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Substrate-Specificity Studies on Protochlorophyllide Reductase in Barley (*Hordeum vulgare*) Etioplast Membranes

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1. The substrate specificity of the enzyme protochlorophyllide reductase in barley (*Hordeum vulgare*) etioplasts was investigated. 2. It was shown that naturally occurring esterified protochlorophyllide and chemically prepared protochlorophyllide methyl ester are not substrates for the enzyme, suggesting an important role for the C-7 carboxylic acid group in binding of the porphyrin to the enzyme. 3. Removal of magnesium from the protochlorophyllide leads to inactivity of the compound as a substrate for the enzyme. However, activity can be restored by replacing the magnesium with zinc, whereas nickel, copper or cobalt failed to restore substrate activity. 4. Binding of the second substrate, NADPH, to the enzyme probably occurs through the 2′-phosphate group in the coenzyme.

Chlorophyll *a*, owing to its key role in photosynthesis, has been described as one of the most important compounds to life on earth. One of the unique reactions involved in chlorophyll *a* formation by higher plants is the reduction of ring D of the porphyrin protochlorophyllide, to give chlorophyllide *a*, the non-esterified precursor of chlorophyll *a*. This reaction is unusual in that it shows an absolute requirement for light that is absorbed by the protochlorophyllide. This phenomenon explains the absence of chlorophyll *a* from dark-grown etiolated higher plants. Simple plant forms, however, such as certain algae and gymnosperms, can synthesize chlorophyll *a* and become green in total darkness (Bogorad, 1976).

We remain largely ignorant of the mechanism of chlorophyll *a* formation in darkness. In contrast, however, an enzyme has recently been described in etiolated tissues of higher plants (Griffiths, 1975*a*, 1978) that can carry out the photoreduction of protochlorophyllide. This enzyme, NADPH–protochlorophyllide oxidoreductase (referred to in the present paper simply as 'protochlorophyllide reductase') catalyses the NADPH-dependent photoreduction of protochlorophyllide and is spectrophotometrically assayable by measuring the chlorophyllide accumulation induced by illumination in the presence of the two substrates, protochlorophyllide and NADPH (Griffiths, 1978). Evidence for two steps in the overall mechanism was proposed, these involving an initial light-independent reaction forming a photoactive enzyme–protochlorophyllide–NADPH ternary complex followed by the rapid photoinduced hydrogen transfer from the NADPH to the protochlorophyllide.

There is well-documented spectral evidence that a photoactive complex of protochlorophyllide is formed as an intermediate during chlorophyll formation by whole leaves (Shibata, 1957; Gassman & Bogorad, 1967; Kahn *et al.*, 1970; Sundquist, 1970; Dujardin & Sironval, 1970). However, owing to the nature of the experimental material, it has only been possible to speculate on the nature of the photoactive complex *in vivo*. Thus both pigment–protein (Boardman, 1966) and pigment–pigment interactions (Seliskar & Ke, 1968; Mathis & Sauer, 1972) have been proposed to account for the structure of the photoactive intermediate *in vivo*. Further, protochlorophyllide alone (Sironval *et al.*, 1965; Godnev *et al.*, 1968) or both protochlorophyll and protochlorophyllide (Godnev *et al.*, 1963; Lancer *et al.*, 1976; Sasa & Sughara, 1976) have been described as the photoconvertible pigments in experiments with cells or whole tissue, perhaps further illustrating the limitations of the *in vivo* approach.

There is every indication (Griffiths, 1978) that the mechanism of the enzymic photoreduction *in vitro* involves reactions identical with those occurring *in vivo*, so that conclusions reached about the former should be equally applicable to the process *in vivo*. Conclusions drawn from work with the isolated system described in the present paper thus provide evidence for the photoreductive mechanism occurring *in vivo*. Results are presented suggesting that esterified protochlorophyll is unlikely to be a chlorophyll

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precursor. Further, evidence obtained while studying photoconvertibility of chemically modified analogues of protochlorophyllide suggests that pigment–pigment interaction alone, that is, aggregation, is not sufficient to account for the structure of the photoactive intermediate.

Experimental

Biological material

Etiolated barley (*Hordeum vulgare* L., cultivar Proctor), 7 days old, was cultivated as previously described (Griffiths, 1975*a*). Etioplasts were isolated from this tissue by differential centrifugation of a leaf homogenate as described previously (Griffiths, 1975*a*,*b*). Prolamellar-body membranes and substrate-depleted prolamellar-body membranes were prepared respectively from etioplasts, and etioplasts flashed exhaustively in the presence of an NADPH-regenerating system, by water-lysing the organelles followed by centrifugation as described previously (Griffiths, 1978).

Chemicals

The reagents used in the various isolation buffers and for enzymic analyses were from the same sources as previously described (Griffiths, 1975*a*), as also were the various thin-layer adsorbents used here. Organic solvents were dried and redistilled immediately before use by routine procedures.

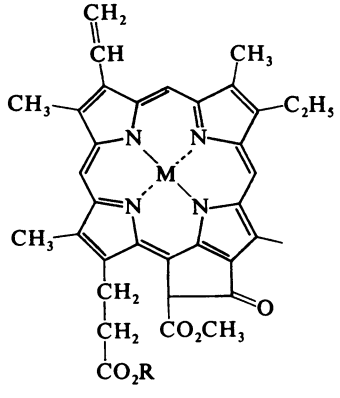
Preparation of substrates

Protochlorophyllide (I) and protochlorophyll (II) (Fig. 1) were isolated from etiolated-barley-leaf tissue. The tissue was extracted by homogenization in acetone in a cold-room under a green light. Total lipid in the extract was transferred into ether and protochlorophyllide separated from this solution by phase separations into aq. methanol/10mM-NH_3 and finally washed, concentrated and stored in ether as described previously (Griffiths, 1978).

