Chloroplast Biogenesis: Biosynthesis of Protochlorophyll(ide) via Acidic and Fully Esterified Biosynthetic Branches in Higher Plants†

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ABSTRACT: The operation of two parallel biosynthetic pathways during the biosynthesis of the protochlorophyll(ide) pool of cucumber cotyledons was probed with the use of several [^{14}C]tetrapyrroles in vitro. A comparison of the ratio (R) of δ-amino[^{14}C]levulinic acid incorporation into protochlorophyllide/^{14}C incorporation into protochlorophyllide ester with other incorporation ratios, R_X, where X represents any of several [^{14}C]tetrapyrrole substrates, allowed us to determine which exogenous ^{14}C-labeled substrate was common precursor of protochlorophyllide and protochlorophyllide ester and which one was not. From such comparisons, a biosynthetic pathway of protochlorophyll(ide) biosynthesis is inferred. According to this pathway, protochlorophyllide is formed via an acidic (mono/dicarboxylic) biosynthetic branch, while protochlorophyllide ester is formed via a fully esterified (neutral) biosynthetic branch. The two biosynthetic branches are linked together by porphyrin ester synthetases at the level of protochlorophyllides and possibly at the level of the photoporphyrin IX and the magnesium protoporphyrin monoester pools.

On the basis of several studies which were performed during the past decase (Rebeiz et al., 1978), it has been proposed that chlorophyll (Chl)[1] *a* is formed via two distinct and parallel biosynthetic routes in plants (a) via the conventional acidic (di/monocarboxylic) route that converts Proto into Chl *a*, that is, Proto → Mg-Proto → MPE → Pchlide → Chlide *a* → Chl *a*, and (b) via a fully esterified (neutral) route that converts an unknown precursor "X" into Pchlide ester and the latter into Chl *a*, that is, X →→→ Pchlide ester → Chl *a*. In this scheme, "X" could conceivably be a common precursor of the acidic and fully esterified pathways (Rebeiz et al., 1978).

The proposal of the above pathway was based on the following observations. (a) Kinetic analysis of [^{14}C]ALA incorporation into the Pchlide and the Pchlide ester pools[2] in vivo was only compatible with biosynthetic models that involved the formation of Pchlide and Pchlide ester in parallel from a common precursor (Rebeiz et al., 1970). (b) Investigations of a possible precursor–product relationship between [^{14}C]Pchlide and [^{14}C]Pchlide ester in crude homogenates (Ellsworth & Nowak, 1973) and in isolated etiochloroplasts (Mattheis & Rebeiz, 1977a) failed to demonstrate the conversion of [^{14}C]Pchlide into [^{14}C]Pchlide ester and vice versa. These results led in turn to the conclusion that the Pchlide and Pchlide ester pools were not metabolically interconnected in greening tissues (Mattheis & Rebeiz, 1977a). (c) The very recent discovery of a fully esterified MPE-7 ester pool in plant tissues (Figure 1) suggested that the tetrapyrrole precursors of Pchlide ester may be fully esterified Mg-porphyrins (McCarthy et al., 1981). (d) Finally the photoconversion of Pchlide ester into Chl *a* has been demonstrated in several laboratories (Liljenberg, 1974; Cohen & Schiff, 1976; Sasa & Sugahara, 1976; Belanger & Rebeiz, 1980a–d), which in turn suggested that Pchlide ester *a* was not a metabolically inactive pool as was previously assumed (Bogorad, 1966).

Since ALA is a common precursor of both Pchlide and Pchlide ester (Rebeiz et al., 1970; Rebeiz & Castelfranco, 1971), the branch for the acidic and fully esterified biosynthetic branches could be at any point between ALA and Pchl(ide). For a more precise location of the common precursor of the acidic and fully esterified biosynthetic branches, a detailed kinetic study of the incorporation of [^{14}C]ALA and of ^{14}C-labeled exogenous tetrapyrrole intermediates into the Pchlide and Pchlide ester pools was undertaken. It is shown that Pchl(ide) biosynthesis in higher plants proceeds via acidic and fully esterified biosynthetic branches which are interconnected and which appear to originate from a common precursor at the level of the protogen or Proto pools.

Materials and Methods

Radiochemicals. [^{14}C]ALA was purchased either from New England Nuclear, Boston, MA (25 mCi/mmol), or from Research Products International, Elk Grove, IL (59 mCi/mmol).

Isolation of Plastids. Etiochloroplasts were prepared from 4-day-old etiolated cucumber cotyledons harvested under laboratory light. The tissue was hand homogenized in a cofactor-enriched medium adjusted to pH 8.0 (Rebeiz et al., 1975a). The homogenate was passed through four layers of cheesecloth and centrifuged, first at 200*g* for 3 min and then at 1500*g* for 7 min in order to obtain the plastid pellet. The latter was then suspended in the same cofactor-enriched medium adjusted to pH 7.7 (Rebeiz et al., 1975a).

