Reconstitution of Chlorophyllide Formation by Isolated Etioplast Membranes

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1. The reconstitution of chlorophyllide biosynthesis by barley etioplast membranes is described. 2. The process is dependent on the addition of NADPH and protochlorophyllide and on illumination, which can be either continuous or intermittent. 3. The reconstituted process involves spectroscopically similar intermediates to the native reaction in whole leaves. 4. Steps in the process are an initial enzymic formation in the dark of a photoactive complex, $P_{638/652}$ (probably a ternary protochlorophyllide–NADPH–enzyme complex), followed by a very rapid light-dependent hydrogen transfer from the NADPH to the protochlorophyllide giving chlorophyllide, finally releasing the enzyme for repeating the process. 5. A continuous assay for the system regenerating complex $P_{638/652}$ was devised on the basis of monitoring chlorophyllide formation. 6. The pH optimum of the reaction is at 6.9 and K_m values for protochlorophyllide and NADPH are 0.46 and $35\mu\text{M}$ respectively. 7. The reaction is associated specifically with the etioplast membrane fraction. 8. Activities of the system assayed *in vitro* are more than adequate to account for rates of chlorophyll formation *in vivo*.

The formation of chlorophyll *a* by higher plants has been extensively studied at the whole-plant level by using a variety of experimental approaches (see Kirk & Tilney-Basset, 1967) and these have culminated in detailed formulations of the biosynthetic route (Jones, 1976). However, reliable data on the individual enzymic reactions from studies *in vitro* have been reported far less frequently (see Rebeiz & Castelfranco, 1973). Thus, even though the operation of the complete pathway *in vitro* is implied from the work of Rebeiz & Castelfranco (1971*a,b*), to date only three of the enzymes thought to be specific to the chlorophyll-biosynthetic pathway have been assayed in cell-free preparations. These are magnesium protoporphyrin methyltransferase (EC 2.1.1.11) (Radmer & Bogorad, 1967), magnesium protoporphyrin methyl ester vinyl reductase (Ellsworth & Hsing, 1974) and chlorophyllase (EC 3.1.1.14) (Holden, 1961). Perhaps also to be included in this list should be the extensive experiments carried out *in vitro* on protochlorophyll(ide) holochrome. This is a photoactive pigment–protein complex that retains the ability to photoreduce its protochlorophyll(ide) to chlorophyll(ide) *in vitro* (see Boardman, 1966). Although this reaction certainly does display some properties of enzyme catalysis, e.g. sensitivity to heat, pH and protein-denaturing agents, it also shares

some characteristics with strictly photochemical reactions in that it proceeds extremely rapidly (complete in less than $10\mu\text{s}$; see Schopfer & Siegelman, 1969) even at very low temperatures (Boardman, 1966).

The photoconversion of protochlorophyll(ide) together with the actual formation of the photoactive complex has again been studied in whole leaves (Granick & Gassman, 1970; Sundquist, 1970). It has been possible to measure formation of the photoactive complex, since *in vivo* this has distinct light-absorption characteristics, absorbing maximally at 638 and 652 nm in both whole leaves (Kahn *et al.*, 1970; Dujardin & Sironval, 1970; Dujardin, 1975) and isolated etioplast membranes (Griffiths, 1975*a*) compared with the non-active pigment, which absorbs at approx. 630 nm. Formation of the photoactive complex in whole plants, in contrast with the actual photoconversion, shows typical enzymic properties. Thus from the limited experimental versatility offered by whole plants it has been shown that the reaction is temperature-sensitive (Granick & Gassman, 1970), is inhibited by a range of poisons (Gassman, 1973*a,b*) and proceeds at readily measurable rates, which vary with the age of the tissue (Akoyunoglou & Siegelman, 1968).

Recently an etioplast preparation was described as being capable of mimicking the whole leaf in terms of carrying out the terminal reactions of chlorophyllide biosynthesis (Griffiths, 1975*a*). Endogenous normally non-photoactive protochlorophyll(ide) in this preparation could be converted into the photoactive form (complex $P_{638/652}$) by an NADPH-

Abbreviations used: complexes P_{630} and $P_{638/652}$, protochlorophyll(ide) pigment complexes showing light-absorption maxima at the wavelengths indicated. Protochlorophyll(ide) refers to protochlorophyllide and/or protochlorophyll. Chlorophyll(ide) refers to chlorophyllide and/or chlorophyll.

Scheme 1. Sequence of reactions leading to chlorophyll *a* formation

requiring reaction. Thus addition of NADPH to this system resulted in a decrease in absorption at approx. 630nm accompanied by an increase at 638 and 652nm, a reaction strictly analogous to the regeneration process occurring *in vivo*. A study of the properties of this reaction (Griffiths, 1975a) was compatible with the sequence of events shown in Scheme 1, to describe the terminal steps of chlorophyll biosynthesis.

In the present paper the complete reconstitution of this activation process by adding protochlorophyllide and NADPH to etioplast membranes is described and a convenient procedure for assaying the reaction *in vitro* is developed. Some of this work has been reported in a preliminary manner (Griffiths, 1977).

Experimental

Biological material

All the experiments described here were carried out with preparations from 7-day-old etiolated barley (*Hordeum vulgare* L., cultivar Proctor) plants. They were cultivated as previously described (Griffiths, 1975a).

Chemicals

Most of the chemicals were readily available from commercial sources and were of A.R. grade or the highest grade obtainable. Sodium cholate was a product of Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Glucose 6-phosphate, NADP⁺ and glucose 6-phosphate dehydrogenase were obtained from Boehringer, Mannheim, Germany. Cellulose-MN for t.l.c. was purchased from BDH Chemicals, Poole, Dorset, U.K.