Protochlorophyll remaining in the original ether extract was isolated by preparative t.l.c. after first evaporating off the ether and redissolving the oily lipid residue in cyclohexane to a concentration suitable for chromatography. Initial chromatography of the mixture was carried out on preparative (0.5mm) plates coated with a mixture of kieselguhr/Kieselgel/$CaCO_3$/$Ca(OH)_2$ (200:500:500:3, by wt.) and developed with light petroleum (b.p. 60–80°C) propan-2-ol/water (400:20:1, by vol.) as previously described (Griffiths, 1975*b*). Final purification of the eluted protochlorophyll was carried out by preparative rechromatography on thin (0.5mm) layers of cellulose (Whatman) developed with light petroleum/acetone/propan-1-ol (1800:200:9, by vol.). In this system protochlorophyll migrated with an R_F of approx. 0.6 and could be eluted into ether free of any spectroscopic impurities. The pigment was stored in a desiccated state, anaerobically at −20°C. This product was spectroscopically identical with protochlorophyllide isolated from the same tissue, with principal wavelength absorption maxima in ether at 623 and 432nm (Fig. 3 below).

Protochlorophyllide methyl ester (*III*). This was prepared from protochlorophyllide by reaction with diazomethane as described by Seely (1966). The product was purified and separated from unchanged protochlorophyllide by chromatography on paper (Whatman no. 1) developed with chloroform. In this system the esterified product migrated with an R_F of approx. 0.9 compared with the unchanged free acid, which remained at the origin. The sample eluted from the paper was finally desiccated *in vacuo* and stored at −20°C.

2-*Vinylphaeoporphyrin* a_5 (*IV*). An ether solution of protochlorophyllide (approx. 0.5mg in 25ml of ether) was shaken vigorously with 10ml of 25% (w/v) HCl and immediately neutralized with solid



		M	R
(I)	Protochlorophyllide	Mg	H
(II)	Protochlorophyll	Mg	?Phytol
(III)	Protochlorophyllide methyl ester	Mg	CH_3
(IV)	2-Vinylphaeoporphyrin a_5	—	H
(V)	2-Vinylphaeoporphyrin a_5 zinc(II)	Zn	H
(VI)	2-Vinylphaeoporphyrin a_5 copper(II)	Cu	H
(VII)	2-Vinylphaeoporphyrin a_5 nickel(II)	Ni	H
(VIII)	2-Vinylphaeoporphyrin a_5 cobalt(II)	Co	H

Fig. 1. *Structural formulae of protochlorophyllide and some of its derivatives*

Table 1. *Spectral characteristics of porphyrin substrates and expected absorption properties of the corresponding chlorins*

	Compound	Absorption maxima (nm)			
		Porphyrin		Chlorin (expected)	
		In methanol	*In vivo*	In methanol	*In vivo*
(I)	Protochlorophyllide	623	632	663	672
(II)	Protochlorophyll	623	632	663	672
(III)	Protochlorophyllide methyl ester	623	632	663	672
(IV)	2-Vinylphaeoporphyrin a_5	638	646	678	686
(V)	2-Vinylphaeoporphyrin a_5 zinc(II)	621	626	661	669
(VI)	2-Vinylphaeoporphyrin a_5 copper(II)	602	608	642	648
(VII)	2-Vinylphaeoporphyrin a_5 nickel(II)	594	600	634	639
(VIII)	2-Vinylphaeoporphyrin a_5 cobalt(II)	601	606	641	646

sodium acetate. The purple phaeoporphyrin solution was washed free of acid, and evaporated to dryness by distillation *in vacuo*. The residue, when dissolved in methanol, exhibited spectral properties identical with those of authentic phaeoporphyrin (Jones, 1966) (see Table 1).

Zinc and copper derivatives of the phaeoporphyrin (V and VI). These were prepared by the metal acetate method as described by Fuhrhop & Smith (1975). The phaeoporphyrin (about 250μg) in methanol was evaporated to dryness in a round-bottom flask and redissolved in 5ml of boiling chloroform. Saturated zinc acetate or copper acetate (1ml) was added and the mixture refluxed for 2 and 5min respectively, followed by removal of the chloroform by rotary evaporation *in vacuo*. The residues were solubilized in a trace of pyridine, diluted with ether, washed with water and dried over anhydrous Na_2SO_4, and finally after evaporating to dryness resolubilized in methanol and used without further purification.

Nickel and cobalt derivatives of the phaeoporphyrin (VII and VIII). These were prepared as described above, except that before the refluxing in the presence of the corresponding metal acetate, 2ml of acetic acid was added and the flasks were flushed with N_2. Refluxing with the cobalt acetate was continued for 30min, whereas 50min refluxing was necessary for preparing the nickel porphyrin from the nickel acetate (Boucher & Katz, 1967).

Two different methods were used of presenting the various compounds for enzymic photoconversion. Solubilization in cholate (Griffiths, 1978) was found to be a suitable procedure for presenting protochlorophyll, protochlorophyllide, protochlorophyllide methyl ester and the phaeoporphyrin derivative of protochlorophyllide. The various metal derivatives of protochlorophyllide, however, could only be used as solutions in methanol, a technique that, as far as the enzyme is concerned, suffers no disadvantages compared with cholate solubilization (W. T. Griffiths, unpublished work).

Assay of protochlorophyllide reductase activity

Continuous recording of flash-induced reduction of the various substrates was carried out by using a dual-wavelength spectrophotometer equipped with a xenon photographic lamp as described previously (Griffiths, 1978). The reference wavelength in all cases was kept at 710nm, but the measuring beam was varied to coincide with the expected wavelength of maximum absorption of the different photoreduced products. Positions of the latter were estimated from the spectra of the original substrates (see Table 1), assuming, by analogy with the protochlorophyllide-to-chlorophyllide transformation, that photoreduction of the various metalloporphyrins and derivatives is accompanied by a hypsochromic shift of the α-band by approx. 40nm.

As a matter of routine, absorption spectra of the incubation mixtures before and after flash illumination in the dual-wavelength spectrophotometer, were recorded as described previously (Griffiths, 1975*a*,*b*), on a split-beam spectrophotometer. This was done to provide confirmation of whether any reaction had occurred with various substrates, to support the kinetic data.

Incubations were always carried out in the assay buffer previously described (Griffiths, 1978). Assays were carried out with substrate-depleted membranes or, in some instances, whole etioplasts. In the latter case an optimal concentration of Triton X-100 (usually 0.3mM; the exact value was predetermined for each membrane preparation) was added to overcome any permeability barriers to the access of substrates. A regenerating system, made up of 0.25mM-$NADP^+$, 5 units of glucose 6-phosphate dehydrogenase and 5mM-glucose 6-phosphate was always used as a source of NADPH unless indicated otherwise.

