8-Vinyl reduction and chlorophyll *a* biosynthesis in higher plants

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A technique involving solid-phase extractions and polyethylene h.p.l.c suitable for the routine compositional analysis of the total protochlorophyllide pool of plants is described. The resynthesis kinetics of the individual components of the pool have been studied in briefly illuminated etiolated tissue of wheat (*Triticum aestivum*) and cucumber (*Cucumis sativus*) during subsequent redarkening. The data are interpreted in terms of a precursor–product relationship between the di- and mono-vinyl analogues of protochlorophyllide during their reaccumulation in darkness. The interconversion is assumed to be catalysed by an 8-vinyl reductase, which shows greater activity in wheat than in cucumber. Analyses of the redox state of the nicotinamide nucleotide of the pool during the process are compatible with NADPH as the cofactor of the putative reductase.

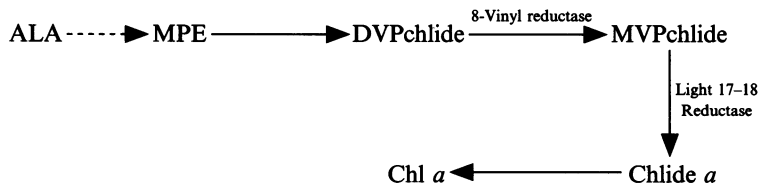
INTRODUCTION

The original chlorophyll biosynthetic pathway proposed by Granick (1950) involves at one stage (see Scheme 1) the reduction of 3,8-divinylprotochlorophyllide (DVPchlide) catalysed by a putative 8-vinyl reductase enzyme. The product, 3-vinyl protochlorophyllide (MVPchlide), acts as a substrate for the light-dependent step of the pathway, namely the 17–18 reduction, evidenced by accumulation of MVPchlide at relatively high levels when higher plants are grown in darkness (Loeffler, 1955) and its subsequent conversion into chlorophyll *a* on illumination (Wolff and Price, 1957).

This simple scheme, however, had to be reconsidered when developments in analytical techniques, especially low-temperature spectrofluorimetry, showed that varying amounts of DVPchlide accompanied the MVPchlide in etiolated and greening tissues of some plants (Belanger and Rebeiz, 1980; Bazzaz et al., 1982; Belanger et al., 1982). Following the detection of further MV- and DV-tetrapyrrole intermediates and their biosynthetic interconversion in tissues and extracts of different plants, a multi-branched chlorophyll biosynthetic pathway leading to the formation of several chemically distinct chlorophyll species, each having a different role in photosynthesis, was proposed [see Rebeiz et al. (1986, 1988) for reviews]. The extent to which these various pathways contribute to chlorophyll formation and hence to the composition of the final chlorophyll pool is governed by both the plant species and its particular response to periods of light and darkness (Carey and Rebeiz, 1985; Carey et al., 1985).

Although this multi-branched scheme purports to account for the undisputed heterogenous tetrapyrrole composition of different plant tissues under various conditions, many of the individual reactions of the scheme have not been demonstrated unambiguously, and none of the enzymes have been identified. Again, if such distinct routes are involved in chlorophyll biosynthesis, it makes it difficult to reconcile the well-documented (Gough, 1972) inhibition of chlorophyll biosynthesis due to specific genetic lesions, since inhibition of a reaction in one branch would still leave the remaining branches operational.

The current multi-branch model includes six main branches with four minor esterification routes and two major mono- and di-vinyl free acid branches (Rebeiz et al., 1988). In the present paper we reappraise the multi-branched pathway, concentrating on the two major free acid branches. The experimental evidence against the esters being photoconvertible *in vivo* (Bovey et al., 1974; Schoch et al., 1977; Shioi & Sasa, 1983) or *in vitro* (Schoch et al., 1977; Griffiths, 1980; Rudiger and Schoch, 1991) is quite compelling, so the routes involving such esters will not be considered in the present paper. As to the major mono- and di-vinyl free acid branches, the presence in etiolated tissue of a mixed pool of DVPchlide and MVPchlide with varying proportions of the two forms depending on species as originally demonstrated (Carey and Rebeiz, 1985) is confirmed in the present study. From a kinetic analysis of the resynthesis of this pool during the redarkening of briefly illuminated cucumber (*Cucumis sativus*) and wheat (*Triticum aestivum*), we conclude a precursor–product relationship between DVPchlide and MVPchlide participating in the light in a single pathway to chlorophyll *a*, as originally proposed by Granick (1950).



