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Protochlorophyll Biosynthesis in a Cell-free System from Higher Plants[1]

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CONSTANTIN A. REBEIZ AND PAUL A. CASTELFRANCO
WITH THE TECHNICAL ASSISTANCE OF IRMGARD LINDEMANN
Department of Botany, University of California, Davis, California 95616

ABSTRACT

A cell free system prepared from etiolated cucumber (*Cucumis sativus*, L) in tris-sucrose buffer is able to incorporate δ-aminolevulinic acid-4-^{14}C into the two components of protochlorophyll: protochlorophyllide and protochlorophyllide ester. The activity is associated with the etioplasts. Optimal incorporation is obtained at pH 7.7. For the formation of protochlorphyllide ester, oxygen, reduced glutathione, methyl alcohol, magnesium, inorganic phosphate, and nicotinamide adenine dinucleotide are required. For the formation of ^{14}C-protochlorophyllide, adenosine triphosphate, and coenzyme A are required in addition to the above. The requirement for methyl alcohol is highly specific, and the methyl group appears to be incorporated into the protochlorophyll molecules. A biosynthetic scheme resulting in the parallel production of ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester from ^{14}C-Mg protoporphyrin monoester is presented.

Recently, Rebeiz *et al.* (28) reported the incorporation of δ-aminolevulinic acid-4-^{14}C into the protochlorophylls of etiolated cucumber cotyledons. Kinetic analysis of specific radioactivities of the ^{14}C-protochlorophyll pools suggested that protochlorophyllide and protochlorophyllide ester[2] originated simultaneously from a common precursor, probably Mg protoporphyrin monoester (28). In order to gain better understanding of the biosynthetic steps between Mg protoporphyrin monoester and protochlorophyll, the authors attempted to develop a cell-free system capable of protochlorophyll biosynthesis (21) and proceded to investigate the biosynthetic activity of cotyledonary homogenates incubated with δ-aminolevulinic acid. They found that these homogenates accumulated uroporphyrin, coproporphyrin, Mg coproporphyrin, and traces of protoporphyrin (21). In the absence of added cofactors, Mg protoporphyrin monoester appeared to be the last intermediate in the biosynthetic sequence leading to protochlorophyll.

During the incubation most of the endogenous protochlorophyll disappeared (21). It was suggested that this loss of biosynthetic capacity by the cell-free system might be attributed to structural disorganization, enzyme destruction, and loss or dilution of needed cofactors (21). In the present study we have shown that by fortifying the cell-free homogenates prepared from etiolated cucumber cotyledons with the proper cofactors it is possible to enhance the incorporation of ^{14}C-ALA[3] into ^{14}C-Mg protoporphyrin monoester and also to biosynthesize labeled protochlorophylls.

When the cell-free homogenate was prepared from cucumber cotyledons in which the lag phase had been removed by the appropriate light treatment chlorophyll *a* and *b* biosynthesis could be demonstrated. The biosynthesis of chlorophyll in such a cell-free system forms the object of another article (23).

Two preliminary communications based on this research were presented to the Western Section of the American Society of Plant Physiologists, Berkeley, California, in June 1970 (24) and to the annual meetings of the American Society of Plant physiologists, Bloomington, Indiana, in August 1970 (25).

MATERIALS AND METHODS

Growing and Harvesting Cucumber Cotyledons. Cucumber seeds (*Cucumis sativus* L. var Alpha green), a gift of the Niagara Chemical Division, FMC Corporation, Modesto, California, were used. They were germinated in vermiculite (Terra Lite) at 24 C in complete darkness (8).

Chemicals and Radiochemicals. ^{14}C-ALA (33.8 μc/μmole) and ^{14}C-methanol (1 μc/μmole) were purchased from Tracerlab, Waltham, Massachusetts. The following compounds were also obtained from commercial sources: GSH, from Eastman Organic Chemicals; CoA, ATP, NAD, from Nutritional Biochemicals Corporation; KH_2PO_4 and methyl alcohol, analytical reagent grade, from Mallinckrodt. The petroleum ether used was a hydrocarbon fraction boiling from 60 to 90 C.

Preparation of the Crude Homogenate. Five grams of 4.5-day-old etiolated cotyledons were macerated gently with mortar and pestle in 7.5 ml of 0.5 M sucrose, 0.2 M tris-HCl, pH 7.7 or 7.9, under a green safelight at 1 C. The slurry was filtered through four layers of cheese cloth. The resulting filtrate is called the "crude homogenate" (C); it contains intact etioplasts and no detectable intact cells as evidenced by light and phase contrast microscopy (21). Total proteins were determined by biuret as described previously (27).

Preparation of Crude Etioplasts. The crude homogenate was centrifuged at 0 C for 3 min at 200*g* and the resulting pellet (P_1) was discarded. The supernatant (S_1) was centrifuged at 0 C for 7 min at 1500*g* (Falk, R., personal communication). The resulting

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[2] The same terminology for porphyrin derivatives is used in this paper as in the preceding articles of this series (21, 28).

[3] Abbreviation: ^{14}C-ALA: δ-aminolevulinic acid-4-^{14}C.

crude etioplast pellet (P_2) was suspended in the homogenizing buffer and adjusted to the same volume as the resulting supernatant (S_2). Equal volumes of 2 ml of the various fractions were assayed.

Preparation of Fortified Etioplasts. Five grams of etiolated cotyledons were ground in 7.5 ml of 0.2 M tris-HCl, pH 7.7, 0.5 M sucrose containing 37.5 μmoles of GSH, 2.25 μmoles of CoA, 3.75 μmoles of $MgCl_2$, 375 μmoles of potassium phosphate buffer, pH 7.7, and 180 μmoles of methanol. Fortified etioplasts (FP_2) were prepared from the fortified crude homogenate (FC) as described above for crude etioplasts. Washed fortified etioplasts (WFP_2) were prepared by suspending FP_2 in 9 ml of the fortified grinding buffer and centrifuging once again at 1500*g* for 7 min. Both FP_2 and WFP_2 were resuspended in 9 ml of buffer.

Incubation of the Cell-Free Preparation with ^{14}C-ALA. Volumes of 2.0 ml of crude homogenate (C), crude etioplasts (P_2), fortified etioplasts (FP_2), washed fortified etioplasts (WFP_2), or supernatant (S_2) were incubated on a metabolic shaker in glass tubes wrapped in aluminum foil, with 2 μc (59 mμmoles) of ^{14}C-ALA. The incubation was carried out in the dark at 28 C with moderate shaking speed for 16 hr. In a total volume of 2.5 ml, the incubation medium contained 400 μmoles of tris-HCl, pH 7.7 or 7.9, 1 mmole of sucrose, 100 μmoles of potassium phosphate buffer pH 7.7 or 7.9, 1 μmole of $MgCl_2$, 10 μmoles of GSH, 0.6 μmole of CoA, 1.2 mmole of methyl alcohol, and the following amounts of protein; 100 mg of C, 40 mg of S_2, 7 to 10 mg of P_2 or FP_2, 3.5 mg of WFP_2.