Protein assays

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

The etiolated-barley total lipid fraction, on partitioning between ammoniacal aqueous methanol and ether/light petroleum (1 : 1, v/v) gave a lower green polar phase and an intense-orange–brown highly pigmented non-polar upper phase. Spectral analysis revealed (Fig. 2) the presence of the characteristic protochlorophyllide absorption with a peak in the red at approx. 623 nm in both fractions. The absorbing pigment in the more polar layer has already been identified as non-esterified protochlorophyllide, which is readily purified from this fraction (Griffiths, 1978). The more hydrophobic component in the upper phase corresponds to esterified protochlorophyllide or protochlorophyll, the purification of which represents a more formidable task, owing to the large amounts of other compounds present in this fraction. The two components are present in 9-day-old etiolated barley to the extent of 1250 nmol of protochlorophyllide and 320 nmol of protochlorophyll/kg fresh weight.

Repeated t.l.c. of the non-polar fraction yielded a spectroscopically pure form of the protochlorophyll displaying absorption characteristics (Fig. 3) in ether indistinguishable from those already published for pure protochlorophyllide (Griffiths, 1978; Jones, 1969). A typical final yield of pure protochlorophyll is 250 nmol recovered from about 2.5 kg fresh weight of etiolated barley. Solubilization of this sample in cholate gave a crystal-clear solution of the pigment that initially adsorbed maximally in the red at 630 nm (Fig. 3*b*). On prolonged standing the position of this absorption maximum shifted to about 646 nm (Fig. 3*c*) in a manner analogous to the previously reported behaviour of protochlorophyllide (Griffiths, 1978), except that the shift with protochlorophyll is much slower, taking approx. 12 h for completion.

On testing this sample as a substrate for protochlorophyllide reductase in the chlorophyll(ide) synthesis assay using substrate-depleted etioplast membranes and a supply of NADPH (Fig. 4), no increase in absorbance at 672 nm results on flash-illumination, indicating no synthesis of chlorophyll(ide). Subsequent addition of cholate-solubilized protochlorophyllide to the incubation mixture, however, gave a large flash-induced absorbance increase at 672 nm, due to the formation of chlorophyllide. This occurs at a rate of 4.20 nmol/min per mg of protein, which is identical with the control rate given by this particular membrane preparation, when supplemented with protochlorophyllide but in the absence of protochlorophyll. Protochlorophyll therefore, besides not being utilized for chlorophyll synthesis by the membranes, does not in addition inhibit the photoreduction of added protochlorophyllide.

Diazomethane treatment of protochlorophyllide results, as expected, in a product that was less polar than free protochlorophyllide during chromatography with the paper-chromatographic system mentioned in the Experimental section. Preparative purification of the derivative by this same chromatographic system gave a preparation of the methyl ester displaying spectroscopic properties identical with those of pure protochlorophyllide. Solubilization of the ester in aqueous cholate buffer, as was the case with the free acid, produced a clear solution with a red absorption maximum at around 630 nm.

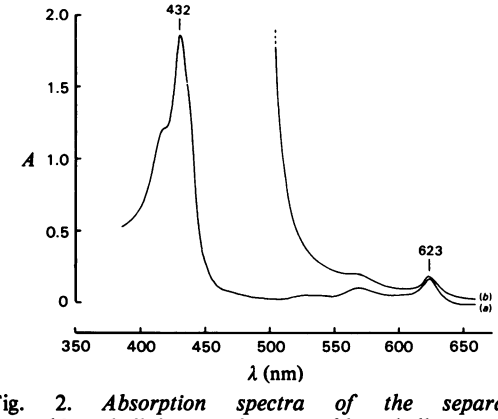


Fig. 2. *Absorption spectra of the separated protochlorophyllide- and protochlorophyll-containing phases of barley lipids*

The total lipids from etiolated barley were separated into polar (*a*) and non-polar (*b*) phases as described in the text, and their absorption spectra were recorded in ether with a Unicam SP. 800 spectrophotometer.

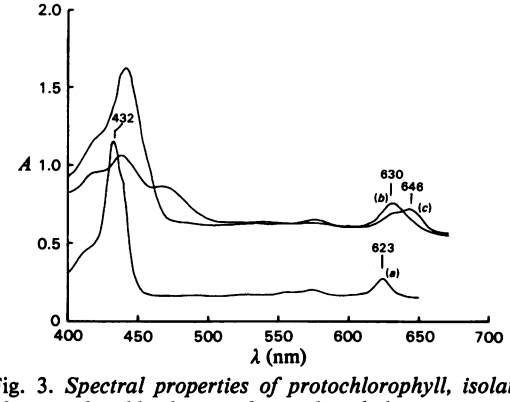


Fig. 3. *Spectral properties of protochlorophyll, isolated from etiolated barley, in ether and in cholate suspension*

Ether (*a*) and cholate (*b*) solutions of highly purified barley protochlorophyll were prepared as described in the text and their absorption spectra recorded. The spectrum of the cholate solution was also re-recorded (*c*) after standing for 12 h to allow aggregation of the pigment.

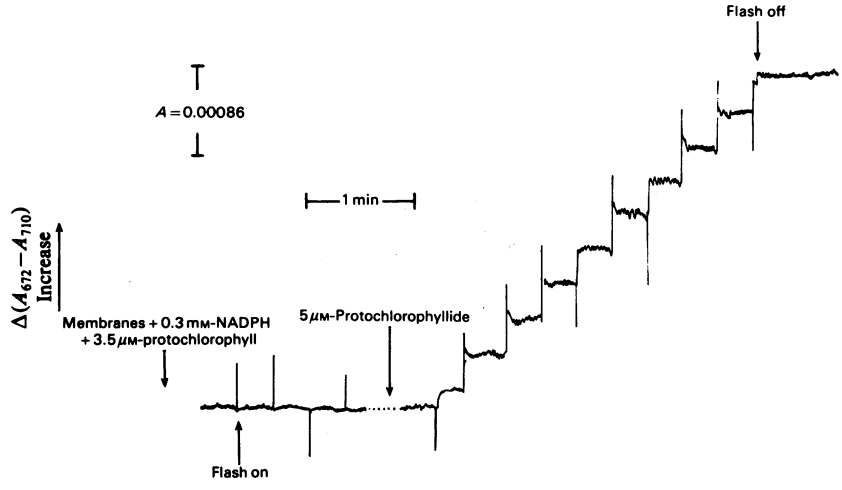


Fig. 4. *Protochlorophyll from barley as a substrate for protochlorophyllide reductase*
Substrate-depleted etioplast membranes, prepared from etioplasts that had been flashed at 1 flash/20 s for 15 min, were incubated in a dual-wavelength-spectrophotometer cuvette and the absorbance changes at 672 nm minus 710 nm recorded with the addition of an NADPH-regenerating system (0.25 mM-$NADP^+$, 5 units of glucose 6-phosphate dehydrogenase and 5 mM-glucose 6-phosphate) and cholate-solubilized protochlorophyll and protochlorophyllide as indicated. Flash-illumination of the sample, to promote photoreduction, was through a 1 cm saturated solution of $CuSO_4$ from above, with the photomultiplier protected from actinic light by a layer of Kodak Wratten no. 70 gelatin filter.