Scheme 1 Sequence of intermediates in the synthesis of chlorophyll *a* (adapted from Granick, 1950)

Abbreviations: MPE, Mg-protoporphyrin IX monomethyl ester; Chlide *a*, chlorophyllide *a*; Chl *a*, chlorophyll *a*.

Abbreviations used: ALA, 5-aminolaevulinic acid; Pchlide, unspecified mixture of the 3,8-divinyl and 3-vinyl-8-ethyl analogues of Mg-phaeoporphyrin a_5; DVPchlide, Mg-3,8-divinylphaeoporphyrin a_5; MVPchlide, Mg-3-vinyl-8-ethylphaeoporphyrin a_5.

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MATERIALS AND METHODS

Plant material

Wheat (*Triticum aestivum* var. Avalon), oats (*Avena sativa* var. Axona) and barley (*Hordeum vulgare* L. var. Proctor) were purchased from the British Seed Houses, Avonmouth, Bristol, U.K. Cucumber (*Cucumis sativus* L. var Beit Alpha) seeds and peas (*Pisum sativum* var. Feltham first) were purchased from local seed merchants. Seeds were imbibed in running tap water for 18 h with constant aeration and germinated between layers of damp tissue for 24 h. They were then planted in Levington potting compost (Fisons, Harston, Cambridge, U.K.) and grown at 25 °C in darkness for the appropriate period of time. Plant tissue was either extracted for pigment determinations, as described below, or used for preparations of etioplasts (Griffiths, 1978).

Illumination of whole plants as required was carried out under an array of 40 W fluorescent daylight white tubes supplemented with 60 W tungsten bulbs.

Pigment standards

DVPchlide and MVPchlide standards were purified either from the V3 mutant of *Rhodobacter sphaeroides* initially isolated by Dr. V. A. Saunders as a non-photosynthetic bacteriochlorophyll-free mutant (Saunders, 1973) or from 8-day-old etiolated wheat tissue. The bacterial mutant was grown heterotrophically in darkness under conditions of low aeration to facilitate excretion of pigments into the medium, as previously described (Saunders, 1973). After approx. 3 days the growth medium was separated from the cells by centrifugation at 5000 rev./min for 30 min in a Sorvall RC-3B centrifuge with the H6000A rotor. A diethyl ether extract of the pigments present in the medium was prepared by adjusting the pH to approx. 6.5 with solid KH_2PO_4, addition of approx. 3 vol. of acetone, followed by transfer of the pigment into 0.6 vol. of diethyl ether. The diethyl ether layer was washed extensively with water to remove all traces of acetone and finally the water was removed by freezing at −70 °C. Such an extract provided a rich source of DVPchlide with lesser amounts of the monovinyl analogue (see the Discussion section). A diethyl ether extract, enriched in the latter at the expense of the divinyl analogue, was prepared from the dark-grown wheat tissue as previously described (Griffiths, 1978). Separation and purification of the mono- and di-vinyl Pchlide from the diethyl ether extracts was carried out by preparative t.l.c. (Whyte, 1989). This involved an initial purification on silica gel followed by resolution into the mono- and di-vinyl forms by chromatography on thin layers of polyethylene (B. J. Whyte and W. T. Griffiths, unpublished work; details are available from W.T.G. on request).

Pigment extraction and assay

Forty 5 cm leaves of 5-day-old wheat seedlings or 40 pairs of cucumber/pea cotyledons were homogenized in 10 ml of methanol/1 M aq. NH_3 (19:1, v/v) using a Polytron blender at half maximum setting for 2 × 50 s. Extracts were clarified by centrifugation at 6000 *g* for 10 min. The resulting pellet was re-extracted with a further 10 ml of methanol, which was bulked with the original extract and its spectrum recorded using a Pye–Unicam SP.8-200 spectrophotometer, for total pigment estimation. Separation of these extracts into an acid fraction containing the compounds of interest (Pchlides) and a neutral fraction was achieved using Bond Elut cartridges (Analytichem International, Harbor City, CA, U.S.A.) as described below.

