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# Some Observations on Chlorophyll(ide) Synthesis by Isolated Etioplasts

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1. A modified procedure for the isolation of etioplasts from dark-grown barley is described and the regeneration of phototransformable protochlorophyll(ide) was demonstrated in the isolated plastids. 2. On exposure of the etioplasts to a long-term flash illumination, chlorophyll(ide) synthesis from a precursor pool, which includes all the protochlorophyllide, was demonstrated. 3. Added δ-aminolaevulinic acid failed to be significantly incorporated into chlorophyll(ide) in the etioplasts despite its extensive incorporation into porphyrin precursors of chlorophyll and haem compounds. The findings are discussed in terms of the inability of etioplasts to carry out the metal-insertion step in chlorophyll synthesis. 4. An elevated chlorophyll(ide) concentration was attained in the etioplasts by increasing the size of the utilizable precursor pool by pre-feeding whole plants with δ-aminolaevulinic acid, isolating the etioplasts and subjecting them to the flash illumination.

Studies aimed at elucidating the steps involved in the acquisition of photosynthetic activity by higher plants have been carried out by using assays *in vivo* on greening etiolated leaves (Bonner & Hill, 1963; Hiller & Boardman, 1971) and assays *in vitro* on plastids isolated from leaves at different stages of greening (Gyldenholm & Whatley, 1968; Plesnicar & Bendall, 1973; Forger & Bogorad, 1973; Remy, 1973). The picture emerging from this work is that of a stepwise mechanism for the greening process with most of the components required for photosynthesis being already present in etiolated systems; development involves the light-dependent addition of chlorophyll and possibly also some other functional (Plesnicar & Bendall, 1973) and structural (Forger & Bogorad, 1973) components enabling photosynthetic reactions to proceed.

Techniques have been described for the isolation of etioplasts that retain the ability to undergo photomorphological changes *in vitro* (Wellburn & Wellburn, 1971*a*; Horton & Leech, 1972). These changes resemble to some extent the light-induced development occurring *in vivo* in the leaf. It has been the aim of this laboratory to study the extent of acquisition of photosynthetic activity during this development *in vitro*. In the present report, a method of etioplast isolation is described together with techniques for accumulating high concentrations of chlorophyll in these preparations.

## Experimental

### *Materials*

Plant material was grown from barley (*Hordeum vulgare* L. cv. Proctor) seeds. These were sown in moistened seedling compost (Levington) and germinated in complete darkness at 24°C for 7 days. Harvesting of the seedlings at the end of this period was carried out under a dim green safelight.

δ-Aminolaevulinic acid hydrochloride was obtained from Sigma Chemical Company, Kingston-upon-Thames, Surrey, U.K., and was used as a solution buffered at pH6.8 with 2mM-potassium phosphate. The Good buffers (Good *et al.*, 1966) used in this work, Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] and Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] were also purchased from Sigma.

### *Methods*

Etioplasts were isolated by a modification of the method of Horton & Leech (1972). Plant material (approx. 120g) was comminuted briefly (10s) by using an Ato-Mix blender in an isolation medium composed of 0.5M-sucrose, 0.2% (w/v) bovine serum albumin, 5mM-cysteine, 1mM-$MgCl_2$, 1mM-EDTA, 20mM-Tes and 10mM-Hepes adjusted with KOH to pH7.2. The resulting homogenate was filtered through four layers of Mirracloth and four layers of muslin and centrifuged by accelerating to 9000*g* in an MSE 18 centrifuge. On reaching this force the centrifuge was rapidly stopped. The resulting pellet of crude etioplasts was resuspended in isolation medium and cellular debris removed by centrifugation at 1500*g* for 90s followed by sedimentation of the etioplasts from this supernatant by centrifugation at 6000*g* for 90s. The etioplasts were finally resuspended in isolation medium supplemented with 1.5mM-ATP (Horton & Leech, 1972) to a protein concentration of 6–8mg/ml. All these steps were carried out under a dim green safelight and the

temperature during the whole procedure was maintained as close to 0°C as possible.

Etioplasts containing a high concentration of protochlorophyll(ide) were isolated by first immersing the excised ends of the seedlings immediately after cutting in a solution of 10mM-$\delta$-aminolaevulinate buffered at pH 6.8 with 2mM-phosphate. These were then placed in darkness in a gentle air stream for 6–8h to facilitate uptake of the substrate. At the end of this period the leaves appear pale green in colour owing to the $\delta$-aminolaevulinate-induced accumulation of protochlorophyll(ide). Etioplasts were finally isolated from this material exactly as described above.