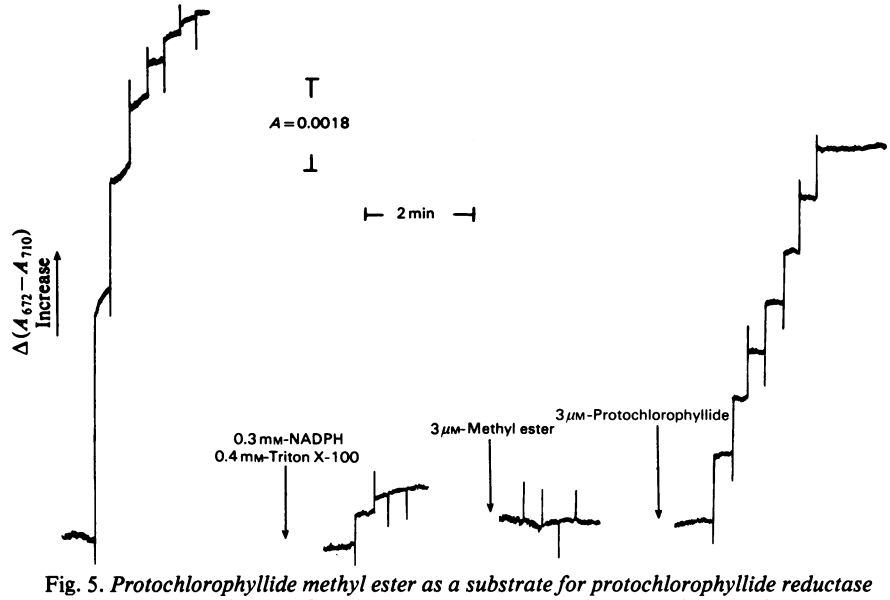


Fig. 5. *Protochlorophyllide methyl ester as a substrate for protochlorophyllide reductase*
The assay was performed essentially as for Fig. 4, except that whole, non-illuminated etioplasts were used as the source of enzyme. These were flash-illuminated to photoconvert the endogenous photoactive pigment, after which 0.4 mM-Triton X-100 and NADPH as in Fig. 4 were added, followed by further flash-illumination to photoconvert endogenous non-photoactive pigment. The methyl ester was then added and flash-illuminated, followed finally by addition of authentic protochlorophyllide.

Aggregation of the pigment in this solution was also evident from the shift in the absorption maximum with time from 630 to 650 nm; however, the rate of this process appeared qualitatively to be much slower than cholate aggregation of the free acid under similar conditions, but faster than aggregation of the protochlorophyll.

Fig. 5 records the results of the attempted enzym-

ic photoreduction of the methyl ester, using isolated etioplasts as the source of the reductase. On flash illumination of the etioplasts in the absence of any additions, chlorophyll is formed in the first few flashes from endogenous photoactive pigment. After approx. five flashes, this is complete. Addition of a supply of NADPH and Triton X-100 at this point results in further small amount of chlorophyll formation on resumption of flash illumination, this utilizing non-photoactive pigment originally present in the membranes. However, again this supply is exhausted after a few flashes. Addition of cholate-solubilized protochlorophyllide methyl ester followed by switching on the flash-lamp failed to restore any photoproduction of chlorophyll indicated by the absence of any flash-induced absorbance increase (Fig. 5). Addition of protochlorophyllide at this stage, however, resulted in rapid chlorophyll synthesis, this proceeding at a rate comparable with control assays performed in the absence of the methyl ester. This implies that, as was the case with protochlorophyll (Fig. 4), the methyl ester is neither photoreduced by the enzyme nor does it inhibit the photoreduction of protochlorophyllide.

The importance of the metal in protochlorophyllide on its activity as a substrate of chlorophyll formation was checked by chemically removing the magnesium and replacing it with other metals and testing the resulting derivatives in the protochlorophyllide reductase assay. Photoreduction was monitored at the wavelengths indicated in Table 1. The synthetic metalloporphyrins resulting from these procedures were found to be poorly solubilized by cholate; therefore additions to the etioplasts had to be made as methanolic solutions. In this respect it was ascertained that methanol up to 5% (v/v) could be tolerated by the enzyme with no adverse effect on its activity. In practice the amounts of methanol used here were always well below this concentration. Again, the assay of each substrate was followed by addition of protochlorophyllide to the assay solution to note the effect of the derivative under test on the normal enzymic activity.

Attempts at the photoreduction of cholate-solubilized 2-vinylphaeoporphyrin a_5 by etioplasts were monitored at 686−710nm. Fig. 6 records the results of one such experiment. Addition of an NADPH-regenerating system and Triton X-100 to isolated etioplasts followed by flash illumination gives incremental absorbance increases at 672nm with each flash, resulting from chlorophyll formation from the endogenous pigment. After exhaustion of this substrate, cholate-solubilized 2-vinylphaeoporphyrin a_5 was added, and the monitoring wavelength changed to 686nm. No absorbance increase at this wavelength results on resumption of flash-illumination, suggesting the lack of photoreduction of this substrate. Addition of cholate-solubilized protochlorophyllide, however, and the return to 672nm as monitoring wavelength, shows that this substrate can still be photoreduced. Calculation of this final rate, however, shows that the presence of the phaeoporphyrin inhibits protochlorophyllide photoreduction by the enzyme. In this particular experiment, protochlorophyllide photoreduction occurred at a rate of 0.55nmol/min per mg of protein for the process in the presence of the phaeoporphyrin. This implies that 2-vinylphaeoporphyrin a_5 is not a substrate for protochlorophyllide reductase and that, furthermore, this compound appears to inhibit protochlorophyllide photoreduction by the enzyme. Confirmation of the lack of photoconversion of this substrate was obtained by comparing the absorption spectrum of the phaeo-