Extraction of ^{14}C-Mg Protoporphyrin Monoester, ^{14}C-Protochlorophyllide, and ^{14}C-Protochlorophyllide Ester. The reaction was stopped in the dark by the addition of 12.4 ml of acetone: 0.1 N NH_4OH (9:1 v/v). The mixture was immediately centrifuged at 30,000*g* for 10 min, and the resulting pellet was washed with 2.0 ml of the acetone:NH_4OH mixture. To the combined supernatant, 1/17 of its volume of saturated NaCl and 1/70 of its volume of 0.5 M KH_2PO_4 were added. At this stage a few micrograms of standard porphyrin pigments were added as carriers. Mg protoporphyrin monoester was added as an acetone:methanol (4:1 v/v) solution. Protochlorophyll (a mixture of protochlorophyllide and protochlorophyllide ester) was added in ether (21). The mixture was extracted once with 5 ml of ether followed by two more extractions of 2.5-ml each. The combined ether extracts containing large amounts of acetone were washed once with 30 ml of cold H_2O saturated with $MgCO_3$. The ether extract was centrifuged and the acetone:H_2O layer removed. The H_2O-washed ether extract was dried under N_2, and the residue from one reaction mixture was transferred quantitatively in about 0.2 ml of acetone to 3 thin layers of Silica Gel H, 500 μ thick, freshly activated at 105 C for 30 min. The chromatograms were developed in the dark at 4 C in benzene:ethyl acetate:ethanol (8:2:2 v/v) (21, 28), The red fluorescent areas corresponding to carrier Mg protoporphyrin monoester, protochlorophyllide, and protochlorophyllide ester and their ^{14}C-counterparts were viewed under ultraviolet light of 366 nm. The protochlorophyllide, Mg protophorphyrin monoester, and protochlorophyllide ester bands were scraped into 25-ml beakers and eluted respectively with: methyl alcohol, acetone:methanol (4:1 v/v), and ether. The suspensions were centrifuged, the silica gel pellets were washed twice with small amounts of eluants, and the total volumes were adjusted to 7 to 8 ml. An aliquot of 0.5 ml was used for determining the quantitative ^{14}C-incorporation into these compounds; the remainder of each sample was concentrated or dried under N_2 for further chromatographic and analytical manipulations.

Conversion of Protochlorophyllide into Protopheophytin. The mixture of carrier protochlorophyllide and ^{14}C-protochlorophyllide eluted in methanol from Silica Gel H was dried under N_2, suspended in cold 4.1 N HCl, (15% HCl w/v) and rapidly mixed with ether (13). The HCl-aqueous layer was neutralized with solid Na-acetate, the pale green ether layer was removed, washed several times with cold H_2O until free of acid, and concentrated under N_2 for further chromatographic manipulations. Alternatively, protochlorophyllide in methanol was made 0.02 M with respect to HCl and dried under N_2 before chromatography.

Conversion of Protochlorophyllide Ester into Protopheophytin Ester. The mixture of carrier protochlorophyllide ester and ^{14}C-protochlorophyllide ester eluted in ether from Silica Gel H was shaken with cold 6.9 N HCl (25% HCl w/v). At this HCl concentration part of the green color was extracted into aqueous phase. The HCl extract, largely free of interfering carotenoids, was removed and the pigment was transferred to ether as described above. The ether extract was concentrated under N_2 for further chromatographic manipulations.

Conversion of Protochlorophyllide Ester into Protopheophytin. The mixture of carrier protochlorophyllide ester and ^{14}C-protochlorophyllide ester eluted in ether from Silica Gel H was thoroughly shaken with 12 N HCl (14) and incubated at 20 C in the dark for 1 hr. The yellow ether phase containing carotenoids was removed, and the acid phase containing unhydrolyzed protopheophytin ester and protopheophytin was mixed with ether; then the acid layer was diluted with H_2O and neutralized with solid Na-acetate. The pigment passed into ether, and the latter was washed to neutrality with H_2O and concentrated under N_2 for further chromatographic manipulations.

Thin Layer Chromatography of Eluted ^{14}C-Protochlorophylls and Their Hydrolysis Products. ^{14}C-Protochlorophyllide and ^{14}C-protochlorophyllide ester were rechromatographed on thin layers of Silica Gel H in benzene:ethyl acetate:ethanol (8:2:2 v/v) in the dark at 4 C. ^{14}C-Protopheophytin, ^{14}C-protopheophytin ester and its hydrolysis product were chromatographed on thin layers of Silica Gel H in the dark at 28 C in benzene: ethyl acetate:ethanol (8:2:5 v/v).

Paper Chromatography of Eluted ^{14}C-Protochlorophylls and Their Mg-free Bases. The chromatographic mobility of ^{14}C-protochlorophyllide and its Mg-free base was determined ascendingly on Whatman No. 1 paper in toluene and on Whatman No. 3 paper in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v) (7,16). That of ^{14}C-protochlorophyllide ester and its Mg-free base was determined on Whatman No. 3 paper in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v) and on Whatman No. 1 paper in acetone: petroleum ether:acetic acid (3:7:0.01 v/v) (7). The chromatograms were developed in the dark at room temperature. The separated compounds were viewed on the chromatogram either under ultraviolet light or visible light.

Determination of ^{14}C-Incorporations. Half-milliliter aliquots of the ^{14}C-Mg protoporphyrin monoester, ^{14}C-protochlorophyllide, and ^{14}C-protochlorophyllide ester eluates were counted in 10 ml of toluene scintillation solution (0.6 g of dimethyl POPOP, 7.0 g of PPO in 1 liter of toluene) for 10 min in a Tri-Carb Packard liquid scintillation counter Model 3310. Under these conditions ^{14}C was counted with an efficiency of about 89%. Radioactive areas on thin layers were localized with a Packard radiochromatogram scanner Model 7201, whereas radioactive areas on the paper chromatograms were localized with a Nuclear Chicago radiochromatogram scanner Model 1025.

Preparation of Porphyrin and Phorbin Standards. Mg protoporphyrin monoester and protoporphyrin monoester were prepared and purified as described in a previous communication (21). Protochlorophyll in ether was prepared from freshly harvested 5-day-old etiolated cucumber cotyledons as described for the extraction of Mg-protoporphyrin monoester (25). ^{14}C-Protochlorophyllide and ^{14}C-protochlorophyllide ester were prepared and purified as described earlier (28).

RESULTS

Radioactive Products of ^{14}C-ALA Incubation. Incubation of ^{14}C-ALA with the crude homogenate for 16 hr at 28 C in the dark in the presence of appropriate cofactors produced a highly radioactive ether extract. Upon chromatography of the latter on thin layers of Silica Gel H, the radioactivity separated into 4 bands (Fig. 1).

The band at the origin consisted probably of free porphyrins (21) and was not investigated any further.

The remaining ^{14}C-bands moved with the same chromatographic mobility as standard protochlorophyllide, Mg protoporphyrin monoester (21), and protochlorophyllide ester (Fig. 1A). Upon elution of these bands and rechromatography on Silica Gel H, they moved again with the same mobility as the standards (Fig. 1, B–D).

The ^{14}C-Mg protoporphyrin monoester band was subsequently submitted to detailed chromatographic analysis as outlined in a previous communication (21). It coincided in every respect with standard Mg protoporphyrin monoester (21). In order to confirm the identity of the radioactive components in the two protochlorophyll bands, the latter were submitted to further chromatographic analysis as described below.

