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Regulation of δ-aminolaevulinic acid synthesis and protochlorophyllide regeneration in the leaves of dark-grown barley (*Hordeum vulgare*) seedlings

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Laevulinic acid (Lev) was used to control the rate of protocholorophyllide (PChl) regeneration in the leaves of dark-grown seedlings of barley (*Hordeum vulgare*) after a brief light treatment. In the leaves given Lev, at concentrations that severely block the resynthesis of protochlorophyllide, there was a massive overproduction of δ-aminolaevulinic acid (AmLev) that was well in excess of that required for the regeneration of PChl observed in the control leaves. Lev, at low concentrations, slightly delayed regeneration and held up, rather than inhibited, the utilization of the AmLev, which accumulated in the tissues. The overproduction and uncontrolled formation of AmLev also occurred in dark-grown leaves treated with a high concentration of Lev and given a light treatment of just sufficient energy to photoreduce only small quantities of the endogenous PChl. Experiments in which a high level of free PChl was induced by incubating the leaves in AmLev indicated that the active species of PChl was that associated with, and bound to, the PChl reductase protein. The results strongly demonstrate a close relationship between the PChl–protein complex and the ability of the leaves to synthesize AmLev.

The regulation of chlorophyll synthesis in the leaves of angiosperms is little understood. The etiolated leaves of dark-grown seedlings, particularly those of cereals, contain a small quantity of PChl that, when associated with a PChl reductase protein complex (Griffiths *et al.*, 1976) is reduced to chlorophyllide in the light (Klein *et al.*, 1977). If these light-treated leaves are returned to the dark, then new PChl is rapidly resynthesized and accumulates to the same level as that found in the leaves before the photoreduction (Bogorad, 1976; Fluhr *et al.*, 1975). The regeneration of PChl is due to promoted synthesis of new AmLev (Stobart *et al.*, 1972; Klein *et al.*, 1977). The most logical candidate to regulate AmLev synthesis is PChl. Many workers have, therefore, suggested that AmLev formation in leaves may be controlled by a negative-feedback mechanism from PChl (Bogorad, 1976; Klein *et al.*, 1977), and Gough (1978) found the AmLev production stopped after a critical level of PChl was reached in the tissues.

It is possible that the light involvement in chlorophyll synthesis is only in the photoreduction of PChl and that the stimulation in AmLev formation is a consequence of this. The present report describes experiments designed to investigate the role of PChl in regulating AmLev production and its relationship to chlorophyll synthesis in the primary leaves of dark-grown seedlings of barley.

Abbreviations used: AmLev, δ-aminolaevulinic acid; Lev, laevulinic acid; PChl, protochlorophyllide.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Proctor) seeds were soaked in water for 16h and, after washing, sown in trays of moist Vermiculite. Germination and growth were allowed to proceed at 25°C. At the desired age, the top 3–6cm of the leaves were removed and used in the experiments. Five segments (approx. 0.2g) were routinely used per treatment unless otherwise stated. The segments were placed with their cut ends in the test solution in small glass vials under a gentle airstream to facilitate uptake. All manipulations were carried out in the dark-room under a dim green safelight.

AmLev extraction and estimation

Lev, a competitive inhibitor of AmLev dehydratase, was used to block the utilization of AmLev in the leaf segments (Harel & Klein, 1972). The AmLev that accumulated was extracted from the

segments by boiling for 15min in 4% (w/v) trichloroacetic acid. The extract and leaf material were filtered through a cottonwool pad held in the base of a plastic syringe. The leaves were washed with 1ml of distilled water and the washings pooled with the original filtrate. After the addition of 3ml of sodium phosphate buffer (0.5M, pH7.2), ethyl acetoacetate (5 drops) was added and the reactions mixture boiled for 15min. A 1ml portion of the solution was removed and combined with 1ml of Ehrlich's reagent [1g of dimethylamino-benzaldehyde in 42ml of acetic acid and 8ml of 70% (w/v) perchloric acid]. After 15min, A_{553} was measured. AmLev was determined by using a calculated molar absorption coefficient of 7.45×10^4 litre·mol^{-1}·cm^{-1}. The efficiency of the procedure was never less than 90% when determined with standard AmLev that was added to the leaves just before extraction.