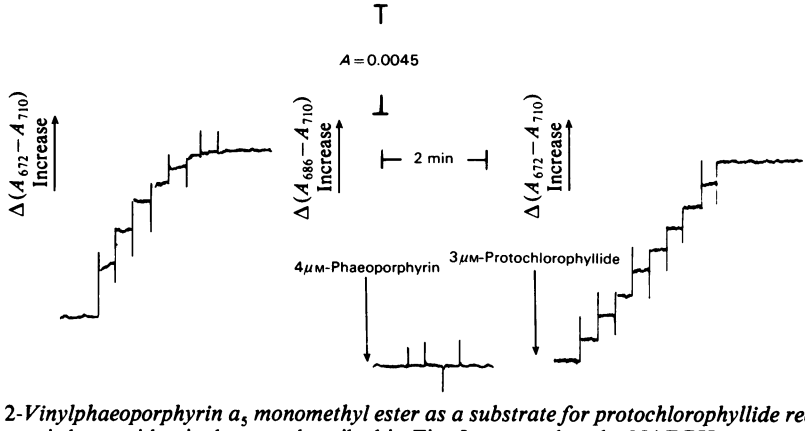


Fig. 6. 2-*Vinylphaeoporphyrin a_5 monomethyl ester as a substrate for protochlorophyllide reductase*
The assay was carried out with etioplasts as described in Fig. 5, except that the NADPH-regenerating system and Triton X-100 were added before any flash-illumination and the monitoring wavelengths changed as indicated during attempts at monitoring photoreduction of the phaeoporphyrin.

porphyrin-absorbance supplemented etioplasts before and after the flash-illumination (Fig. 7). No significant light induced spectroscopic changes are evident in the 686nm region, confirming the kinetic data of Fig. 6. In fact the spectra recorded in Fig. 7 suggest that illumination of chlorophyll-containing etioplasts in the presence of added phaeoporphyrin results in breakdown of the original chlorophyll, since this procedure gives rise to a decreased A_{672} (Fig. 7; compare curves *b* and *c*).

The zinc analogue of protochlorophyllide possesses light-absorption characteristics quite similar to normal protochlorophyllide, with a red absorption maximum in methanol at 621nm. Consequently its photoreduced product might be expected to absorb *in vivo* at approx. 669nm. On monitoring absorbance changes at 672nm of etioplasts in the presence of NADPH and Triton X-100 (Fig. 8), flash-illumination, as expected, results in photoconversion of all the endogenous protochlorophyllide. On addition of the zinc analogue of protochlorophyllide and slightly resetting the monitoring wavelength to 669nm, restoration of the flash-illumination gives a linear increase in absorbance at this wavelength, indicating the possible photoreduction of this derivative. From the extent of this increase, a rate of photoreduction of 1.82nmol/min per mg of protein may be calculated for the zinc derivative by assuming that the absorption coefficients for the zinc and magnesium derivatives are similar.

Confirmation of the photoreduction of the zinc protochlorophyllide analogue is evident from a comparison of the absorption spectrum before and after illumination (Fig. 9). Before illumination, the substrate-depleted membranes on supplementation with the zinc analogue of protochlorophyllide shows absorption maxima at 672nm due to the chlorophyllide formed from endogenous substrates, and also at 626nm as a result of the added zinc analogue (Fig. 9, curve 1). After flash-illumination, in agreement with the kinetic data, a decrease in absorption at approx.. 626nm accompanied by a corresponding increase at approx. 669nm, the absorption maximum of the zinc chlorin *in vivo*, is evident (Fig. 9, curve 2).

In contrast with the positive results obtained with the zinc derivative of protochlorophyllide, none of the remaining metal analogues of protochlorophyllide tested showed any activity in the protochlorophyllide reductase assay. Thus, in the continuous

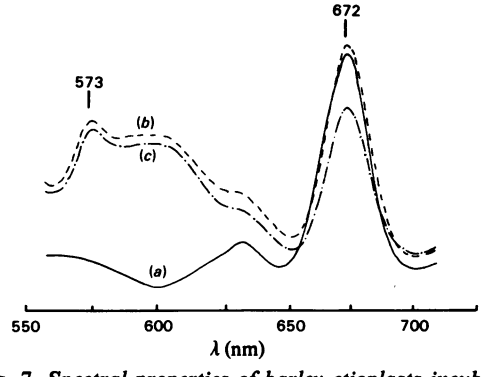


Fig. 7. *Spectral properties of barley etioplasts incubated and flash-illuminated in the presence of 2-vinylphaeoporphyrin a_5*

Pre-illuminated, substrate-depleted barley etioplasts (*a*) were supplemented with a cholate solution of 2-vinylphaeoporphyrin a_5 and the spectrum re-recorded before (*b*) and after (*c*) flash-illumination for 5min at one flash per 20s in the presence of an NADPH supply.

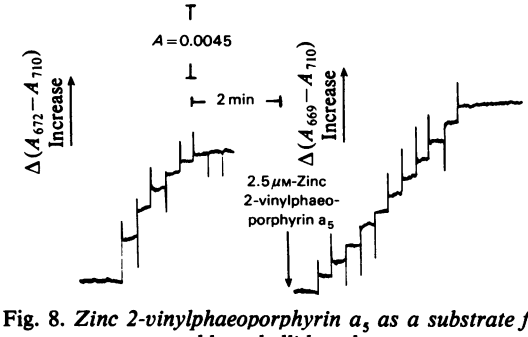


Fig. 8. *Zinc 2-vinylphaeoporphyrin a_5 as a substrate for protochlorophyllide reductase*

The assay was carried out as described in Fig. 6. A methanolic solution of the zinc derivative was added as indicated.