Incubation of the Isolated Plastids. Isolated plastids were routinely incubated in a total volume of 3 mL as previously described unless otherwise indicated (Rebeiz et all., 1975b,c). A final reaction volume of 3.0 mL contained 400 μmol of Tris-HCl, pH 7.7, 1 mmol of sucrose, 100 μmol of potassium phosphate, 1 μmol of $MgCl_2$, 10 μmol of GSH, 0.6 μmol of CoA, 0.8 mmol of methanol, 0.8 μmol of ATP, 0.15 μmol of

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[1] Abbreviations: ALA, δ-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; Proto, protoporphyrin IX; Mg-Proto, magnesium protoporphyrin IX; MPE, magnesium protoporphyrin 6-methyl ester; MPE-7 ester, magnesium protoprophyrin 6-methyl ester 7-ester; Pchlide, protochlorophyllide; Pchlide ester, protochlorophyllide ester; Pchl(ide), a mixture of Pchlide and Pchlide ester; Tris, tris(hydroxymethyl)aminomethane.

[2] The protochlorophyllide pool of etiolated tissues is a mixed pool and consists of monovinyl- and divinylprotochlorophyllide (Belanger & Rebeiz, 1980b). Likewise the protochlorophyllide ester pool consists of monovinyl- and divinylprotochlorophyllide ester components (Belanger & Rebeiz, 1980c).

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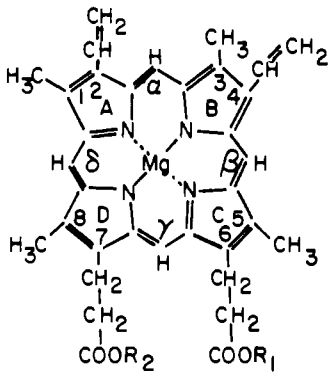

FIGURE 1: Structure of some DV-Mg-porphyrin tetrapyrroles: DV-Mg-Proto, R_1 = H and R_2 = H; DV-MPE, R_1 = CH_3 and R_2 = H; DV-MPE-7 ester, R_1 = CH_3 and R_2 = long chain alcohol.

NAD^+, and, where indicated, 5 μCi of [^{14}C]ALA; the final volume was made up with distilled water. When [^{14}C]tetrapyrroles were used as substrates, they were first dissolved in methanol; 0.03 mL of this solution was then added to the incubations. The incubations were conducted in the dark at 28 °C on a metabolic shaker operated at 10 cycles/min. The assays for the MPE concentration curve, reported in Figure 2, were conducted in a total reaction volume of 1.0 mL in Eppendorf microtubes but under the conditions described above.

Pigment Extraction. After incubation, 3.0 mL of the reaction mixture was mixed with 15 mL of acetone–0.1 N NH_4OH (9:1 v/v) at 0–4 °C and centrifuged at 39000*g* for 10 min (Rebeiz et al., 1975a). The pigments in the acetone extract were transferred to ether prior to chromatographic separation. This was achieved by adding to the acetone extract $^1/_{17}$ of its volume of saturated NaCl and $^1/_{70}$ of its volume of 0.5 M KH_2PO_4. The mixture was extracted once with 7 mL of peroxide-free ether, followed by one extraction with 5 mL of ether. The combined ether extracts were concentrated under N_2 until a distinct water–acetone phase was formed. The latter was then reextracted with approximately 6 mL of ether, and the extracted nonfluorescent aqueous layer was dicarded. This final ether extract was reconcentrated under N_2, and at this point, a few micrograms of unlabeled Chl, Pchlide, and, when needed, MPE were added to the ether as markers for chromatography.

Chromatography. Pchlide was separated from Pchlide ester by chromatography of the ether extracts on thin layers (5 × 20 cm) of silica gel H, 500-μM thick. The volume of the final ether extract was from 2 to 3 mL; of this, 1–2 mL was applied to four thin-layer plates. The plates were developed in toluene–ethyl acetate–ethanol (8:2:2 v/v/v) at 4 °C in the dark. Pigment bands corresponding to the separated tetrapyrroles were located by their red fluorescence, under UV light (366 nm). Pchlide ester was eluted from the silica gel with ether; Pchlide and MPE were eluted with methanol–acetone (4:1 v/v). The silica gel slurries were centrifuged, and the silica gel pellets were washed twice with the appropriate solvents. The [^{14}C]Proto substrate was purified by chromatography either on thin layers of cellulose MN 300 (500-μm thick) developed in 2,6-lutidine–0.05 N NH_4OH (5:3:5 v/v) or on thin layers of silica gel H developed in 2,6-lutidine–H_2O (10:3 v/v) in an ammonia-saturated atmosphere (Jensen, 1963).