Preliminary separations of acid and neutral pigments

Preliminary separation of the pigment extracts prior to h.p.l.c. analysis was achieved using Bond Elut C18 coated silica reverse-phase cartridges. The extracts, in aq. 70% (v/v) methanol were loaded on to the cartridges. These were washed twice with water and twice with aq. 30% methanol. After a further 0.5 ml wash with aq. 50% methanol the samples were left for 2 min and the final Pchlide-containing fraction was eluted with 2 × 1 ml of aq. 90% methanol. This sample was used for h.p.l.c. analysis. Esterified pigments could finally be eluted with 100% acetone.

H.p.l.c. analysis

H.p.l.c. was carried out using a system consisting of two constametric pumps (LDC Milton Roy, Riviera Beach, FL, U.S.A.) and a Cecil CE 515 spectrophotometer fitted with 1 cm-path-length quartz flow cells (Cecil Instruments, Cambridge, U.K.).

Mono- and di-vinyl intermediates were resolved by reversed-phase chromatography using a hand-packed polyethylene column (25 cm × 0.5 cm). Prior to packing the polyethylene was washed five times with pentane, dried under vacuum and slurried with aqueous acetone (Chow et al., 1978). This slurry was used to pack an empty column with the aid of a plastic rod (Shioi and Beale, 1987). Compounds were eluted with the indicated percentages of acetone at a flow rate of 0.5 ml·min^{-1} at room temperature.

5-Aminolaevulinic acid (ALA) feeding of plant tissue

All operations were performed in darkness or under a dim green safelight. Etiolated 7-day-old wheat seedlings were cut to the required length, washed briefly in distilled water and placed in a solution of 10 mM ALA and 1 mM Mes, pH 6.5. Etiolated cucumber cotyledon pairs were incubated in a Petri dish containing the same solution. All plants were placed in a stream of air for 14 h to facilitate the uptake of the ALA solution. Control plants were treated in a similar manner, but placed in a solution containing only the Mes buffer.

Nicotinamide nucleotide determinations

NADPH, $NADP^+$, NADH and NAD^+ were determined in extracts from plastids using the enzyme recycling method of Heber and Santarius (1965). Intact plants exposed to the required light treatment were used as a source of plastids. Nicotinamide nucleotides were extracted from 1 ml aliquots of plastids as described, and the solution was clarified by centrifugation at 30000 *g* for 15 min. To assay NADP(H), a suitable quantity of the extract was made up to 2 ml by the addition of Tris buffer, pH 7.6, and mixed with 200 μl of glucose 6-phosphate (30 mM), 100 μl of phenazine methosulphate (0.3 mg/ml), 100 μl of dichlorophenol-indophenol (0.6 mg/ml) and 100 μl of EDTA (70 mM). The mixture was divided equally between the sample and reference cuvettes, and the assay was started by the addition of glucose 6-phosphate dehydrogenase (0.1 unit) to the sample cuvette. The rate of change of absorption at 600 nm was monitored using a Pye–Unicam SP.1800 spectrophotometer connected to a chart recorder, the nucleotide concentration being determined by reference to a standard curve obtained using known concentrations of nucleotides in the assay mixture. NAD(H) was determined in a similar manner using 600 mM ethanol instead of glucose 6-phosphate and the rate of dye

reduction monitored after the addition of an elevated amount (45 units) of alcohol dehydrogenase.

Enzyme assay

Light-dependent 17–18-Pchlide reductase assay was carried out using a dual-wavelength spectrophotometer as previously described (Griffiths, 1978).

Protein determination

Etioplast protein was determined by the method of Bramhall et al. (1969), using BSA albumin as a standard.

Chemicals

The source of chemicals used in this work was as previously described (Griffiths, 1978) and they were of AnalaR or the highest purity grade available.

RESULTS AND DISCUSSION

In recent years advances in separation techniques have considerably simplified what traditionally have been laborious and difficult procedures. Pchlide analysis in our work has been improved by the adoption of a preliminary solid-phase separation step involving Bond Elut cartridges for the separation of the free acid Pchlide fraction from extracts of different organisms. The technique is very reproducible and gives excellent recovery of Pchlide. On loading increasing amounts, from 4 to 15 nmol, of the Pchlide mixture extracted from the *Rb. sphaeroides* V3 mutant, approx. 97% of the material applied to that cartridge could, on average, be removed in a form suitable for direct spectrophotometric analysis, indicating the suitability of the technique as a rapid routine procedure for Pchlide estimation.

