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CONVERSION OF DIVINYL PROTOCHLOROPHYLLIDE TO MONOVINYL PROTOCHLOROPHYLLIDE IN GREEN(ING) BARLEY, A DARK MONOVINYL/LIGHT DIVINYL PLANT SPECIES

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ABSTRACT

In higher plants, most of the chlorophyll *a* is formed via the divinyl and monovinyl chlorophyll monocarboxylic biosynthetic routes. These two routes are strongly interconnected prior to protochlorophyllide formation in barley (*Hordeum vulgare* L. cv Morex), a dark monovinyl-light divinyl plant species, but not in cucumber (*Cucumis sativus* L. cv Beit Alpha MR), a dark divinyl-light divinyl plant species (BC Tripathy, CA Rebeiz, 1986 J Biol Chem 261: 13556–13564). It is shown that in dark monovinyl-light divinyl plant species such as barley, the divinyl and monovinyl monocarboxylic routes become interconnected at the level of protochlorophyllide during transition from the divinyl to the monovinyl protochlorophyllide biosynthetic mode. In cucumber, a dark divinyl-light divinyl plant species, in which the monovinyl monocarboxylic biosynthetic route becomes preponderant only after an abnormally long sojourn in darkness, the conversion of divinyl to monovinyl protochlorophyllide does not take place on the barley time-scale of incubation.

The discovery of the ubiquitous occurrence of DV[3] and MV Pchlides in higher plants (1, 3) has led to a reevaluation of the Chl biosynthetic pathway (14). Protochlorophyllide is the main precursor of Chl in green plants. Considerable experimental evidence now indicates that, in higher plants, MV and DV Pchlides are formed from MV and DV Proto, respectively. via two parallel MV and DV monocarboxylic Chl biosynthetic routes (15, 17). Furthermore, on the basis of the MV or DV monocarboxylic biosynthetic routes that predominate at night or in daylight, higher plants have been observed to fall into one of four greening groups (3, 4, 15), namely: dark divinyl/light divinyl (DDV/LDV), dark monovinyl/light monovinyl (DMV/LMV), DMV/LDV, and DDV/LMV. It has also been demonstrated that in etiolated DMV/LDV plant species such as barley, which are poised in the MV Pchlide biosynthetic mode (3, 4), the DV and MV monocarboxylic biosynthetic routes are strongly interconnected prior to DV Pchlide formation (15, 17). On the other hand, in etiolated DDV/LDV plant species such as cucumber in which the MV monocarboxylic biosynthetic route is not as preponderant as the DV monocarboxylic route, the DV and MV monocarboxylic routes were found to be weakly interconnected (15, 17). From *in vitro* investigations, the DV and MV monocarboxylic routes did not appear to be interconnected at the level of Pchlide, either in etiolated barley or in etiolated cucumber (15, 17). Indeed, although it is firmly believed that DV Pchlide is convertible to MV Pchlide in higher plants (5) by conversion of the vinyl group at position 4 of the macrocycle to an ethyl group (Fig. 1), this hypothesis has not yet been corroborated by experimental evidence (15, 17). During ongoing investigations of the regulation of the MV and DV Chl monocarboxylic biosynthetic routes in higher plants, preliminary experimental evidence suggested that in some plant species, DV Pchlide may be convertible to MV Pchlide, under certain conditions.

In this work, it is shown that during the transition from the DV Pchlide to the MV Pchlide biosynthetic state, DV Pchlide can be partially converted to MV Pchlide in barley, a DMV/LDV plant species. Under similar incubation conditions, the conversion of DV Pchlide to MV Pchlide did not appear to take place in cucumber, a DDV/LDV plant species.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Barley (*Hordeum vulgare* L. cv Morex) and cucumber (*Cucumis sativus* L. cv Beit Alpha MR) were grown either in darkness, in moist vermiculite at 28°C, or in the greenhouse under a 14 h light/10 h dark photoperiod.