Preparation of Tetrapyrrole Substrates. ^{14}C-Labeled substrates were prepared from cucumber cotyledons that were induced to accumulate various [^{14}C]tetrapyrroles by incubation with [^{14}C]ALA. Three grams of 3-day-old etiolated cotyledons were incubated in the dark for 16–20 h at 28 °C with 25 μCi of [^{14}C]ALA in a total volume of 5.5 mL (Mattheis & Rebeiz, 1977a). In preparing [^{14}C]MPE, the incubation also included 20 μmol of α,α'-dipyridyl dissolved in 0.03 mL of methanol (Mattheis & Rebeiz, 1977b). When [^{14}C]Pchlide was to be prepared, etiolated cotyledons were irradiated for 5 min with 250 ft-c of white fluorescent light prior to the addition of [^{14}C]ALA. The [^{14}C]MPE and [^{14}C]Pchlide substrates were extracted and transferred to ether as described elsewhere for MPE and Pchlide standards (Rebeiz et al., 1975a). They were purified on thin layers of silica gel H which were developed in toluene–ethyl acetate–ethanol as described above.

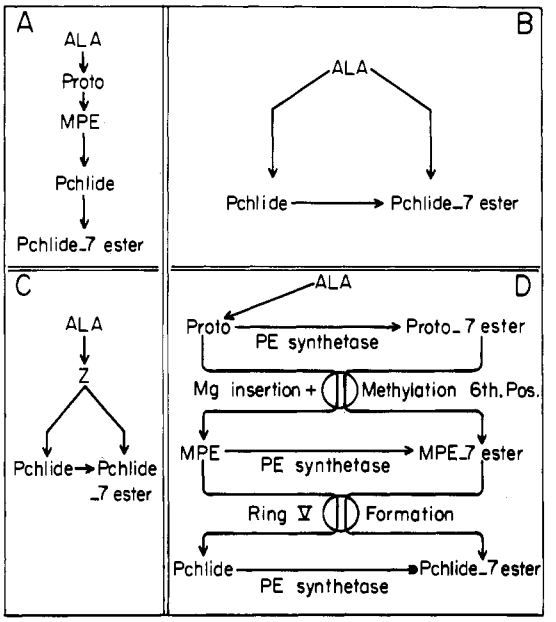

FIGURE 2: Four possible models of Pchl(ide) biosynthesis. ALA, δ-aminolevulinic acid; Proto, protoporphyrin IX; Proto 7-methyl ester, protoporphyrin IX 7-methyl ester; MPE, magnesium protoporphyrin 6-methyl ester; MPE-7 ester, magnesium protoporphyrin 6-methyl ester 7-ester; Pchlide, protochlorophyllide; Pchlide-7 ester, protochlorophyllide 7-ester.

[^{14}C]Proto was prepared from [^{14}C]MPE by acid hydrolysis: the [^{14}C]MPE was first dissolved in a small volume of acetone, and then this was made 7 N in HCl. The hydrolysis was carried out at room temperature in the dark for 2 h. Following this treatment, the [^{14}C]Proto was transferred to ether by neutralizing the acid with solid $NaHCO_3$ (Mattheis & Rebeiz, 1977b) and was further purified by chromatography as described above for [^{14}C]Proto.

All ^{14}C-labeled substrates were dried under N_2 and were used immediately or were stored dry at −20 °C in the dark until used.

Determination of ^{14}C Incorporation. The eluted Pchlide ester band was concentrated under N_2 to a volume of 1 mL or less, and the radioactivity of the entire sample was determined. For determination of the ^{14}C incorporation into Pchlide, aliquots of 1.0 mL were counted. The samples were counted in 15 mL of toluene scintillation solution [0.6 g of 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene and 7.0 g of 2,5-diphenyloxazole in 1 L of toluene]. A Packard Tri-Carb scintillation counter Model 3375 or Model 3255 was used. Quenching was determined by using the instrument internal standard. Samples were counted to a SD of ±5% or less.

Protein Determination. Proteins were determined by the standard biuret protein assay (Rebeiz et al., 1965).