The composition, i.e. mono- and di-vinyl protochlorophyllide, of this fraction was estimated by direct application to h.p.l.c. analysis on columns of polyethylene, a technique specific for this application pioneered by Shioi and Beale (1987). Figure 1 shows the elution pattern obtained from analysis of the Pchlide fraction isolated from 5-day-old etiolated cucumber cotyledons. The data indicate an MVPchlide/DVPchlide ratio for this tissue of approx. 2.5 obtained in under 20 min. This value is in excellent agreement with data published by Hanamoto and Castelfranco (1983) using C18 coated silica reversed-phase h.p.l.c.; however,

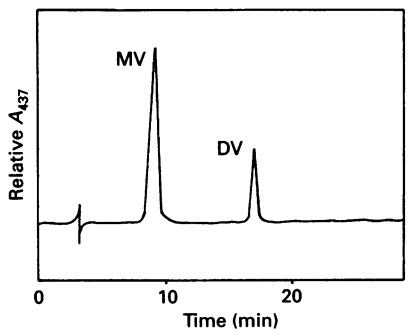


Figure 1 H.p.l.c. analysis of the Pchlide fraction from etiolated cucumber cotyledons

A crude methanol extract of 5-day-old cucumber cotyledons was partially purified using Bond Elut cartridges (see the Materials and methods section). The fraction was then chromatographed on a polyethylene h.p.l.c. column, using 70% (v/v) aq. acetone as the mobile phase. Peaks were detected by absorption measurements at either 437 or 623 nm.

Table 1 Distribution of MV- and DV-pchlides in different etiolated tissues

Species	Total Pchlide (nmol/g fresh weight)	Composition of Pchlide pool (%)	
		MV	DV
Wheat	14.1	100	0
Oat	10.1	100	0
Pea	10.3	70.3	29.7
Cucumber	10.9	71.6	28.4

their method required protracted elution times and was thus unsuitable for routine analysis.

Table 1 records the result of our analysis of the composition of the Pchlide pool from a limited selection of etiolated plants. In general agreement with a more extensive analysis based on low-temperature spectrofluorimetry carried out by Rebeiz and co-workers (Rebeiz et al., 1986, 1988), Table 1 shows that plants can roughly be divided into two main groups on the basis of the composition of their etiolated Pchlide pool, namely the MV- and DV-Pchlide-accumulating (generally dicotyledonous plants) and the MVPchlide-accumulating (typically cereals). Such a pattern has been interpreted in terms of branching of the chlorophyll biosynthetic pathway into two major mono- and di-vinyl branches with an uneven distribution of the branches among different plant groups (Rebeiz et al., 1986, 1988).

In the present work we have investigated other factors that could influence the pattern of Pchlide accumulation in representative members of these two basic plant groups, namely etiolated cucumber (MV- and DV-Pchlide-accumulating) and etiolated wheat (MVPchlide-accumulating). In preliminary experiments no major effect of plant age, between 5 and 9 days, on the total Pchlide content per g fresh weight or its divinyl/monovinyl composition in either wheat or cucumber was observed (Whyte, 1989). In contrast, as previously reported (Akoyunoglou & Argyroudi-Akoyunoglou, 1969), tissue age

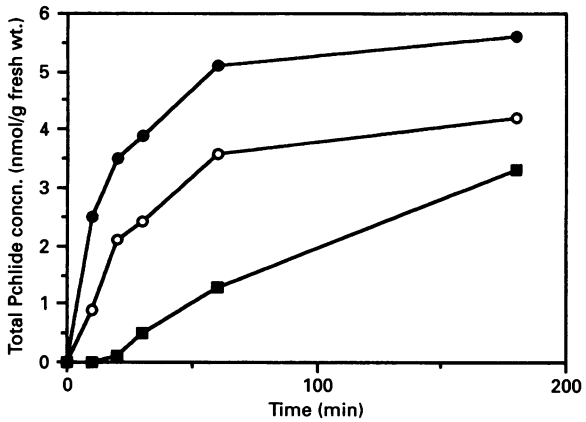


Figure 2 Age-dependence of total Pchlide resynthesis in briefly (1 h) illuminated wheat during subsequent redarkening

Wheat seedlings of different ages were illuminated for 1 h and returned to darkness. At the indicated time points 40 5 cm shoots were extracted twice with 10 ml of methanol/aq. 0.1 M NH_3 (19:1, v/v) using a Polytron homogenizer. Spectra of the methanol extracts were recorded on a Pye–Unicam spectrophotometer (SP.8200) before and after passing a known volume through the Bond Elut cartridges, and total Pchlide concentration was determined from the absorbance at 628 nm using an ϵ_{mM} of 35.5 litre·mmol^{-1}·cm^{-1}. Symbols: ●, 5-day-old plants; ○, 7-day-old plants; ■, 9-day-old plants.