PChl extraction and determination

Leaf segments, after light or dark treatment, were extracted in cold acetone/water (4:1, v/v) in the dark. The total PChl was determined by using the equations of Anderson & Boardman (1964).

PChl regeneration and spectroscopy of the leaves in vivo

The relative PChl regeneration was measured by the direct spectroscopy of the leaves. Leaf segments were inserted in a special glass cuvette, in the dark, to give a light-path of about one leaf thick. The loaded cuvette was placed in a double-beam spectrophotometer and the absorption recorded from 600 to 700nm against a filter-paper blank. The leaves were then given a light treatment, to photoreduce the endogenous PChl, and the spectrum recorded immediately and at regular intervals thereafter. Baseline drift was adjusted to an isosbestic point at 682nm. The increase in absorption at 652nm was taken as a measure of PChl regeneration (Stobart *et al.*, 1972).

Light treatment

For the full phototransformation of PChl to chlorophyllide leaves were exposed to a 3min treatment with white fluorescent light (2.31W·m^2). Brief light exposures, for the partial photoreduction of PChl, were provided from an electronic computerized flash and timing unit (Mecablitz 402). An output of 0.49W·m^{-2} was sufficient to bring about a 30–40% photoconversion of PChl.

Results

Effect of Lev on the accumulation of AmLev and the regeneration of PChl

The leaves of 8-day-old dark-grown seedlings were preincubated in Lev for 4h in the dark and then given a light treatment to photoconvert almost all the PChl. After a further 7h in the dark the AmLev and PChl in the leaves were determined. The results (Table 1) show that Lev, up to a concentration of 60mM, brought about an increased accumulation of AmLev in the tissues. Lev at 100mM was, however, slightly inhibitory and decreased somewhat the AmLev that accumulated. The PChl that was regenerated in the 7h dark period after the light treatment is presented as nmol equivalents of AmLev (Table 1). The PChl content of the leaves treated with up to 20mM-Lev was similar to the control values. In leaves given high concentrations of Lev the PChl that regenerated was less than that in the control tissues. However, even at 60mM- and 100mM-Lev the regeneration of PChl was never completely prevented, and 7h after the light treatment was 80 and 50% respectively of the control values. The total AmLev, comprising the AmLev that accumulated together with that equivalent to the newly generated PChl, (Table 1) increased by over 300% between the control values and that found in leaves treated with 60mM-Lev.

Table 1. *Effect of Lev on AmLev accumulation and the regeneration of PChl in dark-grown leaves of barley*
Leaves of 8-day-old dark-grown seedlings were preincubated in Lev in the dark for 4h. The leaves were then given a white-light treatment of sufficient energy to photoreduce all the phototransformable PChl. After a further 7h in the dark the AmLev and 'regenerated' PChl were determined. PChl is presented as nmol equivalents of AmLev. Total AmLev is the AmLev that accumulated together with the PChl that regenerated. The results are means (±S.D.) for three replicates.

[Lev] (mM)	[AmLev] (nmol/g fresh wt.)	8×[PChl] (nmol/g fresh wt.)	Total [AmLev] (nmol/g fresh wt.)	Excess AmLev (% of control)
0	36±3	121±17	157	0
5	50±9	136±4	186	19
10	74±6	148±10	222	41
20	173±9	132±6	305	94
60	413±3	96±4	509	224
100	360±38	62±3	422	169

It is evident that Lev, particularly at the higher concentrations, brings about an overproduction of AmLev well in excess of that required for the resynthesis of PChl after the light treatment.