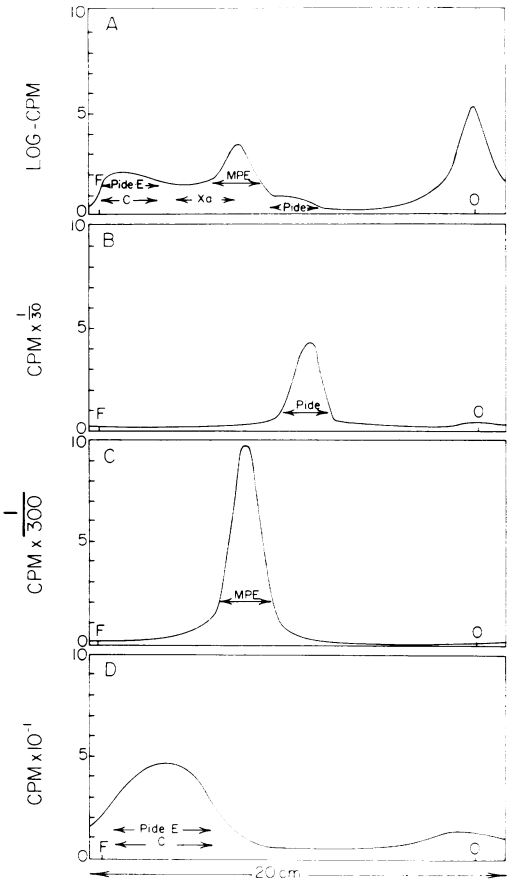

FIG. 1. Radiochromatogram tracings of the products of ^{14}C-ALA incubation with the crude homogenate, cochromatographed with standard protochlorophyllide, Mg protoporphyrin monoester, and protochlorophyllide ester on Silica Gel H in benzene:ethyl acetate:ethanol (8:2:2 v/v). The crude homogenate was incubated in the dark at 28 C for 16 hr. In a total volume of 2.5 ml the incubation medium contained: 2 μc (59 mμmoles) of ^{14}C-ALA, 400 μmoles of tris-HCl, pH 7.9, 1 mmole of sucrose, 100 μmoles of potassium phosphate buffer pH 7.9, 1 μmole of $MgCl_2$, 10 μmoles of GSH, 0.6 μmole of CoA, 1.2 mmole of methyl alcohol, and about 100 mg protein. A: Ether extract of the incubation medium; B: protochlorophyllide eluted from A and rechromatographed on Silica Gel H; C: Mg protoporphyrin monoester eluted from A and rechromatographed on Silica Gel H; D: protochlorophyllide ester eluted from A and rechromatographed on Silica Gel H. O: Origin; F: front; Xa: xanthophylls; C: carotene; Pide: protochlorophyllide; MPE: Mg protoporphyrin monoester; Pide E: protochlorophyllide ester.

Confirmation of the Nature of ^{14}C-Protochlorophyllide. The ^{14}C-protochlorophyllide band was eluted from Silica Gel H and rechromatographed as such in a variety of solvents on paper, and after acidification on paper and on Silica Gel H. In toluene the ^{14}C-protochlorophyllide band remained at the origin with standard protochlorophyllide while standard Mg protoporphyrin monoester moved slightly from the origin and standard protochlorophyllide ester moved a little further (Fig. 2). In this solvent the carotenoids move near the front (Fig. 2A). Upon acidification and chromatography in toluene, the ^{14}C-protochlorophyllide band cochromatographed with standard protopheophytin. (Fig. 2B),

In 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v) the ^{14}C-protochlorophyllide band cochromatographed with standard protochlorophyllide (Fig. 2C). Upon acidification its chromatographic mobility decreased as expected for the Mg-free base in this solvent (7), and it cochromatographed with standard protopheophytin (Fig. 2D).

In acetone:petroleum ether:acetic acid (3:7:0.01 v/v), spectroscopically pure, standard ^{14}C-protochlorophyllide and ^{14}C-protopheophytin (19, 20) give rise to two major bands and one minor band (Fig. 2A, C). Here too *in vitro*-biosynthesized ^{14}C-protochlorophyllide chromatographed in this solvent, before and after acidification, as standard ^{14}C-protochlorophyllide and ^{14}C-protopheophytin respectively (Fig. 3). In this solvent Mg protoporphyrin monoester, protochlorophyllide ester, protoporphyrin monoester, and protopheophytin ester had chromatographic mobilities strikingly different than ^{14}C-protochlorophyllide and ^{14}C-protopheophytin (Fig. 3). No efforts were made to determine whether the segregation of protochlorophyllide and its Mg-free base into multiple bands is due to degradation of the pigments or to a separation of closely related, spectroscopically identical, compounds. A similar case was reported for radioisotopically and spectroscopically pure ^{14}C-pheophorbide *a* and *b* chromatographed on icing sugar (17). After acidification the ^{14}C-protochlorophyllide band cochromatographed on Silica Gel H in benzene:ethyl acetate:ethanol (8:2.5:5v/v) with standard protopheophytin (Fig. 5A).

The foregoing results strongly suggest that the cell-free system is indeed synthesizing ^{14}C-protochlorophyllide.

Confirmation of the Nature of ^{14}C-Protochlorophyllide Ester. The ^{14}C-protochlorophyllide ester band was eluted in ether from Silica Gel H and rechromatographed as such and after acidification on paper. It was also chromatographed on Silica Gel H after partial acid hydrolysis.

In 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v) it moved differently than standard protochlorophyllide and Mg protoporphyrin monoester and with the same mobility as standard protochlorophyllide ester (Fig. 4). In this solvent some standard protochlorophyllide ester and ^{14}C-protochlorophyllide ester remain at the origin together with some carotene (Fig. 4A). It is felt that this might be due to the interference of excess carotene in this solvent. After acidification the ^{14}C-protochlorophyllide ester band moved with standard protopheophytin ester ahead of protoporphyrin monoester and protopheophytin (Fig. 4B).

In acetone:petroleum ether:acetic acid (3:7:0.01 v/v) the ^{14}C-protochlorophyllide ester band moved with standard protochlorophyllide ester, ahead of Mg protoporphyrin monoester (Fig. 4C) and ahead of the multiple bands of protochlorophyllide (compare Fig. 4C and Fig. 3, A and B). After acidification it cochromatographed with standard protopheophytin ester (Fig. 4D) ahead of protoporphyrin monoester (Fig. 4D) and the multiple bands of protopheophytin (Compare Fig. 4D and 3, C

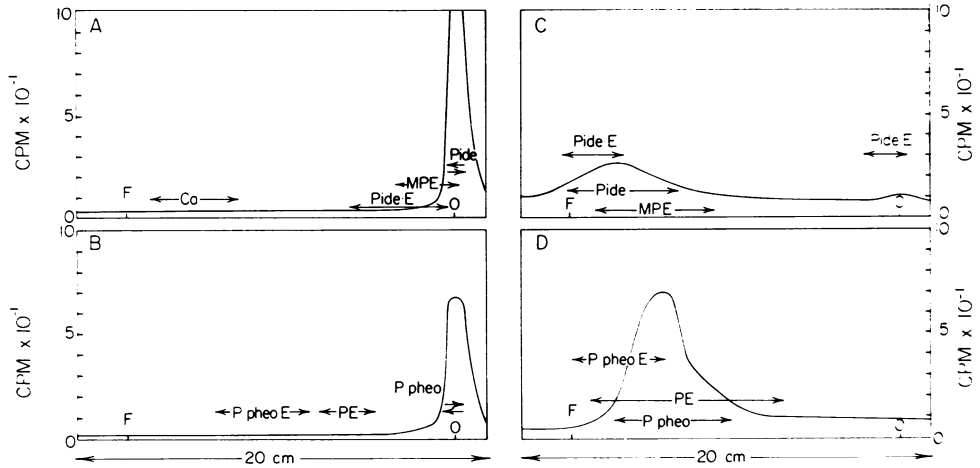

FIG. 2. Radiochromatogram tracings of the ^{14}C-protochlorophyllide band eluted from Silica Gel H and rechromatographed on Whatman paper. A: Before acidification on Whatman No. 1 paper in toluene; B: after acidification in 15% HCl on Whatman No. 1 paper in toluene; C: before acidification on Whatman No. 3 paper in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v); D: after acidification in 15% HCl on Whatman No. 3 paper in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v). P.pheo: Protopheophytin. The other symbols are the same as in Figure 1.

