Protochlorophyll Biosynthesis in a Cell-free System from Higher Plants

CONSTANTIN A. REBEIZ AND PAUL A. CASTELFRANCO

WITH THE TECHNICAL ASSISTANCE OF IRMГARD 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-4-14C into the two components of protochlorophyll: protochlorophyllide and protochlorophyllide. The activity is associated with the etioplast. Optimal incorporation is obtained at pH 7.7. For the formation of protochlorophyllide ester, oxygen, reduced glutathione, methyl alcohol, magnesium, inorganic phosphate, and nicotinamide adenine dinucleotide are required. For the formation of 14C-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 14C-protochlorophyllide and 14C-protochlorophyllide ester from 14C-Mg protochlorophyll monoester is presented.

Recently, Rebeiz et al. (28) reported the incorporation of δ-aminolevulinic acid-4-14C into the protochlorophylls of etiolated cucumber cotyledons. Kinetic analysis of specific radioactivities of the 14C-protoporphyrin pools suggested that protochlorophyllide and protochlorophyllide ester2 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 proceeded 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 bio-

1 This work was supported in part by Research Grants GM-07532 from the United States Public Health Service and GB-12906 from the National Science Foundation.
2 The same terminology for porphyrin derivatives is used in this paper as in the preceding articles of this series (21, 28).

synthetic 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 14C-ALA into 14C-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. 14C-ALA (33.8 μμ/mole) and 14C-methanol (1 μμ/mole) 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; KH2PO4 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 (P1) was discarded. The supernatant (S1) was centrifuged at 0 °C for 7 min at 1500 g (Falk, R., personal communication). The resulting
crude etioplast pellet (P3) was suspended in the homogenizing buffer and adjusted to the same volume as the resulting supernatant (S0). 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 MgCl2, 375 μmoles of potassium phosphate buffer, pH 7.7, and 180 μmoles of methanol. Fortified etioplasts (FP3) were prepared from the fortified crude homogenate (FC) as described above for crude etioplasts. Washed fortified etioplasts (WF3) were prepared by suspending FP3 in 9 ml of the fortified grinding buffer and centrifuging once again at 1500g for 7 min. Both FP3 and WF3 were resuspended in 9 ml of buffer.

Incubation of the Cell-Free Preparation with 14C-ALA. Volumes of 2.0 ml of crude homogenate (C), crude etioplasts (P3), fortified etioplasts (FP3), or supernatant (S0) were incubated on a shaker 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 μmoles of MgCl2, 10 μmoles of GSH, 0.6 μmoles of CoA, 1.2 mmole of methyl alcohol, and the following amounts of protein; 100 mg of C, 40 mg of S0, 7 to 10 mg of P3 or FP3, 3.5 mg of WF3.

Extraction of 14C-Mg Protoporphyrin Monoester, 14C-Protoporphyrinides, and 14C-Protoporphyrinylide Ester. The reaction was stopped in the dark by the addition of 12.4 ml of acetone: 0.1 N NH4OH (9:1 v/v). The mixture was immediately centrifuged at 3000g for 10 min, and the resulting pellet was washed with 2.0 ml of the acetone: NH4OH mixture. To the combined supernatant, 1/17 of its volume of saturated NaCl and 1/70 of its volume of 0.5 M K2HPO4 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. Protoporphyrin (a mixture of protoporphyrinylide and protoporphyrinide 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 H2O saturated with MgCO3. The ether extract was centrifuged and the acetone:H2O layer removed. The H2O-washed ether extract was dried under N2, 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:ether (8:2.2 v/v) (21, 28). The red fluorescent areas corresponding to carrier Mg protoporphyrin monoester, protoporphyrinylide, and protoporphyrinide ester and their 14C-counterparts were viewed under ultraviolet light of 366 nm. The protoporphyrinylide, Mg protoporphyrin monoester, and protoporphyrinide 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 14C-incorporation into these compounds; the remaining 0.5 ml of the acetone sample was concentrated or dried under N2 for further chromatographic and analytical manipulations.

Conversion of Protoporphyrinylide into Protoporphyrin. The mixture of carrier protoporphyrinylide and 14C-protoporphyrinylide eluted in methanol from Silica Gel H was dried under N2, 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 H2O until free of acid, and concentrated under N2 for further chromatographic manipulations. Alternatively, protoporphyrinylide in methanol was made 0.02 M with respect to HCl and dried under N2 before chromatography.