Light-absorption characteristics of the preparations after dilution with resuspending medium were recorded on a split-beam spectrophotometer described by Jones & Saunders (1972) with a dilute solution of milk as a blank to compensate for the scattering effect of the preparation. Chlorophyll concentrations in the samples were determined from the peak height of the red chlorophyll absorption band, assuming linearity between absorption and chlorophyll concentration (Boardman, 1962).

Spectroscopic measurement of the regeneration of phototransformable protochlorophyll(ide) (pigment P-650*) after illumination of etioplasts was achieved by using a dual-wavelength spectrophotometer equipped with facilities for cross-illumination (Jones, 1969). Samples were illuminated *in situ* in the spectrophotometer with light from a 55W quartz–halogen lamp filtered through an interference filter (Balzers, B20) with maximum transmission at 440nm giving an illumination intensity of 0.75mW/cm$^2$. The photomultiplier (EMI type 6255B) was protected during this cross-illumination by two layers of Kodak Wratten gelatine filters no. 92, transmitting light of wavelength greater than 615nm only. For the assay, etioplasts were illuminated to completely convert all the endogenous pigment P-650 into chlorophyll(ide). Pigment P-650 regeneration was then measured, after incubation in darkness, from the change in the absorption difference, $E_{652}-E_{620}$, induced in the samples by a subsequent illumination. When studying the kinetics of the regeneration a fresh etioplast sample had to be used for each dark-incubation time assayed. Incubations at 22°C were carried out in the spectrophotometer, whereas for incubations at 0°C the samples were kept on ice and transferred in darkness to the spectrophotometer for assay of the regeneration.

Illumination of the etioplasts was achieved by one of two methods. For a single illumination to determine the photoconvertibility of protochlorophyll(ide) in preparations the sample, usually in the spectrophotometer, was illuminated at an intensity of 15mW/cm$^2$ for 90s by a 100W tungsten-filament lamp held at a distance of 10cm away. For flash illumination the samples were exposed to flashes of 1ms from a photographic xenon lamp (Mecablitz) supported 10cm from the sample. The light-intensity in this case was 4mJ/flash. The lamp was triggered electronically and the time between successive flashes could also be electronically controlled, but in the experiments reported here a 15min dark-interval between successive flashes was adopted. During these prolonged incubations the samples were gently shaken on a shaking water bath.

Protein concentrations in samples were assayed by the Folin method (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

* Abbreviations: P-650 and P-630, protochlorophyll-(ide) pigment complexes occurring *in vivo* and showing light-absorption maxima at the wavelengths indicated.

## Results and Discussion

The absorption spectrum of an etioplast suspension, isolated from 7-day-old dark-grown barley, before (curve *a*) and after (curve *b*) 90s of illumination from a 100W tungsten filament lamp is shown in Fig. 1. Protochlorophyll(ide) in the non-illuminated etioplasts absorbs maximally at 652nm, with a shoulder at approx. 633nm. After photoconversion the 652nm peak disappeared and was replaced by chlorophyll(ide) absorption at 683nm. If the sample was left in darkness for 25min and then the spectrum redetermined (curve *c*) a build-up of pigment P-650 peak reappeared together with a shift towards the blue of the chlorophyll(ide) peak. Re-illumination of the sample at this stage would have resulted in an increase in the chlorophyll(ide) peak at the expense of the regenerated protochlorophyll(ide). This indicated that, as in whole leaves, these etioplasts were capable of regenerating photoconvertible protochlorophyll(ide) (pigment P-650), a phenomenon which until now has not been reported in a cell-free system.

A more convincing demonstration of the pigment-P-650-regenerating ability of etioplasts was seen if spectra were recorded at 77°K in liquid $N_2$. Fig. 2 (curve *a*) gives such a spectrum of a flashed etioplast preparation which had been incubated in darkness at room temperature for 25min and then frozen in liquid $N_2$. The regenerated pigment P-650 is now clearly seen as a sharp peak at 654nm. Illumination of another portion of the same sample followed by freezing demonstrated (curve *b*) the complete conversion of pigment P-650 into chlorophyll(ide).