Time course for AmLev production in Lev-treated leaves

The relationship between PChl regeneration and AmLev production was further investigated in time-course studies. The leaves of 8-day-old dark-grown seedlings were treated with Lev (20 and 100mM) for 4h in the dark. A concentration of Lev was selected that was high enough to ensure a substantial inhibition in PChl regeneration and at the same time still compatible with adequate AmLev synthesis. The leaves, after the laevulinate treatment, were exposed to light to photoreduce all the endogenous PChl and then sampled at regular intervals. The PChl in the leaves after the photoreduction was approx. 20% of that before the light treatment (Fig. 1*a*). The regeneration of PChl was similar in the water-control leaves and those treated with 20mM-Lev and reached a maximum 3h after the light treatment. The PChl in the 100mM-Lev treatments, however, increased for 1h after the light and then slightly decreased. The PChl that regenerated in the 100mM-Lev-treated leaves, within 3h of the light, was about 40% of that in the equivalent 20mM-Lev- and water-treated tissues. The PChl in leaves given Lev and not exposed to light remained similar throughout the experiment.

The amount of AmLev that accumulated in the leaves after the photoreduction of PChl is shown in Fig. 1(*b*). The AmLev content of the leaves in both the Lev treatments was similar 2h after exposure to light. However, in the 20mM-Lev-treated tissue the AmLev stopped accumulating and was similar throughout the remaining period. The AmLev, on the other hand, in the leaves treated with 100mM-Lev, continued to increase at an almost linear rate, and 6h after the light was nearly twice that found in the 20mM-Lev treatments. There was a slight accumulation of AmLev in leaves given Lev and not exposed to light. This production of AmLev in the dark has now been observed in many experiments.