Results

Demonstration of the Conversion of Small Amounts of [^{14}C]Pchlide into [^{14}C]Pchlide Ester in Vitro. In the past,

Table I: Conversion of [^{14}C]Pchlide into [^{14}C]Pchlide Ester in Vitro[a]

expt	substrate	amount of substrate added[b]	net ^{14}C recovered in the following pools[b]	
			Pchlide	Pchlide ester
1	[^{14}C]ALA	125.3	1.000	0.037
	[^{14}C]Pchlide	21.3	6.840	0.046
2	[^{14}C]ALA	88.1	0.928	0.031
	[^{14}C]Pchlide	10.1	4.831	0.020
3	[^{14}C]ALA	81.7	0.883	0.036
	[^{14}C]Pchlide	11.9	6.514	0.017

[a] Etiochloroplasts (9–13 mg of proteins) were incubated for 2 h in the dark with exogenous [^{14}C]Pchlide or with [^{14}C]ALA. Following the incubation, the pigments were extracted and separated by chromatography on thin layers of silica gel H. Net ^{14}C incorporation into Pchlide ester was determined by subtracting the ^{14}C incorporation into heat-inactivated controls from the ^{14}C incorporation into the active preparations. Incorporation of [^{14}C]ALA into the Pchl(ide) pools of the heat-inactivated controls was negligible. Incorporation of [^{14}C]Pchlide into the Pchlide ester pool of the heat-inactivated controls for experiments 1–3 amounted to 4%, 11%, and 18%, respectively, of the incorporations into the Pchlide ester pool of the active preparations. [b] In units of 10^{-6} × dpm/100 mg of plastid protein.

well-established cell-free systems have been successfully used in demonstrating the conversion of various tetrapyrroles into Pchlide *a* and Chl *a* (Mattheis & Rebeiz, 1977b–d). Since it was not possible to demonstrate with these same cell-free systems a precursor–product relationship between Pchlide and Pchlide ester (Mattheis & Rebeiz, 1977a), this was taken as further proof for the operation of a two-branched Pchl(ide) biosynthetic pathway in plants (Rebeiz et al., 1978). It was therefore conjectured that a detailed cell-free study of the precursor–product relationships among various intermediates of the Pchl(ide) pathway might help to determine the precise location of the branching point in the proposed two-branched pathway.

When experimental evidence was sought for such a branch point in the pathway, it became apparent that the situation was more complex than previously realized. Indeed improved chromatographic separation and recovery of ^{14}C-labeled reaction products and the use of much higher concentrations of ^{14}C-labeled substrates leads to the conclusion that small amounts of exogenous [^{14}C]Pchlide are indeed converted into [^{14}C]Pchlide ester during cell-free incubations (Table I). It is still impossible, however, to demonstrate the conversion of exogenous [^{14}C]Pchlide ester into [^{14}C]Pchlide (data not shown). These results indicate nevertheless that contrary to previous assertions (Mattheis & Rebeiz, 1977a), a slow reaction that converts small amounts of Pchlide into Pchlide ester irreversibly does link these two pools in plants. This, in turn, makes the interpretation of ^{14}C incorporation into Pchlide and Pchlide ester more difficult and prompted us to reconsider in more detail the impact of such a Pchlide to Pchlide ester conversion on the nature of the previously proposed two-branched Pchl(ide) biosynthetic pathway (Rebeiz et al., 1978).

Theoretical Analysis of Different Possible Routes of Pchl(ide) Biosynthesis from Various ^{14}C-Labeled Precursors. Because of the complications that arose from the demonstration in vitro of a reaction that linked the Pchlide pool to the Pchlide ester pool, an appropriate method that allows the meaningful interpretation of ^{14}C incorporation into [^{14}C]-Pchl(ide) was sought. An ideal method of ^{14}C-labeled analysis would allow us to distinguish between ^{14}C incorporation into Pchlide ester that arose from the esterification of [^{14}C]Pchlide and those which may arise from putative intermediates of the fully esterified biosynthetic branch. A method of analysis that nearly achieves this objective was finally developed. It was based on the observation that since [^{14}C]ALA is the earliest possible specific precursor to any possible branch point (Rebeiz et al., 1970), its relative incorporation into [^{4}C]Pchlide and [^{14}C]Pchlide ester could be used as a useful reference yardstick. Comparison of the relative incorporation of other ^{14}C-labeled intermediates into Pchlide and Pchlide ester to ALA incorporation may tell us whether any particular metabolic precursor is located before or after a putative branching point.

More specifically the reference yardstick was taken as the ratio "R_{ALA}" which is the net [^{14}C]ALA incorporation into Pchlide ester/net [^{14}C]ALA incorporation into Pchlide. The relative incorporation of any other ^{14}C-labeled intermediate "X" into Pchlide ester and Pchlide was denoted by the ratio "R_X". In this context "X" refers to the ^{14}C-labeled intermediate which is being used as a substrate in any particular experiment and "R" is the net ^{14}C incorporation of substrate X into Pchlide ester/net ^{14}C incorporation of substrate X into Pchlide. A closer examination of R_{ALA} and R_X values reveals that these ratios can assume different relative values depending on (a) whether the ^{14}C-labeled substrate X is metabolized via a branched or linear Pchl(ide) biosynthetic pathway, (b) if the pathway is actually branched, whether X belongs to the acidic or fully esterified Pchl(ide) biosynthetic branches, and (c) whether X is a metabolic intermediate which is located before or after a branch point in the pathway.