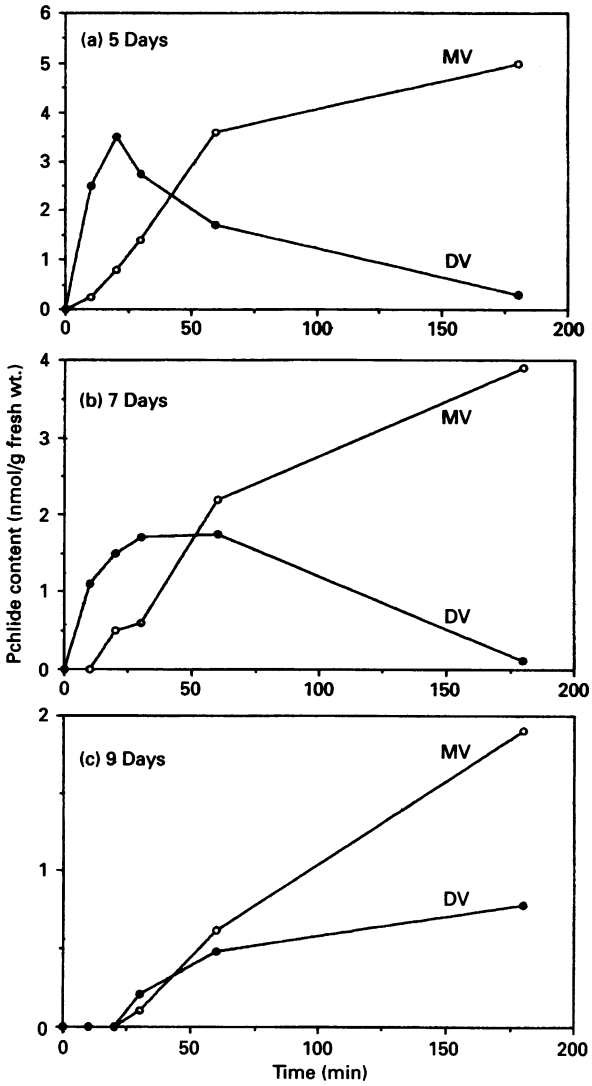


Figure 3 Compositional analysis of the Pchlide pool during resynthesis in wheat of differing ages (a, 5 days; b, 7 days; c, 9 days)

The aq. 70% methanol fractions from the Bond Elut cartridges in Figure 2 were analysed by polyethylene h.p.l.c. The mobile phase was aq. 70% (v/v) acetone. Pchlides were detected at 437 nm. Results are the means for two duplicate determinations.

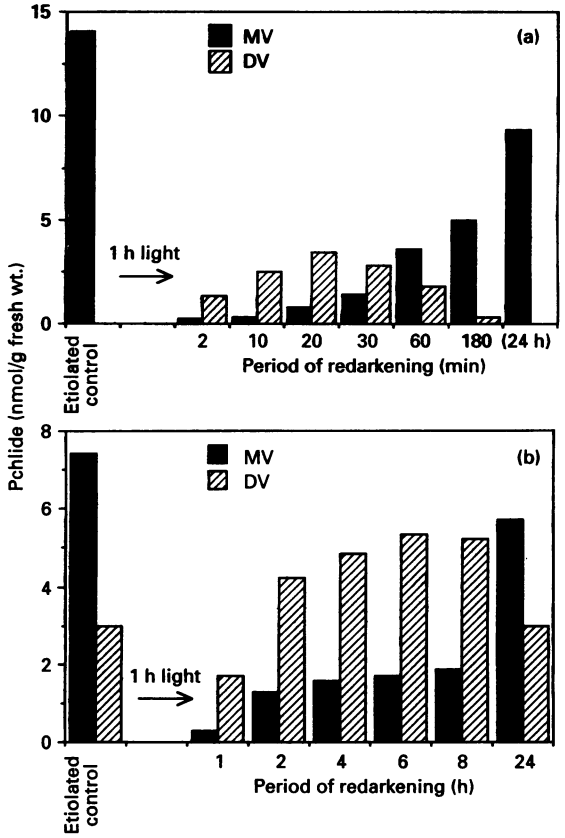


Figure 4 Effect of prolonged redarkening on the composition of the Pchlide pools of (a) wheat and (b) cucumber

