Characterization of the Terminal Stages of Chlorophyll(ide) Synthesis in Etioplast Membrane Preparations

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(Received 18 July 1975)

1. Chlorophyll(ide) formation from protochlorophyll(ide) that is normally inactive was demonstrated in etioplast membranes isolated from maize and barley plants, the process being dependent on intermittent illumination and the addition of NADPH. 2. The addition of NADPH to the membranes was shown to result in the conversion of inactive protochlorophyll(ide) absorbing at about 630nm into a form(s) with light-absorption maxima at about 640 and 652nm, both of which disappear when chlorophyll(ide) is formed on illumination. 3. The temperature-dependence of the activation process and its response to a variety of reagents were examined. From these, the conclusion is drawn that –SH groups are involved in the activation but in the active complex these are unavailable for reaction with –SH reagents. 4. Evidence is presented for the occurrence of glucose 6-phosphate dehydrogenase activity within etioplasts and the suggestion is made that the oxidative pentose phosphate pathway can provide the NADPH required for chlorophyll biosynthesis during the early stages of greening.

The development of energy-conserving membranes is currently attracting much interest. In higher plants, chlorophyll synthesis is a prerequisite to the development of the photosynthetic apparatus, its formation leading to the conversion of the etioplast, present in dark-grown tissues, into mature chloroplasts. Thus the well known dependence of higher-plant-chloroplast formation on light can largely be accounted for by the light requirement of the terminal reaction of chlorophyll(ide) biosynthesis, namely the photoreductive transformation of protochlorophyll(ide) to chlorophyll(ide). A study of this and other reactions involved in chlorophyll biosynthesis therefore holds special interest in that they may provide evidence of the mechanism by which light regulates chloroplast development.

Our knowledge of the compounds involved in chlorophyll biosynthesis is based largely on identification of intermediates produced by cells in which their accumulation has been enhanced by techniques such as prior feeding with the specific porphyrin precursor δ -aminolaevulinic acid (Granick, 1959), or else by blocking the normal biosynthetic pathway either genetically (see Gough, 1972) or chemically (Duggan & Gassman, 1974). Such studies have led to detailed schemes for chlorophyll formation (Jones, 1973), which must, however, remain tentative pending the development of cell-free systems in which the individual reactions may be demonstrated. Some progress in this direction has been achieved by Rebeiz & Castelfranco (1973) and by Ellsworth & Hsing (1973, 1974).

From studies on etiolated whole leaves it has been

shown that protochlorophyll(ide) *in vivo* exists as a number of spectroscopically distinct forms. Thus a non-photoconvertible form absorbing light maximally at about 630nm (pigment P-630*) and a photoactive form with absorption maximum at 650nm *in vivo* (pigment P-650) have been described (Shibata, 1957; Gassman & Bogorad, 1967). Various suggestions to explain the differences between these two forms have been made (Shibata, 1957; Boardman, 1966; Mathis & Sauer, 1973) and the physical separation of the two forms by sucrose-density-gradient centrifugation has been claimed (Murray & Klein, 1971). Techniques for the isolation of soluble protochlorophyll(ide) protein complexes retaining photoconvertibility have also been described (Smith & Kupke, 1956; Boardman, 1962a; Schopfer & Siegelman, 1968; Henningsen *et al.*, 1974). Photo-transformation of the protochlorophyll(ide) in these preparations was found to be particularly resistant to a wide range of inhibitors and was inactivated only by protein-denaturing agents such as urea and sodium dodecyl sulphate (Boardman, 1962b).

We described the preparation of a membrane fraction from barley etioplasts in which the protochlorophyll(ide) exists almost completely as the non-photoconvertible form (pigment P-630). Supplementation of this preparation with NADPH results in the dark formation of photoconvertible pigment P-650 at the expense of pigment P-630, pigment

* Abbreviations: pigments P-630, P-640, P-650, protochlorophyll(ide) pigment complexes occurring *in vivo* and showing light-absorption maxima at the wavelengths indicated.

P-650 being completely photoconvertible to chlorophyll(ide) by flash illumination (Griffiths, 1974). In the present paper further characteristics of this system are described. It is shown that NADPH is highly specific as a reductant for chlorophyll(ide) formation in this system *in vitro*. The sensitivity of the reaction to various inhibitors is described and the origin of the NADPH in the 'native' system is briefly investigated.

Experimental

Materials

Etiolated 7-day-old barley (*Hordeum vulgare* L., cultivar Proctor) seedlings were obtained as previously described (Griffiths, 1975a). Etiolated maize (*Zea mays*, var. 'Golden Bantam') shoots were grown in an identical manner but harvested after 9 days of growth.

Sodium dithionite solution was prepared by dissolving the solid in 50 mM-Tris-HCl solution, pH 7.4, which had been rendered anaerobic by preflushing with high-purity argon gas (British Oxygen Co., Deer Park Road, London, U.K.) from which final traces of O₂ were removed by passage through a column of Fieser's solution (Fieser, 1924). Horse heart cytochrome *c* from Boehringer, Mannheim, Germany, was reduced with sodium ascorbate, excess of reagent being removed by gel filtration through a column (4 cm × 1 cm diam.) of Sephadex G-25 (coarse). *N*-Ethylmaleimide, 2'-AMP and 5'-AMP were obtained from BDH Chemicals, Poole, Dorset, U.K. and were used as aqueous solutions neutralized with NaOH.

5,5'-Dithiobis-(2-nitrobenzoic acid) was a product from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. and was used as a solution adjusted to pH 7.4 with solid Tris. Trimethylhydroquinone, from Ralph N. Emanuel, Alpert, Middx., U.K., was used as an ethanolic solution. Dibromothymoquinone, a gift from Dr. Jerome Laverne, CNRS Laboratory for Photosynthesis, Gif-sur-Yvette, France, was used as a solution in tetrahydrofuran. Disalicylidenepropanediamine, prepared by the method of Trebst & Burba (1967), was a gift from Dr. F. Hawkes, Glamorgan Polytechnic, Treforest, Glamorgan, U.K. Marrow ferredoxin was a gift from Dr. E. J. Hewett, Long Ashton Research Station, Bristol, U.K. All other chemicals were from commercial sources and were of AR grade or the highest grade obtainable.