It is interesting to note the positions of the chlorophyll(ide) peaks in Figs. 1 and 2. Within approx. 2min of illumination, the chlorophyll(ide) absorbs maximally at 683–684nm at room temperature, whereas if it is left in darkness for 30min or so, the peak shifts to the blue, and now absorbs maximally at 674nm. This phenomenon, the Shibata shift, has been well

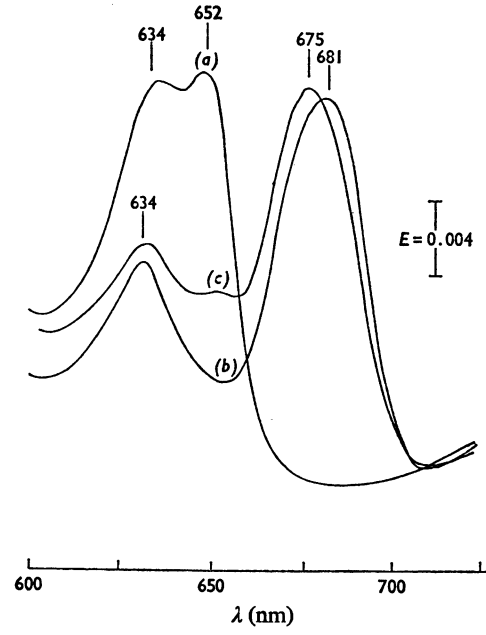


Fig. 1. *Phototransformation and dark regeneration of photoactive protochlorophyll(ide) in isolated barley etioplasts*

Freshly isolated non-illuminated etioplasts were diluted with resuspending medium in darkness and their absorption spectrum was recorded before any illumination (curve *a*). Illumination of the sample in the spectrophotometer was carried out for 90s with a 100W tungsten lamp. The spectrum was recorded immediately after illumination (curve *b*) and after further incubation in darkness for 25min (curve *c*).

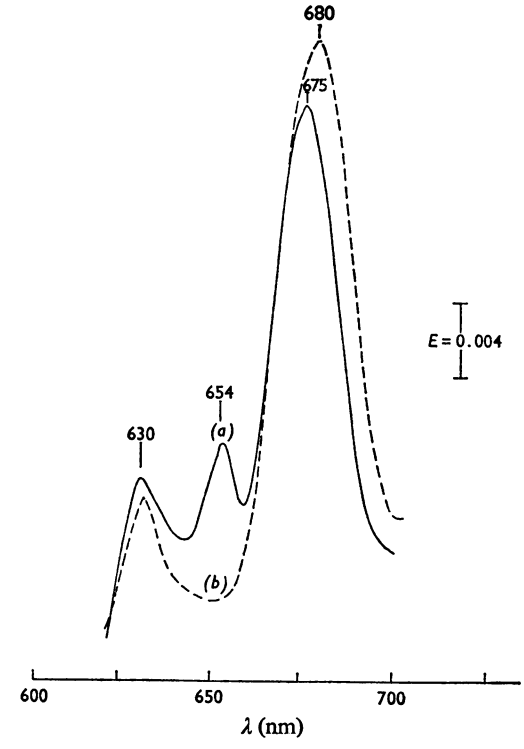


Fig. 2. *Low-temperature (77°K) spectra of etioplasts*

Freshly isolated etioplasts were diluted and illuminated for 90s with a 100W lamp as in Fig. 1. They were then kept in darkness, and without any exposure to light a sample was removed, frozen to 77°K in liquid $N_2$ and the spectrum recorded (curve *a*). The remainder was given a second 90s illumination from a 100W lamp before being frozen and the spectrum being recorded (curve *b*).

documented in whole leaves (Shibata, 1957), where the correlation between the shift and phytylation has been made (Sironval *et al.*, 1965). However, the occurrence of a similar shift (678–674nm) in isolated protochlorophyll(ide) holochrome (Smith, 1960; Schultz & Sauer, 1972) and an identical shift in isolated etioplasts (Figs. 1 and 2) makes it unlikely that phytylation is the cause, and evidence presented by Sauer and co-workers (Mathis & Sauer, 1972, 1973; Schultz & Sauer, 1972), from circular-dichroism and fluorescence spectroscopy of holochrome from bean, points to the shift as being caused by dissociation of the dimeric form of chlorophyll(ide) into monomeric chlorophyll(ide) structural units.