The above statements will now be illustrated by reference to four possible models of Pchl(ide) biosynthesis which are depicted in Figure 2. Model A (Figure 2A) depicts a linear unbranched Pchl(ide) biosynthetic pathway. In order for any intermediate to serve as a precursor of Pchlide ester, it would first have to be converted into Pchlide. The latter would then be converted, by esterification, into Pchlide ester. Model B (Figure 2B) depicts an extreme case of biosynthetic branching, where ALA is the only common precursor of the Pchl(ide) species. On the other hand, model C (Figure 2C) represents a branched pathway through which Pchlide ester can be formed from two different sources: (a) from Pchlide by direct esterification and (b) from a common precursor "Z" via a second biosynthetic branch. Model D (Figure 2D) is essentially a refined version of model C. It implies the operation of distinct porphyrin ester synthetases which connect the two Pchl(ide) biosynthetic branches at several points. To simplify the analysis, it is assumed that the enzymes responsible for the conversion of Proto and Proto-7 ester into MPE and MPE-7 ester, respectively (Figure 1) and those responsible for the formation of the cyclopentanone ring are nonspecific and cannot therefore recognize the presence or absence of a free carboxylic group at the seventh position of the macrocycle (Figure 1). In model D, then (Figure 2D), the esterification of the seventh position of the macrocycle may take place at any step of the pathway past Proto.

Finally, it is essential to recognize that mathematically speaking, three relationships between R_{ALA} and R_X values are of interest, namely, (a) $R_X = R_{ALA}$, (b) $R_X < R_{ALA}$, and (c) $R_X > R_{ALA}$. The biosynthetic consequences of these three relationships are summarized in Table II.

Experimental Strategy. So that the various Pchl(ide) biosynthetic models that were just described could be tested, the following experimental approach was adopted. Various [^{14}C]tetrapyrrole intermediates (X) of the Pchl(ide) biosynthetic pathway were prepared as described under Materials and Methods and were incubated with isolated plastids. The cell-free systems that were used had been shown earlier to be

Table II: Biosynthetic Consequences of Various R_X/R_{ALA} Relationships[a]

R_X/R_{ALA} relationships	biosynthetic consequences
$R_X = R_{ALA}$	1. relationship incompatible with model B 2. X can be any precursor of Pchlide in model A 3. X can only be an intermediate prior to or at the branching point Z in model C 4. X can only be an intermediate of the acidic biosynthetic branch in model D and is more likely to be Proto than a tetrapyrrole past Proto
$R_X > R_{ALA}$	1. relationship incompatible with model A 2. if R_X is infinite, then intermediate X is located past the branching point Z in model B, C, or D and belongs to the fully esterified biosynthetic branch 3. if R_X is finite, then X belongs to the acidic biosynthetic branch in model D at or past Proto and past any putative rate-limiting reaction
$R_X < R_{ALA}$	1. relationship incompatible with model A 2. if R_X is finite and assumes a constant value which is independent of the nature of the intermediate X, then X is located past the branching point in models B and C and on the acidic biosynthetic branch 3. if R_X is finite but assumes a variable value which depends on the nature and concentration of intermediate X, then X is located on the acidic biosynthetic branch in model D at or past Proto but prior to any putative rate-limiting reaction

[a] R_X refers to the net ^{14}C incorporation of substrate X into Pchlide ester/net ^{14}C incorporation of substrate X into Pchlide. In R_{ALA}, the ^{14}C-labeled substrate is ALA.

capable of metabolizing exogenous tetrapyrrole intermediates (Mattheis & Rebeiz, 1977a–d; Smith & Rebeiz, 1977). In parallel reaction mixtures, [^{14}C]ALA was also incubated with the isolated plastids under the same conditions as the substrate X. For each substrate, background counts were determined by heating one reaction mixture for 5 min in a boiling water bath before adding the ^{14}C-labeled substrate to the inactivated plastids. These inactivated reaction mixtures were then incubated alongside the active ones. Following incubation, the pigments were extracted and chromatographed on thin layers of silica gel H, an the net ^{14}C incorporations into Pchl(ide) were then determined by subtracting the amount of label recovered in the Pchl(ide) bands of the heat-inactivated samples from the amount of label recovered in the active samples. The R_X and R_{ALA} ratios, for a particular experiment, were then calculated, and the appropriate conclusions were drawn from the R_X/R_{ALA} relationship, as was just described.