Methods

The various cell-free preparations used in the present work were prepared as described previously (Griffiths, 1975a). Briefly this involved homogenization of the plant material followed by filtration to

give the cell-free homogenate. Etioplasts were isolated from the homogenate by differential centrifugation. Osmotic lysis of the etioplasts by a 10-fold dilution with water gave the etioplast membrane fraction corresponding to the prolamellar-body membranes of the original etioplasts, and this was separated again by centrifugation.

The fractions, etioplasts or prolamellar-body membranes were resuspended as a routine in the isolation buffer free of cysteine but supplemented with 1.5 mM-ATP (Horton & Leech, 1972). Membrane fractions enriched in protochlorophyll(ide) were prepared by an identical procedure except that the shoots were pre-fed for 6–8 h on a solution of 10 mM- δ -aminolaevulinic acid as described previously (Griffiths, 1975a) and then subjected to the usual isolation procedure.

Samples, cell-free homogenates, etioplasts or prolamellar-body membranes, for incubation under different experimental conditions, were diluted as a routine with the above resuspending medium to a total volume of 2–5 ml with incubation usually carried out at 20°C in a thermostatically heated shaking water bath. A temperature of 0°C used in some experiments was achieved by placing the samples on ice in a laboratory ice-container and placing the whole in the shaker. Incubations in darkness, to study the progress of pigment P-650 formation in prolamellar-body membranes were performed under a dim green safelight and were carried out after first illuminating each sample by exposure to a 100 W tungsten lamp for 45 s. This treatment photoconverts all the endogenous pigment P-650 already present in the membranes. Pigment P-650 formation was then initiated by the addition of NADPH to the sample followed by incubation in the shaker. Termination of the reaction was achieved by adding 5,5'-dithiobis-(2-nitrobenzoic acid) to the sample to a final concentration of 40 μ M, to prevent further build-up of pigment P-650 followed by illumination for 15 s with the tungsten lamp to photoconvert the formed pigment P-650. Finally, Triton X-100 to a concentration of 0.4 mM was added and each sample stored in darkness on ice before spectrophotometric assay for chlorophyll(ide) concentration.

Flash illumination of samples during incubation was by a photographic xenon lamp (Mecablitz) supported 10 cm from the sample as described by Griffiths (1975a). The lamp was triggered electronically to produce a flash of energy 4 mJ every 40 s. Additions under test, unless otherwise noted, were usually made immediately before the start of the illumination. Routine checks to see that flash-saturating conditions prevailed were carried out by using neutral-density filters between the light-source and sample and also by varying the concentration of material used.

Light-absorption spectra of samples at both room temperature (18–22°C) and 77°K were recorded by using a sensitive split-beam spectrophotometer (Jones & Saunders, 1972) as described previously (Griffiths, 1975a). When such spectra were recorded for the purpose of measuring the chlorophyll(ide) concentration, Triton X-100, to a final concentration of 0.4 mM, was added as a routine to each sample before scanning. Chlorophyll(ide) concentration was then calculated from the extent of the absorption at 672 nm by using the millimolar extinction coefficient value of 91.2 litre·mmol⁻¹·cm⁻¹ (see below).

Absorption spectra of ether extracts of samples were recorded on the same instrument as described above. The extracts were prepared by diluting each sample, usually 3.0 ml, in a separating funnel with 4 vol. of acetone followed by approx. 4 vol. of peroxide-free ether, and the mixture was shaken. On further addition of water, an upper organic phase containing all the pigments separated from a colourless aqueous acetone phase. The latter was discarded and the ether layer washed several times with water to remove all traces of acetone, dried over anhydrous Na₂SO₄, dried under N₂ and finally redissolved in 3.0 ml of ether and the spectrum recorded immediately. Chlorophyll(ide) concentrations were calculated from the resulting spectra using published millimolar-extinction-coefficient values (Jones, 1969).

Enzyme activity assays

Glucose 6-phosphate dehydrogenase activity in various fractions was assayed by the method of Glock & McLean (1953), with corrections applied for the activity of 6-phosphogluconate dehydrogenase. Reactions were carried out in a total volume of 2.8 ml made up with 0.8 mM-MgCl₂ and 50 mM-Tris-HCl, pH 7.4, and with 3.5 mM-glucose 6-phosphate, 2.0 mM-6-phosphogluconate and 2.7 mM-NADP⁺ as substrates. NADP⁺ reduction was measured as absorbance change at 340 nm in a Unicam SP.1800 recording spectrophotometer.

Protein assay

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

Results and Discussion

One problem encountered in studies on chlorophyll biosynthesis in cell-free preparations is that of quantifying the trace amounts of chlorophyll encountered. Thus the usual technique of extracting the preparations, normally present as a suspension of membranes in 3 ml of incubating solution, with 4 vol. of acetone (Arnon, 1949) invariably cannot be

applied, owing to the dilution involved. Further, during our studies we have found it impractical to try and overcome this dilution by attempting to sediment the pigment-containing membranes by centrifugation, and adding a suitable volume of acetone-water (4:1, v/v) to the pellet. Such a centrifugation procedure invariably leads to a greater or lesser amount of pigment remaining in the supernatant.

Previous studies have relied on the extent of the chlorophyll absorption *in vivo* to provide a measure of chlorophyll(ide) concentration (Boardman, 1962b; Nielsen & Kahn, 1973; Griffiths, 1975a). However, in the absence of an extinction coefficient *in vivo* for the pigment, such values can only remain relative and cannot be converted into absolute amounts. Another serious drawback of working from chlorophyll absorption *in vivo* arises from the fact that several spectroscopically distinct forms of the pigment exist *in vivo* (Virgin & French, 1973) and further, the relative proportions of some of these probably change in a time-dependent manner giving the so-called 'Shibata shift' (Shibata, 1957).