Purification of solvents

Peroxide-free diethyl ether was prepared by drying the commercial product over sodium/lead alloy

overnight followed by distillation over reduced iron immediately before use. Light petroleum (b.p. 60–80°C) was also dried over sodium/lead alloy and again redistilled before use.

Methods

Etioplasts were isolated from 7-day-old dark-grown barley by differential centrifugation of a leaf homogenate as previously described (Griffiths, 1975a). Osmotic lysis of the etioplasts followed by centrifugation gave the etioplast membrane fraction, corresponding to the prolamellar-body membranes. 'Proto-pigment'-free membranes, depleted of their endogenous chlorophyll precursors, were prepared in the same way as used for the prolamellar-body membranes, except that before lysis of the etioplasts they were flash (4mJ) illuminated (1 flash/40s; Griffiths, 1975a) at 0°C for 20min on a shaker in the presence of an NADPH-regenerating system [0.5 unit (1 μmol of substrate converted/min) of glucose 6-phosphate dehydrogenase, 5mM-glucose 6-phosphate and 0.5mM-NADP⁺].

Protochlorophyllide was isolated from dark-grown 8–9-day-old etiolated barley plants by a modification of a previously described method (Griffiths, 1975b). The tissue was macerated in acetone in an Ultra-Turrax homogenizer. The resulting homogenate was filtered through glass wool and the residue washed with peroxide-free ether till no further coloured pigment was extracted. Water was added to the pooled acetone and ether extracts until two phases were obtained, an upper organic phase containing pigments and a lower colourless aqueous acetone phase.

The lower phase was discarded and the ether layer was washed with tepid (approx. 30°C) water several times to remove last traces of acetone. The ether extract at this stage was mixed with an equal volume of redistilled light petroleum (b.p. 40–60°C), and finally approx. 0.1 vol. of methanol/0.01 M-NH₃ (4:1,

v/v) was added. This resulted in the formation of two phases once more, a lower ammoniacal methanol phase, pale-green in colour, due to protochlorophyllide, and an upper highly pigmented non-polar phase. This procedure was repeated a further three times till no more protochlorophyllide appeared in the ammoniacal methanol layer. The latter extracts were pooled and back-washed with diethyl ether/light petroleum (b.p. 40–60°C) (1:1, v/v) to remove any traces of non-polar pigments carried over into the protochlorophyllide extract. The protochlorophyllide solution was then concentrated *in vacuo*, diluted with water and the protochlorophyllide extracted into ether. The ether extract was back-washed with water, dried over anhydrous Na_2SO_4 , and finally concentrated by distillation *in vacuo* and stored at -20°C . Protochlorophyllide was solubilized for addition to enzymic incubations by evaporating a portion of the ether stock solution to dryness under N_2 followed by addition of a methanolic cholate solution and re-evaporation to dryness. Final traces of solvent were removed from the cholate/protochlorophyllide oily residue by desiccating the mixture *in vacuo* followed by resuspension in 50 mM-Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.2. This procedure gave a crystal-clear suspension of the pigment/cholate mixture, this being normally about $25\ \mu\text{M}$ with respect to protochlorophyllide, and had 2.0 mg of cholate/ml. In this form the pigment was stable at 0°C for at least 48 h. Fresh substrates were usually prepared daily.

Absorption spectra of substrates or incubation mixtures for the purpose of assaying chlorophyll(ide) concentrations were recorded by using a split-beam spectrophotometer (Jones & Saunders, 1972) as described earlier (Griffiths, 1975a). Difference spectra at 77 K were similarly recorded with the cuvette holder immersed in liquid N_2 . Continuous recording of flash-induced chlorophyll(ide) formation was performed by using a dual-wavelength spectrophotometer set with the measuring beam at 672 nm and the reference beam at 710 nm. Flash illumination was from a xenon photograph lamp (Mecablitz) placed 3 cm above the cuvette holder; the light was filtered through 1 cm of saturated CuSO_4 solution. Formation of chlorophyllide under continuous illumination was carried out in an identical manner except that the xenon flash lamp was replaced by a 55 W quartz/halogen lamp arranged to illuminate the sample from one side. The light-beam in this case was passed through an interference filter (Balzers, B40) transmitting maximally at 440 nm, and the light intensity at the cuvette was $0.75\ \text{mW}/\text{cm}^2$. The photomultiplier during both of these assays was protected from the actinic light by two layers of Wratten no. 70 gelatine filters transmitting only light of wavelength greater than approx. 650 nm. Rates of chlorophyllide formation were calculated from the extent of the $\Delta(A_{672}-$

$A_{710})$ assuming an ϵ_{mM} of $91.2\ \text{litre}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$ for the pigment at these wavelengths (Griffiths, 1975a).

Protein assays

Protein concentrations in suspensions of cell-free preparations, etioplasts or prolamellar-body membranes were assayed by the Folin method (Lowry *et al.*, 1951), with bovine serum albumin (Sigma) as standard. Total plant protein concentration was assayed as inorganic nitrogen by using Nessler's reagent on samples of dried plant material after digestion at 300°C with conc. H_2SO_4 and H_2O_2 (Johnson, 1964).

Results and Discussion

The procedure described for isolating protochlorophyllide produced a product with spectroscopic properties (Fig. 1) agreeing with the published data for this compound (Houssier & Sauer, 1969), and analysis of this preparation by t.l.c. on cellulose (Griffiths, 1975b) showed it to be essentially free of any impurities. On solubilization of the sample in aqueous cholate the absorption, if measured immediately, was shifted by about 9 nm towards longer wavelength, but the shape of the spectrum was qualitatively similar to that of the methanolic solution (Fig. 2a). When the sample was left in cholate, however, a progressive shift in absorption towards even longer wavelengths occurred, this being complete after about 3 h, with the α -peak of the sample now at approx. 653 nm (Fig. 2b) compared with its original position at 632 nm. An additional Soret peak at 473 nm was also evident in the final spectrum. Isosbestic points for the shift occurred at 458, 609 and 637 nm.