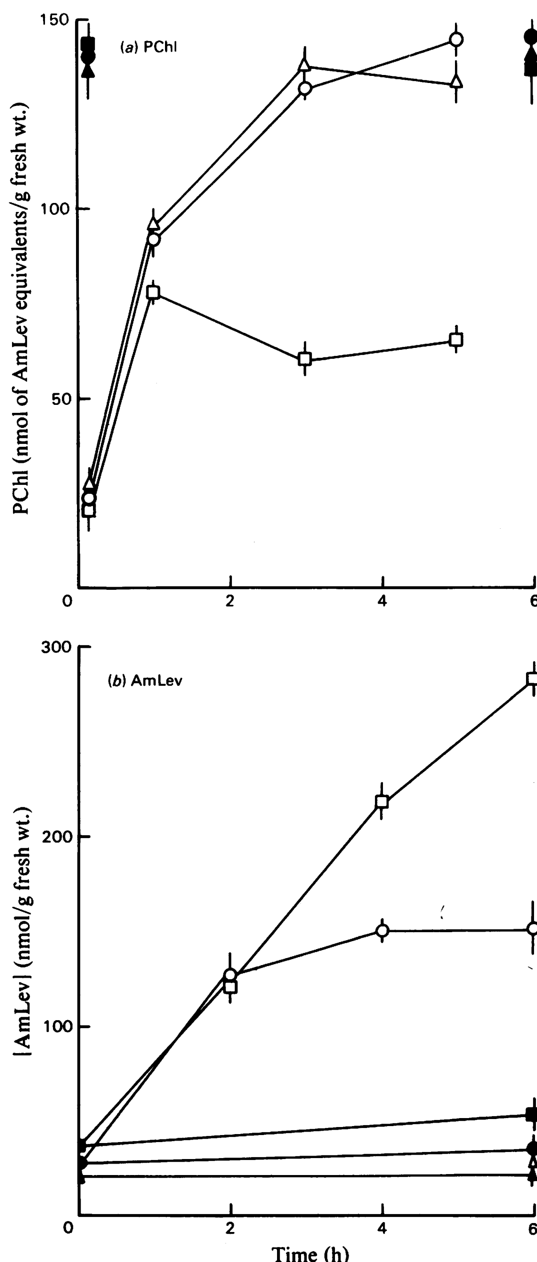

Fig. 1. *Regeneration of PChl (a) and the production of AmLev in dark-grown leaves given a light treatment (b)*
Leaves from 8-day-old dark-grown seedlings were preincubated in the dark in Lev (20 and 100mM) for 4h and then given a light treatment to photoreduce all the photoconvertible PChl. At regular intervals thereafter the regenerated PChl (*a*) and AmLev (*b*) in the leaves were determined. Samples of leaves were also kept in complete darkness throughout the experiments. The results are means (±S.D.) for three replicates. Symbols used: ○ and □, 20mM- and 100mM-Lev, light treatments; ● and ■, 20mM- and 100mM-Lev, dark treatments; △ and ▲, no Lev, light and dark treatment respectively.

AmLev production in leaves that rapidly regenerate PChl

The above results indicate a close relationship between PChl regeneration and the ability of the leaves to synthesize AmLev. In leaves, where the full regeneration of PChl is limited by a sufficiently high Lev concentration, the accumulation of AmLev progresses at an almost unimpeded rate. However, in leaves treated with Lev, at concentrations (20mM) that delay slightly the regenera-

tion of PChl, the AmLev accumulates only until the newly synthesized PChl has reached a critical level. To substantiate these findings, the production of AmLev was investigated in leaves that rapidly regenerate PChl. The age of the dark-grown seedlings that are used in regeneration studies has a pronounced effect on the rate of PChl synthesis after a light treatment, and the younger the leaf the more rapid is the regeneration (Stobart *et al.*, 1972).

The relative regeneration of PChl (measured by spectroscopy *in vivo*) in the leaves of 5- and 7-day-old dark-grown seedlings treated with 20mM-Lev is shown in Fig. 2. The rate of PChl resynthesis in the 5-day-old Lev-treated leaves was very rapid and reached a maximum within 30min of the light treatment, after which any further accumulation stopped abruptly and at a level slightly greater than the PChl in the leaves before the light treatment. The regeneration of PChl in the 7-day-old Lev-treated leaves was slower than in the 5-day-old tissue, and reached a maximum 75min after the light treatment. The pattern of AmLev accumulation in the leaves reflected the rate of PChl regeneration (Fig. 2). In the 5-day-old leaves, in which the regeneration was extremely rapid, the AmLev reached a maximum at almost the same time as complete PChl resynthesis had occurred and then declined to a constant level after 60min. Similar results were obtained with the 7-day-old leaves, but at a slightly later time in the experiment. These observations have been confirmed in many different experiments.

The results confirm that AmLev production is 'switched off' when the PChl has reached a critical level in the leaves. Whether at that point the small quantity of AmLev in the pathway is destined for PChl is unclear. However, it is noteworthy that in these and many other experiments the final content of the regenerated PChl is often slightly greater than that found before the photoreduction.

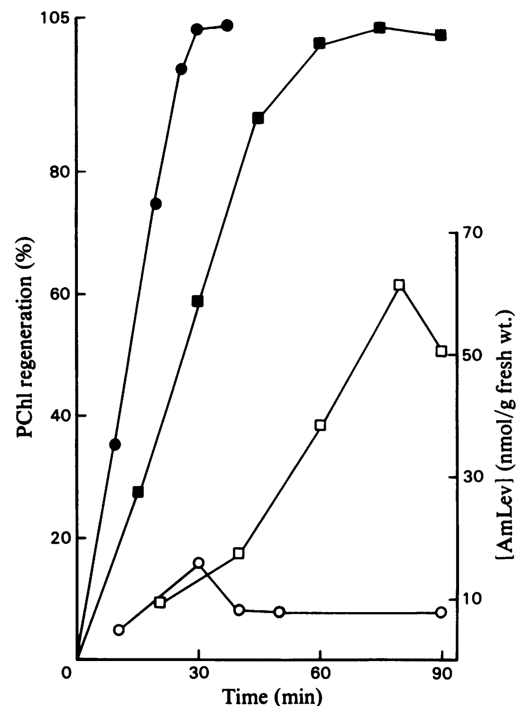

Fig. 2. *Effect of rapid PChl regeneration on the accumulation of AmLev*

Leaves 5- and 7 days old were preincubated in Lev (20mM) for 2h in the dark, after which they were given a light treatment of sufficient energy to photoreduce all the photoconvertible PChl. At regular intervals thereafter the AmLev and relative PChl regeneration (as measured by spectroscopy *in vivo*) were determined. Values for AmLev are corrected for the small quantity present in the dark control leaves. Symbols used: ○ and □, AmLev in 5- and 7-day-old leaves respectively. ● and ■, PChl in 5- and 7-day-old leaves respectively.