These imprecisions were effectively overcome during the present work simply by the addition of the non-ionic detergent Triton X-100 to samples before spectroscopic assay. This treatment results in a narrowing of the chlorophyll(ide) absorption *in vivo* and a shift in the wavelength of maximum absorption to 672 nm (cf. curves *a* and *b*, Fig. 1). The absorption of freshly illuminated etioplasts can be resolved by plotting the fourth derivative of the absorption, into several (at least three) spectroscopically distinct species. On treatment with Triton, however, only a single form, absorbing at 672 nm, can be seen by this technique (W. T. Griffiths, N. Holmes & A. R. Crofts, unpublished work). Extraction of the pigments from a Triton-treated sample, followed by dissolving the extract in a volume of ether identical with the original sample volume, gave on analysis a spectrum (Fig. 1, curve *c*), quantitatively identical with the original spectrum but qualitatively shifted to the blue region by 10 nm. On repeating this comparison over a wide range of chlorophyll concentrations (from 0.01–2.0 nmol/3.0 ml) the equivalence of the absorbance of the Triton-treated sample (at 672 nm) and its ether extract (in the same volume at 662 nm) was always borne out. This makes it possible to extend the millimolar extinction coefficient of 91.2 ascertained for chlorophyll in ether solution, to a Triton solution of membranes containing chlorophyll, thus enabling quantification of the chlorophyll in such a solution. This procedure was used as a routine in the experiments reported in the present paper.

The interconversion of the two protochlorophyll(ide) forms, the photoconvertible pigment P-650 and the non-photoconvertible pigment P-630, has been frequently observed in etiolated leaves (e.g. Gassman,

Fig. 1. Effect of different environments on the absorption of etioplast pigments

Freshly isolated barley etioplasts diluted with resuspending medium to give 0.85 mg of protein/3.0 ml were flash-illuminated with 0.1 ms xenon flashes (1 flash/40 s) for 30 min, and the absorption spectra recorded. Curve (a), before and curve (b), after the addition of Triton X-100 to 0.4 mM. Curve (c) is the absorption of an identical sample after extraction into acetone and redissolving in an equal volume (3.0 ml) of ether.

Fig. 2. NADPH-dependent formation of chlorophyll(ide) by flash-illuminated etioplast membranes

Prolamellar-body membranes, prepared from barley etioplasts (0.46 mg of protein/3.0 ml of resuspending medium) were flash-illuminated as in Fig. 1 for 1 h at 20°C, and spectra were recorded. Curve (a) no additions; curve (b), in the presence of an NADPH-regenerating system (0.5 unit of glucose 6-phosphate dehydrogenase, 5 mM-glucose 6-phosphate and 0.2 mM-NADP⁺).

1973; Sundqvist, 1970). In cell-free extracts, however, although the conversion of pigment P-650 into pigment P-630 can readily be demonstrated (Horton & Leech, 1972), a reversal of this reaction has, to our knowledge, only been described by this laboratory (Griffiths, 1974) where the NADPH-dependence of the reaction was observed. In this context Nikolaeva and co-workers had earlier shown that maize homogenates, deliberately aged at room temperature (thereby converting the endogenous pigment P-650 into the P-630 form) could, on incubation with NADPH, photoconvert this P-630 form into chlorophyll(ide) by flash illumination (Nikolaeva *et al.*, 1972). Again, Horton & Leech (1972) reported that similar accumulation of chlorophyll(ide) from pigment P-630 could be induced in isolated maize etioplasts. The P-630 pigment in this case was derived from pigment P-650, originally present, by a process of aging as before. Further, chlorophyll(ide) formation from this P-630 pigment was found to be completely dependent on added ATP.

Fig. 2 confirms our earlier observation of the NADPH-dependent chlorophyll(ide) formation in

etioplast prolamellar-body membranes containing pigment P-630. This process, as mentioned above, has been shown to involve the dark NADPH-dependent conversion of pigment P-630 into pigment P-650, the latter being an obligate intermediate of chlorophyll(ide) formation. Fig. 2 (curve a) shows that when prolamellar-body membranes were flash-illuminated (1 flash/40 s) for 1 h in the absence of any additions, only a trace (0.093 nmol/mg of protein) of chlorophyll was formed, as evidenced from the extent of the 672 nm peak. In this sample most of the pigment at the end of the incubation was present as protochlorophyll(ide) as seen from the high absorption at 633 nm. In the presence of NADPH, however, incubation under otherwise similar conditions as before results in a greatly increased chlorophyll(ide) concentration (0.748 nmol/mg of protein), accompanied by complete disappearance of the absorption due to protochlorophyll(ide) (Fig. 2, curve b).

The specificity of this chlorophyll(ide) synthesis in terms of source of reducing equivalents is shown in Table 1. Data for this Table were obtained by exposing prolamellar-body membranes to flash-illumination as described above for 45 min in the presence of

Table 1. *Alternate sources of reducing equivalents for chlorophyll(ide) synthesis by barley etioplast membranes*

Etioplast membranes were prepared by water lysis of barley etioplasts by diluting tenfold with water as described in the text. Preparation 1 was made from normal etiolated barley and preparation 2 from shoots preincubated with 10mM- δ -aminolaevulinate before etioplast isolation. Incubations, containing 0.2–0.4mg of protein/3 ml, were carried out for 45 min at room temperature with intermittent illumination (1 flash/40s) from a xenon lamp, at the end of which period chlorophyll(ide) concentrations were determined as described in the text. The trimethylhydroquinone sample was in ethanol, and was added to a final concentration of 0.5% (v/v).

Additions	Chlorophyll(ide) yield			
	Preparation 1		Preparation 2	
	(nmol/mg of protein)	(%)	(nmol/mg of protein)	(%)
0.1 mM-NADPH	0.624	100	2.244	100
No additions	0.046	7.4	0.028	1.2
0.1 mM-NADH	—	—	0.032	1.4
25 mM-NADH	0.046	7.4	—	—
0.4 mM-Dithionite	0.038	6.1	0.028	1.2
1 mM-Ferrocyanide	0.014	2.2	—	—
20 μ M-Cytochrome <i>c</i> (reduced)	0.036	5.7	—	—
1 mM-Ascorbate	0.036	5.7	—	—
1 mM-Trimethylhydroquinone	0.042	6.7	—	—

possible donors, followed by spectral analysis to determine the chlorophyll(ide) concentration. Membranes from both normal etiolated plants (preparation 1) and from plants enriched in protochlorophyll(ide) obtained by prior feeding with δ -aminolaevulinate (preparation 2) were used. This difference in protochlorophyll(ide) concentration in the two samples is reflected in the final chlorophyll(ide) concentration found after flash-illumination for 45 min in the presence of NADPH. Thus 2.24 nmol of chlorophyll/mg of protein was found in preparation 2 compared with 0.624 nmol/mg of protein in preparation 1. It is noteworthy that we always find most (approx. 95%) of the δ -aminolaevulinate-induced protochlorophyll(ide) to be sedimented with the prolamellar-body membranes implying, in contrast with earlier suggestions (Granick & Gassman, 1970; Sundqvist, 1970), that the extra pigment is also membrane-bound, probably as a protein–protochlorophyll(ide) complex.