Conversion of Protoporphyrinylide Ester into Protoporphyrin Ester. The mixture of carrier protoporphyrinylide ester and 14C-protoporphyrinylide 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 N2 for further chromatographic manipulations.

Conversion of Protoporphyrinylide Ester into Protoporphyrin. The mixture of carrier protoporphyrinylide ester and 14C-protoporphyrinylide 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 protoporphyrinylide ester and protoporphyrin ester was mixed with ether; then the acid layer was diluted with H2O and neutralized with solid Na-acetate. The pigment passed into ether, and the latter was washed to neutrality with H2O and concentrated under N2 for further chromatographic manipulations.

Thin Layer Chromatography of Eluted 14C-Protoporphyrinds and Their Hydrolysis Products. 14C-Protoporphyrind and 14C-protoporphyrinylide ester were rechromatographed on thin layers of Silica Gel H in benzene:ethyl acetate:ether (8:2.2 v/v) in the dark at 4 C. 14C-Protoporphyrin, 14C-protoporphyrinylide ester and its hydrolysis product were chromatographed on thin layers of Silica Gel H in the dark at 28 C in benzene:ethyl acetate:ether (8:2.5 v/v).

Paper Chromatography of Eluted 14C-Protoporphyrind and Their Mg-free Bases. The chromatographic mobility of 14C-protoporphyrind 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 NH4OH (5:3.5 v/v) (7, 16). That of 14C-protoporphyrinylide ester and its Mg-free base was determined on Whatman No. 3 paper in 2,6-lutidine:0.05 N NH4OH (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 14C-Incorporations. Half-milliliter aliquots of the 14C-Mg protoporphyrin monoester, 14C-protoporphyrind, and 14C-protoporphyrinylide 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 14C 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 Porphyrins and Phorbin Standards. Mg protoporphyrin monoester and protoporphyrin monoester were prepared and purified as described in a previous communication (21). Protoporphyrin in ether was prepared from freshly harvested 5-day-old etiolated cucumber cotyledons as described for the extraction of Mg-protoporphyrin monoester (25). 14C-Protoporphyrind and 14C-protoporphyrinylide 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 protorhophyllide, Mg protoporphyrin monoster (21), and protorhophyllide ester (Fig. 1A).

Upon elution of these bands and recomatography on Silica Gel H, they moved again with the same mobility as the standards (Fig. 1, B-D).

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

Confirmation of the Nature of $^{14}$C-Pschorophyllide. 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 monoster 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 protoporphyrin (Fig. 2B).

In acetone:petroleum ether:acetic acid (3:7:0.01 v/v), spectrophotometrically pure, standard $^{14}$C-protochlorophyllide and $^{14}$C-protoporphyrin (19, 20) give rise to two major bands and one minor band (Fig. 2A, C). Here the $^{14}$C-protochlorophyllide chromatographed in this solvent, before and after acidification, as standard $^{14}$C-protochlorophyllide and $^{14}$C-protoporphyrin respectively (Fig. 3). In this solvent Mg protoporphyrin monoster, protochlorophyllide ester, protoporphyrin monoster, and protoporphyrin ester had chromatographic mobilities strikingly different than $^{14}$C-protochlorophyllide and $^{14}$C-protoporphyrin (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, spectrophotometrically identical, compounds. A similar case was reported for radioisotopically and spectrophotometrically pure $^{14}$C-porphorbide a and b chromatographed on iceing 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 protoporphyrin (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-Protoporphyrin 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 acetone:petroleum ether:acetic acid (3:7:0.01 v/v) the $^{14}$C-protoporphyrin ester band moved differently than standard protochlorophyllide and Mg protoporphyrin monoster and with the same mobility as standard protochlorophyllide ester (Fig. 4). In this solvent some standard protochlorophyllide ester and $^{14}$C-protoporphyrin 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 protoporphyrin ester ahead of protoporphyrin monoster and protoporphyrin (Fig. 4B).

In acetone:petroleum ether:acetic acid (3:7:0.01 v/v) the $^{14}$C-protoporphyrin ester band moved with standard protochlorophyllide ester, ahead of Mg protoporphyrin monoster (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 protoporphyrin ester (Fig. 4D) ahead of protoporphyrin monoster (Fig. 4D) and the multiple bands of protoporphyrin (Compare Fig. 4D and 3, C.