Chemicals. δ-Amino[4-^{14}C]levulinic acid (46 Ci/mol) was purchased from Research Product International, Elk Grove, IL. ALA was purchased from Sigma, St. Louis, MO.

Light Pretreatment of Etiolated Seedlings. Etiolated cucumber and barley seedlings were poised in the DV Pchlide biosynthetic state by illumination with 320 μW/cm^2 of cool-white fluorescent light at 22°C for 5 h.

Incorporation of [^{14}C]ALA into Pchlide. Green cucumber cotyledons and barley leaves poised in the DV Pchlide biosynthetic state were excised from photoperiodically grown seedlings during the middle of the light phase of the photoperiod (4). Greening cucumber cotyledons and barley leaves poised in the DV Pchlide biosynthetic state were excised from etiolated seedlings illuminated with cool-white fluorescent light for 5 h (3, 4). Two to 2.5 g batches of excised tissues were floated on 10 ml of distilled H_2O in deep Petri dishes, 9 cm in diameter. Five μCi of [^{14}C]ALA were added to each Petri dish. In order to induce the accumulation of DV and MV [^{14}C]Pchlide, the Petri dishes were wrapped in aluminum foil and were incubated at 28°C in darkness for 0 to 4 h (barley) or for 0 to 10 h (cucumber). At regular intervals, tissue samples were homogenized in acetone: 0.1 N NH_4OH

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[3] Abbreviations: DV, divinyl; MV, monovinyl; ALA, δ-aminolevulinic acid; D, dark; L, light; Proto, protoporphyrin IX. Unless preceded by MV or DV, the terms Proto, Pchlide, and Chl are used generically to designate metabolic pools that may consist of MV and DV components.

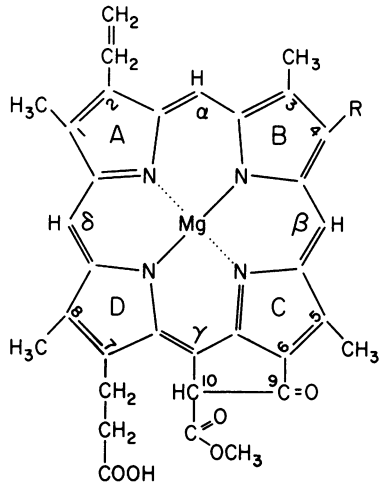

FIG. 1. Chemical structures of DV and MV Pchlide.

(9:1,v/v) at a ratio of 6.7 ml/g of tissue.

Preparation of DV Pchlide Substrate. DV Pchlide was prepared from etiolated cucumber cotyledons. The cotyledons were induced to accumulate μg amounts of DV Pchlide by subjecting 2 g batches of excised 4 d old etiolated cotyledons to three 2.5 ms pulses of actinic white light. The light pulses were separated by 60 min of darkness (8). DV Pchlide was extracted and purified as described below.

Isolation of Plastids. Plastids poised in the DV Pchlide biosynthetic mode were isolated either from green photoperiodically grown tissues or from etiolated tissues partially greened for 5 h under white fluorescent light (4) (*vide supra*). Plastids were isolated as described elsewhere (7, 17).

Incubation of Plastids with ALA or DV Pchlide. Incubation of isolated plastids was carried out at 28°C in darkness for 1 h in a reciprocating water bath at 50 oscillations per min (17). Incubation was terminated by the addition of 15 ml of acetone: 0.1 N NH_4OH (9:1, v/v) to 3 ml of incubation medium.

Pigment Extraction. Partition of fully esterified and monocarboxylic tetrapyrroles between hexane and hexane-extracted acetone and transfer of Pchlide from hexane-extracted acetone to diethyl ether was described elsewhere (17).