These gradual spectral shifts are identical with the changes described by Seliskar & Ke (1968) for proto-

Fig. 1. Absorption spectrum of purified protochlorophyllide. The pigment, isolated as described in the text, was dissolved in methanol and the spectrum recorded on a Unicam SP.800 recording spectrophotometer. The value in parentheses is the dilution factor.

Fig. 2. Absorption spectra of cholate-solubilized protochlorophyllide under different conditions

Protochlorophyllide was solubilized in cholate as described in the text and spectra were recorded after approx. 2 min (curve *a*, ---), after 3 h (curve *b*, —) and immediately after the addition of 0.1 mM-Triton X-100 to the solution shown in curve *b* (curve *c*, - - -).

Fig. 3. Effects of sodium cholate on chlorophyllide synthesis and retention by etioplast membranes

Prolamellar-body membranes from barley etioplasts were flash-illuminated for 20 min at 1 flash/40 s in the presence of NADPH and a range of cholate concentrations. Centrifugation of the mixture at the end of the incubation was followed by assaying the chlorophyllide in the resulting pellet and soluble fractions by sensitive split-beam spectrophotometry. ○, Chlorophyll(ide) formed; □, chlorophyllide in supernatant/chlorophyllide in pellet.

chlorophyllide in solvents of low dielectric constants (see also Brouers, 1972) and were ascribed by Seliskar & Ke (1968) to pigment aggregation. This phenomenon probably also occurs in aqueous cholate, since addition of 0.1 mM-Triton X-100 instantaneously reversed the shift (Fig. 2*c*), possibly as a result of the breakdown of the polymeric form.

Apart from the complications caused by these spectral shifts, cholate solubilization of protochlorophyllide offers a convenient procedure for preparing

Fig. 4. Chlorophyllide synthesis from added substrates by prolamellar-body membranes

Membranes were isolated from etioplasts that had been depleted of substrate by flashing for 20 min at 1 flash/40 s. These were diluted in resuspension medium (1.2 mg of protein/3.0 ml) in the presence of cholate-solubilized protochlorophyllide and an NADPH-regenerating system, and spectra were noted before (*a*) and after (*b*) flash illumination (1 flash/40 s) for 20 min. Finally, the incubation mixture was extracted into acetone/water (4:1, v/v) and the absorption spectrum again recorded (*c*).

water-'soluble' protochlorophyllide as a substrate for chlorophyllide synthesis *in vitro*. To test whether cholate has an inhibitory effect on chlorophyllide formation from endogenous pigment by prolamellar-body membranes, samples of the latter pigment were flash illuminated for 20 min at 1 flash/40 s in the presence of NADPH and with various cholate additions. At the end of this period the samples were centrifuged at 15000*g* for 10 min to sediment the membranes, and finally chlorophyll(ide) was assayed spectrophotometrically in the resulting supernatants and resuspended pellets. Fig. 3 shows that increasing the cholate concentration increased the amount of chlorophyll(ide) appearing in the final supernatant: only 36% was solubilized at a cholate concentration of 1 mg/ml, whereas 65% was solubilized when the cholate concentration was 8.5 mg/ml. Apart from this pigment-solubilizing effect, however, a cholate concentration as high as 12 mg/ml has no inhibitory effect on chlorophyllide formation from endogenous pigment, this concentration being in excess of the amount required to solubilize the purified protochlorophyllide (Fig. 2*a*).

By way of confirmation of my earlier results (Griffiths, 1975*b*), when substrate-depleted prolamellar-body membranes (prepared by water lysis of previously extensively flashed etioplasts) (Fig. 4*a*) are flash illuminated for 20 min in the presence of exogenously added protochlorophyllide and NADPH, a product absorbing at 672 nm accumulates, concomitant with a decrease in the absorption at approx. 632 nm (Fig. 4*b*). This change does not occur in the absence of either the protochlorophyllide

or NADPH (Griffiths, 1975b). Extraction of such an incubation sample into acetone/water (4:1, v/v) gives an extract displaying the characteristic chlorophyll(ide) absorption at 663 nm (Fig. 4c), with a further peak at 623 nm indicative of residual non-photoconverted protochlorophyllide. Identification of the synthesized product as unesterified chlorophyllide has been ascertained by t.l.c. of the extract on cellulose plates developed with methanol/dichloromethane/water (200:36:45, by vol.), when fluorescent pigment spots corresponding in position to authentic chlorophyllide and protochlorophyllide were seen. We have never detected any trace of compounds with the chromatographic properties of esterified chlorophyllides appearing as products in this synthesis *in vitro*. However, Rüdiger *et al.* (1977) have described a membrane fraction separated from an etiolated-maize-leaf homogenate, which on supplementation with [^{14}C]isopentenyl pyrophosphate incorporated radioactivity into chlorophyll pigments esterified with unsaturated isoprenoid alcohols, which are considered as possible intermediates in the formation of the final phytylated chlorophyll *a*. Chromatographic and mass-spectroscopic evidence for the transient appearance of compounds similar to the latter has also been obtained from analysis of whole leaves (Liljenberg, 1974; Ogawa *et al.*, 1975; Wellburn, 1976).