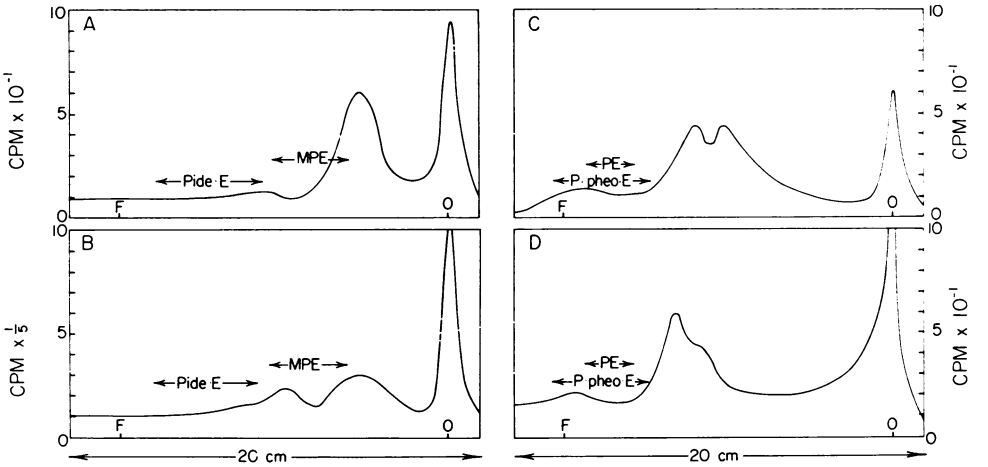

FIG. 3. Radiochromatogram tracings of standard ^{14}C-protochlorophyllide synthesized *in vivo* and of *in vitro* formed ^{14}C-protochlorophyllide, eluted from Silica Gel H, and rechromatographed on Whatman No. 1 paper in acetone:petroleum ether:acetic acid (3:7:0.01 v/v). A: Standard ^{14}C-protochlorophyllide before acidification; B: *in vitro* formed ^{14}C-protochlorophyllide before acidification; C: standard ^{14}C-protochlorophyllide after acidification to 0.02 M HCl; D: *in vitro* formed ^{14}C-protochlorophyllide after acidification to 0.02 M HCl. The symbols are the same as in Figure 1.

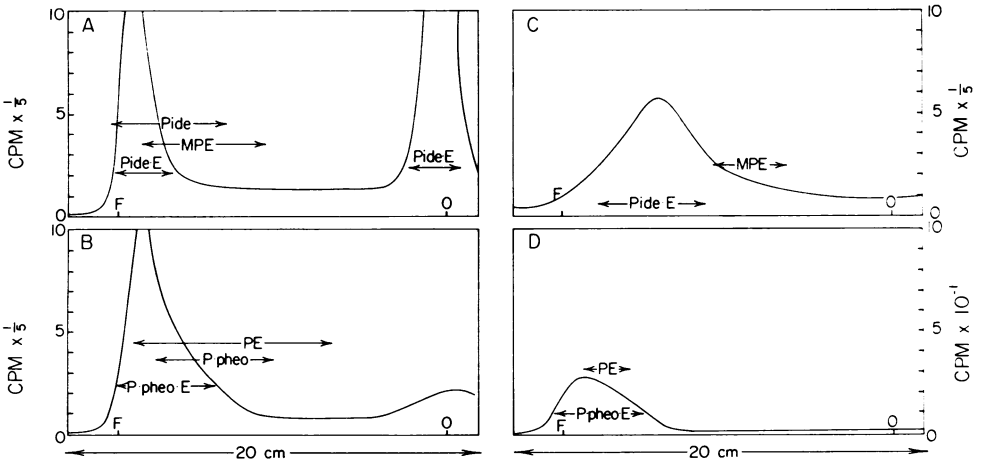

FIG. 4. Radiochromatogram tracings of the ^{14}C-protochlorophyllide ester band eluted from Silica Gel H and rechromatographed on Whatman paper. A: Before acidification on Whatman No. 3 in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v); B: after acidification in 25% HCl, in 2,6-lutidine: 0.05 N NH_4OH (5:3.5 v/v); C: before acidification, on Whatman No. 1 paper in acetone:petroleum ether:acetic acid (3:7:0.01 v/v); D: after acidification in 25% HCl on Whatman No. 1 paper in acetone:petroleum ether:acetic acid (3:7:0.01 v/v). P.pheo.E: Protopheophytin ester. The other symbols are the same as in Figure 1.

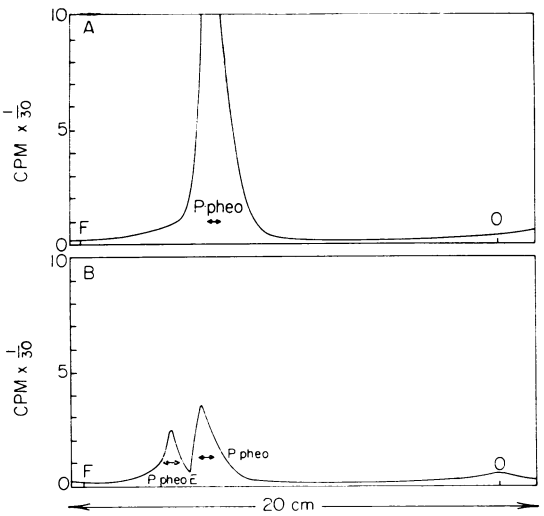

FIG. 5. Radiochromatogram tracing of the ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester bands eluted from Silica Gel H and rechromatographed on Silica Gel H in benzene:ethyl acetate:ethanol (8:2:5 v/v). A: ^{14}C-Protochlorophyllide after acidification with 15% HCl; B: ^{14}C-protochlorophyllide ester after hydrolysis in 12 N HCl. The symbols denote the following standards: P.pheo: Protopheophytin; P.pheo.E: protopheophytin ester. Other symbols are as in Figure 1.

Table I. *Cofactor Requirements for the Biosynthesis of Protochlorophyllide and Protochlorophyllide Ester by the Crude Homogenate*

The complete reaction mixture contained in a total volume of 2.5 ml: 2 μc (59 mμmoles) of ^{14}C-ALA, 400 μmoles of tris-HCl, pH 7.9, 1 mmole of sucrose, 100 μmoles of potassium phosphate buffer, pH 7.9, 1 μmole of $MgCl_2$, 10 μmoles of GSH, 0.6 μmole of CoA, 1.2 mmole of methyl alcohol, and about 100 mg of protein. When the phosphate buffer was omitted, it was replaced by an equal amount of the tris-HCl buffer. Time of incubation: 16 hr in the dark at 28 C.

Experiment	Components	^{14}C Incorporated		
		Mg protoporphyrin monoester	Protochlorophyllide	Protochlorophyllide ester
		dpm per 100 mg protein		
A	Complete	72900	11000	11800
	Without CoA, without GSH	46100	6600	5800
	Without methanol	51500	7800	7000
	Without potassium phosphate	59900	8800	4200
	Without Mg^{2+}	67500	8000	4100
B	Complete	97300	14800	11100
	No cofactors	14390	3900	1300
C	Complete	95400	26600	7000
	Complete under N_2[1]	600	300	40

[1] The incubation was carried out in a two-armed Warburg vessel continuously flushed with N_2 gas.

and D). Upon partial hydrolysis of the ^{14}C-protochlorophyllide ester band in 12 N HCl and chromatography on Silica Gel H in benzene:ethyl acetate:ethanol (8:2:5 v/v), the radioactivity exhibited the same mobility as standard protopheophytin ester and its hydrolysis product protopheophytin (Fig. 5).

Table II. *Effect of GSH and CoA on the Biosynthetic Activity of the Crude Homogenate*

The complete reaction mixture and incubation conditions were the same as in Table I, except that the reaction was carried out at pH 7.7.

Experiment	Components	^{14}C Incorporated		
		Mg protoporphyrin monoester	Protochlorophyllide	Protochlorophyllide ester
		dpm per 100 mg protein		
A	Complete	167000	13900	10400
	Without GSH, without CoA	38900	5900	6900
	Without GSH	19100	2700	5000
B	Complete	86400	13180	14500
	Without CoA	88700	8900	14400
	Without GSH	52000	7600	9200

Here too the results strongly suggest that the cell-free system is indeed synthesizing ^{14}C-protochlorophyllide ester.