Separation of MV from DV Pchlide. The ether fraction containing Pchlide was dried under N_2 gas, and the pigment was redissolved in 1 ml of ether. Methylation of Pchlide was achieved by adding 3 ml of freshly prepared diazomethane in ether to the Pchlide solution (2). The reaction was allowed to proceed for 6 min at 1 to 4°C, after which the ether was evaporated under N_2 gas; the methylated Pchlide was dissolved in 90% acetone and was chromatographed on thin layers of polyethylene developed in 90% acetone. Methylated MV Pchlide migrated faster (R_F = 0.56) than methylated DV Pchlide (R_F = 0.41). The segregated MV and DV Pchlides were eluted in methanol:acetone (4:1, v/v).

Quantitative Determination of MV and DV Pchlide. The amounts of MV and DV Pchlide were determined by spectrofluorometry with a precision of about 5% (13, 16).

Measurement of ^{14}C Incorporation into MV and DV Pchlide. Small aliquots of the MV and DV [^{14}C]Pchlide fractions were dissolved in 6 ml of Beckman Ready-Solv CP scintillation cocktail. Radioactivity was determined in a liquid scintillation counter, Beckman model LS 3800, operated in the automatic quench compensation mode (17).

Spectrofluorometry. Fluorescence spectra were recorded on a fully corrected, photon-counting spectrofluorometer model SLM 8000 DS, equipped with two red-sensitive, extended S20 photomultipliers (EMI 9658) and interfaced with a Hewlett-Packard microcomputer system, model 9825. Pigment solutions were monitored at room temperature in cylindrical microcells 3 mm in diameter. Conversion of the digital spectral data to quantitative values was performed automatically by the microcomputer, following the recording of the pertinent spectra. Low temperature fluorescence emission and excitation spectra of the ether extracts were recorded at 77 K in cylindrical sample tubes as described elsewhere (6). The emission and excitation spectra were recorded at excitation and emission bandwidths of 4 nm unless otherwise indicated.

Spectrophotometry. Absorption spectra were recorded on an Aminco model DW-2 spectrophotometer, operated in the split beam mode. All spectra were recorded at a bandwidth of 2 nm.

Protein Determination. Total proteins were determined by biuret on aliquots of the plastid suspensions, after delipidation (11).

RESULTS

Experimental Strategy. Because of the kinetics of DV and MV Pchlide biosynthesis and accumulation in plant tissues (Fig. 2), a direct demonstration of DV Pchlide conversion to MV Pchlide *in vivo* is not possible. In investigating this issue, we have therefore adopted a two-tiered approach. In a first step, it was determined whether the plastids extracted from the tissue under investigation can convert exogenous DV Pchlide to MV Pchlide. The results derived from such *in vitro* experiments are usually relevant to the biosynthetic activities of the tissue *in vivo*. This is a consequence of having demonstrated earlier that the biosynthetic activity of plastids extracted from a particular tissue, poised in a particular MV or DV Pchlide biosynthetic state, reflected adequately the biosynthetic activity of the tissue (4). Since the demonstration of a DV to MV Pchlide conversion *in vitro* gave no indication, however, of the extent of DV Pchlide conversion to MV Pchlide *in vivo*, this was investigated in a