Effect of a partial photoreduction in PChl on AmLev synthesis

It is possible to induce a partial reduction in the endogenous PChl in the leaves by regulating the duration of the light treatment. Leaves given a partial reduction normally regenerate PChl to a similar level to that found in tissues given a full reduction. Experiments were carried out to determine whether a partial reduction in PChl could also enhance the accumulation of AmLev in the presence of Lev. Leaves of 8-day-old dark-grown seedlings were incubated in 80mM-Lev for 2h in the dark. The leaves were then given a brief light treatment to bring about a partial reduction in PChl and returned to the dark. The levels of PChl in the treatments are given in Table 2. Leaves treated with Lev or water and given light to induce a partial reduction still contained over 60% of the endogenous PChl. Leaves treated with Lev and light to bring about a full or partial reduction exhibited only a slight regeneration in PChl. However, the control leaves, given only water before the photoreduction, fully regenerated the PChl during the experiment.

The AmLev that accumulated in the Lev-treated leaves after the partial reduction in PChl was determined at regular intervals (Fig. 3). AmLev was detectable within 10min of the light treatment, and its accumulation continued at an almost linear rate for the further 120min in the dark. The PChl required for full regeneration after the partial reduction is maximally about 7nmol (equivalent to about 60nmol of AmLev). At the end of the experi-

Table 2. *PChl in dark-grown leaves exposed to light to give partial or full photoreduction*
Leaf-segments of 8-day-old dark grown seedlings were treated with Lev (80mM) for 2h in the dark and then given a light-treatment to photoreduce partially or fully the photoconvertible PChl. PChl was determined immediately after the light treatment and after a further 2h in the dark. PChl was also determined in leaves given no light treatment ('Dark leaves'). Values given in parentheses are the observed relative (percentage) photoconversions. Results are means (±S.D.) for three replicates.

Treatment (and time after light exposure)	[PChl] (nmol/g fresh wt.)		
	Partial photoreduction	Full photoreduction	Dark leaves
Water			
0min	9.5±0.8 (33.6%)	1.8±0.9 (86.7%)	14.3±2.1
120min	15.6±1.7	14.1±1.2	15.7±2.3
Lev			
0min	9.3±1.1 (31.1%)	2.3±1.2 (82.9%)	13.5±1.6
120min	10.6±1.1	3.7±1.7	14.1±1.9