Attempts at following the kinetics of the dark pigment P-650 regeneration by continuously monitoring absorbance changes at 650nm before and after illumination proved unsuccessful owing to the complex and extensive light-scattering changes induced in the etioplasts by illumination. The post-illumination chlorophyll(ide) band shift (Fig. 1) also resulted in absorbance changes at 650nm, and these further complicated the measurement of pigment P-650 regeneration. It should be mentioned, however, that this method for studying regeneration has been used extensively with whole leaves. Despite this, in our hands we find the technique unsatisfactory with both whole leaves and etioplast preparations and note that Mathis & Sauer (1972) also found that after illumination of whole leaves absorbance increases occurred at all wavelengths between 665 and 700nm. It has been our experience that this non-specific absorption change after illumination of isolated etioplasts extends even further into the blue. Thus in etioplasts the process of regeneration after illumination was studied initially by recording spectra of the etioplasts after increasing time-intervals in darkness with manual compensation for scattering-induced baseline drift (Fig. 3).

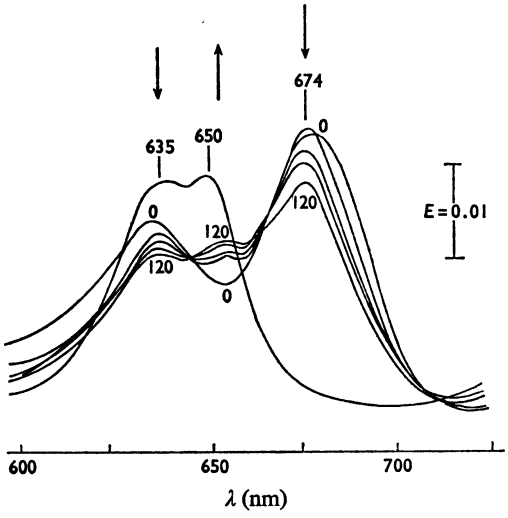


Fig. 3. *Time-course of the dark regeneration of photo-transformable protochlorophyll*

Isolated non-illuminated etioplasts were exposed to a 90s illumination from a 100W lamp and their spectra recorded after 0, 14, 30, 60 and 120min in darkness. The arrows indicate the directions in which the absorbance changed during the successive recordings.

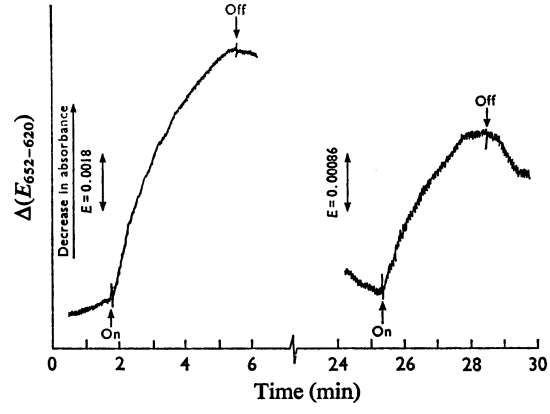


Fig. 4. *Dual-wavelength recording of photoconversion of pigment P-650 into chlorophyll(ide)*

Freshly isolated non-illuminated etioplasts diluted as in Fig. 1 were transferred in darkness to a dual-wavelength spectrophotometer set to measure absorbance changes at 652 minus 620nm. Actinic light, filtered through a 440nm interference filter, was turned on/off where indicated. The first such period of illumination converted the endogenous photoconvertible pigment P-650 into chlorophyll(ide), seen as a decrease in absorbance at 652–620nm. After 20min in darkness a second period of illumination induced a further absorbance change at 652nm, the extent of which reflects the amount of pigment P-650 that had been formed during the dark interval.

Regeneration of pigment P-650 can clearly be seen in these spectra and it is apparent that the process was initially quite rapid (note the extent of the 650nm peak after only 15min) but also continued over a longer period at a somewhat slower rate. It should be noted at this stage that these incubations were carried out at 22°C. If the process was repeated at 0°C then the initial relatively fast regeneration of pigment P-650 occurred as before; however, at this temperature the long-term slow regeneration was inhibited (see below). Another feature of incubations carried out at 0°C was that no loss of the chlorophyll(ide) absorption occurred at this temperature, in contrast with incubations carried out at 22°C (see Fig. 3). This loss of absorption must represent chlorophyll(ide) degradation and it was particularly noticeable when incubations were carried out at higher temperatures in the presence of continuous light.