It should be made clear from the onset that the R_X and R_{ALA} values for any particular experiment are meaningful only if the Pchlide and Pchlide ester pools did not become saturated with substrate during the cell-free incubations. Under our incubation conditions, substrate saturation of the Pchl(ide) pool biosynthesis would result in a sharp decline in the rate of ALA or X conversion into Pchl(ide), as a function of time or as a function of substrate concentration. We have already demonstrated elsewhere that this condition did not prevail during ALA incubations in vitro (McCarthy & Rebeiz 1980). Instead, ALA conversion into Pchlide increased during the first 2 h of incubation while its rate of conversion to Pchlide ester increased during the first hour of incubation and underwent only an insignificant decrease during the second hour of incubation (Figure 1 in McCarthy & Rebeiz, 1980).

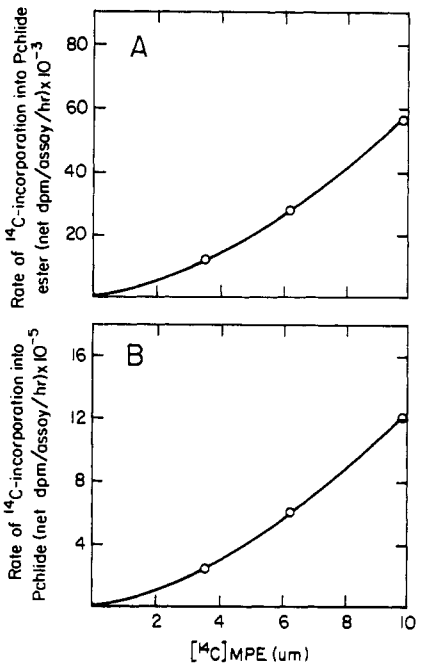

FIGURE 3: Rate of [^{14}C]Pchlide-7 ester (A) and [^{14}C]Pchlide synthesis from [^{14}C]MPE, as a function of [^{14}C]MPE concentration. Etiochloroplasts (6–8 mg of proteins) were incubated with increasing concentrations of [^{14}C]MPE for 2 h in the dark. Pigment separation and the determination of net ^{14}C incorporations were as described in Tables I and III.

The possible Pchl(ide) biosynthesis saturation with exogenous tetrapyrroles was tested in a fixed time assay by using various concentrations of exogenous [^{14}C]MPE (Figure 3). The latter is a tetrapyrrole that was shown earlier to be very rapidly converted into Pchlide in vitro (Mattheis & Rebeiz, 1977b). As shown in Figure 3, under these incubation conditions, the rate of [^{14}C]MPE conversion into Pchlide and Pchlide ester in vitro did not decline as the concentration of added [^{14}C]MPE was increased. Instead the rate of [^{14}C]MPE incorporation into [^{14}C]Pchl(ide) increased accordingly.

Altogether the above results indicated that under the incubation conditions used in this work, the Pchl(ide) pools did not become saturated during the first 2 h of incubation. Therefore, meaningful R ratios could be derived from such incubations.

Biosynthetic Route of [^{14}C]*MPE Conversion into* [^{14}C]-*Pchl(ide)*. [^{14}C]MPE was the first tetrapyrrole substrate that was used in order to probe the R_X–R_{ALA} relationship and to help visualize a logical Pchl(ide) biosynthetic route. This tetrapyrrole was shown earlier to be very readily convertible into Pchlide in vitro (Mattheis & Rebeiz 1977b). Thus active and heat-inactivated etiolchloroplasts were incubated with [^{14}C]MPE and with [^{14}C]ALA. Net ^{14}C incorporations into Pchlide and Pchlide ester were determined, and the R_{MPE} and R_{ALA} values were calculated.

A small but significant net ^{14}C-label incorporation into Pchlide ester was consistently observed (Table III). This was expected regardless of the biosynthetic route via which [^{14}C]MPE was metabolized since this substrate was readily converted into [^{14}C]Pchlide (Table III) and the latter could be consequently converted into Pchlide ester via the Pchlide/Pchlide ester reaction which is common to all biosynthetic models (Figure 2).

The differences between the R_{MPE} and the R_{ALA} values of five different experiments were subjected to a paired variates Student t distribution analysis (Alder & Roessler, 1961) in order to first determine whether the R_{MPE} and R_{ALA} values

Table III: Comparison of R_{MPE} to R_{ALA} Values[a]

expt	substrate	amount of substrate added[e]	net ^{14}C incorporation into[e]: Pchlide ester (A)	Pchlide (B)	$10^2 \times R_X$[b] (A/B)	Student's t distribution[f]: calcd[c]	tabulated[d]
1	[^{14}C]ALA	126.1	0.0178	0.5136	3.47		
	[^{14}C]MPE	30.0	0.0220	4.4628	0.49		
2	[^{14}C]ALA	126.1	0.0162	0.4689	3.45	3.952	$t_{0.05} = 2.776$
	[^{14}C]MPE	61.1	0.0695	8.2703	0.84		$t_{0.02} = 3.747$
3	[^{14}C]ALA	131.1	0.0165	0.7922	2.08		
	[^{14}C]MPE	26.0	0.0034	4.3471	0.08		
4	[^{14}C]ALA	131.0	0.0037	0.3962	0.93		
	[^{14}C]MPE	46.3	0.0219	16.5000	0.13		
5	[^{14}C]ALA	269.1	0.0032	0.2618	1.22		
	[^{14}C]MPE	23.7	0.0122	2.4400	0.50		