Earlier experiments (Griffiths, 1975a) had indicated that the accumulation of chlorophyllide *in vitro* by etioplast membranes in flashing light is the result of flash photoconversion of a photoactive complex, $\text{P}_{638/652}$, formed repeatedly after a flash by a NADPH-requiring reaction. Further, the kinetics of formation of this complex were also reported as comprising an initial quite rapid linear phase followed by a phase of gradually decreasing rate. From a consideration of these regeneration kinetics it should be possible to assay the initial rate of regeneration of complex $\text{P}_{638/652}$ *in vitro* by measuring the yield of chlorophyllide formed as a result of a saturating light flash given within the initial linear phase of the regeneration. Reference to Fig. 4 shows that the assay of such a chlorophyllide formation can be readily achieved *in vitro* by dual-wavelength spectrophotometry by measuring flash-induced absorbance changes at 672 nm relative to 710 nm. The latter wavelength is chosen as one at which no specific flash-induced absorbance changes occur and thus compensates for any non-specific changes caused by, e.g., scattering.

Fig. 5 depicts a typical dual-wavelength trace to illustrate this complex $\text{P}_{638/652}$ regeneration assay by using prolamellar-body membranes prepared from barley etioplasts. Immediately before assay the membranes were given a saturating flash of light from a 100 W lamp for 20 s to photoconvert endogenous complex $\text{P}_{638/652}$, which is always present to a greater

or lesser extent in these membranes. These were then transferred into the spectrophotometer and flash illuminated at the rate of one flash/20 s while absorbance changes $\Delta(A_{672} - A_{710})$ were monitored. Initially, under these conditions, no chlorophyllide synthesis occurs, i.e. no absorbance increase at 672 nm is seen (Fig. 5). However, on addition of a NADPH supply each flash now produces an incremental rise in absorption at 672 nm, indicating chlorophyllide formation from the newly synthesized complex $\text{P}_{638/652}$, the latter being formed in the dark from the endogenous pigment and the added NADPH. The extent of the increase in A_{672} per flash, although initially linear with respect to time, starts to decrease exponentially after the third flash or so, and finally ceases altogether. This probably represents complete photoconversion of the endogenous pigment, since subsequent addition of protochlorophyllide (see Fig. 5) results in a restoration of the flash-induced change to its original extent, and this is sustained for a prolonged period as a result of the saturating protochlorophyllide concentration used.

The slope of this final trace (Fig. 5) can be converted into a rate of chlorophyllide accumulation by using a $\Delta\epsilon_{\text{mM}}$ of $91.2 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ for the absorbance change. This is permissible since it has been shown (Griffiths, 1975a) that the A_{672} corresponding to chlorophyllide *in vivo* is quantitatively the same as the A_{672} of the extracted pigment in ether. This illustration (Fig. 5) offers convincing confirmation of the utilization of protochlorophyllide for

Fig. 5. Continuous assay of chlorophyllide synthesis by membranes from barley etioplasts

Prolamellar-body membranes (0.12 mg of protein) were diluted in 3.0 ml of resuspension medium and exposed to a 100 W lamp for 20 s to photoconvert endogenous complex $\text{P}_{638/652}$. They were then exposed to 0.1 ms xenon flashes every 20 s while absorbance changes $\Delta(A_{672} - A_{710})$ were monitored continuously before and after addition of NADPH and cholate-solubilized protochlorophyllide.

Fig. 6. Effect of intervening dark period on the chlorophyllide yield per flash by substrate-supplemented etioplast membranes

Prolamellar-body membranes (0.27 mg of protein/3.0 ml) were incubated as in Fig. 5 and the mean yield of chlorophyllide/flash was measured at the different flash rates indicated. Fresh samples were used for each of the selected dark intervals.

Fig. 7. Protein-dependence of the chlorophyllide synthesis by isolated etioplast membranes

Rates of chlorophyllide synthesis by etioplast membranes were assayed as described in Fig. 5, but with different amounts of membranes as indicated.

chlorophyllide synthesis by a system *in vitro*, first described by this laboratory (Griffiths, 1975b).

The variation in extent of the flash-induced chlorophyllide synthesis with different dark intervals between successive flashes is illustrated in Fig. 6. The data were obtained by incubating prolamellar-body membranes in the presence of protochlorophyllide and NADPH and noting spectrophotometrically the extent of chlorophyllide formation per flash with various dark times between successive flashes. The plot illustrates that for dark times up to approx. 60 s the flash yield of chlorophyllide, measured as $\Delta(A_{672} - A_{710})$, is directly proportional to the length of the dark interval between flashes, implying that formation of complex $P_{638/652}$ must also be linear with time over this period, in agreement with earlier experiments (Griffiths, 1975a). Therefore under these incubation conditions a flash rate with a dark time of anything less than 60 s should give kinetics of chlorophyllide synthesis reflecting accurately the initial phase of complex $P_{638/652}$ accumulation in the dark. In our assays of this rate, a dark time of 20 s is usually adopted.

Fig. 7 shows the relationship between the measured rate of chlorophyllide formation and the protein concentration used in the assay, and Fig. 8 shows how the rate varies with the intensity of the flash illumination. Thus Fig. 7 shows that over the protein-concentration range investigated, up to 1.25 mg/2.5 ml of final assay volume, the rate of chlorophyllide synthesis, as expected, is linearly related to the

Fig. 8. Effect of the intensity of flash illumination on chlorophyllide synthesis by etioplast membranes

Chlorophyllide-synthesis rates by isolated prolamellar-body membrane suspensions (0.23 mg of protein/3.0 ml) were measured as described in Fig. 5 by using fresh samples for each illumination intensity investigated. The light intensities were varied by means of neutral-density filters placed in the light path as described in the Experimental section.

Fig. 9. Double-reciprocal plot of chlorophyllide-synthesis rates by etioplast membranes at various protochlorophyllide concentrations

Prolamellar-body membranes (0.19mg/3.0ml) were flash illuminated as described in Fig. 5 and rates of chlorophyllide synthesis noted at different protochlorophyllide concentrations, but in the presence of excess NADPH as indicated.