5-day-old etiolated plants of cucumber and wheat were illuminated for 1 h and returned to darkness. At various time points Pchlides were extracted, partially purified on Bond Elut columns, and finally analysed by h.p.l.c. The composition of the endogenous pool prior to illumination is also shown.

greatly influenced the resynthesis of Pchlide in darkness following the illumination of such tissues. Figure 2 gives the result of such an experiment for wheat and indicates a rapid decline in both rate and extent of Pchlide resynthesis on aging from 5 to 9 days. The composition of the Pchlide pool was also analysed during its resynthesis in this experiment, and the results are shown in Fig. 3. These clearly indicate that the early stage of resynthesis in both 5- and 7-day-old wheat tissue is dominated by the presence of DVPchlide (Figures 3a and 3b). Accumulation of DVPchlide, however, is transient, and with increasing time MVPchlide accumulation becomes increasingly more apparent, exceeding the DVPchlide level after approx. 50 min, with the latter barely detectable after approx. 3 h. In the 9-day-old plants a similar trend can be seen, but now the changes occur at a much lower rate (Figure 3). We interpret these data as indicating a product–precursor relationship between MV- and DV-Pchlide during Pchlide synthesis with the DVPchlide-into-MVPchlide conversion, catalysed by the putative enzyme 8-vinyl reductase, becoming progressively slower in older tissues, in accord with the overall decline in the rate of Pchlide synthesis with age (Figure 2).

Figure 4 shows the results of the analysis of Pchlide resynthesis under similar conditions, but over an extended time course, in 5-day-old wheat and cucumber tissue. As already indicated above, in wheat the rise in level of MVPchlide at the expense of DVPchlide continues over a 24 h period, at the end of which the Pchlide pool contains exclusively the monovinyl analogue as in the original pre-illuminated material (Figure 4a). The final level of this pool is smaller than that present originally, in keeping with previous results (Sundqvist, 1974). The 5-day-old etiolated cucumber tissue contains (Figure 4b), as already mentioned, a Pchlide pool with 72% monovinyl and 28% divinyl analogues. Initial resynthesis following illumination, as in wheat, is again dominated by the presence of DVPchlide. In contrast with the latter, however, in cucumber DVPchlide is still present after 24 h (Figure 4b). Here again MV/DV-Pchlide ratios in an extended period of darkness appear to approach that present in the original etiolated tissue, in a manner analogous to that already observed for wheat above. Broadly speaking, this pattern of Pchlide resynthesis in cucumber can again be reconciled with a single biosynthetic branch pathway involving a DV/MV-Pchlide sequence, but with this reaction, for some unknown reason, e.g. reductase activity or cofactor availability, slower in cucumber than in wheat. It should be mentioned that similar experiments to those reported in Figure 4 have been carried out in which the illumination period was varied from 15 min to 3 h using both wheat and cucumber. In all cases subsequent dark resynthesis

was essentially as described in Figure 4, with an initial transient accumulation of DVPchlide followed by a steady increase in the level of MVPchlide (results not shown). What these data highlights is the importance of kinetic considerations and sampling time on interpretation of the composition of the Pchlide pool.

Somewhat similar data to those shown in Figure 4, but carried out mostly on a range of photoperiodically grown plants, have already been reported by Rebeiz and co-workers [see Rebeiz (1986, 1988) for reviews]. They ascribe the observed differences in "greening patterns" to separate monovinyl and divinyl branches of chlorophyll biosynthesis, with the two branches not only showing differences in activity in darkness in different plants, but also responding differently to initial and prolonged periods of illumination. In contrast, we prefer a simpler interpretation of the data based upon a single unbranched pathway, essentially as originally proposed by Granick (1950), but one in which the enzymes display a broad substrate specificity range, i.e. during chlorophyll synthesis in the light this would proceed as follows (MgProto is Mg-protoporphyrin IX):