In view of the difficulties presented by scattering changes during efforts to continually monitor pigment P-650 regeneration at a single wavelength, an attempt was made to measure the process by using a dual-wavelength assay, a technique which should compensate for non-specific light-scattering changes (Chance, 1951). The results are included here, as they reinforce the observations made above. Fig. 4 shows the change in absorbance at 652nm relative to 620nm occurring in etioplasts as a result of illumination with light at 440nm. A reference wavelength of 620nm was adopted, as it had been shown that no specific absorbance changes occur at this wavelength during the process of photoconversion. The light-induced decrease (Fig. 4) indicates the photoconversion of pigment P-650 into chlorophyllide, which at this illumination intensity was complete within 2.5min. Dark regeneration was then monitored by leaving the sample in darkness for a fixed time-interval, after increasing the sensitivity of measurement. This was followed by once more taking the illumination-induced decrease in absorbance as a reliable measure of the amount of pigment P-650 present. This procedure was then repeated on fresh samples, each time the dark-incubation time being varied. In Fig. 5 the progress of pigment P-650 regeneration, as measured by this method at 22° and 0°C, is represented. The curves confirm the observations commented on above, i.e. that at 22°C the regeneration of pigment P-650 is biphasic, comprising an initial fast phase followed by a slower more prolonged phase. At 0°C, Fig. 5 clearly shows that there is very little change in the extent of the fast phase, whereas the slow phase is almost completely abolished.

The data suggest that the etioplast preparation, besides regenerating pigment P-650, must also be

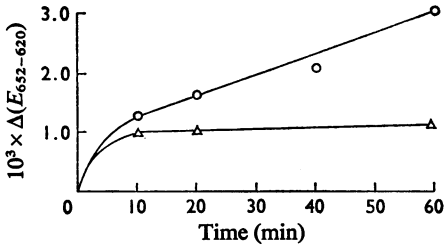


Fig. 5. *Time-course of pigment P-650 regeneration at 22° and 0°C*

The extent of regeneration of pigment P-650 in etioplasts after different dark-incubation times was obtained from recordings similar to those described in Fig. 4 carried out at 22° (○) and 0°C (△).

capable of forming pigment P-630, as it has been shown that pigment P-650 regenerated in darkness does so at the expense of pigment P-630 (see below). Therefore in a preparation in which extensive regeneration of pigment P-650 has occurred there should be a corresponding decrease in the pigment P-630 content. However, Fig. 1 demonstrates that this is not so, as there is approximately the same amount of pigment P-630 present in the etioplasts before and also after a period during which pigment P-650 regeneration has occurred. This implies that in the etioplast a re-formation of pigment P-630 occurs to make good that which is used to regenerate pigment P-650.

Fig. 6 shows the results of an attempt to increase the chlorophyll(ide) concentration in etioplasts by a series of 1 ms light-flashes given every 15 min. Flash illumination was adopted to try and minimize the chlorophyll(ide) degradation known to occur in continuous light. The incubation was also carried out by shaking at 0°C. This would again minimize chlorophyll(ide) breakdown while allowing the initial fast phase of pigment P-650 regeneration to proceed unimpaired. The dark time-interval of 15 min between flashes was adopted, since it had already been shown that the initial fast regeneration of pigment P-650 was complete in this time, even at 0°C. A somewhat similar flashing arrangement had also been used earlier by Sundquist (1969) to photo-transform normally non-phototransformable protochlorophyll(ide) into chlorophyll(ide) at 22°C in young etiolated wheat leaves.

It can be seen (Fig. 6) that after an overnight (10 h) incubation under these conditions chlorophyll(ide) had been formed, its formation being accompanied by the now more-or-less complete disappearance of pigment P-630. The small absorption remaining at 630 nm at the end of the incubation can most probably be accounted for by a minor band of chlorophyll.