The amount of chlorophyll(ide) formed in the absence of NADPH in both preparations was less than 10% of the concentration present in the incubations containing NADPH (cf. 7.3% in preparation 1 and only 1.25% in preparation 2). Addition of NADH to samples at the same concentration as the NADPH (0.1 mM) or at 250 times this amount (25 mM), failed to stimulate significantly the yield of chlorophyll(ide) over that present in the unsupplemented sample (Table 1), illustrating the very specific nature of the reaction for NADPH. This narrow specificity is further borne out by the fact that all of the electron donors or hydrogen donors tried, covering a wide range of E_0 values and of widely differing structures and solubilities, again failed to

produce any significant increase in the chlorophyll(ide) concentration over the unsupplemented sample (Table 1), in marked contrast with the incubation in the presence of NADPH.

The time-course of the NADPH-induced chlorophyll(ide) synthesis by prolamellar-body membranes under our standard illumination conditions is indicated in Fig. 3. It must be emphasized that data for this Figure were obtained under flash-saturating conditions so that the curve reflects the formation of the photoactive pigment P-650 complex under conditions of intermittent illumination. The curve shows an initial rate which progressively decreases with time. This decreasing rate we attribute to the fact that as the reaction proceeds the decreasing protochlorophyll(ide) concentration limits the rate. Confirmation of this conclusion has come from my experiments in which, with exogenously added protochlorophyllide as a substrate for chlorophyllide formation in this system, the linear rate of the reaction was greatly extended (Griffiths, 1975*b*, and unpublished work).

It is obvious from Fig. 3 that definition of chlorophyll(ide) synthesis under different experimentally defined conditions requires information on both the initial rate and final extent of its formation. Such information can be obtained by noting the chlorophyll(ide) concentration in an incubation after 5 min, as an indication of the initial 'rate', and again at 30 min, as a measure of the total extent of chlorophyll(ide) formation. By using values of chlorophyll(ide) concentrations observed after 5 min as representing initial rates of chlorophyll(ide) synthesis in incubations containing various amounts of NADPH, we have derived a K_m value for NADPH of 35 μ M for

this reaction. The effect of various reagents on the progress of chlorophyll(ide) synthesis in membranes as defined by these two terms is shown in Table 2. The chlorophyll(ide) concentrations present after incubation of the different samples for 5 min and 30 min are expressed as percentages of the chlorophyll(ide) found in controls incubated for 30 min under similar conditions in the presence of NADPH. The various reagents were selected on the basis of previous claims regarding their effect on chlorophyll(ide) formation in different systems (see, e.g. Kirk & Tilney-Bassett, 1967; Boardman, 1962b).

A lack of inhibition of chlorophyll(ide) formation by $\alpha\alpha'$ -dipyridyl makes it unlikely that any iron compound is involved in the protochlorophyll(ide) reaction. Failure of the chelating agent EDTA to inhibit extends this conclusion to include most common metal ions. The failure of FAD, FMN or ferredoxin to effect either the 'rates' or extents of the reaction suggests that none of these compounds mediates in the transfer of reducing equivalents from the NADPH to the protochlorophyll(ide). However, at this stage the possibility cannot be eliminated that tightly bound forms of these compounds still persist on the membranes, and participate in the reaction.

The involvement of -SH groups in the activation of protochlorophyll(ide) to pigment P-650 for

photoconversion is implied from the marked inhibition of chlorophyll(ide) synthesis (Table 2) in the presence of the -SH reagents, 5,5'-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide. Further, the absence of any inhibition by arsenite implies that

Fig. 3. Time-course of chlorophyll(ide) synthesis by illuminated-etioplast membranes

Prolamellar-body membranes from barley etioplasts were given a saturating illumination (45 s from a 100 W tungsten lamp) to photoconvert endogenous photoactive pigment, and then incubated under intermittent illumination in the presence of NADPH as in Fig. 2. Samples were removed after various time-intervals, Triton X-100 was added to 0.4 mM and finally chlorophyll(ide) concentrations were determined spectroscopically.

Table 2. Possible effectors of chlorophyll(ide) synthesis by barley etioplast membranes

Samples were incubated in the usual resuspending medium containing added ATP but without cysteine, and flash-illuminated at room temperature. After 5 and 30 min 5,5'-dithiobis-(2-nitrobenzoic acid) and Triton X-100 were added, followed by spectroscopic assay of chlorophyll(ide) concentrations. As the data were obtained on different occasions, by using preparations of varying activity, the results have been made directly comparable by expressing the chlorophyll(ide) yield obtained by a preparation in a particular incubation as a percentage of the chlorophyll(ide) formed by the same preparation illuminated in the presence of NADPH. The complete incubation system was made up of prolamellar-body membranes (0.2–0.4 mg of protein) incubated in 3.0 ml of resuspending medium containing 1.5 mM-ATP with added NADPH-regenerating system (0.5 unit of glucose 6-phosphate dehydrogenase, 5 mM-glucose 6-phosphate and 0.5 mM-NADP⁺).