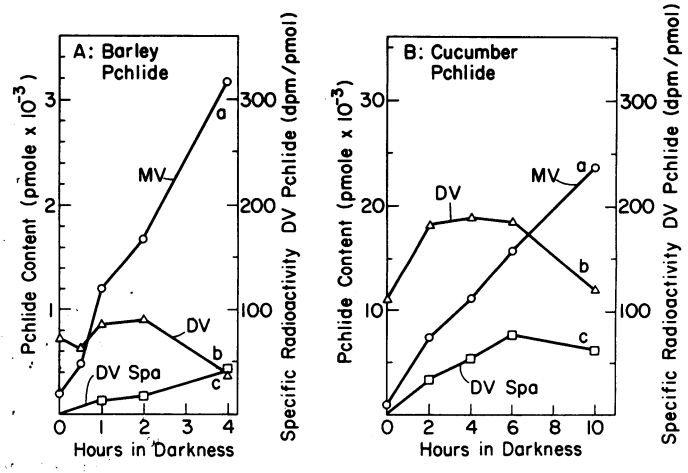

FIG. 2. Time course of MV and DV Pchlide accumulation and time course of the change in specific radioactivity in (A) barley and (B) cucumber seedlings. The seedlings were grown under a 14 h light-10 h dark photoperiod. Leaves were excised during the middle of the light phase of the photoperiod and were incubated in darkness with 5 μCi of [^{14}C]ALA for the indicated times. a, MV Pchlide content; b, DV Pchlide content; c, specific radioactivity of DV Pchlide (DV spa).

second set of experiments as described below.

Indeed about 17 years ago we reported that it is possible to determine whether a compound '*B*' can be formed exclusively from compound '*A*' *in vivo* by radiotracer kinetic analysis (12). The equations derived in Rebeiz *et al.* (12) have now been adapted for more general cases. It has been shown that for any number of time intervals t_1 to t_2, the following equation describes the relationship between radiolabel incorporation into a compound *B*, its net synthesis from compound *A* and the specific radioactivity of *A* (Rebeiz and Tripathy, unpublished data):

$$Q_{B2} = \frac{\gamma_{A1} + \gamma_{A2}}{2} \cdot \Delta B_2 \qquad (1)$$

where Q_{B2} = amount of radiolabel incorporated into compound *B* during time inter t_1 to t_2; γ_{A1}, γ_{A2} = specific radioactivity of compound *A* at the beginning and end of time interval $t_1 - t_2$, respectively; and ΔB_2 = amount of *B* synthesized during time interval $t_1 - t_2$.

By comparing expected radiolabel incorporation into compound *B*, as calculated from Eq. 1, with experimentally determined incorporations, it is possible to tell whether compound *B* is formed exclusively from compound *A* or not. If compound *B* is formed exclusively from compound *A*, then within the range of experimental error, the theoretical and experimental radiolabel incorporations into *B* should be identical or reasonably similar. On the other hand, if compound *B* is not formed from compound *A* or is only partially formed from *A*, then the calculated and experimental radiolabel incorporations into *B* will be different. The difference between the calculated and experimental values may then depend among other things on the extent of the partial contribution of compound *A* to the synthesis of compound *B*.

The above approach was successfully used in Ref. (12) to predict that in etiolated cucumber cotyledons, esterified Pchlide (i.e. Pchlide ester) was not derived from Pchlide. Later on, this conclusion was corroborated by cell-free investigations (9).

Conversion of Exogenous DV Pchlide to MV Pchlide in Barley but Not in Cucumber Plastids. The dark conversion of exogenous DV Pchlide to MV Pchlide in barley plastids poised in the DV monocarboxylic biosynthetic mode was investigated with etiochloroplasts as well as with chloroplasts. It has been our experience that etiochloroplasts prepared from plant tissues exposed to about 5 h of continuous illumination are usually capable of converting exogenous ALA to Pchlide at rates that range from about 10 to 30 nmol/100 mg of plastid proteins (10). These rates are usually significantly lower than those exhibited by etioplasts prepared from kinetin + gibberellic acid (GA_3)-pretreated tissues (7, 10), but much higher than the biosynthetic rates exhibited by mature chloroplasts. The latter are usually in the picomole range.

Table I describes the conversion of exogenous DV Pchlide to MV Pchlide in etiochloroplasts prepared from etiolated barley seedlings, greened for 5 h under white fluorescent light (Table I, A and B), as well as in mature chloroplasts prepared from photoperiodically grown barley seedlings (Table I, C). The inherent activity of the plastid preparations was determined from their ability to convert exogenous ALA to MV and DV Pchlide. In barley etiochloroplasts, poised in the DV Pchlide biosynthetic mode, the conversion of exogenous DV Pchlide to MV Pchlide in darkness was very pronounced (Table I, A and B). Barley chloroplasts (Table I, C), poised in the DV Pchlide mode, were also capable of converting exogenous DV Pchlide to MV Pchlide at rates commensurate with their ALA-dependent MV Pchlide biosynthetic capabilities.