Contrary to these findings, a non-phototrans-

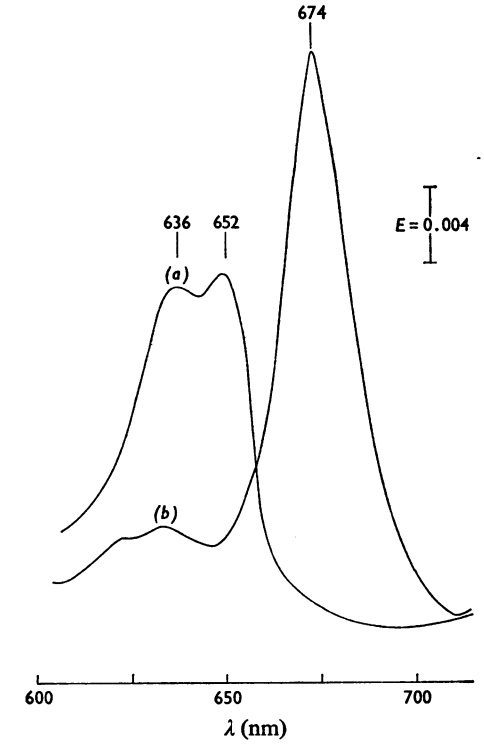


Fig. 6. *Effect of flash illumination on chlorophyll(ide) formation in isolated etioplasts*

Freshly isolated non-illuminated etioplasts were diluted in resuspending medium and their spectrum was recorded (curve *a*). They were then incubated on ice with exposure to a 1 ms xenon flash every 15 min for 10 h, after which their spectrum was re-recorded (curve *b*).

formable protochlorophyll(ide) fraction was identified in maize etioplasts by Horton & Leech (1972). The suggestion was made that this fraction was esterified with phytol, as earlier work (Godnev *et al.*, 1968) had already suggested protochlorophyll to be non-phototransformable. Recent work from this laboratory (W. T. Griffiths, unpublished work) has provided a direct demonstration of the inability of barley etioplasts to convert added protochlorophyll into chlorophyll, whereas added protochlorophyll(ide) is readily transformed. This then suggests that the protochlorophyll(ide) in the preparations described in the present paper, in view of its complete transformation to chlorophyll(ide), exists exclusively as the unesterified form. Incubation of etioplasts isolated from older barley plants, which are known to have a higher protochlorophyll concentration, under similar conditions to those described in the present paper results in some of the protochlorophyll

Table 1. *Effect of various substrates and cofactors on chlorophyll(ide) accumulation by flashed etioplasts*

Isolated etioplasts were incubated in resuspension medium for 10h at 22°C under the flashing regime described in Fig. 6 in the presence of various additives. At the end of this period the relative chlorophyll(ide) concentrations of the samples were determined spectrophometrically from the intensities of the red chlorophyll(ide) band at 673nm *in vivo* assuming linearity between chlorophyll(ide) absorption and concentration. Results obtained from two separate experiments on different etioplast preparations are given. The cofactors added were 5mM-reduced glutathione, 0.3mM-CoA, 50mM-potassium phosphate, 25mM-methanol and 0.06mM-$NAD^+$.

| Incubation conditions | Chlorophyll (% of control) Expt. 1 | 2 |
|---|---|---|
| Etioplasts, no additions | 100 | 100 |
| Etioplasts+cofactors | 114 | 138 |
| Etioplasts+1.0mM-δ-aminolaevulinate | 92 | 102 |
| Etioplasts+1.0mM-δ-aminolaevulinate+cofactors | 116 | 142 |
| Etioplasts+1.0mM-δ-aminolaevulinate+cofactors+4mM-laevulinic acid | 110 | — |

Table 2. *Effect of different cofactors and incubation conditions on chlorophyll(ide) assimilation by flashed etioplasts*

Etioplasts were incubated at 22°C in resuspending medium supplemented with 1.0mM-δ-aminolaevulinate under flash illumination as in Fig. 6 in the presence of various cofactors. After 10h the relative chlorophyll(ide) concentrations of the samples were determined spectroscopically as in Table 1. These values are expressed as a percentage of the concentration found in the sample incubated in the presence of δ-aminolaevulinate and the complete cofactors. The complete cofactor mixture is as described in Table 1. Incubations were carried out under aerobic conditions except where noted differently.

| Incubation conditions | Chlorophyll (% of control) |
|---|---|
| δ-Aminolaevulinate+cofactors | 100 |
| δ-Aminolaevulinate+cofactors (anaerobically) | 118 |
| δ-Aminolaevulinate+cofactors−methanol | 116 |
| δ-Aminolaevulinate+cofactors−$NAD^+$ | 110 |
| δ-Aminolaevulinate+cofactors−CoA | 91 |
| δ-Aminolaevulinate+cofactors−glutathione | 66 |

remaining non-phototransformed (W. T. Griffiths, unpublished work).