Incubation conditions	Chlorophyll(ide) yield (% of the control)	
	5 min	30 min
Complete	63	100
–NADPH-regenerating system	7.5	7.0
–ATP	61	100
–ATP+2 mM-2'-AMP	63	100
–ATP+2 mM-5'-AMP	63	100
+25 μ M-dibromothymoquinone	17	29.2
+50 μ M-disalicylidene-propanediamine	53	89
+40 μ M-5,5'-dithiobis-(2-nitrobenzoic acid)	13.7	6.5
+2 mM- <i>N</i> -ethylmaleimide	11.3	9.7
+0.1% sodium dodecyl sulphate	0	0
+1.5 mM-arsenite	62	100
+0.2 mM- $\alpha\alpha'$ -dipyridyl	57	95
+1 mM-EDTA	57	97
+20 μ M-FMN	44	97
+20 μ M-FAD	55	93
+25 μ M-ferredoxin	52	97

lipoic acid -SH groups are not involved. It is noteworthy that although *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid) effectively inhibited the NADPH-dependent regeneration of active pigment P-650 [see the greatly decreased chlorophyll(ide) concentrations in these samples after 30 min, being only 6.5%, with *N*-ethylmaleimide and 9.7% with 5,5'-dithiobis-(2-nitrobenzoic acid) respectively of the corresponding NADPH-incubated control sample], they failed to inactivate the trace of endogenous pigment P-650 already present in the prolamellar-body membranes at the start of the experiment. Thus this endogenous pigment P-650 represented by the incubation carried out in the absence of NADPH, gave 7.5% of the control chlorophyll(ide) concentration whereas in the *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid) samples, the corresponding values were increased to 11.3 and 13.7% respectively. This slight increase is readily explained by the well known instability of pigment P-650 at room temperature, with NADPH [present in the *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid) incubations] acting as a stabilizing agent.

The inhibition of pigment P-650 regeneration by 5,5'-dithiobis-(2-nitrobenzoic acid), although having no effect on already existing concentrations of pigment P-650, explains the basis of adding this reagent to terminate further P-650 pigment regeneration in our standard procedure for determining rates of P-650 pigment formation (see below). The lack of an effect of -SH reagents on photoconvertible protochlorophyll(ide) phototransformation has already been previously demonstrated in protochlorophyll(ide)-holochrome preparations by Boardman (1962*b*) who concluded that -SH groups were not involved in the actual phototransformation. Although our data agree with this, we would add, however, that -SH groups are essential for the formation of the active complex, these becoming modified/protected during the process so as to be unavailable for reaction with reagents subsequently added. Sodium dodecyl sulphate, as in isolated holochrome preparations (Boardman, 1962*b*), completely inhibited chlorophyll(ide) formation, indicating the requirement for native protein conformation for the process. A role for plastoquinone in chlorophyll formation was first suggested by Oku & Tomita (1970), but was subsequently modified (Oku & Tomita, 1971). The presence of this compound in etiolated plants, including barley, has already been well documented (Griffiths *et al.*, 1967). The plastoquinone analogue, dibromothymoquinone has been shown to inhibit competitively photosynthetic electron transport in higher-plant chloroplasts at the plastoquinone site (Trebst *et al.*, 1970; Bohme *et al.*, 1971). Inclusion of this compound at 25 μ M in our standard chlorophyll(ide)-biosynthesis system resulted in approx.

75% inhibition of the 'rate' of chlorophyllide synthesis (Table 2), complete inhibition occurring at a concentration of 90 μ M in maize preparations (see Table 3).

Neither 2'-AMP nor 5'-AMP produced any inhibition of chlorophyll(ide) formation in our system either in terms of 'rate' or in the extent of synthesis (Table 2). This is in contrast with the results obtained by Horton & Leech (1972) or Nikolaeva *et al.* (1972). The former group described an ATP-dependent conversion of pigment P-630 into chlorophyll(ide) on flash illumination of maize etioplasts or etioplast membranes. The pigment P-630 for this reaction was formed by deliberately aging the etioplasts, which results in the conversion of pigment P-650 into P-630. Further, chlorophyll(ide) formation in this system was reported to be sensitive to AMP. In the aged total maize homogenate used by Nikolaeva *et al.* (1972) a flash-dependent chlorophyll(ide) formation was seen on NADPH supplementation, the process being inhibited by 2'-AMP.

The chlorophyll(ide) formation by both barley (Table 2) and maize (Table 3) etioplast membranes prepared by our procedure always fails to show any increased chlorophyll(ide) synthesis on supplementation with ATP, but consistently does so on addition of NADPH whether in the presence or absence of ATP (see Tables 2 and 3). I feel the discrepancy between these results could lie in the different procedures employed by the various groups to prepare samples in which the pigment P-650 of the original etioplasts has been more or less completely converted to the inactive pigment P-630 form. Thus if as one of several possible working models it is assumed (see below) that pigment P-650 represents a membrane-bound protochlorophyll(ide)-protein-NADPH ternary complex, then aging of isolated etioplasts at room temperature (Horton & Leech, 1972) might result in the dissociation of the complex, as a result of degradative enzyme(s) action, giving the completely dissociated form absorbing now at 630 nm. The original complex may then possibly be reassembled from the components still present, but with the reaction requiring the addition of ATP (Horton & Leech, 1972). In this context it is noteworthy that an ATP-dependent reconstitution of functional oxidase activity by added protohaem has been described in depleted membranes of a δ -aminolaevulinate-requiring *Escherichia coli* mutant (Haddock, 1973).

The technique of water lysing of etioplasts to give prolamellar-body membranes enriched in pigment P-630 used in this laboratory can, by using the same model, be visualized as washing away NADPH from the original native form, leaving the protochlorophyll(ide) still as a protein complex but again absorbing at 630 nm. Reconstitution of pigment P-650 then occurs merely by the binding of NADPH

Table 3. *Effects of inhibitors on chlorophyll(ide) synthesis in extracts from etiolated maize*

Total homogenate and prolamellar-body-membrane fractions were prepared from maize shoots by the routine procedures used for barley. The fractions (0.2–1.0 mg of protein/3.0 ml of resuspending medium) were incubated at 20°C under flash-illumination as defined in Table 2 with additions as indicated. Samples representing each of the conditions under test were analysed for chlorophyll(ide) concentration after 5 and 30 min incubations. NADPH was added as a regenerating system as in Table 2.