Fig. 10. Double-reciprocal plot of chlorophyllide-synthesis rates by etioplast membranes at various NADPH concentrations

Prolamellar-body membranes were flash illuminated as described in Fig. 5 and rates of chlorophyllide synthesis measured at different NADPH concentrations as indicated.

protein concentration. Increasing the concentration of protein beyond this range was not attempted, since problems due to light saturation arising from the shadowing effect of pigments on the membranes might be encountered at higher concentrations. The effect of light intensity on the measured chlorophyllide synthesis rate (Fig. 8) shows that at lower intensities the rate increases linearly with increasing light intensity, but becomes independent of intensity at approx. 60% transmission, which indicates the light saturation of the actual photoconversion step. It is of course essential that conditions of light saturation must prevail to give meaningful rates of regeneration of complex $P_{638/652}$ by this procedure. In routine assays the fulfilment of this condition is always checked by using neutral density filters to lower the light intensity by approx. 20%, when no decrease in recorded rate of chlorophyllide synthesis should occur. Further, to check that assays are being carried out under optimal conditions a doubling of the observed rate in response to a 2-fold increase in protein concentration was usually ascertained.

Fig. 9 records a double-reciprocal plot of the rate of flash-induced chlorophyllide synthesis as a function of protochlorophyllide concentration in the presence of a saturating concentration of NADPH, and Fig. 10 records corresponding data obtained for saturating protochlorophyllide concentration but with various NADPH concentrations. Despite the fact that unfractionated etioplast membranes were used to collect the data the resulting plots show typical enzymic kinetics. K_m values for the two sub-

strates, computed from Figs. 9 and 10, are K_m (protochlorophyllide) $0.46\mu\text{M}$ and K_m (NADPH) $35\mu\text{M}$. It is uncertain whether these concentrations of the two substrates are ever exceeded within the etioplast membrane system *in vivo*. Thus very low amounts of protochlorophyllide are found in leaves of plants grown under normal illumination conditions (Kaler & Shlyk, 1962), which suggests that under these conditions the regeneration reaction is out of equilibrium and as such may represent a potential regulatory step in the chlorophyllide-biosynthetic pathway. A different conclusion regarding this reaction has, however, been arrived at by Sundquist (1973) from spectroscopic experiments *in vivo*. Thus he showed that in whole leaves the reaction leading to complex P_{650} formation, corresponding to complex $P_{638/652}$ of the present paper, is faster than the rate of protochlorophyllide formation from its precursors.

In addition to the dependence of initial reaction rates on substrate concentration, the extent of chlorophyllide accumulation resulting from extended flashing of the system to completion also varied with substrate concentrations. Thus on prolonged (approx. 30min) flash illumination of barley etioplast membranes supplemented with a saturating NADPH concentration but with various protochlorophyllide contents, the final chlorophyllide concentration attained (Fig. 11) was, as expected, directly related to the protochlorophyllide concentration used. Final chlorophyllide concentrations in this experiment were estimated from the $\Delta(A_{672} - A_{710})$ kinetic traces

Fig. 11. *Dependence of the extent of chlorophyllide synthesis by flash-illuminated etioplast membranes on the protochlorophyllide added*

Prolamellar-body membranes (0.19 mg of protein/3.0 ml) were exhaustively flashed at 1 flash/20 s while chlorophyllide synthesis was monitored in the presence of a saturating concentration of NADPH and of various amounts of protochlorophyllide as indicated. The extent of chlorophyllide synthesis was calculated from the total change, $\Delta(A_{672} - A_{710})$ and confirmed by measuring spectra of the incubated samples on the split-beam spectrophotometer.

and were confirmed by the absorption spectra of the final reaction mixtures determined on the split-beam spectrophotometer. The stoichiometry of the process, obtained after correction for the chlorophyllide formed from the endogenous substrate, corresponds to 0.5 mol of chlorophyllide formed/mol of protochlorophyllide added. When this same technique was applied in attempts to obtain the corresponding ratios for the second substrate, NADPH, very inconsistent results were obtained. Thus extensive flashing of etioplast membranes supplied with saturating amounts of protochlorophyllide but various NADPH concentrations led to ratios of chlorophyllide synthesized/mol of NADPH added ranging from 0.5 to 0.01. This unsatisfactory relationship can only mean that non-specific NADPH-binding sites occur on the etioplast membranes which can scavenge the added NADPH, rendering it unavailable for the regeneration of complex $P_{638/652}$ and subsequent chlorophyllide synthesis. In contrast, no such extensive non-specific protochlorophyllide-binding sites can occur on these membranes, in view of the good stoichiometry obtained with this substrate. In view of the results with NADPH, the K_m for this substrate noted above should therefore be considered as a tentative value only, and a more reliable value will have to await further purification of the system.

The effect of various pH values on the chlorophyllide-synthesizing activity of the etioplast membranes, as determined by the spectrophotometric

Fig. 12. *pH-dependence of the chlorophyllide-synthesis activity of etioplast membranes*

Prolamellar-body membranes were resuspended in assay medium containing 20 mM each of Tricine [N -[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine], Bes [2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid] and Mes (4-morpholine-ethanesulphonic acid) with saturating concentrations of protochlorophyllide and NADPH. The pH of the incubation mixture was varied with KOH additions as indicated and chlorophyllide-synthesis rates were measured as described in Fig. 5.

assay, is recorded in Fig. 12. Thus a single pH optimum for the reaction occurs at pH 6.9. This differs from the more alkaline pH optimum reported for protochlorophyllide photoconversion in isolated holochrome (see Boardman, 1966); however, the latter may merely reflect the stability of the active pigment-protein at various pH values, with an alkaline environment tending to favour its stability.