By contrast during 1 h of dark incubation, cucumber etiochloroplasts, poised in the DV Pchlide biosynthetic mode, converted exogenous ALA mainly to DV Pchlide (Table I, D and E). This was in line with previously reported results (4). Furthermore, in such plastids, the conversion of exogenous DV Pchlide to MV Pchlide was not observed (Table I, D and E).

***In Vivo* Kinetics of MV and DV Pchlide Biosynthesis and Accumulation during Incubation in Darkness.** Since the aforementioned results suggested that DV Pchlide may be convertible to MV Pchlide in green and greening barley seedlings, we undertook to investigate the possible extent of this DV to MV Pchlide *in vivo* conversion in barley. In deriving Eq. 1, it was assumed that for any dark time-interval t_1 to t_2, the change in specific radioactivity of DV Pchlide and the formation of MV Pchlide were linear functions of time.

Figure 2 depicts the changes of MV and DV Pchlide in green barley and cucumber seedlings during dark-incubation. In both photoperiodically grown barley and cucumber seedlings, the level of DV Pchlide underwent a significant decrease after an initial increase (Fig. 2, Ab and Bb). On the other hand, the level of MV Pchlide increased continuously with time (Fig. 2, Aa and Ba). Actually, over the full length of the dark-incubation period, the accumulation of MV Pchlide was quasi-linear with time. As a consequence, the assumption that, during any short dark-incubation interval t_1 to t_2, the formation and accumulation of MV Pchlide was a linear function of time is a reasonable one. The same applies for the increase in specific radioactivity of DV Pchlide (Fig. 2, Ac and Bc). Likewise, there was no reason to suspect a serious deviation from linearity for the decrease in specific radioactivity of DV Pchlide in cucumber after 6 h of dark incubation (Fig. 2, Bc).

Determination of the Extent of *in Vivo* DV to MV Pchlide Conversion in Barley, a DMV/LDV Plant Species. Green barley leaves, poised in the DV Pchlide biosynthetic mode, were excised from photoperiodically grown seedlings in the middle of the light phase of the photoperiod (3). In order to induce the biosynthesis of [^{14}C]MV and DV Pchlide, the excised leaves were incubated in darkness with [^{14}C]ALA. At regular intervals, the amounts and specific radioactivities of [^{14}C] incorporations into MV Pchlide were then compared to the theoretical incorporations, which were to be expected if [^{14}C]MV Pchlide was formed exclusively from [^{14}C]DV Pchlide. The expected theoretical ^{14}C incorporations into MV Pchlide were calculated with Eq. 1.

The results of two experiments with green barley seedlings are reported in Table II, A and B. At all time intervals, the theoretical ^{14}C incorporations into MV Pchlide that were to be expected if MV Pchlide was formed exclusively from DV Pchlide were much lower than the experimentally determined values. This in turn suggested either (a) that MV Pchlide was not being formed from DV Pchlide or (b) that MV Pchlide was being only partially formed from DV Pchlide, the balance being formed from other sources.

Since isolated barley chloroplast and etiochloroplasts poised in the DV Pchlide biosynthetic mode were capable of converting exogenous DV Pchlide to MV Pchlide in darkness (Table I, A–C), alternative (b) was considered more plausible than alternative (a). In this latter case, the proportion of MV Pchlide that was formed from DV Pchlide could not have exceeded, however, the maximum theoretical ^{14}C incorporation values reported in Table II, A and B. Under these conditions, the maximum possible proportion of MV Pchlide formed from DV Pchlide could be calculated from the theoretical and experimental ^{14}C incorporation values as described below. For example, in experiment A (Table II), after 1 h of dark incubation, the maximum possible proportion of MV Pchlide that could have been formed from DV Pchlide would have amounted to 52% (100 − [(116.7 − 60.5)/116.7 × 100] = 52%). Thus, the maximum possible proportions of MV Pchlide that were probably formed from DV Pchlide in green barley seedlings during various dark-incubation time intervals are reported in the last column of Table II, A and B. The proportion of MV Pchlide formed from DV Pchlide ap-

