protohaem by 12%. This increase, however, was more or less equally distributed throughout the leaf.

Table 3. The distribution of chlorophyll in the primary leaf after a 7 hr light treatment

Leaf section	% of total chlorophyll in leaf section	
	Dark-grown 7 days	Dark-grown 10 days
Top third	44.4	52.1
Middle	38.3	35.4
Bottom third	14.6	11
Coleoptile region	2.7	1.5

Barley seedlings were grown in the dark for 7 to 10 days and then given a 7 hr light treatment. The leaves were divided into 4 sections and chlorophyll determined in each section.

For comparison the distribution of chlorophyll in 7- and 10-day-old dark-grown leaves subjected to 7 hr light is given in Table 3. The top and middle portions of the leaves accounted for over 80% of the total chlorophyll formed in the light.

DISCUSSION

Protochlorophyllide (P650) and protohaem reach their maxima in 6 to 8-day-old dark-grown barley leaves. Henningsen and Boynton [7] reported that total protochlorophyll(ide) (photo and non-photo convertible forms) in a barley cultivar (Svalofs Bonus) reached a maximum at day 7 and declined rapidly to day 11. On illumination, both the lag phase and the doubling period of chlorophyll formation was shortest in 7-day-old leaves. Even in plants grown continuously in the light maximum amounts of chlorophyll were reached shortly after the 6th day of growth [18]. The light-induced increase in protohaem was also greatest in 6-day-old material. Virgin [19] has shown that water stress in leaves affected the rate of P650 formation. In the present investigation however, the leaf tissue showed no sign of a water deficit during growth in the dark [18]. A more likely explanation for the decline in P650 after day 8 is

Table 2. The distribution of protohaem in the primary leaf before and after a 7 hr light treatment

Leaf section	% of total protohaem in leaf section			
	Dark-grown 7 days		Dark-grown 10 days	
	Control	After 7 hr light	Control	After 7 hr light
Top third	50.7	56	36.7	36.9
Middle	14.1	15.9	23.4	23.3
Bottom third	12	11	39.9	39.8
Coleoptile region	23.2	17.1	0	0

Barley seedlings were grown in the dark for 7 or 10 days. Half the samples were given a 7 hr light treatment and the remainder left in the dark for 7 hr. The leaves were divided into 4 sections and protohaem determined in each section.

limited ALA synthesis. In fact Stobart *et al.* [8] found a 66% decrease in the rate of P650 formation from endogenous ALA between day 7 and 12 of dark growth. On the other hand the formation of protochlorophyll (P630) from exogenous ALA was much less affected over this period [8].

ALA is the primary precursor of both P650 and protohaem [2]. In dark-grown leaves, however, protohaem, unlike P650, does not decline rapidly at day 8. If P650 and protohaem share a common pool of ALA then the decrease in amount of one porphyrin at a greater rate than the other suggests two possibilities: (a) that ALA is formed, in the dark, in ever declining amounts and that after day 8 there is a selective diversion of ALA to protohaem at the expense of P650, or (b) the destruction rates of the two porphyrins are different. If the two porphyrins are produced from different pools of ALA then there must be two sites of ALA synthesis which show different kinetics in ageing leaves.

On illumination the levels of chlorophyll and protohaem change in slightly different ways. Both exhibit a lag phase which suggests that the subsequent increase in the two porphyrins is dependent on the light induction of ALA synthesis. In 7-day-old dark-grown leaves chlorophyll formation begins 15 min before that of protohaem. This implies that the light-induced increase in ALA is initially almost entirely diverted to the formation of chlorophyll. The level of protohaem actually falls (observed in many experiments [18]) 15 min after the start of chlorophyll formation suggesting that all the ALA had been channeled towards chlorophyll.

We consider the decline in protohaem shortly after the initiation of chlorophyll synthesis a constant feature during the period of limited ALA availability. The data indicates that there is only one common pool of ALA serving all biosynthetic pathways in barley leaves. The increase in protohaem 30 min afer the start of chlorophyll synthesis suggests a restoration of a restricted supply of ALA for protohaem formation.

With prolonged growth in the dark the rate of chlorophyll production declines on illumination. The increase in protohaem in the light was not seriously affected for at least 13 days of dark-growth. Because the rate of ALA synthesis in ageing leaves was diminished it would seem that protohaem has 'first call' on the supply of ALA. This would be in accord with the suggestion that protohaem may have a regulatory role in chlorophyll synthesis. From the data presented, however, it is not clear whether protohaem is playing a role in the fine control of the Mg-porphyrin pathway.

EXPERIMENTAL

Barley seeds (*Hordeum vulgare*, cv Proctor) were stored at room temp. in bins. Seeds were imbibed for 16 hr in tap H_2O, planted in trays of vermiculite, and germinated at 25° in the dark. Illumination was at 3500–4000 lx.

Porphyrin extraction and estimation. Chlorophyll was determined in 80% Me_2CO using the extinction coefficients of ref. [20]. Photoconvertible protochlorophyllide (P650) was estimated by first exposing the dark-grown leaves to light for 3 min before extraction in 80% Me_2CO. The A at 672 nm was measured and related to the concentration of P650 [21]. Protohaem was extracted and estimated by the methods of refs [22] and [16]. The leaves were ground in a mortar in liquid N_2 and extracted twice in ice-cold 90% ammoniacal Me_2CO (10 ml/g fr. wt). The pellet, after centrifugation (3000 *g*, 10 min) was further extracted twice with 5 ml lots of Me_2CO containing HCl (49:1) and the supernatant after centrifugation reduced to dryness at 27° under vacuum. The residue was dissolved in 3 ml alkaline Py (20 ml Py in 30 ml 0.2M KOH). The sample was divided between two cuvettes, solid Na-dithionite being added to one cuvette, and after 2 min the difference-spectrum of the oxidised and reduced Py–haemochrome measured at 557 and 540 nm. Protohaem was determined using nmol difference extinction coefficients [22].

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