Table 1 records specific-activity values of the complex $P_{638/652}$ -regenerating system as found in the various cell fractions obtained during the course of isolation of prolamellar-body membranes. The data were obtained by our usual assay and the results are expressed as rates of chlorophyllide formation per mg of protein. There is an approx. 34-fold increase in specific activity of the system in the etioplast compared with the cell homogenate, whereas the specific activity is increased a further 1.6-fold in the prolamellar-body membranes. This would imply that the activity within the cell is associated with an integral component of the etioplast membrane. Perhaps the fact should not be ignored, however, that this membrane fraction, obtained as it is by lysis of etioplasts, represents a mixed membrane population, comprising besides genuine prolamellar-body membranes, other insoluble etioplast components such as, for example, etioplast outer envelopes. Thus again further purification of the system

Table 1. *Specific activity of the complex $P_{638/652}$ -regenerating system in different subcellular fractions*

Samples of the different fractions produced during isolation of prolamellar-body membranes from etiolated barley plants were assayed for rates of chlorophyllide synthesis as in Fig. 5.

Fraction	Specific activity of complex- $P_{638/652}$ regeneration (nmol of chlorophyllide synthesis/ min per mg of protein)
Total homogenate	0.086
Whole etioplasts	2.98
Prolamellar-body membranes	4.91

is awaited before its exact sub-etioplast location can be described unambiguously. Assuming, however, that the system is associated with the prolamellar-body membranes, its location here would complement the similarly located methyltransferase (Radmer & Bogorad, 1967) and chlorophyllide-esterifying system (Rüdiger *et al.*, 1977), but would contrast with the apparent soluble location of the vinyl-side-chain-reducing enzyme (Ellsworth & Hsing, 1974).

Spectral characterization of the product ($P_{638/652}$) formed in the dark from endogenous pigment and added NADPH has already been done (Griffiths, 1975a). The corresponding properties for the reaction involving exogenously added protochlorophyllide were investigated by difference spectra recorded at 77K. Fig. 13 records such a spectrum obtained from these substrate-depleted membranes prepared as described below, after supplementation with protochlorophyllide and incubated in darkness for 20min in the presence or absence of NADPH. The spectrum shows maxima at approx. 638nm and at 652nm and a minimum at 631nm. This spectrum gives the absorption of the intermediate formed from the added protochlorophyllide and NADPH, which absorbs at 638 and 652nm and has been formed at the expense of a pigment absorbing at 631nm, the added protochlorophyllide. The additional positive peak in the difference spectrum at 673nm (Fig. 13) probably corresponds to the degradation of chlorophyllide occurring in these membranes over the 20min incubation period; this process was considerably decreased in the presence of the NADPH in the test sample.

Fig. 13 shows the spectrum obtained when membranes supplemented with NADPH plus protochlorophyllide incubated as above were illuminated at room temperature, then frozen in liquid N_2 and the spectrum read against that of a similarly incubated sample that received no illumination. The resulting light-minus-dark difference spectrum shows that, as with the complex $P_{638/652}$ formed from the endogenous pigment (Griffiths, 1975b), the inter-

Fig. 13. *Low-temperature (77 K) spectra of the formation and photoconversion of the photoactive complex synthesized from added protochlorophyllide by etioplast membranes* Substrate-depleted prolamellar-body membranes were prepared from exhaustively flashed etioplasts. Samples (1.32mg of protein/3.0ml) were then incubated in darkness for 20min in the presence and absence of NADPH and the difference spectrum (---) between the two samples was recorded at 77K in liquid N_2 . Another sample incubated with NADPH as above was subdivided into two; one sub-sample was illuminated for 20s with a 100W lamp and the other was kept in darkness, after which both were frozen immediately in liquid N_2 and the illuminated minus non-illuminated difference spectrum was recorded (—).

mediate formed from the exogenously added protochlorophyllide in the presence of NADPH completely disappears on illumination, its absorption being replaced by a new absorption peak at approx. 673nm, indicating the formation of chlorophyllide on illumination. Therefore as far as it is possible to show by spectroscopic means chlorophyllide synthesis from added protochlorophyllide involves the same intermediates as does the process proceeding from the endogenous pigment in either isolated etioplasts (Griffiths, 1975a) or whole leaves (Kahn *et al.*, 1970). Various postulates have been put forward in attempts at defining the nature of the photoactive pigment complex $P_{638/652}$, and its formation from the non-photoactive pigment absorbing at lower wavelength. These include the suggestion that the latter represents 'free' pigment, which on association with a specific protein-photoenzyme becomes the photoactive species (Granick & Gassman, 1970). Again, by analogy with the behaviour of isolated protochlorophyllide in different organic solvents (Seliskar & Ke, 1968) or in aqueous cholate (see

Fig. 14. Chlorophyllide synthesis by etioplast membranes under flash and continuous illumination

(a) Prolamellar-body membranes were incubated in the presence of saturating concentrations of protochlorophyllide, and NADPH and chlorophyllide synthesis was assayed by flash illumination (1 flash/20s) as in Fig. 5. (b) As in (a) but with continuous illumination through an interference filter transmitting maximally at 440nm to excite chlorophyllide synthesis. ↓ Light on. ↑ Light off. Initial incubation was set up without NADPH, this being added as indicated.

above), it has been widely suggested that the wavelength shift accompanying activation of protochlorophyll(ide) *in vivo* may be related to the aggregation of the pigment. However, despite superficially resembling the physiological activation process, the various spectra recorded during the aggregation of the pure pigment *in vitro* intersected at an isosbestic point at 673 nm, whereas the physiological activation does not give rise to such a feature. In fact, there is an accessory peak or shoulder at 638 nm in the absorption spectrum of the biosynthetic product (see Fig. 13). Again, of course, the pigment aggregated *in vitro* undergoes no change on illumination, in contrast with the enzymically formed complex, which gives chlorophyllide under these conditions. Brouers & Sironval (1975) have also succeeded in reproducing *in vitro* the spectroscopic shifts accompanying formation of complex $P_{638/652}$ by freeze-drying isolated holochrome samples. However, whether the changes reported correspond to the genuine formation of photoactive complex $P_{638/652}$ or are due to aggregation phenomena analogous to those described above is difficult to decide from the data.