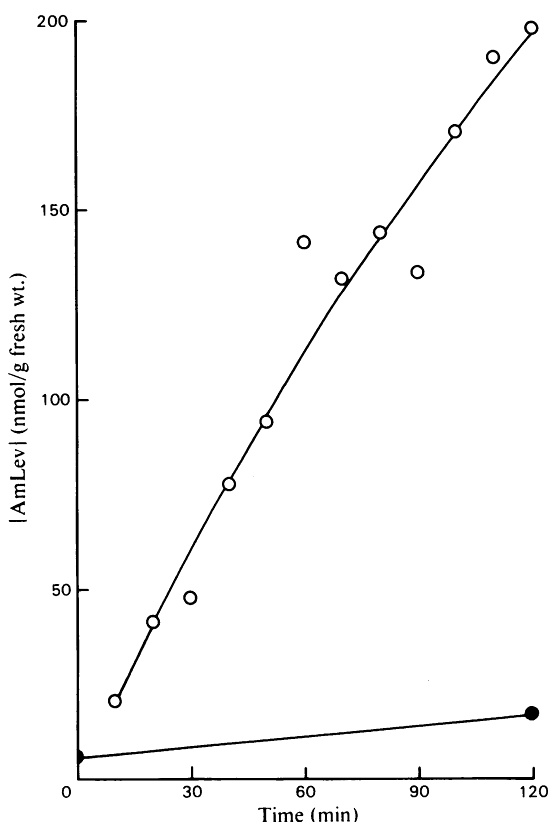

Fig. 3. *AmLev accumulation after a partial photoreduction of PChl*
Leaf segments of 8-day-old dark-grown seedlings were treated with Lev (80mM) for 2h in the dark and then given a light-treatment to partially photoreduce the PChl (see Table 2). AmLev was determined immediately after the light-treatment and at regular intervals thereafter (○). AmLev was also determined in leaves given no light but treated similarly to those above (●).

ment, however, some 200nmol of AmLev had accumulated in the tissues and represented an overproduction three times in excess of that based on the PChl that could normally regenerate.

Effect of free PChl on PChl regeneration and AmLev synthesis

Attempts were made to determine the species of PChl that appeared to regulate the synthesis of AmLev. The 4cm leaf tips of 7-day-old dark-grown seedlings were left in AmLev (1.0mM, pH6.9) for 135min in the dark and then transferred to Lev (80mM) for a further 2h in the dark. Control segments were similarly treated with water. Fig. 4 shows the spectra *in vivo* of the AmLev-treated leaves, together with that of the water-control tissue. The major absorption band in the AmLev treatments is at 630nm (Fig. 4*a*) and corresponds to an excess of free PChl compared with the bound photoconvertible PChl, with a maximum absorption at 652nm, in the water-control leaves (Fig. 4*b*). Figs. 4(*c*) and 4(*d*) give the absorption spectra *in vivo* of leaves treated with AmLev and water respectively and given light to photoreduce the bound PChl. PChl regeneration was almost unaffected in the AmLev-treated leaves given Lev (Fig. 4*e*), whereas very little, if any, occurred in the water-control tissue with Lev (Fig. 4*f*). The regeneration of PChl took place in leaves treated with water only and no Lev (Fig. 4*g*).

The leaves treated with AmLev and Lev, followed by light, were returned to the dark and their AmLev content determined over a 2h period. The results (Fig. 5) show that the AmLev in the AmLev-treated leaves immediately after the light treatment was similar to the leaves given water only. AmLev accumulation in the water control tissues was linear throughout the 120min dark period and reached a final level of some 250nmol/g fresh wt. On the other hand, AmLev production in

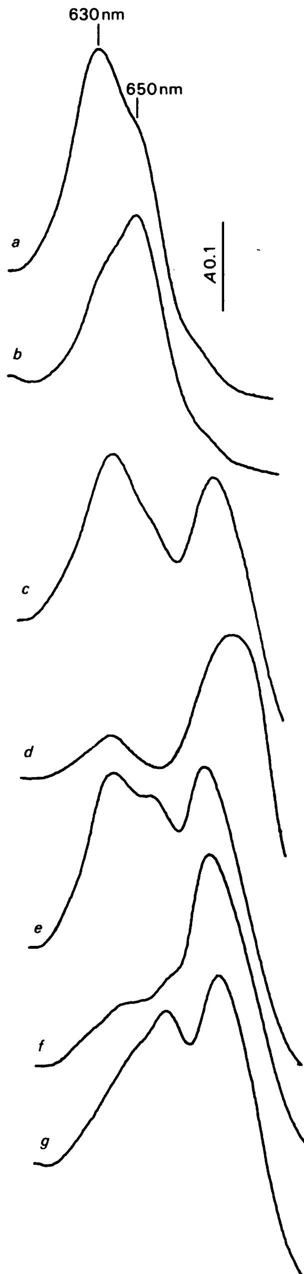

Fig. 4. *Absorption spectra in vivo of leaves incubated in AmLev or water followed by a light treatment*
Leaf segments from 7-day-old dark-grown leaves were left in AmLev (1.0mM) for 135min in the dark and then transferred to Lev (80mM) for a further 2h in the dark. Leaves were given a light-treatment to photoreduce the bound PChl and returned to the dark. *a* and *b*, AmLev- and water-treated leaves. *c* and *d*, AmLev- and water-treated leaves immediately after the light treatment. *e* and *f*, AmLev- and water-treated leaves 120min after the light treatment. *g*, Leaves given no AmLev or Lev; 120min after the light treatment.