Table 1 shows the effect of adding various cofactors and substrates on the chlorophyll-biosynthetic activity of etioplasts under incubation conditions as described above. The data from two separate experiments are included. δ-Aminolaevulinic acid when given to etiolated leaves gives rise to an accumulation of protochlorophyll(ide) (Granick, 1959), which can by flashing illumination be converted into chlorophyll(ide) (Sundquist, 1969). Table 1 shows that δ-aminolaevulinate added to etioplasts under similar illumination failed to produce a significant increase in the chlorophyll(ide) concentration in the absence of any added cofactors. Extensive accumulation of porphyrin intermediates, identified by standard extraction procedures as mixtures of uro-, copro- and proto-porphyrin with the uroporphyrin predominating, was observed, however, in the δ-aminolaevulinate-supplemented incubations. Addition of the cofactors demonstrated by Rebeiz & Castelfranco (1971*a*) to be required for the synthesis of Mg–protoporphyrin monoester and protochlorophyll from added δ-aminolaevulinate in cell-free systems from etiolated and greening cucumber cotyledons (Rebeiz *et al.*, 1972) resulted in a marginal increase in the chlorophyll(ide) concentration only in our preparation, with the increase being of the same order in either the presence or absence of added δ-aminolaevulinate.

It should be noted that the incubations in the presence of δ-aminolaevulinate were carried out at 22°C, since at 0°C δ-aminolaevulinate, perhaps not unexpectedly, failed to be utilized by the etioplasts. However, as mentioned earlier, incubation at 22°C has the disadvantage of leading to increased chlorophyll(ide) breakdown. Thus it may be that the effect of the added cofactors in raising the chlorophyll(ide) concentration might be explained by their effectiveness as inhibitors of chlorophyll(ide) breakdown. Evidence for this suggestion can be seen in Table 2. Here the effectiveness of individual components in the mixture can be seen from the effect of their omission from the incubation on the chlorophyllide concentration.

Anaerobic conditions and omission of $NAD^+$ gave an increased chlorophyll(ide) concentration, the presence of air and $NAD^+$ probably producing oxidative conditions for chlorophyll(ide) breakdown. Methanol, which might also be expected to render membrane-bound components more labile, produced a similar apparent rise in chlorophyll(ide) concentration when omitted. Omission of glutathione, however, on account of its reducing and thiol-conserving properties led to a drastically decreased concentration of chlorophyll(ide). Thus it is suggested that the added cofactors are having an indirect effect only on chlorophyll(ide) biosynthesis, mediated through stabilization of the chlorophyll(ide) or, perhaps, other substrates normally destined for incorporation into chlorophyll(ide). The results of Rebeiz and co-workers also attribute a role of protecting membrane structure for these cofactors during incubations *in vitro* (Rebeiz *et al.*, 1973).

In summary, it appears that in isolated etioplasts δ-aminolaevulinate fails to significantly enhance protochlorophyll(ide) concentrations, in contrast with its effect on whole leaves. Thus the synthesis seen in these etioplasts (Fig. 6) must depend for substrates on a pool of late precursors which cannot be replenished by added δ-aminolaevulinate. In agreement with this, added laevulinic acid failed to inhibit chlorohyll(ide) formation in etioplasts (Table 1). Added δ-aminolaevulinate, however, was effectively incorporated into porphyrins by etioplasts (see above). It appears therefore that the substrate pool utilized for chlorophyll(ide) biosynthesis by etioplasts must be restricted to postporphyrin type of intermediates, and the inability of etioplasts to convert δ-aminolaevulinate into protochlorophyll(ide) must be due to a deficiency in some reaction(s) lying between the porphyrins and the photoconvertible substrate pool. The reaction most likely to be limiting in the etioplasts is the $Mg^{2+}$-insertion reaction, a step which has so far evaded detection in a cell-free system. However, incorporation *in vivo* of $Mg^{2+}$ into whole cells of *Rhodopseudomonas spheroides* has been shown by Gorchein (1973), and it is significant that this activity was lost on subjection of the cells to the mildest of treatments in attempts to demonstrate the reaction in cell-free preparations.

In contrast with our data, as mentioned above, a cofactor-stimulated light-dependent incorporation of δ-amino[$^{14}$C]laevulinate into chlorophyll(ide) has been observed with isolated plastids from etiolated greening cucumber cotyledons (Rebeiz & Castelfranco, 1971*a*,*b*). Similar results were obtained by Wellburn & Wellburn (1971*b*) with etioplasts isolated from dark-grown wheat. This work has been reviewed by Rebeiz & Castelfranco (1973). Our failure to observe a stimulation of chlorophyll(ide) synthesis from added δ-aminolaevulinate under apparently similar conditions can probably be accounted for by the increased sensitivity of the radiochemical assays used by these workers compared with our spectroscopic techniques.