Table I. *Metabolism of Exogenous DV Pchlide in Isolated Etiochloroplasts in Darkness*

Etiochloroplasts were prepared either: (A, B) from etiolated barley seedlings, which were greened for 5 h under white fluorescent light; (C) from photoperiodically grown barley seedlings; or (D, E) from etiolated cucumber cotyledons, which were greened for 5 h under white fluorescent light. After 1 h of incubation in darkness, the disappearance of the DV Pchlide substrate and the formation of MV Pchlide were monitored. The ALA-dependent formation of MV and DV Pchlides was also determined. Numbers in parentheses refer to nmoles of substrate added per 100 mg plastid protein.

Plastids	Experiment	Substrate	Exogenous Substrate-Dependent Changes in the MV and DV Pchlide Pools[a]	
			DV Pchlide	MV Pchlide
			nmol/100 mg plastid protein	
Barley etiochloroplasts		ALA (20×10^3)	9.2	6.8
	A	DV Pchlide (85)	−28.0	23.8
Barley etiochloroplasts		ALA (20×10^3)	16.4	27.3
	B	DV Pchlide (293)	−60.2	41.1
Barley chloroplasts		ALA (20×10^3)	1.5	1.5
	C	DV Pchlide (88)	−2.5	0.9
Cucumber		ALA (20×10^3)	11.6	0.5
	D	DV Pchlide (68)	−9.0	−0.8
Cucumber		ALA (20×10^3)	11.5	0.1
etiochloroplasts	E	DV Pchlide (126)	−33.3	−0.5

[a] The exogenous substrate-dependent changes in the MV and DV Pchlide pools were taken as the amount of MV or DV Pchlide detected at the end of incubation minus the amount of MV or DV Pchlide present before incubation, and minus the amount of MV or DV Pchlide formed from endogenous substrates. The latter were determined from 1 h etiochloroplast incubations to which no exogenous substrates were added.

Table II. *Comparison of Theoretical and Experimental ^{14}C Incorporations into MV Pchlide in Green and Greening Barley Leaves*

Photoperiodically grown barley leaves or etiolated leaves, which were illuminated for 5 h with white fluorescent light, poised in the DV Pchlide biosynthetic mode were incubated with 5 μCi of [^{14}C]ALA in darkness. After various periods of incubation, the MV and DV [^{14}C]Pchlides were purified by separation on thin layers of polyethylene. ^{14}C Incorporations were determined as described in "Materials and Methods."

Experiment	Tissue	Length of Dark-Incubation with [^{14}C]ALA	Time Interval (X) under Consideration	Specific Radioactivity (Y_{Ax}) of DV Pchlide at End of Time Interval X	Increase in MV Pchlide (ΔB_x) during Time Interval X	Theoretical ^{14}C Incorporation (QB_x) into MV Pchlide during Time Interval X	Experimental (Exp) 14C Incorporation into MV Pchlide during Time Interval X	Maximum Possible Percent Conversion of DV Pchlide to MV Pchlide during Time Interval X[a]
		h		*dpm/pmol*	*pmol*	*calculated from Eq. 1 ($dpm \times 10^{-3}$)*	*$dpm \times 10^{-3}$*	*%*
A	Green	0	t_0	0	0	0	0	0
		1	t_0–t_1	121.0	1000	60.5	116.7	52
		2	t_1–t_2	163.0	435	61.8	131.7	47
		4	t_2–t_4	418.0	1490	432.8	1346.5	32
B	Green	0	t_0	0	0	0	0	0
		1	t_0–t_1	37.0	1449	26.8	44.7	60
		2	t_1–t_2	50.0	389	16.9	56.5	30
C	Greening	0	t_0	0	0	0	0	0
		1	t_0–t_1	15.2	7592	57.7	47.7	79
		2	t_1–t_2	41.1	2037	57.2	151.3	38

[a] Maximum possible % conversion = $100 - [(|QB_x - \text{Exp}|)/\text{Exp} \times 100]$.

peared to rise during the first hour of dark-incubation after which it decreased.