Finally, it thus appears unlikely that pigment aggregation alone (Butler & Briggs, 1966; Mathis & Sauer, 1972) can account for the physiological NADPH-dependent activation reaction, especially in view of the fact that subunits of holochrome prepared by saponin treatment of barley leaves retain 'photoconvertibility' yet contain only a single protochlorophyllide molecule per 63 000 mol.wt. protein molecule (Henningsen & Kahn, 1971). Recent experiments using $[4\text{-}^3\text{H}]\text{NADPH}$ carried out in our laboratory (W. T. Griffiths & D. Wiles, unpublished

work) have indicated that the $P_{638/652}$ complex contains bound NADPH rather than reduced protein as immediate hydrogen donor for the photoreduction. It is concluded that within the photoactive complex there is direct juxtapositioning of the NADPH with the β -position of ring 4 of the protochlorophyllide, thereby facilitating the photoinduced hydrogen transfer.

The exact mechanism of photoreduction of the protochlorophyllide resulting from illumination of the photoactive complex has yet to be elucidated. The fact that the complete process can occur in less than 10 μs (Schopfer & Siegelman, 1969) implies that no dark reaction of significant lifetime can be involved. However, the reaction is not strictly photochemical, since it is greatly inhibited at low temperatures, e.g. only 40% phototransformation occurs in barley at -60°C (Smith & Beneitz, 1954), suggesting that the process must also involve some restricted molecular collisions (Boardman, 1966). It is probable that the photoconversion involves an initial, temperature-independent, photoexcitation of the pigment complex, followed by a hydrogen-transfer step showing a much higher temperature coefficient. Thus at temperatures between -120 and -40°C , Goedheer & Verhulsdonk (1970) were able to demonstrate fluorimetrically in whole leaves the formation of chlorophyll(ide) in the post-illumination period. Below this temperature presumably the dark reaction did not proceed or proceeded at a rate too low to measure, whereas at temperatures higher than -40°C the dark reaction was so fast as to be unresolved from the light step.

Despite this, however, attempts at resolving the light and dark steps, e.g. by illumination at low temperature and warming in darkness while monitoring chlorophyllide formation, have, with one exception (Sironval & Kuyper, 1972) consistently failed (Goedheer & Verhulsdonk, 1970; Canaani, 1975). The most probable reason for these failures is that the lifetime of the excited state produced in the light is too short to enable it to survive the time needed to warm up to the temperature where the hydrogen transfer can occur. We have repeated these experiments with isolated prolamellar-body membranes in the presence of a large excess of NADPH, but again failed to detect any evidence of dark chlorophyllide synthesis on warming to room temperature in darkness after illumination at 77 K (W. T. Griffiths & N. L. Morgan, unpublished work).

Regarding the validity of the flash-induced chlorophyllide assay as a measure of the physiological rate of regeneration of complex $P_{638/652}$, Fig. 14 shows recorder tracings of chlorophyllide synthesis by prolamellar-body membranes measured both under our normal standard flash-illumination conditions (Fig. 14a) and also under continuous illumination (Fig. 14b). Illumination in the latter case had to be

through a narrow band-pass interference filter to eliminate light-induced artefacts. The rate is somewhat slower in the continuous light, owing to a non-saturating light intensity. However, repeating the latter and varying the light intensity with neutral-density filters enables a rate extrapolated to saturating intensity to be calculated. The extrapolated rate (1.32 nmol/min per mg of protein) is always identical with the rate determined under saturating flash-illumination conditions, implying that no artefacts are introduced as a consequence of using flash illumination. The latter procedure, however, has the great advantage of not causing any photo-oxidation of pigments as occurs with continuous illumination, especially at the higher intensities. The comparison (Fig. 14) also shows that regeneration of complex $P_{638/652}$ in isolated membranes proceeds at the same rate in the light or in the dark. Despite this, however, it should be mentioned that a dramatic decrease in the regeneration activity is found in plastids isolated from dark-grown plants given a brief exposure (30 min) to continuous illumination immediately before etioplast isolation (Maplestone & Griffiths, 1977), a phenomenon that is largely reversed if the illumination is followed by a return of the plants to darkness before isolating the plastids.

As it is our belief that the complex- $P_{638/652}$ -regeneration reaction described here represents an obligate step in the route to chlorophyllide *a* in higher plants then it follows that the rate of this reaction measured *in vitro* must be adequate to account for the whole plant rate of chlorophyll synthesis *in vivo*. The rate of chlorophyll synthesis achieved by illumination of 7-day-old etiolated barley leaves was determined, per mg of total leaf protein, as described in the Experimental section by exposing etiolated plants to illumination and at intervals by taking samples for chlorophyll and protein assays. During illumination for 12 h the protein concentration varied between 1.66 and 1.96 mg/shoot, so for the purpose of calculation an average value of 1.8 mg of protein/shoot was taken. The chlorophyll content increased from zero to 0.027 mg/shoot in the 12 h of illumination, giving a mean rate of chlorophyll synthesis over the 12 h period of 0.023 nmol/min per mg of protein. For comparison the rate of the photo-reduction of protochlorophyllide to chlorophyllide, as assayed under our standard conditions in plastids isolated from the etiolated plants, was 2.7 nmol of chlorophyllide formed/min per mg of protein.