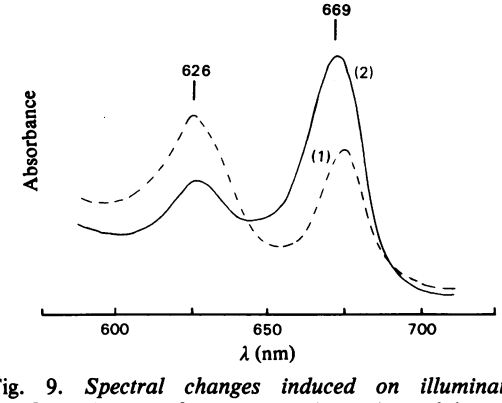


Fig. 9. *Spectral changes induced on illumination of etioplasts in the presence of zinc 2-vinylphaeoporphyrin a_5*

Preilluminated substrate-depleted barley etioplasts were supplemented with a methanolic solution of zinc 2-vinylphaeoporphyrin a_5 and the spectrum recorded (1). An NADPH supply was then added and the sample flash-illuminated for 5min at one flash per 20s as in Fig. 7; finally the spectrum was re-recorded (2).

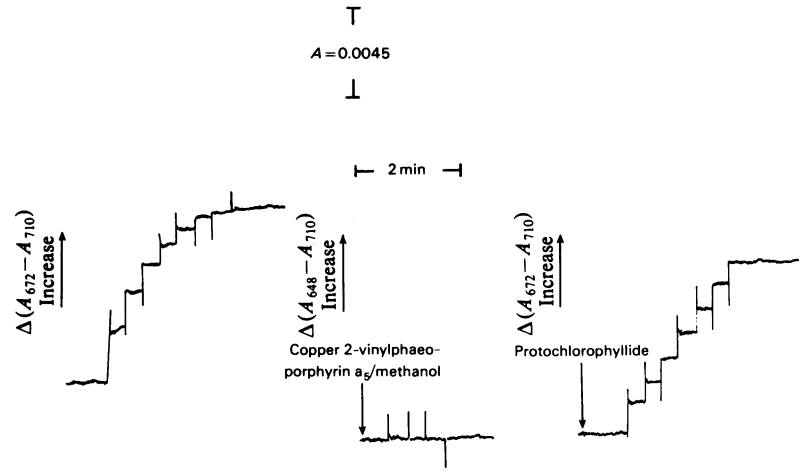


Fig. 10. *Assay of protochlorophyllide reductase in the presence of copper 2-vinylphaeoporphyrin* a_5
The copper porphyrin dissolved in methanol was added to etioplasts and enzymic photoreduction monitored by flash-illumination as described in Fig. 6, except that the measuring wavelengths were set at 648 nm minus 710 nm. Finally cholate-solubilized protochlorophyllide was added and, after changing the measuring wavelengths to 672 nm minus 710 nm, photoreduction of this substrate was monitored.

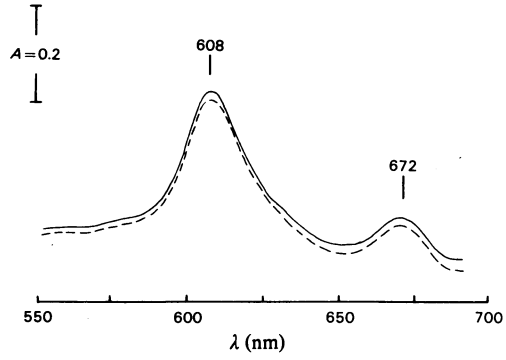


Fig. 11. *Spectral characteristics of etioplasts illuminated in the presence of copper 2-vinylphaeoporphyrin* a_5
Etioplasts were flash-illuminated to photoconvert their endogenous substrates, then the copper derivative was added and the absorption spectrum recorded before (——) and after (– – – –) flash-illumination at one flash per 20 s for 5 min.

kinetic assay, no increase in absorption was seen at the expected wavelengths of metal chlorin absorption for the copper, nickel or cobalt 2-vinylphaeoporphyrin a_5 supplemented incubations. Further, no differences were noted between the spectra of the incubation mixtures containing these derivatives recorded before and after flash-illumination.

This is illustrated for the case of the copper derivative of protochlorophyllide in Figs. 10 and 11. Reference to Table 1 shows that copper 2-vinylphaeoporphyrin a_5 absorbs maximally at approx. 602 nm in methanol, this shifting to approx. 608 nm on adsorption to the membranes. Consequently reduction should result in a product absorbing *in vivo* at approx. 650 nm. In the kinetic trace of Fig. 10, illumination of isolated etioplasts in the presence of NADPH and Triton X-100 results in chlorophyllide formation from endogenous substrates, as noted from the absorbance increase at 672 nm. After about seven flashes the endogenous substrate supply becomes exhausted. Supplementation of the etioplasts at this stage with a methanolic solution of copper protochlorophyllide and changing the monitoring wavelength to 648 nm, followed by resumption of the flash-illumination, produced no increase at this wavelength, indicating no photoreduction of this derivative. Addition of cholate-solubilized protochlorophyllide at this point, however, followed by a return of the monitoring wavelength to 672 nm, results in an increase in absorbance at this wavelength, indicating photoreduction of the protochlorophyllide in the presence of the copper derivative. The final rate of protochlorophyllide reduction calculated from the absorbance increase was 0.73 nmol/min per mg of protein compared with a control rate of 1.85 nmol/min per mg of protein obtained with this substrate in the absence of added copper protochlorophyllide, indicating, as already observed with the metal-free analogue, an inhibition of protochlorophyllide photoreduction with the copper derivative.

In agreement with the kinetic findings, the absorption spectrum of the etioplast suspension incubated and illuminated with added copper protochlorophyllide plus NADPH and Triton X-100

possesses absorption peaks (Fig. 11) corresponding to chlorophyllide at 672nm, formed from endogenous protochlorophyllide, and at approx. 608nm, corresponding to the added (but non-photoconverted) copper protochlorophyllide. Comparison of this spectrum with one recorded after copper protochlorophyllide addition but before the second period of illumination in the presence of this substrate, indicated that the addition of this substrate gave no illumination-induced absorbance changes. As mentioned above, qualitatively similar results were obtained for the other metal derivatives tested; i.e., neither the nickel nor the copper derivatives could be photoreduced by the enzyme, whereas both inhibited the rate of protochlorophyllide reduction to varying extents.