Minimal Cofactor Requirement for the Biosynthetic System. Table I shows the minimal cofactor requirement for the incorporation of ^{14}C-ALA into ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester by the crude homogenate. The corresponding incorporations into ^{14}C-Mg protoporphyrin monoester are also listed. It appears from Table I, experiment A, that CoA plus GSH, potassium phosphate, methyl alcohol, and Mg^{2+} are needed for a maximal aerobic biosynthetic activity of the crude homogenate. The absolute requirement for oxygen is also evident (Table I, experiment C). The unknown level of endogenous cofactors in the crude homogenate is expressed by the biosynthetic activity of the system in the absence of any added cofactors (Table I, experiment B). Other chemicals were tested for their effect on the biosynthetic activity of the crude homogenate: ATP, NAD, NADP, thiamine pyrophosphate, cytidine triphosphate, FAD, pyridoxal phosphate, NADPH, NADH, L-ascorbic acid, dehydroascrobic acid, D,L-methionine, cytochrome *c*, D-glucose + glucose oxidase, Fe^{3+}, Fe^{2+}, Zn^{2+}, Co^{2+}, vitamin B_{12}, and mannitol. None of these produced any stimulation; some were slightly inhibitory.

Effect of GSH and CoA on the Biosynthetic Activity of the Crude Homogenate. The individual effects of added GSH and CoA on the biosynthetic activity of the system are reported in Table II. The omission of exogenous GSH from the reaction mixture depresses the ^{14}C-incorporations into ^{14}C-Mg protoporphyrin monoester, ^{14}C-protochlorophyllide, and ^{14}C-protochlorophyllide ester (Table II) regardless of the presence or absence of CoA. This finding might suggest a general protective effect of GSH on sulfhydryl enzymes and porphyrinogen intermediates. However, the omission of exogenous CoA from the reaction mixture containing GSH resulted in decreased ^{14}C-protochlorophyllide biosynthesis (Table II, experiment B) without interference with ^{14}C-protochlorophyllide ester. These results suggest that the CoA site of action along the biosynthetic pathway is probably located after Mg-protoporphyrin monoester and is involved in the production of ^{14}C-protochlorophyllide but not of ^{14}C-protochlorophyllide ester. These data support the hypothesis that protochlorophyllide and protochlorophyllide ester are produced from a common precursor by two parallel and distinct pathways (28).

Effect of K^+ and P_i on the Biosynthetic Activity of the Crude Homogenate. In order to determine which component of the potassium phosphate buffer had an effect on the biosynthetic activity of the system, the potassium phosphate buffer was replaced by a

Table III. *Effect of Phosphate and Potassium on the Biosynthetic Activity of the Crude Homogenate*

The reactions were carried out in 0.2 M tris-phosphate, 0.5 M sucrose, pH 7.7, or in 0.2 M tris-HCl, 0.5 M sucrose, pH 7.7. All concentrations and other incubation conditions were the same as in Table I except for the ionic components indicated in the table.

Components	Concentration of Major Ionic Components				^{14}C Incorporated		
	Tris	P_i	K^+	Cl^-	Mg protoporphyrin monoester	Protochlorophyllide	Protochlorophyllide ester
	μmoles per 2.5 ml				*dpm per 100 mg protein*		
Complete	400	267	100	100	185500	18900	18000
Without K^+, without P_i	500	0	0	333	89100	9000	3600
Without P_i	400	0	100	367	80600	9700	7100
Without K^+	500	267	0	67	152200	14400	20600
Complete	400	100	189	267	178500	19200	17000

tris-P_i buffer and K^+ was added back to the reaction mixture as KCl.

The omission of exogenous phosphate has a depressing effect on the biosynthesis of both ^{14}C-Mg protophyrin monoester, ^{14}C-protochlorophyllide, and ^{14}C-protochlorophyllide ester (Table III). On the other hand, the omission of exogenous potassium from the complete mixture does not appear to affect the biosynthesis of ^{14}C-protochlorophyllide ester but depresses the biosynthesis of ^{14}C-Mg protoporphyrin monoester and ^{14}C-protochlorophyllide (Table III). The endogenous K^+ level in these crude homogenates is, of course, unknown. Although these results do suggest an involvement of K^+ in our system, more experimental work with washed etioplasts preparations is needed before a specific cofactor role can be assigned to K^+ in protochlorophyll biogenesis. The concentration of Cl^- is without appreciable effect on the ^{14}C-ALA incorporation into Mg protoporphyrin monoester, protochlorophyllide, or protochlorophyllide ester (Table III).

Effect of Methyl Alcohol and Other Aliphatic Alcohols on the Biosynthetic Activity of the Crude Homogenate. In order to study the alcohol specificity in this system, methanol was replaced in the reaction mixture by a short chain primary alcohol (ethanol) a secondary alcohol (isopropanol) or a tertiary alcohol (*t*-butanol). In all cases these alcohols were unable to substitute for methanol (Table IV, experiment A). The effect of higher concentrations of methanol on the biosynthetic activity of the system was subsequently investigated. It appeared that higher concentrations of methanol were inhibitory (Table IV, experiment B). To determine whether methanol acted as a catalyst or a substrate, the system was incubated with ^{14}C-methanol. Mg-protoporphyrin monoester as well as protochlorophyllide and protochlorophyllide ester eluted from Silica Gel H were labeled (Table IV, experiment C). Although these compounds were not purified to constant specific radioactivity, they remained radioactive after elution from Silica Gel H and rechromatography on paper in 2,6-lutidine:0.1 N NH_4OH (5:3.5 v/v) or acetone:petroleum ether:acetic acid (3:7:0.01 v/v). These results suggest a substrate role for methanol in this system.

The differential incorporation of ^{14}C-methanol into ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester (Table IV, experiment C) might also support the hypothesis that these two compounds are produced via two separate pathways from a common precursor (28). Alternatively, it might also indicate a certain degree of transesterification of the alcohol of ^{14}C-protochlorophyllide ester with ^{14}C-methanol. The possibility that methanol might be preferentially incorporated into the phytol of protochlorophyllide ester would also explain the observed incorporation data. However, we are not aware of any direct pathway leading from methanol to polyisoprenoids. The lower incorporation of ^{14}C-methanol into ^{14}C-Mg protoporphyrin monoester and ^{14}C-protochlorophyllide compared to the incorporation of ^{14}C-ALA (Table IV, experiment C) is expected under our experimental conditions. Assuming that methanol esterifies the propionic residue at the seventh position of the macrocycle, 8 molecules of ALA are incorporated into the macrocycle for every molecule of methanol utilized. Moreover, the specific radioactivity of the ^{14}C-methanol used was about 34 times lower than that of ^{14}C-ALA.

Table IV. *Effect of Aliphatic Alcohols on the Biosynthetic Activity of the Crude Homogenate*

The reaction mixture and incubation conditions were the same as in Table I, except for the alcohols added. In experiment C, bottom line, ^{14}C-ALA was replaced by 59 mμmoles of unlabeled ALA and the usual 1.2 mmole of methanol was replaced by 50 μc (50 μmoles) of ^{14}C-methanol. Experiment C was run at pH 7.7 instead of 7.9.

Experiments	Alcohol	Alcohol added	^{14}C Incorporated		
			Mg protoporphyrin monoester	Protochlorophyllide	Protochlorophyllide ester
		μmoles	*dpm per 100 mg protein*		
A	Methanol	1200	118300	18100	13480
	Ethanol	1200	18500	2900	4700
	Isopropanol	1200	7300	1600	2800
	t-Butanol	1200	7800	2200	3400
B	Methanol	none	16300	4000	3000
		1200	54800	7700	6200
		2400	46200	4700	4400
		4800	25500	3300	2900
C	Methanol	1200	92500	12700	8600
	50 μc-^{14}C-methanol	50	2400	2600	62000

Effect of pH and Temperature on the Biosynthetic Activity of the Crude Homogenate. The multienzyme system appears to have a pH optimum of about 7.7 for the biosynthesis of Mg-protoporphyrin monoester, protochlorophyllide, and protochlorophyllide ester (Fig. 6). Some of the initial experiments reported in this paper were carried out at pH 7.9 before it was recognized that the system is more active and the results more reproducible at pH 7.7.