It should be mentioned that it is not strictly justifiable to compare these two values, e.g. the assay *in vivo* takes into account the total leaf protein, whereas in the rate *in vitro* only the etioplast protein is considered. Again, a comparison assumes that the rate measured in plastids from dark-grown plants *in vitro* is maintained on illumination of the plants; this may not be so (Maplestone & Griffiths, 1977).

Despite these shortcomings, however, the data illustrate that the activity of the complex $P_{638/652}$ -regenerating system as assayed *in vitro* under optimal substrate concentrations is adequate to account for the capacity of the whole plant for chlorophyll synthesis.

We therefore conclude that the system described here represents an addition to the list of enzymes of chlorophyll biosynthesis that are assayable *in vitro*. From its characteristics described here an appropriate name for the enzyme is NADPH-protochlorophyllide oxidoreductase. We have recently completed a survey of the distribution of this enzyme (W. T. Griffiths & D. Wiles, unpublished work) and found it in all the species investigated, including tissues capable of chlorophyll synthesis in the dark, such as gymnosperm cotyledons and various algae.

References

- Akoyunoglou, G. & Siegelman, H. W. (1968) *Plant Physiol.* **43**, 66–68
- Boardman, N. K. (1966) in *The Chlorophylls* (Vernon, L. P. & Seely, G. R., eds.), pp. 437–439, Academic Press, New York
- Brouers, M. (1972) *Photosynthetica* **6**, 415–423
- Brouers, M. & Sironval, C. (1975) *Plant Sci. Lett.* **4**, 175–181
- Butler, W. L. & Briggs, W. R. (1966) *Biochim. Biophys. Acta* **112**, 45–53
- Canaani, O. D. (1975) Ph.D. Thesis, University of California, Berkeley
- Dujardin, E. (1975) *Photosynthetica* **9**, 283–287
- Dujardin, E. & Sironval, C. (1970) *Photosynthetica* **4**, 129–138
- Ellsworth, R. K. & Hsing, A. S. (1974) *Photosynthetica* **8**, 228–234
- Gassman, M. L. (1973a) *Plant Physiol.* **51**, 139–145
- Gassman, M. L. (1973b) *Plant Physiol.* **53**, 590–594
- Goedheer, J. C. & Verhulsdonk, C. A. H. (1970) *Biochem. Biophys. Res. Commun.* **39**, 260–266
- Granick, S. & Gassman, M. (1970) *Plant Physiol.* **45**, 201–205
- Griffiths, W. T. (1975a) *Biochem. J.* **152**, 623–635
- Griffiths, W. T. (1975b) *FEBS Lett.* **49**, 196–200
- Griffiths, W. T. (1977) *Biochem. Soc. Trans.* **5**, 88–90
- Henningsen, K. W. & Kahn, A. (1971) *Plant Physiol.* **47**, 685–690
- Holden, M. (1961) *Biochem. J.* **78**, 359–364
- Houssier, C. & Sauer, K. (1969) *Biochim. Biophys. Acta* **172**, 476–491
- Johnson, M. J. (1964) in *Manometric Techniques and Related Methods for the Study of Tissue Metabolism* (Umbreit, W. W., Burris, R. H. & Stauffer, J. F., eds.), 4th edn., pp. 208–209, Burgess Publishing Co., Minnesota
- Jones, O. T. G. (1976) *Philos. Trans. R. Soc. London Ser. B* **273**, 207–225
- Jones, O. T. G. & Saunders, V. A. (1972) *Biochim. Biophys. Acta* **275**, 427–436

- Kahn, A., Boardman, N. K. & Thorne, S. W. (1970) *J. Mol. Biol.* **48**, 85–101
- Kaler, V. L. & Shlyk, A. A. (1962) *Biochemistry (Engl. Transl.)* **27**, 506–512
- Kirk, J. T. O. & Tilney-Basset, R. A. E. (1967) *The Plastids*, W. H. Freeman, San Francisco
- Liljenberg, C. (1974) *Physiol. Plant.* **32**, 208–213
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maplestone, R. E. & Griffiths, W. T. (1977) *Biochem. Soc. Trans.* **5**, 319–321
- Mathis, P. & Sauer, K. (1972) *Biochim. Biophys. Acta* **267**, 498–511
- Ogawa, T., Bovey, F. & Shibata, K. (1975) *Plant Cell Physiol.* **16**, 199–202
- Radmer, R. J. & Bogorad, L. (1967) *Plant Physiol.* **42**, 463–465
- Rebeiz, C. A. & Castelfranco, P. A. (1971a) *Plant Physiol.* **47**, 24–32
- Rebeiz, C. A. & Castelfranco, P. A. (1971b) *Plant Physiol.* **47**, 33–37
- Rebeiz, C. A. & Castelfranco, P. A. (1973) *Annu. Rev. Plant Physiol.* **24**, 129–172
- Rüdiger, W., Hedden, P., Kost, H. P. & Chapman, D. J. (1977) *Biochem. Biophys. Res. Commun.* **74**, 1268–1272
- Schopfer, P. & Siegelman, H. W. (1969) in *Prog. Photosynth. Res. Proc. Int. Congr. 1st* **11**, 611–617
- Seliskar, C. J. & Ke, B. (1968) *Biochim. Biophys. Acta* **153**, 685–691
- Sironval, C. & Kuyper, Y. (1972) *Photosynthetica* **6**, 254–275
- Smith, J. H. C. & Beneitz, A. (1954) *Plant Physiol.* **29**, 135–143
- Sundquist, C. (1970) *Physiol. Plant.* **23**, 412–424
- Sundquist, C. (1973) *Physiol. Plant.* **29**, 434–439
- Wellburn, A. R. (1976) *Biochem. Physiol. Pflanzen.* **169**, 265–271