Regarding reductants (other than NADPH) that can be utilized by protochlorophyllide reductase, Fig. 12 illustrates that the structurally related NADH even at very high concentrations shows no activity in the reaction. Addition of NADH (2.5mM) to substrate-depleted etioplasts in the presence of protochlorophyllide and Triton X-100 failed, on flash-illumination, to photoreduce any of the protochlorophyllide as estimated from the flash-induced absorbance change at 672nm relative to 710nm. Subsequent addition of NADPH (0.25mM), however, produced the characteristic flash-induced absorbance increase at these wavelengths, due to chlorophyllide formation, thereby confirming previous data (Griffiths, 1975*a*,*b*) obtained from a non-continuous assay of chlorophyllide formation. Further, a comparison of the rate of reductase activity assayed with NADPH in the presence and absence of NADH indicates that the latter has no effect of the NADPH-induced activity.

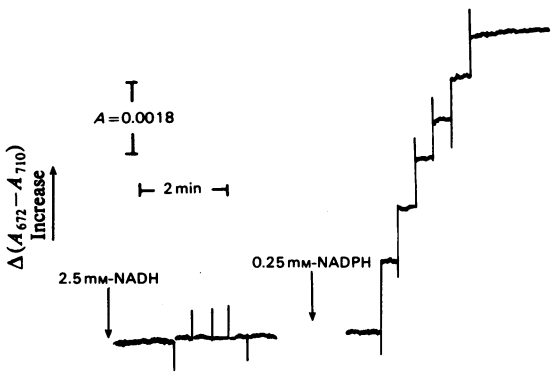


Fig. 12. *NADH and NADPH as hydrogen donors for protochlorophyllide reductase*

Barley etioplast membranes supplemented with cholate-solubilized protochlorophyllide were pre-flash-illuminated as described in Fig. 4 to photoconvert any endogenous photoactive complex; this was followed by addition of solutions of the reduced coenzymes NADH and NADPH, as indicated, while illumination-induced absorbance changes at 672nm minus 710nm were monitored.

Discussion

Chlorophyll *a*-containing tissues of higher plants can readily achieve the reduction of a porphyrin, i.e. protochlorophyllide, to a chlorin, i.e. chlorophyllide, during the normal course of chlorophyll synthesis. Two separate mechanisms appear to be available to plants for carrying out this reduction, namely (1) a reduction involving light, which occurs in higher plants and in some algae and gymnosperms, and (2) a purely enzymic reduction, which is confined to some algae and gymnosperms only. In contrast, the chemical or photochemical reduction of porphyrins *in vitro* is far more difficult to carry out, especially for labile complex porphyrins like protochlorophyllide. In fact, significant progress in the photochemical reduction of protochlorophyllide lagged behind the complete chemical synthesis of this compound by several years (see Fischer & Stern, 1940; Suboch *et al.*, 1970).

The work described in the present paper concerns the substrate specificity of the recently identified enzyme protochlorophyllide reductase (Griffiths, 1978). The enzyme has been shown to catalyse the photoreduction of protochlorophyllide to chlorophyllide *in vitro*, and normally functions in the biosynthetic pathway leading to chlorophyll *a*. In contrast with the strictly photochemical procedures for porphyrin reduction that have been described and that produce variable yields of reduced products, enzymically quantitative photoconversion of protochlorophyllide into chlorophyllide as the sole product can be achieved. Again, as expected, enzymic photoreduction gives a *trans* chlorin, whereas ascorbic acid in pyridine effects the photochemical reduction, protochlorophyll giving a *cis* chlorin (Gurinovich *et al.*, 1972).

There is still some controversy as to the substrate used for the enzymic photoreduction during chlorophyll synthesis in higher plants. It might be expected that, because of the obligate light requirement for photoreduction, if higher plants are kept in darkness, a limited build-up of the chlorophyll precursor preceding the light reaction should occur. In practice, however, dark-grown plants accumulate various amounts of both non-esterified protochlorophyllide and protochlorophyllide ester in darkness, both of which have been implicated in photoreduction. These two compounds further possess identical light-absorption characteristics and are distinguished solely on the basis of chromatographic mobilities.

Although it is widely assumed that the esterifying alcohol in protochlorophyllide ester is C_{20} phytol, in our experience with the material from etiolated

barley we have routinely failed to confirm this. Thus, despite several attempts, we have been unable to identify phytol as one of the hydrolysis products of barley protochlorophyllide ester. In one such experiment, rigorously purified (by t.l.c., see the Experimental section) protochlorophyllide ester (approx. 60μg) was subjected to mild alkaline hydrolysis and the ester group recovered by ether extraction. On analysis of this fraction by t.l.c. (adsorption and partition, see the Experimental section) along with various isoprenoid-alcohol marker compounds (geraniol, farnesol, geranylgeraniol and phytol) on no occasion was there observed in the ether extract a compound with chromatographic properties comparable with the authentic samples. Further, extensive mass-spectrophotometric analysis of the hydrolysis products of the ether fraction once more failed to produce any mass ions characteristic of an identifiable isoprenoid alcohol. Despite our failure with the protochlorophyllide ester from barley, however, when identical hydrolytic and identification procedures were repeated with purified chlorophyll *a*, isolated and purified from spinach (*Spinacia oleracea*) leaves by methods identical with those used for protochlorophyllide ester isolation (see the Experimental section), then phytol was very easily identified, even starting with as small an amount as 10μg of pure chlorophyll *a*.

Despite the lack of positive identification of the esterifying alcohol in barley protochlorophyllide ester, it is considered justifiable to mention this experience here, if only to endorse similar doubts on this point that have already been raised previously (see Fischer & Rudiger, 1959; Rebeiz & Castelfranco, 1973). In contrast with the uncertainty of the exact chemical nature of the protochlorophyllide ester, there is no doubt at all that this compound is not photoreduced to chlorophyll by the reductase in barley etioplast membranes (Fig. 4) under conditions when the non-esterified protochlorophyllide is efficiently photoreduced. It is also noteworthy that the presence of the ester at the concentration used (0.5μM) in no way inhibited photoconversion of subsequently added protochlorophyllide. It was not possible to check inhibition by higher concentrations of the ester, since problems of light limitations are encountered at high pigment concentrations.