The effect of two temperatures on the biosynthetic activity of the system is presented in Table V. This table suggests that at 20 C more ^{14}C-Mg protoporphyrin monoester accumulates than at 28 C whereas the incorporations into protochlorophyllide and protochlorophyllide ester are slightly depressed.

Intracellular Localization of Mg Protoporphyrin and Protochlorophyll Biosynthesis. In order to establish a connection between the porphyrin and phorbin biosynthetic activities and various subcellular fractions, the crude homogenate (C) was fractionated by differential centrifugation into a crude etioplast preparation (P_2) and a supernatant (S_2), enriched in soluble proteins, microsomes, mitochondria, and microbodies. The biosynthetic activities of the various fractions are reported in Table VI, experiment A. The crude etioplast preparation was more active than either the crude homogenate (C) or the supernatant (S_2). No recombination effect could be demonstrated (Table VI, experiment A). A crude homogenate (FC) was prepared from

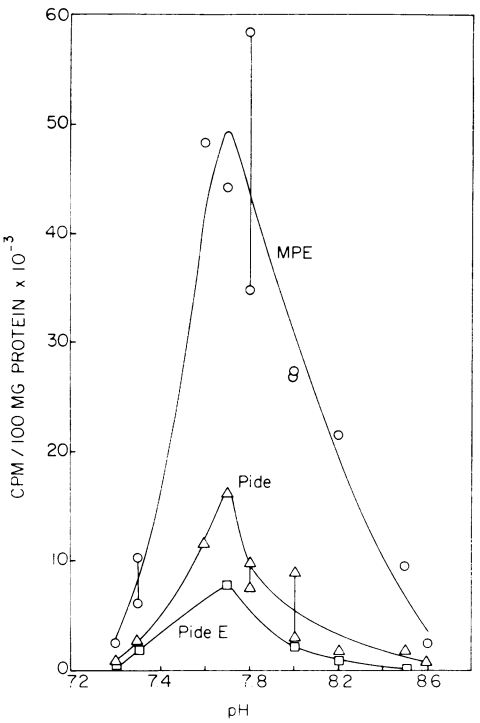

FIG. 6. The effect of pH on the biosynthetic activity of the crude homogenate. The reaction mixtures and incubation conditions were the same as in Figure 1 except for pH which was varied as indicated; ○: specific radioactivity of Mg protoporphyrin monoester; △: specific radioactivity of protochlorophyllide; □: specific radioactivity of protochlorophyllide ester.

Table V. *Effect of Temperature on the Biosynthetic Activity of the Crude Homogenate*

The reaction mixture and incubation conditions were the same as in Table I except for temperature. The reaction was run at pH 7.7.

Temperature	^{14}C Incorporated		
	Mg Protophyrin monoester	Protochlorophyllide	Protochlorophyllide ester
	dpm per 100 mg protein		
28 C	75300	11200	12000
20 C	101800	9300	9700

etiolated cotyledons in a grinding buffer containing all the cofactors needed for maximal protochlorophyll activity. From this homogenate a fortified etioplast pellet (FP_2) was obtained which was much more active than anything we had previously encountered and had a specific radioactivity much higher than the fortified crude homogenate (FC) (Table VI). Upon washing these crude fortified etioplasts (FP_2) with the fortified grinding buffer their activity was remarkably well preserved (Table VI, experiment C). These results strongly suggest that the *in vitro* biosynthesis of Mg protoporphyrin monoester, protochlorophyllide, and protochlorophyllide ester is associated with the etioplasts.

ATP and NAD Requirement for Maximal Biosynthetic Activity of Washed Fortified Etioplasts. The ability to prepare washed, active etioplasts presented a good opportunity for further studies of cofactor requirements in the presence of reduced levels of endogenous cofactors.

Although a limited amount of experimentation was performed

Table VI. *Biosynthetic Activity of Various Subcellular Fractions Isolated by Differential Centrifugation*

The reaction mixtures and other incubation conditions were the same as in Table I, except that the incubations were run at pH 7.7. Equivalent volumes of the fractions were assayed as described in "Materials and Methods."

Experiment	Fraction	^{14}C Incorporated		
		Mg Protoporphyrin monoester	Protochlorophyllide	Protochlorophyllide ester
		dpm per 100 mg protein		
A	C	93300	12700	3500
	S_2	67500	8000	2000
	P_2	103200	19200	8800
	$S_2 + P_2$	90300	23600	7500
B	FC	51100	8700	10000
	FP_2	176000	93300	16000
C	WFP_2	177000	58800	17300

Table VII. *ATP and NAD Requirements for Maximal Biosynthetic Activity of Washed Fortified Etioplasts*

The standard reaction mixture was identical in composition and other conditions to the complete reaction mixture in Table I, except that the incubation was run at pH 7.7. ATP, 1 μmole, and NAD, 0.15 μmole, were added as indicated. The reaction mixture contained 3.5 mg of protein of washed fortified etioplasts (WFP_2).

Components	^{14}C Incorporated		
	Mg protoporphyrin monoester	Protochlorophyllide	Protochlorophyllide ester
	dpm per 100 mg protein		
Standard cofactors	177500	58800	17280
+ ATP	189500	120000	13500
+ NAD	148500	85300	23200
+ ATP + NAD	114500	298000	15100

on this particular system, a requirement for ATP and NAD was established (Table VII). It appears from Table VII that in the presence of ATP and NAD, the utilization of ^{14}C-Mg protoporphyrin monoester is increased as evidenced by reduced levels of the latter and increased levels of ^{14}C-protochlorophyllide. Both ATP and NAD seem to be required for maximal accumulation of ^{14}C-protochlorophyllide by the washed fortified etioplasts (Table VII). The presence of ATP does not seem to stimulate ^{14}C-protochlorophyllide ester accumulation. On the other hand, NAD alone, in the absence of ATP, resulted in a marked increase of ^{14}C-protochlorophyllide ester (Table VII). These observations might indicate that although both ATP and NAD are required for ^{14}C-protochlorophyllide biosynthesis, only NAD might be needed for ^{14}C-protochlorophyllide ester biosynthesis.

DISCUSSION

The foregoing results clearly demonstrate the *in vitro* biosynthesis of ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester from added ^{14}C-ALA, by cell-free preparations from etiolated cucumber cotyledons. These two compounds are chromatographically similar in every respect to protochlorophyllide and protochlorophyllide ester isolated from etiolated cucumber cotyledons. The two protochlorophylls of etiolated cucumber cotyledons have already been characterized by their chroma-

tographic behavior and absorption spectra in various solvents, in the Mg and Mg-free forms (20, 21). The possible contamination of ^{14}C-Mg porphyrins and ^{14}C-Mg phorbins synthesized from a specific tetrapyrrole precursor such as ^{14}C-ALA is discussed at length in another article (23). Were ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester contaminated to any significant extent by ^{14}C-colorless lipids (12), the chromatographic mobility of the latter would remain unchanged after pheophytinization (17); this is obviously not the case as evidenced by Figures 2, C and D; 3; 4; and 5B.

The activity which is responsible for the biosynthesis of Mg protoporphyrin monoester, protochlorophyllide, and protochlorophyllide ester appears to be associated with the crude etioplast pellet (Table VI). It must be stressed, however, that crude etioplast preparations are contaminated with other subcellular constituents such as nuclear fragments, mitochondria, microbodies and with bacteria as shown by electron microscopic examination (C. A. Rebeiz, T. E. Weier, and P. A. Castelfranco, unpublished results). Indeed the uro- copro-, and protoporphyrin which appear in these crude cell free systems upon incubation with δ-ALA (21) could be related to the synthesis of mitochondrial cytochromes or glyoxysomal catalase, as well as to the protochlorophyll biosynthesis in the etioplasts.