[a] Etiochloroplasts (8–9 mg of proteins) were incubated for 2 h in the dark with added [^{14}C]MPE or with [^{14}C]ALA. Following the incubation, the pigments were extracted and separated by chromatography on thin layers of silica gel H. Net ^{14}C incorporations were determined by subtracting the ^{14}C incorporation into heat-inactivated controls from the ^{14}C incorporation into the active preparations. Incorporations of [^{14}C]ALA into the Pchl(ide) pools of the heat-inactivated controls were negligible. Incorporation of [^{14}C]MPE into the Pchl(ide) pools of the heat-inactivated controls for experiments 1–5 amounted to 9%, 6%, 7%, 1%, and 2%, respectively, of the incorporations into the Pchlide pool of the active preparations and to 50%, 48%, 92%, 31%, and 28%, respectively, of the incorporations into the Pchlide ester pool of the active preparations. [b] R_X refers either to R_{MPE} or to R_{ALA}, depending on the substrate used; it is defined as in Table II. [c] t (calcd) = $|\bar{D} - O|/S_{\bar{d}}$, where $S_{\bar{d}} = S_d/n^{1/2}$ and where $n = 3$ = number of paired variates, $\bar{D}$ = mean of the differences between the paired variates = $\Sigma_{i=1}^{3} R_{ALA} - R_{MPE}/5$, and S_d = standard deviation of the differences between the paired variates (Alder & Roessler, 1961). [d] $t_{0.05}$ and $t_{0.02}$ = tabulated values for 4° of freedom, at the 95% and 98% confidence levels, respectively (Alder & Roessler, 1961). [e] In units of 10^{-6} × dpm/100 mg of plastid protein. [f] Conclusion: $R_{MPE} < R_{ALA}$.

Table IV: Comparison of R_{Proto} to R_{ALA} Values[a]

expt	substrate	amount of substrate added	net ^{14}C incorporations into[e]: Pchlide ester (A)	Pchlide (B)	$10^2 \times R_X$[b] (A/B)	Student's t distribution[d]: t calcd	$t_{0.1}$[c] tabulated
1	[^{14}C]ALA	755.0	0.2579	8.5871	3.00		
	[^{14}C]Proto	34.66	0.0155	0.3867	4.01		
2	[^{14}C]ALA	1563.0	0.4628	11.4094	4.06	1.045	2.35
	[^{14}C]Proto	114.05	0.1111	1.2797	8.68		
3	[^{14}C]ALA	742.0	0.0415	5.9609	0.70		
	[^{14}C]Proto	120.0	0.0263	3.2932	0.80		
4	[^{14}C]ALA	1283.0	0.0862	4.9598	1.74		
	[^{14}C]Proto	32.4	0.0103	1.0332	1.00		

[a] Etiochloroplasts (8–14 mg of proteins) were incubated for 2 h in the dark, with added [^{14}C]Proto or with [^{14}C]ALA. Pigment separation and the determination of net ^{14}C incorporations were as described in Table III. Incorporations of [^{14}C]ALA into the Pchl(ide) pools of the heat-inactivated controls were negligible. Incorporation of [^{14}C]Proto into the Pchl(ide) pools of the heat-inactivated controls for experiments 1–4 amounted to 18%, 9%, 8%, and 6%, respectively, of the incorporations into the Pchlide pool of the active preparations and to 24%, 18%, 44%, and 49%, respectively, of the incorporations into the Pchlide ester pool of the active preparations. [b] R_X refers either to R_{Proto} or to R_{ALA}; these were defined in Table II. [c] $t_{0.1}$ = tabulated value for 3° of freedom, at the 90% confidence level (Alder & Roessler, 1961). [d] Conclusion: $R_{Proto} = R_{ALA}$. [e] In units of 10^{-5} × dpm/100 mg of plastid protein.