Incubation	Chlorophyll(ide) concentration (nmol/mg of protein) after incubation for	
	5 min	30 min
Homogenate		
No additions	36	52
+NADPH	47	110
+NADPH+0.2 mM-Triton X-100	76	114
+NADPH+0.2 mM-Triton+2 mM-2'-AMP	79	112
Prolamellar-body membranes		
No additions	67	53
+NADPH	316	458
+NADPH+2'-AMP	324	420
-ATP	43	37
-ATP+NADPH	320	446
-ATP+NADPH+2 mM-2'-AMP	327	446
+NADPH+90 μ M-dibromothymoquinone	63	52
+NADPH+150 μ M-disalicylidenepropanediamine	302	397

to re-form the original ternary complex. The results of Nikolaeva *et al.* (1972) may perhaps also be explained as the complete dissociation of the active ternary-complex pigment P-650 during aging of the homogenate. However, in this case the released NADPH would become oxidized by the mitochondrial-membrane population in the total homogenate. Reconstitution of chlorophyll(ide) synthesis in aged homogenates would now have to rely on exogenously provided NADPH but could perhaps use an existing endogenous ATP supply. A very active NADPH oxidase activity in maize and barley homogenates has been demonstrated, the activity being completely sensitive to added cyanide, implying mitochondrial involvement (W. T. Griffiths, unpublished work).

We have consistently failed with our preparations, homogenates, etioplasts or prolamellar-body membranes, both from barley and from maize, to repeat the 2'-AMP inhibition of chlorophyll(ide) synthesis seen by Nikolaeva *et al.* (1972) in homogenates of etiolated maize (see Tables 2 and 3). We can offer no explanation for this discrepancy. Table 3 shows that, in our hands, addition of NADPH to maize homogenates stimulates both the 'rate' and extent of chlorophyll(ide) synthesis, the 'rate' reflected by the chlorophyll(ide) concentration present after 5 min (Table 3) being enhanced even more in the presence of 0.4 mM-Triton X-100, probably owing to a greater exposure of the chlorophyll(ide)-synthesizing system to the NADPH. Addition of 2'-AMP to a concentra-

tion of 2 mM under these conditions failed to inhibit the reaction, expressed either as a 'rate' or total extent of chlorophyll(ide) formation. Similarly, chlorophyll(ide) synthesis in the prolamellar-body membranes, either in the presence or in the absence of ATP, remained unaffected by 2'-AMP addition (Table 3). It should be noted, however, that the presence of ATP appears to stimulate the 'rate' of chlorophyll(ide) synthesis [67 nmol of chlorophyll(ide)/mg of protein present after a 5 min incubation with ATP, compared with 43 nmol of chlorophyll(ide)/mg of protein in its absence]. This again confirms the earlier observation (Griffiths, 1975a) that ATP helps to stabilize existing pigment P-650 in samples, which underlies the reason for the routine addition of ATP to resuspended samples.

The inhibition of chlorophyll(ide) synthesis by 2'-AMP was seen by Nikolaeva *et al.* (1972), [considering the known inhibitory effect of this compound on the flavoprotein ferredoxin-NADP reductase (Shin & Arnon, 1965)] as indicating this enzyme to be involved with hydrogen transfer in the process. However, in view of my experiences with this compound, I question this suggestion. Further, disalicylidenepropanediamine, another well known inhibitor of the flavoprotein (Trebst & Burba, 1967; Robinson *et al.*, 1975), failed in my hands to inhibit chlorophyll(ide) synthesis from NADPH by etioplast membranes (Table 2). Again, involvement of the flavoprotein might be expected to result in some

Fig. 4. Time-course of the NADPH-dependent dark formation of pigment P-650 by etioplast membranes at 0–20°C

Prolamellar-body membranes from barley etioplasts were first pre-illuminated as in Fig. 3 to photoconvert residual endogenous pigment P-650. Incubations in darkness were then initiated by the addition of NADPH as in Fig. 2. Samples were removed after different time-intervals; 5,5'-dithiobis-(2-nitrobenzoic acid) was added to 40 μ M, followed by illumination for 15s to photoconvert the regenerated P-650 pigment, and then Triton X-100 added to 0.4 mM. Finally chlorophyll(ide) concentrations were assayed spectroscopically and these, after first subtracting the chlorophyll(ide) concentration found in a zero-time sample, were plotted to illustrate the progress of pigment P-650 regeneration. The experiment was carried out at 0°C on ice (○), and at 22°C (●). Half-rise times were 35s (●) and 8.5 min (○).

chlorophyllide synthesis if a sufficiently high concentration of NADH were added, since the enzyme does in fact reduce NAD^+ but with a high K_m (Nelson & Neumann, 1969; Shin & Arnon, 1965). However, addition of NADH at 250 times the concentration of NADPH required to saturate the reaction (Table 1) still failed to give any trace of chlorophyll(ide) synthesis. Finally, one of the last photosynthetic activities to be acquired during the greening of etiolated plants is that of NADP^+ reduction via the flavoprotein enzyme (Glydenholm & Whatley, 1968), making it highly unlikely, in view of the massive chlorophyll synthesis already occurring over this period, that this enzyme is involved in the process, or at least during the early stages of greening.

It is noteworthy that as in barley preparations, dibromothymoquinone at 90 μ M completely inhibited the regeneration of active P-650 pigment in maize membranes, without apparently inhibiting photoconversion of existing P-650 pigment (Table 3), since the chlorophyll(ide) concentration in this sample after 5 min of incubation (63 nmol/mg of protein) was approximately the same as the amount derived solely from existing P-650 pigment in the ATP-supplemented membranes (67 nmol/mg of protein). Although it

would be wildly speculative to suggest plastoquinone involvement in chlorophyll(ide) formation from these results, the mechanism of dibromothymoquinone inhibition of the reaction certainly warrants further investigation.