Previous work has shown that the bacterial population contaminating these crude etioplast preparations is not capable of significant porphyrin biosynthesis (21). Since the chloroplast outer membranes are permeable to ALA whereas they are completely inpermeable to porphobilinogen (3), and since we have no reason to suspect that etioplast membranes behave differently in this respect, we feel that the whole metabolic sequence from ALA to protochlorophyll takes place in the etioplasts.

Electron microscopic observation and pigment analysis agree that in order to enhance protochlorophyll biogenesis in isolated etioplast preparations, it is essential to prevent the destruction of these organelles *in vitro* (C.A. Rebeiz, T.E. Weier and P.A. Castelfranco, unpublished). Indeed the cofactors which have been found to stimulate the incorporation of ^{14}C ALA into Mg protoporphyrin monoester and protochlorophyll also appear to stabilize the etioplasts during the 16-hr incubation. One could speculate whether the action of the required cofactors is a direct one on protochlorophyll biogenesis or rather an indirect one mediated through organelle stablilization and maintenance. Of course, since no individual enzymatic reactions have been studied yet, any assignment of cofactors for any one reaction is at best tentative. We hope, however, that a discussion of the possible role of these cofactors might suggest some general guidelines for future research.

Our list of required cofactors may not be complete, since most of our determinations were performed on crude homogenates, which possibly contained a number of cofactors in sufficiently high concentration to elude detection.

The absolute requirement for O_2 by the system (Table I, experiment C) might be attributed to the O_2 requirement of coproporphyrinogen oxidase (29). Indeed, Sano and Granick (29) have clearly demonstrated the absolute requirement of molecular O_2 for the decarboxylation of coproporphyrinogen by a beef liver enzyme prepared from acetone powders of mitochondria. That O_2 is required in our system before Mg protoporphyrin monoester biosynthesis is evidenced by the practically complete lack of ^{14}C-ALA incorporation in this compound under anerobic conditions (Table I, experiment C).

The requirement for Mg^{2+} (Table I, experiment A) is best justified by the structural role of Mg^{2+} as part of protochlorophyll. However, other catalytic functions are not ruled out, considering the ubiquitous catalytic activities of this metal in phosphorylation and activation reactions (22, 26).

As suggested earlier, GSH might provide a general reductant protective effect for sulfhydryl functional groups and porphyrinogen intermediates.

At this stage we find it difficult to assign a specific role for inorganic phosphate in the system. However, protochlorophyll biosynthesis and accumulation are probably coupled to membrane biogenesis. Because of its important role as a lipid and membrane constituent, phosphate could have an essential role on membrane biogenesis (15) and therefore on protochlorophyll biosynthesis and accumulation.

The specific requirement for methyl alcohol is worth noting. That methanol acts as a substrate rather than a catalyst in this system is established by the incorporation of ^{14}C-methanol into Mg protoporphyrin monoester and protochlorophylls (Table IV, experiment C). Radmer and Bogorad (16) demonstrated in crude chloroplast pellets the incorporation of the methyl group from S-adenosyl-L-methionine into Mg protoporphyrin to form Mg protoporphyrin monomethyl ester. It appears likely that methyl alcohol is used to esterify the carboxyl at the 10 position of the macrocycle possibly via an S-adenosyl methionine type mechanism.

The involvement of NAD in protochlorophyllide and protochlorophyllide ester biosynthesis (Table VII) might be viewed as a requirement for a β-oxidation type dehydrogenase (analogous to hydroxyacyl dehydrogenase), acting on the propionic residue at the sixth position of the macrocycle, and preceding the cyclopentanone ring formation (5). This modified β-oxidation sequence could be expected to take place on the methylated propionic residue of Mg protoporphyrin monoester. In this case, since the carbon undergoing oxidation is conjugated with the Mg tetrapyrrole system, the removal of a hydrogen α to the macrocycle would be facilitated and the thio ester linkage would not be needed (L. L. Ingraham, personal communication). Possibly a flavoprotein enzyme analogous to the acyl CoA dehydrogenase in the conventional β-oxidation scheme might also be involved.

ATP and CoA are required for the biosynthesis of protochlorophyllide, but not of Mg protophyrin monoester or protochlorophyllide ester (Table II, VII). These findings suggest that the synthesis and accumulation of protochlorophyllide involves the activation of the free propionic residue at the seventh position of the macrocycle. In this case an activation of protochlorophyllide might be viewed as a prerequisite for positioning this molecule at a specific site on the membranes while the ester group in protochlorophyllide ester positions the latter at other sites, thus contributing to the membranes heterogeneity (28).

Recently, Ellsworth and Aronoff (5, 6) using chromatographic, spectrophotometric, and mass spectroscopic techniques detected compounds in various *Chlorella* mutants which may be involved in the formation of the cyclopentanone ring between Mg protoporphyrin monoester and protochlorophyllide. Most intermediates existed in both the monovinyl and divinyl forms (6). These authors postulated a scheme for chlorophyll biosynthesis beginning at Mg protoporphyrin monoester and leading to the formation in parallel of protochlorophyllide (Mg-vinyl pheoporphyrin a_5) and vinyl protochlorophyllide (Mg-divinyl pheoporphyrin a_5) (6). In their scheme, the cyclopentanone ring formation is preceded by a β-type oxidation of the methyl propionate residue at the sixth position of the macrocycle in both branches of the pathway (6). Extramitochondrial β-oxidation activity in plants is no novelty, since it was first demonstrated by Rebeiz and Castelfranco (22, 27) in 1964 and has been subsequently confirmed by others (4, 10, 30).

The analytical techniques used in this work are not sufficiently refined to detect the intermediates of the vinyl and divinyl branches of such a pathway (if operative in our system). It is very possible that our ^{14}C-protochlorophyllide fraction consisted of ^{14}C-protochlorophyllide and closely related mono- and divinyl

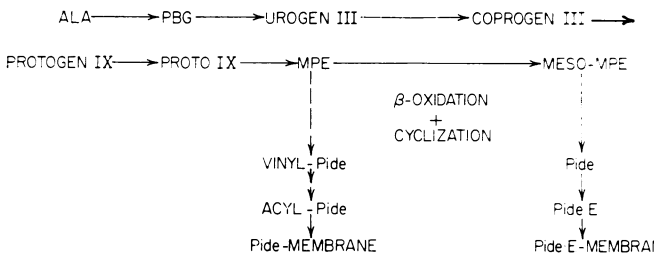

FIG. 7. Proposed pathway to membrane-bound protochlorophylls. ALA: δ-Aminolevulinic acid; PBG: porphobilinogen; UROGEN III: Uroporphyrinogen III; COPROGEN III: coproporphyrinogen III; PROTOGEN IX: protoporphyrinogen IX; PROTO IX: protoporphyrin IX; MPE: Mg protoporphyrin monomethyl ester; MESO-MPE: Mg 2-vinyl-4-ethylprotoporphyrin IX monomethyl ester; VINYL-Pide: 2,4-divinyl pheoporphyrin a_5; Pide: protochlorophyllide; ACYL-Pide: acylprotochlorophyllide; Pide.E: protochlorophyllide phytyl ester; Pide-MEMBRANE: membrane bound protochlorophyllide; Pide.E-MEMBRANE: membrane bound protochlorophyllide phytyl ester.

intermediates of the β-oxidation sequence. The same might be true for the ^{14}C-Mg protoporphyrin monoester fraction.

The production of protochlorophyllide and protochlorophyllide ester in parallel from Mg protoporphyrin monoester was suggested earlier by Rebeiz et al. (28). This hypothesis was based on the kinetics of ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester accumulation *in vivo* and was suggested as a means of selectively polarizing certain sites of the prolamellar body in conjunction with the future buildup of chlorophyll heterogeneity *in situ* (28). As has been pointed out, the difference in the cofactor requirement for the biosynthesis of protochlorophyllide and protochlorophyllide ester seems to support this hypothesis.