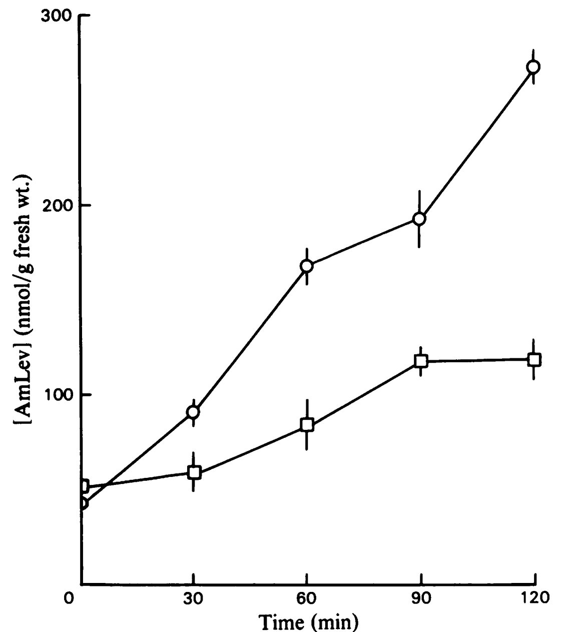

Fig. 5. *AmLev accumulation in leaves with a high content of free PChl after a brief light treatment*
Leaf segments from 7-day-old dark-grown leaves were stood in AmLev (1.0mM) for 135min in the dark and then transferred to Lev (80mM) for a further 2h in the dark. The leaves were given a light treatment to photoreduce the bound PChl and returned to the dark. The AmLev in the leaves was determined at regular intervals thereafter. Symbols used: □, leaves pretreated with AmLev followed by Lev and a light treatment. ○, leaves given only water before Lev and the light treatment.

the leaves with the relatively greater free PChl exhibited an initial delay, after which accumulation occurred and reached a maximum within 1.5h and stopped.

The results show that AmLev production, in leaves in which PChl regeneration is severely restricted by Lev, continues unchecked and apparently out of control. AmLev synthesis, on the other hand, in leaves in which the regeneration of bound PChl can only occur from excess free PChl (i.e. those leaves that have had a previous incubation in exogenously supplied AmLev) and not from newly synthesized AmLev, is rapidly turned off. The described effect of excess free PChl on AmLev synthesis has now been observed in some ten experiments.

Discussion

The results strongly indicate that the regulation of AmLev production in the leaves of dark grown seedlings after a light induction is controlled by

PChl. In leaves in which the regeneration of PChl is prevented, or severely restricted, by Lev, there is a massive synthesis and accumulation of AmLev. The AmLev produced under these conditions is well in excess of that necessary for the observed regeneration of PChl in the control leaves. In tissue, however, that has been treated with Lev at concentrations that hold up to some extent the utilization of AmLev and delay, rather than inhibit, the regeneration of PChl, there is a close relationship between the PChl that accumulated and the synthesis of AmLev. This was particularly evident in leaves that were rapidly regenerating PChl and also in those in which a partial photoreduction in PChl had occurred. Even in leaves given light to only partially reduce the PChl, but Lev to inhibit the regeneration severely, there was an uncontrolled, overproduction of AmLev.