Reliable results for the time-course of pigment P-650 regeneration in darkness proved to be very difficult to obtain and early attempts were greatly hampered by my failure to appreciate that samples could not be stored on ice to stop the reaction while awaiting spectral assay. However, this became recognized and effectively overcome by the use of 5,5'-dithiobis-(2-nitrobenzoic acid), addition of which stopped further pigment P-650 regeneration (see Table 2) so that the time-course of the reaction could now be measured. The regeneration of pigment P-650 as assayed by this method of adding 5,5'-dithiobis-(2-nitrobenzoic acid) to stop the reaction after different incubation times, followed by illumination and assaying the resulting chlorophyll(ide), carried out at 0° and 20°C is shown in Fig. 4. Regeneration of pigment P-650 clearly occurs at 0°C at a rate which is, however, lower than at 20°C. Fig. 4 gives a half-rise time of 35s for pigment P-650 regeneration at 20°C compared with a value of 8.5 min for the process at 0°C. These values are comparable with a half-rise time of 16s determined for the process in leaves at 22°C (Granick & Gassman, 1970).

The shape of the pigment P-650-regeneration curve at 20°C explains how it is possible to get an accelerated rate of flash-induced chlorophyllide synthesis by shortening the dark times between successive flashes to, e.g., 40s. Under these conditions (Fig. 3) photoconversion occurs of pigment P-650 regenerated exclusively by the initial rapid phase of the process, the shape of the pigment P-650 regeneration curve being repeated exactly after each flash, or at least during the early stages of photoconversion before the regeneration becomes limited by protochlorophyll(ide) concentration.

My results suggest that in the presence of NADPH, pigment P-650 formation can proceed in membrane fractions by a reaction that must have a low Q_{10} value and which occurs at quite an appreciable rate at 0°C. In fact, the data on regeneration at 0°C are comparable with published values for the process at this temperature in intact leaves where one-fifth of the regeneration at 22°C can be observed at 0°C (Granick & Gassman, 1970). A similar, apparently temperature-independent formation of pigment P-650 in whole leaves has been reported by Sundqvist (1970). One interpretation of the mechanism of this relatively temperature-insensitive process has already been made (see above); however, further experimentation is required before others, equally applicable at present, can be excluded, e.g. there is no evidence as yet that the process of activation does not involve an actual reduction by the NADPH.

Fig. 5. Low-temperature (77°K) spectrum of photoactive protochlorophyll(ide) complex synthesized *in vitro*

Prolamellar-body membranes from barley etioplasts were pre-illuminated as in Fig. 4 to photoconvert any residual photoactive pigment. A portion (0.94 mg of protein/3 ml) was then incubated in darkness at 20°C for 10 min in the presence of NADPH as in Fig. 4. The difference spectrum between this and a similarly treated sample incubated without NADPH, was then recorded at 77°K in liquid N₂.

Accounts of the existence *in vivo* and *in vitro* of protochlorophyll(ide) as non-photoconvertible (P-630) and photoconvertible (P-650) forms have already been mentioned. This picture was first modified by Boardman and co-workers (Kahn *et al.*, 1970; Boardman *et al.*, 1970) who demonstrated absorption bands due to protochlorophyll(ide) at 637 and 649 nm in etiolated bean leaves at 77°K. Both these bands disappeared on the formation of chlorophyll(ide) by illumination, and the presence of a third non-photoconvertible protochlorophyll(ide) form absorbing at 628 nm was revealed. Energy absorbed by the 637 nm-absorbing form was transferred to pigment P-650 with a very high efficiency and calculation showed that the 637 nm form accounted for up to 40–45% of the total protochlorophyll(ide) present.

Figs. 5 and 6 provide confirmation of these results. Thus Fig. 5 records the difference in absorption at 77°K between barley prolamellar-body membranes pre-illuminated to photoconvert endogenous photoactive pigment and then incubated for 10 min in darkness in the presence of NADPH and (as a reference) a similarly treated sample incubated without NADPH. The spectrum illustrates the appearance of peaks at approx. 642 and 652.5 nm with a trough at approx. 630 nm in the NADPH-incubated sample. This suggests that the presence of NADPH results in the conversion of pigment P-630 into pigment P-640 (which is equivalent to the P-637

Fig. 6. Photoconversion of photoactive protochlorophyll(ide) synthesized *in vitro*

Duplicate samples of barley prolamellar-body membranes were incubated in darkness in the presence of NADPH for 15 min as in Fig. 5. After one had been illuminated for 15 s with a 100 W tungsten lamp, both samples were immediately frozen in liquid N₂ and the illuminated-minus-non-illuminated difference spectrum was recorded.

pigment of bean) and pigment P-650 (which is equivalent to the 649 nm-absorbing form in bean).

Fig. 6 demonstrates that both pigments P-640 and P-650 are photoconvertible. For this experiment, two identical membrane preparations were incubated in darkness in the presence of NADPH for 15 min. One was then illuminated and the light-minus-dark difference spectrum was immediately scanned at 77°K. This results (Fig. 6) in minima appearing at approx. 642 and 652 nm owing to the photoconversion of these two protochlorophyll(ide) forms, their disappearance being accompanied by the appearance of chlorophyllide, which is seen as a positive peak at 681 nm in the illuminated sample.

Thus these Figures provide a convincing illustration of the previously reported existence of two spectroscopically distinct photoconvertible protochlorophyll(ide) forms and further show that both of these result from the dark NADPH activation of an inactive protochlorophyll(ide) absorbing at lower wavelengths. My failure to observe the two photoconvertible forms in my earlier work (Griffiths, 1974, 1975a) undoubtedly stems from the lack of resolution

Table 4. *Localization of glucose 6-phosphate dehydrogenase within etioplasts and recovery of the enzyme in etioplast subfractions*

Total homogenate and etioplast fractions were prepared as described in the Experimental section. Lysis of the etioplasts, to prepare the prolamellar-body membranes, and treatment of these to give once-washed membranes, was achieved by a tenfold dilution with water followed by centrifugation to sediment the membranes. Enzymic activity was measured as described in the text.

Fraction	Glucose 6-phosphate dehydrogenase activity		
	Specific activity (nmol of NADP ⁺ reduced/min per mg of protein)	Total activity (mmol of NADP ⁺ reduced/min per total fraction)	Distribution in etioplast subunits (%)
Homogenate	12.4	19530	—
Etioplasts	26.5	424	100
Prolamellar-body membranes	9.3	96	22
First water extract	37.1	307	72
Once-washed prolamellar-body membranes	—	—	0
First water washings	13.3	73	17

of the inactive pigment P-630 from the active pigment P-640 form in absolute spectra, this being especially true of spectra recorded at room temperature. At present there is no information on the nature of these two photoconvertible forms. Indeed, the suggestion has been made that no two separate forms exist, rather that the absorbances represent twin peaks of a single protochlorophyll(ide) species (Kahn & Nielsen, 1974).