In view of the limited ability of etioplasts to effect the conversion of δ-aminolaevulinate into chlorophyll (Table 2) and bearing in mind their effectiveness in converting endogenous protochlorophyll(ide) into chlorophyll(ide), it was decided to isolate protochlorophyll(ide)-enriched etioplasts from δ-aminolaevulinate-fed leaves and subject these to our flash-incubation procedure. The absorption spectrum of etioplasts, isolated from leaves fed for 6h on δ-aminolaevulinate, flashed continuously overnight at 0°C, is shown in Fig. 7 (curve *a*). This spectrum is to be compared with spectra of etioplasts maintained in darkness overnight before (curve *b*) and after (curve *c*) being given a 90s light-flash from a 100W lamp.

The spectra show almost complete photoconversion of all the δ-aminolaevulinate-protochlorophyll(ide) resulting in a greatly increased chlorophyll(ide) concentration (2.3 μg/mg of protein) in the sample. Greening leaves which have been allowed to accumulate a similar chlorophyll/protein ratio can yield plastids in which photosynthetic reactions can be readily demonstrated (Henningsen & Boardman, 1973). The ability of etioplasts to photoconvert this large δ-aminolaevulinate-protochlorophyll(ide) reserve implies that (1) sufficient reductant for the reaction must have been available in the etioplasts, or, as appears more likely, in the incubation mixture, since Sundquist (1973) has shown that wheat leaves

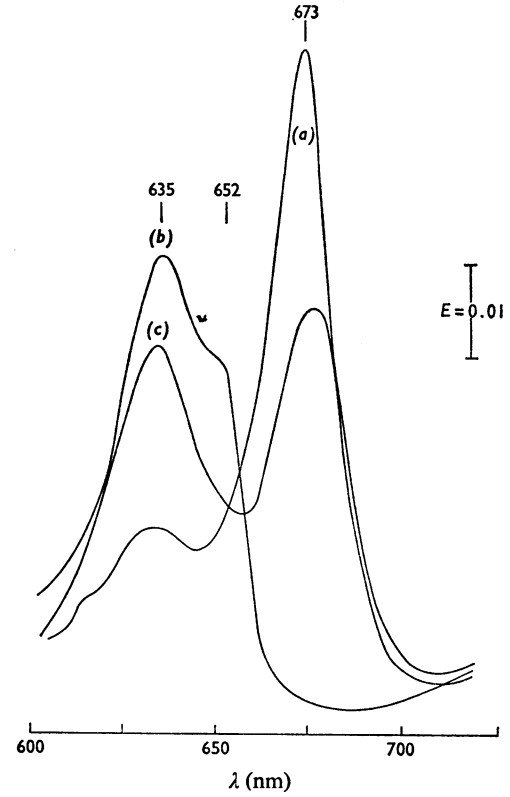


Fig. 7. *Flash phototransformation of δ-aminolaevulinate protochlorophyll(ide) into chlorophyll(ide) in isolated etioplasts*

Etioplasts were isolated from plants that had been fed on δ-aminolaevulinate for 6h in darkness. These were then continuously flashed with 1 ms xenon flashes for 10h as in Fig. 6 and the absorption spectrum was recorded (curve *a*). Spectra of a sample incubated in complete darkness over this period recorded before (curve *b*) and after (curve *c*) a saturating flash from a 100W lamp are also included.

cannot phototransform all their accumulated δ-aminolaevulinate-protochlorophyll(ide), probably owing to a deficiency in hydrogen donors, and (2) the photoenzyme functions as a shuttling holochrome protein, as it is hardly likely that the isolated etioplasts are capable of stoicheiometric holochrome protein synthesis. This suggests that the newly formed chlorophyll(ide) is translocated from the site of photoconversion to some other centre allowing further protochlorophyll(ide) access to the photoconversion site.

A similar conclusion was drawn by Gassman (1973) from experiments on flashed δ-aminolaevulinic acid-fed bean leaves. Evidence was also presented that in this case pigment P-650 was an intermediate in the conversion of the δ-aminolaevulinate-protochlorophyll(ide) into chlorophyll(ide).

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