Essentially the same DV to MV Pchlide conversion pattern was observed in etiolated barley seedlings (Table II, C) poised in the DV Pchlide biosynthetic mode by illumination with white fluorescent light for 5 h (4). In this case, however, after 1 h of dark-incubation, the expected theoretical ^{14}C incorporation into MV Pchlide was higher than the experimental ^{14}C incorporation. In other words, during the first hour of dark-incubation, the actual ^{14}C incorporation into MV Pchlide fell short of the theoretical value that was to be expected if MV Pchlide was formed exclusively from DV Pchlide. Under these conditions the maximum possible conversion of DV Pchlide to MV amounted to about 79% (100 − [(57.7 − 47.7)/47.7 × 100]= 79%).

Is DV Pchlide Convertible to MV Pchlide during Prolonged *in Vivo* Dark-Incubation in Cucumber, a DDV/LDV Plant Species? As may be recalled, cucumber etiochloroplasts, poised in the DV Pchlide mode, were not capable of converting DV Pchlide to MV Pchlide during the initial phases of the dark-incubation period, when the DV Pchlide biosynthetic activity was still very strong (Table I, D and E). What is not clear at this stage is whether DV Pchlide can indeed be converted to MV Pchlide during the more prolonged aphysiological dark-incubations that are needed for the DV to MV Pchlide biosynthesis transition to take place in cucumber. As reported elsewhere, it takes about 18 h of dark-incubation for such a transition to take place in green cucumber cotyledons (Fig. 1 in Ref. 2). Cell-free systems capable of adequate ALA to Pchlide conversion rates on that time scale are not yet available. Therefore, the answer to the aforementioned question will have to await the development of such cell-free systems.

Nevertheless, a comparison of theoretical and experimental ^{14}C incorporation into MV Pchlide during prolonged dark-incubations of green and greening cucumber cotyledons is reported in Table III. With the exception of one case (Table III A, interval t_4-t_6), the theoretical ^{14}C incorporations were quite different from the experimental ones. This, in turn, suggested that if the conversion of DV to MV Pchlide was possible during such prolonged dark-incubations in cucumber, the proportion of DV to MV conversion could not exceed the values reported in the last column of Table III.

DISCUSSION

We have recently demonstrated that in barley and cucumber etioplasts poised in the MV Pchlide state, DV and MV Pchlide were formed from DV and MV Proto via DV and MV monocarboxylic biosynthetic routes, respectively (17). Furthermore, it was demonstrated that while in barley etioplasts, the DV and MV monocarboxylic routes were strongly interconnected prior to DV Pchlide formation, the two routes were very weakly, if at all, interconnected in cucumber etioplasts (17). These conclusions were based (a) on the establishment of precursor-product relationships between various DV and MV porphyrins and Mg porphyrins and (b) on the inability of the barley and cucumber etioplasts to convert DV Pchlide to MV Pchlide (17).

The results reported in this work complement and expand those reported in Tripathy and Rebeiz (17). It is shown for the first time that barley chloroplasts and etiochloroplasts poised in the DV Pchlide biosynthetic mode are perfectly capable of converting exogenous DV Pchlide to MV Pchlide in darkness (Table I). It may be recalled that in such DMV/LDV plastids the transition from a DV Pchlide to a MV Pchlide biosynthetic mode occurs very rapidly in darkness, within the 60 min time frame of the *in vitro*-dark incubations (3, 4).