I have considered the possibility that, as both are formed in an NADPH-requiring reaction, they might represent different states of reduction of a protochlorophyll(ide) complex, with one being an intermediate in the formation of the other. However, I have now abandoned this idea, since on studying the rate of formation of the two forms (by recording spectra at 77°K after different incubation times in the presence of NADPH) it was found that even though total P-640 plus P-650 pigment concentrations increased with time, the relative ratios of the two forms, as judged from peak heights, remained constant (W. T. Griffiths, unpublished work). This I have taken to imply that no product-precursor relationship can be demonstrated for the two forms. In contrast, mention must be made of the claim that pigment P-637 represents the normal native photoconvertible protochlorophyll(ide), with pigment P-650 only appearing (together with crystallization of the prolamellar body) as a consequence of prolonged etiolation (Klein & Schiff, 1972).

One question raised by the results is: what is the origin of the NADPH used in activating the protochlorophyll(ide)? Although photosynthesis probably takes over this role in the mature chloroplast, this supply is not available during the early stages of greening when the demand for NADPH for biosynthetic reactions is probably at its greatest. This, as mentioned above, is due to low activity of the ferredoxin-NADP⁺ reductase enzyme in the early stages of greening (Glydenholm & Whatley, 1968), this enzyme being essential for photosynthetic NADPH formation. My studies have shown that isolated etioplasts are capable, on flash-illumination, of chlorophyll(ide) synthesis from normally inactive pigment P-630 (Griffiths, 1975a), a process for which I have also demonstrated an obligate requirement for NADPH (Griffiths, 1974). This implies that etioplasts, as isolated, must possess the native NADPH-producing system used for pigment P-630 activation.

From early on in my studies it became apparent that isolated prolamellar-body membranes could regenerate pigment P-650 from added glucose 6-phosphate and NADP⁺ alone, implying the possible association of the enzyme glucose 6-phosphate dehydrogenase with the lamellar membranes. In view of the regulatory role this enzyme plays in controlling the pentose phosphate pathway in animals, a pathway believed to function primarily as a source of NADPH for biosyntheses, I decided to investigate the presence of this enzyme in etioplasts and derived membranes. Although it is well known that reactions of the oxidative pentose phosphate pathway occur in the soluble part of animal cells, their localization in plant cells is not so well defined (see Ap Rees, 1974). Table 4 shows the results of my analysis of the presence of glucose 6-phosphate dehydrogenase in etioplasts. It must be emphasized that this experiment was not carried out to define the subcellular localization of the oxidative pentose phosphate pathway, as measured by the distribution of the key enzyme glucose 6-phosphate dehydrogenase, but rather to see if significant amounts of this enzyme occurred within the etioplast.

Table 4 shows that the specific activity of glucose 6-phosphate dehydrogenase in my etioplast preparations, 26.5 nmol/min per mg of protein, is greater than

its specific activity in the total homogenate, 12.4 nmol/min per mg of protein. It must be mentioned, however, that only 2.1 % of the enzyme's activity in the homogenate was recoverable in the etioplast fraction. This value compares with my routine recovery of approx. 15 % of the protochlorophyll(ide) of the total homogenate, as estimated spectrophotometrically in our etioplast fraction. Assuming this pigment occurs exclusively within the etioplasts, then on correcting my values for glucose 6-phosphate dehydrogenase recovery in etioplast, about 14 % of the total enzyme must be present in the etioplast. Further, when the difference in solubilities of the pigment and enzyme are considered it must be realized that even this value must be low.

The soluble nature of the enzyme within the etioplasts can be seen from the data in Table 4. These show that 72 % of the activity in the etioplast was recovered in the soluble fraction after the initial water lysis of the etioplasts, with the residual enzyme (22 %) being completely washed off the membranes during a subsequent water washing. The presence of the enzyme on the prolamellar-body membranes explains my earlier observation (see above) of chlorophyll(ide) synthesis by these membranes in the presence of added glucose 6-phosphate and NADP⁺ only, in this case the NADPH for the reduction being produced by the residual membrane-associated dehydrogenase.

To strengthen the case for the existence of glucose 6-phosphate dehydrogenase within etioplasts my repeated failure to detect any cytoplasmic hexokinase activity in my etioplast fraction can be mentioned, whereas the enzyme is readily assayable in total homogenates. This eliminates the possibility of cytoplasmic contamination accounting for my result. In contrast, the soluble plastid enzyme carboxydismutase in our hands shows a distribution almost identical with the dehydrogenase, with 2.5 % of the activity in the total homogenate being recovered in the etioplast fraction (W. T. Griffiths & R. Mapleston, unpublished work). The conclusion from these data is that glucose 6-phosphate dehydrogenase in etioplasts could, as part of the oxidative pentose phosphate pathway, provide the NADPH required for chlorophyll biosynthesis. A similar location of this pathway has been suggested in chloroplasts of tobacco and spinach and root plastids of wheat where it provides the NADPH required for the similarly located nitrite reductase (Dalling *et al.*, 1972a,b).

The regulatory problem resulting from the presence of both the oxidative and reductive enzymes of the pentose phosphate pathway within plastids of higher plants has already been pointed out (Bassham, 1971). Evidence is accumulating that, as in animal cells (Krebs & Eggleston, 1974), regulation of the oxidative pathway occurs at the glucose 6-phosphate dehydrogenase reaction (Bassham, 1971). In plants,

this enzyme may be regulated indirectly by light through photosynthetically induced fluctuations in the ATP, NADPH or ribulose diphosphate concentrations, or even pH, all of which have been shown to effect the activity of the enzyme (Lendzian & Ziegler, 1972; Muto & Uritani, 1972; Pelroy *et al.*, 1972; Grossman & McGowan, 1975). Biosynthetic reactions through their effects on the ATP and NADPH concentrations also probably contribute to the regulation of the oxidative pentose phosphate pathway.

I am grateful to Mr. Julian Roberts for excellent skilful assistance.

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