It is also of interest that, in many of our experiments, the production of AmLev in leaves after a light treatment was detectable almost immediately and certainly without any appreciable lag. These observations also suggest that the control of the enzyme(s) involved in the synthesis of AmLev is by the activation of a pre-existing protein rather than the complete synthesis *de novo* of enzyme. We also found that AmLev is synthesized to a small extent in dark-grown leaves given no light treatment. At the moment we have no information on the identity of the enzyme(s) involved or whether it (they) contribute(s) AmLev to haem and PChl, which are slowly turned over in leaves in the dark (Hendry & Stobart, 1977, 1978*a*).

Leaves of dark-grown seedlings contain two major species of PChl (Shibata, 1957; Griffiths, 1975). In young leaves the predominant form has a maximum absorption at 652nm and is phototransformable to chlorophyllide. The photoconvertible PChl is known to be bound to a PChl reductase protein in a ternary complex with NADPH (Griffiths *et al.*, 1976). Also present in the leaves, in relatively smaller quantity, is free PChl with a maximum absorption at 630nm (Shibata, 1957; Sundqvist, 1969). The phototransformable PChl is normally limited by the quantity of the reductase protein present in the leaves. Exogenously supplied AmLev is rapidly converted into PChl, which accumulates as the free species. The free PChl is not directly photoconvertible, but becomes bound to the reductase protein, provided that active sites are available, i.e. after a brief light treatment. In leaves, therefore, in which the rate of endogenous regeneration is limited by Lev, but that can still synthesize photoconvertible PChl from excess free PChl (i.e. in those leaves fed AmLev before Lev treatment), there is a more rapid turning-off in AmLev synthesis. The fact that AmLev still accumulates after a light treatment, albeit to a smaller extent, in the Lev-treated leaves with a high free PChl content, indicates that the regulation of AmLev production is controlled by bound PChl (i.e. the reductase ternary complex) and not the free species. Chlorophyllide and chlorophyll are not involved in the regulation of AmLev synthesis, since similar amounts of chorophyllide are present after a light treatment in leaves with a high free PChl content and in control leaves not incubated in AmLev. It should also be noted that no increase in haem was observed in leaves with exogenously supplied AmLev (Castelfranco & Jones, 1975; Hendry & Stobart, 1978*b*).

It is also of interest that only low levels of AmLev were detected in leaves incubated with exogenously supplied AmLev followed by a period in Lev or buffer. It appears that the cells contain an active mechanism for the rapid metabolism of AmLev. The location of the reactions that utilize AmLev, other than those involved in the synthesis of PChl, are probably extraplastidic. This suggestion is supported by the facts that any AmLev reaching the plastid is rapidly converted into PChl and that the AmLev synthesized after a light treatment in the presence of Lev readily accumulates. These observations also suggest that the AmLev that is destined for chlorophyll is synthesized within the plastid and not transported from an extraplastidic site. Other workers have also reported the rapid metabolism of AmLev other than in porphyrin synthesis (Gassman *et al.*, 1978; Duggan *et al.*, 1982).

It is pertinent to speculate on the nature of the control by the bound PChl complex in the synthesis of AmLev. It is possible that the reductase protein, in the presence of its substrates, exerts a direct feedback control on AmLev synthetase protein (Bogorad, 1976), and after photoreduction the restraint is removed and AmLev is synthesized. It is relevant to this proposal that AmLev production is observed almost immediately after a light treatment.

An alternative to the involvement of the bound PChl complex should also be considered. It is possible that the free reductase protein, after discharging its products (Griffiths, 1975; Griffiths *et al.*, 1976) exerts either some conformational control on a spatially related AmLev synthetase or is partially broken down and reorganized into a synthetase protein. The effect of light on the photoreduction of the PChl in the reductase complex, and the subsequent release of the products, provides conditions for a change in the reductase protein that endows it with AmLev synthetase activity. The PChl reductase protein may therefore have a dual enzyme capability that is mediated by light.

It is of interest that the PChl reductase protein substantially declines in leaves in the light owing to the participation of a specific proteinase (Kay & Griffiths, 1983). The products of the reductase protein breakdown, however, remain unidentified.

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