Since the foregoing *in vitro* studies gave no indication of the

Table III. *Comparison of Theoretical and Experimental ^{14}C Incorporation into MV Pchlide in Green and Greening Cucumber Cotyledons*

Photoperiodically grown cucumber cotyledons or etiolated cotyledons, which were illuminated for 5 h with white fluorescent light, poised in the DV Pchlide biosynthetic mode, were incubated with 5 μCi of [^{14}C]ALA in darkness. All experimental and analytical conditions are as in Table II.

Experiment	Tissue	Length of Dark-Incubation with [^{14}C] ALA	Time Interval (X) under Consideration	Specific Radioactivity (Y_{Ax}) of DV Pchlide at the End of Time Interval X	Increase in MV Pchlide (ΔB_x) during Time Interval X	Theoretical ^{14}C Incorporation (QB_x) into MV Pchlide during Time Interval X	Experimental (Exp) ^{14}C Incorporation into MV Pchlide during Time Interval X	Maximum Possible Percent Conversion of DV Pchlide to MV Pchlide during Time Interval X[a]
		h		*dpm/pmol*	*pmol*	*calculated with Eq. 1 (dpm × 10^{-3})*	*dpm × 10^{-3}*	*%*
A	Green	0	t_0	0	0	0	0	0
		2	t_0-t_2	34.1	6526	111.3	262.8	42
		4	t_2-t_4	53.5	3729	163.3	262.3	62
		6	t_4-t_6	76.0	4634	300.3	316.6	95
		10	t_6-t_{10}	61.7	7899	544.2	361.8	50
B	Greening	0	t_0	0	0	0	0	0
		2.5	$t_0-t_{2.5}$	68.7	1392	47.8	442	11
		4.5	$t_{2.5}-t_{4.5}$	91.7	3545	284.3	159.3	22
		6.5	$t_{4.5}-t_{6.5}$	56.9	3119	231.7	444	52

[a] Calculated as in Table II.

possible extent of the DV to MV Pchlide conversion in barley seedlings, they were complemented by *in vivo* investigations. Kinetic analysis of radiolabel incorporation in barley leaves indicated that during the transition from the DV to the MV Pchlide biosynthetic state, MV Pchlide could be formed only in parts from DV Pchlide. The proportion of MV Pchlide formed from DV Pchlide appeared to undergo an initial rise at the beginning of dark-incubation and then decreased after 1 h in darkness (Table II). The decrease in the conversion of DV to MV Pchlide in darkness corresponded to the terminal stages of the DV to MV Pchlide transition, during which the MV monocarboxylic route became the preponderant biosynthetic route. This observation is compatible with the lack of conversion of DV Pchlide to MV Pchlide which is observed in isolated etioplasts poised in the MV Pchlide biosynthetic mode (17).

Altogether, the foregoing *in vitro* and *in vivo* results suggest rather strongly that in DMV/LDV plant species such as barley, the conversion of DV Pchlide to MV Pchlide becomes functional during transition from the DV to the MV Pchlide biosynthetic state. The DV to MV Pchlide conversion may then be visualized as one means of eliminating the excess DV Pchlide which is no longer needed as the plant settles into the MV Pchlide biosynthetic mode.

In cucumber, a DDV/LDV plant species, in which the MV monocarboxylic route does not become preponderant until after abnormally long dark-incubation periods (3), the possible conversion of DV Pchlide to MV Pchlide could not be demonstrated. Indeed, on the barley incubation-time scale, isolated cucumber etiochloroplasts were not capable of converting DV Pchlide to MV Pchlide in darkness. It was not possible to determine, however, whether during much longer dark-incubation periods, isolated cucumber etioplasts would be capable of converting DV Pchlide to MV Pchlide. On this longer incubation-time scale, *in vivo* experiments with cucumber cotyledons suggested that if DV Pchlide was indeed convertible to MV Pchlide, then the conversion of DV Pchlide to MV Pchlide would rise and fall during dark-incubation as was observed for barley (Table III).

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