The parallel production of the two components of protochlorophyll is also compatible with the Ellsworth and Aronoff scheme (6) if one assumes that ^{14}C-protochlorophyllide is derived from one branch of the pathway (divinyl branch?) while the ^{14}C-protochlorophyllide ester originates from the other branch of the pathway (monovinyl branch?). Actual research is under way to test this hypothesis; preliminary spectrophotometric results already suggest that this might be the case.

Finally, we trust that the development of a cell-free system from higher plants capable of protochlorophyll biosynthesis will open the way for detailed enzymological investigations. A most important phase of future research will deal with the relationships between chlorophyll biosynthesis and the biogenesis of thylakoid membranes; and here again we hope that *in vitro* studies with cell-free homogenates will play an important role.

Figure 7 presents a working hypothesis of protochlorophyll biosynthesis in higher plants summarizing the knowledge acquired so far from *in vivo* and *in vitro* studies (21, 28) and taking into account standard concepts of protochlorophyll biosynthesis (2) as well as the effect of a modified β-oxidation upon Mg protoporphyrin monomethyl ester and its 4-meso derivative as proposed by Ellsworth and Aronoff (6).

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LITERATURE CITED

1. BOARDMAN, N. K. AND J. M. ANDERSON. 1964. Studies on the greening of dark-grown bean plants. I. Formation of chloroplasts from proplastids. Aust. J. Biol. Sci. 17: 86–92.
2. BOGORAD, L. 1965. Chlorophyll biosynthesis. *In:* T. W. Goodwin, ed., Chemistry and Biochemistry of Plant Pigments. Academic Press, New York. pp. 29-74.
3. CARREL, E. F. AND I. S. KAHN. 1964. Synthesis of porphyrins by isolated chloroplasts of *Euglena*. Arch. Biochem. Biophys. 180: 1-6.
4. COOPER, T. G. AND H. BEEVERS. Mitochondria and glyoxysomes from castor bean endosperms. J. Biol. Chem. 244: 3507–3513.
5. ELLSWORTH, R. K. AND S. ARONOFF. 1968. Investigations on the biogenesis of chlorophyll *a*. III. Biosynthesis of Mg-vinyl pheoporphine a_5 methylester from Mg-protoporphine IX monomethyl ester as observed in *Chlorella* mutants. Arch. Biochem. Biophys. 125: 269–277.
6. ELLSWORTH, R. K. AND S. ARONOFF. 1969. Investigations of the biogenesis of chlorophyll *a*. IV. Isolation of partial characterization of some biosynthetic intermediates between Mg-protoporphine. IX monomethyl ester and Mg-vinyl pheoporphine a_5, obtained from *Chorella* mutants. Arch. Biochem. Biophys. 130: 374–383.
7. GRANICK, S. 1961. Magnesium protoporphyrin monoester and protoporphyrin monomethyl ester in chlorophyll biosynthesis. J. Biol. Chem. 236: 1168–1172.
8. HARDY, S. I., P. A. CASTELFRANCO, AND C. A. REBEIZ. 1970. Effect of the hypocotyl hook on greening in etiolated cucumber cotyledons. Plant Physiol. 46: 705–707.
9. HINES, G. D. AND R. K. ELLSWORTH. 1969. Methyl chlorophyllide as a probable intermediate in the chlorophyll *a* pathway. Plant Physiol. 44: 1742–1744.
10. HUTTON, D. AND P. K. STUMPF. 1969. Fat metabolism in higher plants. XXXVII. Characterization of the β-oxidation systems from maturing and germinating castor bean seeds. Plant Physiol. 44: 508–516.
11. JACOBSON, A. B. 1968. A procedure for isolation of proplastids from etiolated maize leaves. J. Cell Biol. 38: 238–244.
12. JEFFREY, S. W. 1963. Purification and properties of chlorophyll from *Sargossum flavicons*. Biochem. J. 86: 313–318.
13. JONES, O. T. G. 1963. Magnesium 2,4-divinyl phaeoporphyrin a_5 monomethyl ester, a protochlorophyll-like pigment produced by *Rhodopseudomonas spheroides*. Biochem. J. 89: 182–189.
14. JONES, O. T. G. 1966. A protein-protochlorophyll complex obtained from inner seed coats of *Cucurbita pepo*. Biochem. J. 101: 153–160.
15. LUCY, J. A. 1968. Theoretical and experimental models for biological membranes. *In:* Dennis Chapman, ed., Biological Membranes. Academic Press, New York. pp. 233–285.
16. NICOLAS, R. E. H. AND C. RIMINGTON. 1951. Paper chromatography of porphyrins: some hitherto unrecognized porphyrins and further notes on the method. Biochem. J. 48: 306–309.
17. PERKINS, H. J. AND D. W. A. ROBERTS. 1962. Purification of chlorophylls, pheophytins and phenophorbides for specific activity determinations. Biochem. Biophys. Acta 58: 486–498.
18. RADMER, R. J. AND L. BOGORAD. 1967. (—) S-Adenosyl-L-methionine magnesium protoporphyrin methyl transferase, an enzyme in the biosynthetic pathway of chlorophyll in *Zea mays*. Plant Physiol. 42: 463–465.
19. REBEIZ, C. A. 1967. Studies on chlorophyll biosynthesis in etiolated excised cotyledons of germinating cucumber at different stages of seedling development. Magon, Série Scientifique 13: 1–21.
20. REBEIZ, C. A. 1968. The chloroplast pigments of etiolated and greening cucumber cotyledons. Magon, Série Scientifique 21: 1–25.
21. REBEIZ, C. A. M. ABOU-HAÏDAR, M. YAGHI, AND P. CASTELFRANCO. 1970. Porphyrin biosynthesis in cell-free homogenates from higher plants. Plant Physiol. 46: 543–549.
22. REBEIZ, C. A. AND P. CASTELFRANCO. 1964. An extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of fatty acids. Plant Physiol. 39: 932–938.
23. REBEIZ, C. A. AND P. A. CASTELFRANCO. 1971. Chlorophyll biosynthesis in a cell free system from higher plants. Plant Physiol. 47: 33–37.
24. REBEIZ, C. A. AND P. CASTELFRANCO. 1970. Protochlorophyll biosynthesis in a cell free system from higher plants. Program and Abstracts. Amer. Soc. Plant Physiol. Western Section, Berkeley, California. p. 32.
25. REBEIZ, C. A. AND P. A. CASTELFRANCO. 1970. Protochlorophyllide biosynthesis in a cell free system from higher plants. Plant Physiol. 45: S-226.
26. REBEIZ, C. A., P. CASTELFRANCO, AND R. W. BREIDENBACH. 1965. Activation and oxidation of acetic acid 1-C^{14} by cell free homogenates of germinating peanut cotyledons. Plant Physiol. 40: 286–289.
27. REBEIZ, C. A., P. CASTELFRANCO, AND A. H. ENGELBRECHT. 1965. Fractionation and properties of an extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of palmitic acid. Plant Physiol. 40: 281–286.
28. REBEIZ, C. A., M. YAGHI, M. ABOU-HAÏDAR, AND P. CASTELFRANCO. 1970. Protochlorophyll biosynthesis in cucumber (*Cucumis sativus*, L.) cotyledons. Plant Physiol. 46: 57–63.
29. SANO, S. AND S. GRANICK. 1961. Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J. Biol. Chem. 236: 1173–1180.
30. YAMADA, M. AND P. K. STUMPF. 1965. Fat metabolism in higher plants. XIV. A soluble β-oxidation system from germinating seeds of *Ricinus communis*. Plant Physiol. 40: 653–658.