Conclusions from these data include the possibility that a bulky ester group on the protochlorophyllide ester could sterically hinder the reduction of the C_7–C_8 double bond, in agreement with the suggestion, based on theoretical considerations of molecular dimensions, made by Godnev *et al.* (1968). Alternatively, a free carboxy group on the porphyrin might be an essential requirement for the binding of the substrate to the enzyme. The failure of the synthetic methyl ester of protochlorophyllide to be photoreduced (Fig. 5) would tend to favour the latter suggestion, as it is unlikely that a single methyl group would confer any significant steric hindrance to reduction. As with the native protochlorophyllide ester (Fig. 4), the presence of the synthetic methyl ester of protochlorophyllide did not interfere with the reduction of subsequently added non-esterified protochlorophyllide (Fig. 5). It thus seems very likely that the free C_7 carboxy group is essential for formation of the enzyme–substrate complex, either by H-bonding or salt linkage. Esterification of this group results in failure of the ester to bind, making it inactive as a substrate and, in addition, rendering it unable to interfere with the binding of the true substrate and thereby not inhibiting reduction of the latter. Therefore, as far as it is permissible to extrapolate from studies *in vitro* to the situation *in vivo*, it appears highly unlikely that esterified protochlorophyllide can be photoreduced. To accommodate existing reports contrary to this conclusion the possibility may exist that some species, e.g. *Euglena* (Cohen & Schiff, 1976) and mutant *Chlorella* (Sasa & Sughara, 1976) possess a completely different reductase, which can carry out the reduction of esterified protochlorophyll. It might be significant that the chemical photohydrogenation of analogues of protochlorophyllide by ascorbic acid in pyridine is most efficient with compounds possessing free carboxy groups at C_7 (Suboch *et al.*, 1970).

An as-yet-unanswered question in studies on protochlorophyllide phototransformation is the exact structure of the photoactive pigment complex. In our studies this is seen as a protein(enzyme)–NADPH–pigment (monomeric or aggregated?) complex (Griffiths, 1978). Other interpretations variously ascribe aggregation (Mathis & Sauer, 1972) or disaggregation (Henningsen *et al.*, 1973; Nielson, 1973) of the pigment to the photoactive species. However, in view of the data obtained in the present work, pigment aggregation alone is not a sufficient criterion for photoactivity, since both native protochlorophyllide ester and synthetic protochlorophyllide methyl ester are capable of aggregation, yet do not form photoactive complexes.

The magnesium-free derivative of bacteriochlorophyll, bacteriophaeophytin, is firmly established as a redox intermediate in bacterial photosynthetic electron transfer (Tiede *et al.*, 1976), and there is recent evidence that phaeophytin *a* may play a similar role in higher-plant chloroplasts (see Klimov *et al.*, 1978). Regarding the origin of these compounds, they would logically be expected to arise by loss of magnesium from the corresponding chlorophylls rather than by their synthesis along a parallel biosynthetic route for metal-free compounds. The observation that the metal-free derivative of protochlorophyllide, 2-vinylphaeoporphyrin a_5, is not photoconverted to the corresponding chlorin by

NADPH-supplemented flash-illuminated etioplasts (Fig. 6) might be taken as supporting evidence for this suggestion. Again, all attempts at photochemically reducing this porphyrin in ascorbic acid and in pyridine have also proved unsuccessful (Krasnovskii *et al.*, 1970).

In marked contrast with the behaviour of the esterified protochlorophyllides (see above) 2-vinylphaeoporphyrin a_5 inhibited the photoreduction of protochlorophyllide by the reductase. This suggests, as would be expected, that this compound can occupy the active site on the enzyme (via its CO_2H group ?); however, the absence of magnesium from the molecule makes the association photochemically inert.

The insertion of zinc into 2-vinylphaeoporphyrin results in complete restoration of this compound as a substrate for protochlorophyllide reductase (Fig. 8). This is witnessed by the flash-induced incremental increase in absorption at 669nm seen when this compound is incubated with substrate-depleted membranes in the presence of NADPH. Despite the extensive experiments that have been carried out on the strictly photochemical reduction of various porphyrins, this is the first report of the enzymic photoreduction of a non-naturally-occurring porphyrin in a system *in vitro*. In separate experiments K_m and $V_{max.}$ values for the zinc derivative were estimated as 0.41 μM and 1.91 μmol/min per mg of protein respectively. These values are very similar to the corresponding values obtained previously (Griffiths, 1978) for authentic magnesium-containing protochlorophyllide and indicate that the enzyme works just as efficiently at reducing the zinc analogue as it does with the natural, magnesium-containing, compound.

In contrast with the positive result with the zinc derivative, insertion of cobalt, copper or nickel into 2-vinylphaeoporphyrin produced metal derivatives that failed to act as substrates for protochlorophyllide reductase in that, on incubation with substrate-depleted etioplast membranes in the presence of NADPH and flash-illumination, no absorbance changes indicative of chlorin formation were seen (Fig. 10). It is worthwhile to compare this metalporphyrin-specificity of protochlorophyllide reductase with the corresponding properties of another chlorophyll-biosynthetic enzyme, namely the methyltransferase enzyme, which again can utilize either zinc or magnesium protoporphyrin IX together with calcium protoporphyrin IX as substrates for methylation by *S*-adenosyl-L-methionine (Gibson *et al.*, 1963). Again, the enzyme ferrochelatase is very active at inserting a whole range of different metals, including zinc (Ellsworth & Lawrence, 1973) into protoporphyrin IX, but to date, rather surprisingly, no magnesium chelation has been unequivocally observed.

It is obviously important to identify the specific properties of the zinc and magnesium chelates that enable these compounds to function as substrates for protochlorophyllide reductase. In this respect it is worth recalling that both zinc and magnesium have very similar ionic radii, closed *d*-electron shells, do not show any redox properties and that the metalloporphyrins derived from them have very similar properties, such as electronegativity, ability to aggregate and the formation of strong reductants when photoexcited. However, which, if any, of these properties is important in conferring on those compounds the ability to act as substrates for protochlorophyllide reductase must await elucidation of the enzyme's mechanism of catalysis.

Confirmation of the absolute specificity of protochlorophyllide reductase for NADPH is unambiguously borne out by the data of Fig. 12, which demonstrates the failure of NADH, even at 2.5mM, to act as a reductant. Similarly, all the common reductants tested previously by the discontinuous assay (Griffiths, 1975*a*) again failed to produce any chlorophyllide from protochlorophyllide with the continuous assay used here (W. T. Griffiths, unpublished work), providing further evidence of the absolute specificity of the enzyme for reductant.

As it is possible that the photoactive enzyme–substrate intermediate in the photoreduction of protochlorophyllide is a ternary enzyme–protochlorophyllide–NADPH complex, it is likely that the 2′-phosphate group in NADPH is involved in bonding the reductant in the complex.

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