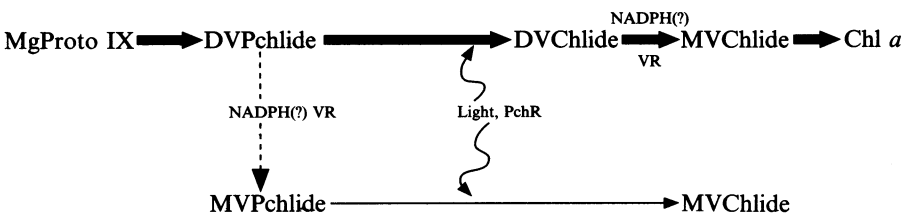
Interrupting the pathway by darkness, however, the system invariably utilized experimentally, leads initially to the accumulation of DVPchlide. However, owing to the broad substrate specificity of the 8-vinyl reductase this can also reduce the DVPchlide to MVPchlide (Scheme 2, broken arrow). The extent of accumulation of the latter would obviously be governed by such factors as activity of the reductase and cofactor availability. In species such as cereals, conditions favouring active 8-vinyl reductase exist to account for the observed accumulation of MVPchlide in preference to DVPchlide, whereas in dicotyledonous species such as cucumber, where such favourable conditions do not prevail, conversion of the DVPchlide into MVPchlide is much slower. Such an explanation, we believe, can simply and logically account for the differences observed in the composition of the Pchlide pool in various species. Obviously the effect of the time of sampling on the analysis, as recorded in Figure 4, can readily be accommodated by this model.

On reillumination of darkened tissue, as in photoperiodically grown plants, the accumulated Pchlide mono- and di-vinyl analogues are photoconverted into the corresponding chlorin derivatives, followed by conversion into chlorophyll *a* (Scheme 2).

This sequence of reactions relies on the assumption that the enzymes involved have a broad range of substrate specificities enabling them to catalyse the various alternative, i.e. mono- or di-vinyl, porphyrin or chlorin conversions. That this assumption is valid for the case of the light-requiring 17–18 protochlorophyllide reductase is already well established. This enzyme can utilise both mono- and di-vinyl Pchlide as substrate. In fact, latest estimations by one of us (B.J.W.) indicates the enzyme to be more efficient with DVPchlide than with MVPchlide, with K_m values of 0.96 and 2.6 μM respectively (Whyte, 1989), highlighting, perhaps, the main reaction in steady-state light conditions (bold arrows in Scheme 2) compared with the situation on initial illumination of a dark sample, i.e. as in photoperiodically grown plants when both branches of Scheme 2 will operate initially as dictated by the composition of the Pchlide pool accumulated in the dark.

Regarding the putative 8-vinyl reductase the situation is not quite so clear. Not only is the cofactor of the enzyme to be confirmed (see below), but also its porphyrin specificity remains to be unambiguously established. Despite earlier evidence to the contrary (Tripathy and Rebeiz, 1988), it now seems that some plants, e.g. barley (Tripathy and Rebeiz, 1988), can effect the reduction of DVPchlide to MVPchlide, as demanded by Scheme 2. Also, as expected from this Scheme, the experimental evidence (Duggan and Rebeiz, 1982; Richards et al., 1992) suggests that reduction of divinyl chlorophyllide is much more active.

By taking such enzymic considerations into account one can simply explain the various patterns of greening observed under different conditions in various species without having to invoke a multibranched pathway where each branch exhibits different activity in different plants under different conditions. According to the latter, wheat has been described as accumulating MVPchlide in darkness, owing to the exclusive operation of the monovinyl branch of the pathway. Figure 5 shows the effect of ALA treatment on the composition of the Pchlide pool in etiolated wheat. In this experiment, 5-day-old etiolated tissue was incubated in 10 mM ALA in complete darkness for 14 h, after which time the level and composition of the Pchlide pool was analysed. The results show, as expected, an increase in Pchlide from 15 to 123 nmol/g fresh weight in the ALA-fed wheat and from 11 to 60 nmol/g fresh weight in cucumber. Significantly the wheat pool contained 8.4 nmol/g fresh weight of DVPchlide after ALA treatment. This cannot be explained by the exclusive operation in darkness of the monovinyl branch in wheat. These observations are consistent with Scheme 2 if it is assumed that, under these conditions of increased flux to Pchlide in darkness, the activity of the 8-vinyl reductase, even in wheat, eventually becomes limiting, resulting in DVPchlide accumulation. Similar reasoning can also accommodate the recorded changes in Pchlide composition observed in the ALA-fed cucumber cotyledons. Here, as anticipated from the lower 8-vinyl reductase activity (see above), an even greater increase in the



Scheme 2 Terminal reactions in the synthesis of chlorophyll *a*

Abbreviations: VR, putative 8-vinyl reductase; PchR, 17–18 Pchlide reductase (light-dependent).