were significantly different from one another. As shown in Table III, the calculated t value (3.952) was higher than the tabulated $t_{0.05}$ (2.776) and $t_{0.02}$ (3.747) values for 4° of freedom and the 95% and 98% confidence levels, respectively. These results indicated that at both the 98% and 95% confidence levels (i.e., at both the 2% and 5% levels of significance), the R_{MPE} and R_{ALA} values were significantly different from one another and were therefore related by an $R_{MPE} < R_{ALA}$ relationship. This in turn led to the following conclusions (Table II): (a) Since R_{MPE} was significantly smaller than RALA, this relationship was incompatible with model A (Figure 2A), which also meant that Pchlide and Pchlide ester were not synthesized via a single-branched, linear biosynthetic pathway. (b) Since R_{MPE} was significantly smaller than R_{ALA} and since R_{MPE} assumed a finite value, MPE must have been processed via a branched pathway such as one of those depicted in Figure 2B–D. Furthermore, it indicated that MPE was located on the acidic biosynthetic branch of any putative branched pathway. (c) On the other hand, since the R_{MPE} values appeared to vary rather widely depending, among other things, on the substrate concentration used in a particular experiment (Table III), it is suggested that MPE was more likely to be metabolized via the monocarboxylic biosynthetic branch of model D than that of model B or C.

Biosynthetic Route of [^{14}C]Proto Conversion into [^{14}C]-Pchl(ide). For further determination of which one of the branched biosynthetic routes depicted in Figure 2 most closely described the flow of intermediates during Pchl(ide) biosynthesis, the metabolism of [^{14}C]Proto in vitro was studied.

The results of four different experiments are depicted in Table IV. As expected, [^{14}C]Proto was incorporated into both Pchlide and Pchlide ester, since Proto is readily converted into Pchlide in vitro (Mattheis & Rebeiz, 1977d). However, statistical analysis of the R_{Proto}–R_{ALA} differences indicated that the R_{ALA} and R_{Proto} values for any particular experiment were not significantly different even at the 90% condifence level (Table IV). These results indicated that an $R_{Proto} = R_{ALA}$ relationship most closely characterized the metabolism of exogenous Proto in vitro. This in turn ruled out the possible operation of model B (Figure 2B; Table II). Since the $R_{MPE} < R_{ALA}$ relationship (Table III) ruled out the operation of model A (Figure A; Table II), this left only models C and D (Figure 2C,D) as possible biosynthetic routes of Pch(ide) biosynthesis. Finally, since it is a Proto substrate that yielded an $R_X = R_{ALA}$ relationship (Table IV), this in turn appeared to favor the operation of model D instead of model C as did

the $R_{MPE} < R_{ALA}$ relationship (Table II).

Discussion

The possible involvement of acidic and fully esterified biosynthetic pathways in the formation of Pchl(ide) (Rebeiz et al., 1978) has been reexamined with the use of well-established cell-free systems (Mattheis & Rebeiz, 1977a–d). Several possible biosynthetic models were tested by comparing the ratio of ^{14}C incorporation of various ^{14}C-labeled substrates into [^{14}C]Pchlide and [^{14}C]Pchlide ester to the [^{14}C]ALA incorporation into [^{14}C]Pchlide/[^{14}C]Pchlide ester. The use of R_X values, that is, of ratios of ^{14}C incorporation into [^{14}CPchlide ester/^{14}C incorporation into [^{14}C]Pchlide, instead of absolute incorporations into either Pchlide or Pchlide ester, proved very convenient. This analytical approach normalized all incorporation data and rendered it independent of the fluctuations in the biosynthetic activity of various plastid preparations. This in turn permitted comparisons between experiments (Tables III and IV) that would have been otherwise impossible. The comparison of R_X values from different ^{14}C-labeled substrates X with R_{ALA} values lent itself admirably for the analysis of biosynthetic branching (Table II).

The biosynthetic models of Pchl(ide) biosynthesis that best fit the experimental data are depicted in parts C or D of Figure 2. According to these pathways, Pchlide is formed via an acidic biosynthetic branch while Pchlide ester is formed via a fully esterified biosynthetic branch. The acidic and fully esterified biosynthetic branches are linked together by an irreversible porphyrin ester synthetase at the level of Pchlide (models C and D) and at the level of Pchlide, Proto, and MPE in model D (Figure 2D). Further experimental evidence in favor of model D instead of model C (Figure 2C,D) is forthcoming (S. A. McCarthy and C. A. Rebeiz, unpublished results). Evidence for the operation of a Pchlide ester synthetase linking the Pchlide and Pchlide ester pools was provided by the successful demonstration of [^{14}C]Pchlide conversion into [^{14}C]Pchlide ester in vitro (Table I).

Interestingly, the Pchl(ide) biosynthetic pathway depicted in Figure 2D predicts the occurrence of several novel tetrapyrrole intermediates that populate the fully esterified biosynthetic branch in plants. One of these, i.e., MPE-7 ester (Figure 1), has been very recently detected in higher and lower plants (McCarthy et al., 1981). Research aimed at detecting Proto-7 ester is in progress. The latter may share a common feature with MPE-7 ester and Pchlide-7 ester, namely, esterification of the carboxylic group at position 7 of the macrocycle with long chain fatty alcohols (Figure 1) (McCarthy et al., 1981).

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