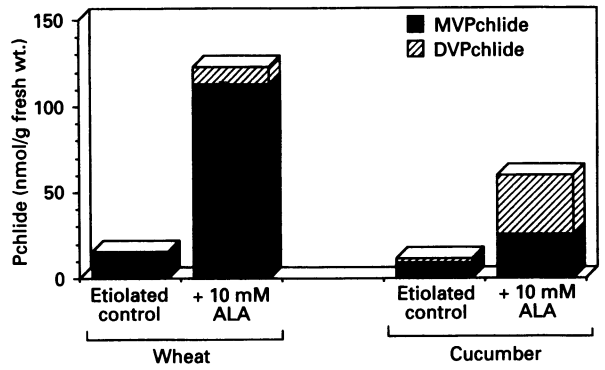


Figure 5 Effect of ALA on the composition of the etiolated Pchlide pool in 5-day-old wheat and cucumber

Forty 5 cm etiolated wheat seedlings or 40 etiolated cucumber cotyledon pairs were placed in either (i) 10 mM ALA/1 mM Mes, pH 6.5, or (ii) 1 mM Mes, pH 6.5 (control). Samples were left overnight (14 h) in darkness and finally extracted and analysed as described in the Materials and methods section.

Table 2 Redox state of nicotinamide nucleotides isolated from dark-grown and 1 h-illuminated wheat and cucumber plants

Light treatments were performed on the intact plant tissue. Plastids were then isolated and the nicotinamide nucleotide ratios were determined using the method of Heber and Santarius (1965). Columns 1 and 2 represent different experiments.

		NADPH/NADP$^+$		NADH/NAD$^+$	
Plant type	Treatment	1	2	1	2
Wheat	Dark	5.5	5.0	0.16	0.17
	60 min illumination	0.53	0.40	0.14	0.16
Cucumber	Dark	4.40	4.21	0.32	0.26
	60 min illumination	1.48	1.51	0.27	0.24

relative proportion of DVPchlide from 23 to 60% of the total Pchlide pool is observed on ALA feeding (Figure 5).

Several factors might influence 8-vinyl reductase activity under different conditions, an obvious one being supply of reductant. The identity of the reductant is uncertain (Ellsworth, 1972; Ellsworth and Hsing, 1973). Table 2 shows changes in the redox states of NAD(P)$^+$/NAD(P)H in plastids from etiolated wheat and cucumber under conditions (i.e. before and after 1 h of illumination) where large differences in 8-vinyl reductase activity would have been anticipated (see Figures 4a and 4b). In both plants the NADPH/NADP$^+$ ratio decreases significantly on light treatment, whereas no corresponding changes in the redox state of NAD$^+$ are observed. Furthermore, there is a much greater apparent oxidation of NADPH in the wheat plastids (see Table 2), which are expected to have the highest activity of reductase. This might indicate an involvement of NADPH in the activity of the reductase; indeed, Richards and co-workers have recently provided evidence for the specific utilization of NADPH in the reduction of divinylchlorophyllide to chlorophyllide *a* in cucumber plastids (Richards et al., 1992). Clarification of the exact role of the putative reductase in chlorophyll synthesis, however, must wait until the enzyme has been purified and fully characterized.

Received 21 August 1992/16 November 1992; accepted 1 December 1992

Conclusions

The apparent different patterns of Pchlide resynthesis in darkened wheat and cucumber previously reported have been confirmed in the present study. Our data are interpreted in terms of different rates of 8-vinyl reduction at the Pchlide level in plants, a reaction catalysed by a putative reductase with a broad range of substrate specificity. The qualitative differences in composition of the Pchlide pool in wheat and cucumber may reflect the more severe limits imposed on the reductase in cucumber compared with wheat. No evidence in support of discrete mono- and di-vinyl branches of chlorophyll biosynthesis was obtained.

Financial support was provided by the Science and Engineering Research Council, which is gratefully acknowledged.

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