![Fig. 1. Radiochromatogram tracings of the products of $^{14}$C-ALA incubation with the crude homogenate, cochromatographed with standard protochlorophyllide, Mg protoporphyrin monoster, 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 μg (59 μmole) of $^{14}$C-ALA, 400 μmole of tris-HCl, pH 7.9, 1 μmole of sucrose, 100 μmole of potassium phosphate buffer, pH 7.9, 1 μmole of MgCl₂, 10 μmole 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 monoster 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; MPF: Mg protoporphyrin monoster; Pide: protochlorophyllide ester.](image-url)
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\(_4\)OH (5:3.5 v/v); D: after acidification in 15% HCl on Whatman No. 3 paper in 2,6-lutidine:0.05 N NH\(_4\)OH (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\(_4\)OH (5:3.5 v/v); B: after acidification in 25% HCl, in 2,6-lutidine:0.05 N NH\(_4\)OH (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.
and D). Upon partial hydrolysis of the $^{14}$C-protoporphyrin 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 protoporphyrin ester and its hydrolysis product protoporphyrin (Fig. 5).

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Components</th>
<th>$^{14}$C Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg protoporphyrin monoester</td>
<td>Protoclorophyllide</td>
</tr>
<tr>
<td></td>
<td>dpm per 100 mg protein</td>
<td></td>
</tr>
<tr>
<td>A Complete</td>
<td>167000</td>
<td>13900</td>
</tr>
<tr>
<td>Without GSH, without CoA</td>
<td>38900</td>
<td>5900</td>
</tr>
<tr>
<td>Without GSH</td>
<td>19100</td>
<td>2700</td>
</tr>
<tr>
<td>B Complete</td>
<td>86400</td>
<td>13180</td>
</tr>
<tr>
<td>Without CoA</td>
<td>88700</td>
<td>8900</td>
</tr>
<tr>
<td>Without GSH</td>
<td>52000</td>
<td>7600</td>
</tr>
</tbody>
</table>

Here too the results strongly suggest that the cell-free system is indeed synthesizing $^{14}$C-protoporphyrin 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-protoporphyrin and $^{14}$C-protoporphyrin ester by the crude homogenate. The corresponding incorporations into $^{14}$C-Mg protoporphyrin monooester 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, dehydroascorbic acid, D,L-methionine, cytochrome c, D-glucose + glucose oxidase, Fe$^{2+}$, Fe$^{3+}$, Zn$^{2+}$, Co$^{2+}$, vitamin B$_6$, 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 monooester, $^{14}$C-protoporphyrin, and $^{14}$C-protoporphyrin 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-protoporphyrin biosynthesis (Table II, experiment B) without interference with $^{14}$C-protoporphyrin ester. These results suggest that the CoA site of action along the biosynthetic pathway is probably located after Mg-protoporphyrin monooester and is involved in the production of $^{14}$C-protoporphyrin but not of $^{14}$C-protoporphyrin ester. These data support the hypothesis that protoporphyrin and protoporphyrin ester are produced from a common precursor by two parallel and distinct pathways (28).

**Effect of K$^+$ and P$^+$ 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 ion components indicated in the table.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration of Major Ionic Components</th>
<th>^14C Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles per 2.5 ml</td>
<td>dpm per 100 mg protein</td>
</tr>
<tr>
<td>Complete</td>
<td>400 267 100 100</td>
<td>185500 18900 18000</td>
</tr>
<tr>
<td>Without K⁺</td>
<td>500 0 0 0</td>
<td>89100 9000 3600</td>
</tr>
<tr>
<td>without P₁</td>
<td>400 0 100 367</td>
<td>80600 9700 7100</td>
</tr>
<tr>
<td>Without K⁺</td>
<td>500 267 0 67</td>
<td>152200 14400 20600</td>
</tr>
<tr>
<td>Complete</td>
<td>400 100 189 267</td>
<td>178500 19200 17000</td>
</tr>
</tbody>
</table>

tris-P₁ 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 ^14C-Mg protoporphyrin monooester, ^14C-protoporphyrin, and ^14C-protoporphyrinylide ester (Table III). On the other hand, the omission of exogenous potassium from the complete mixture does not appear to affect the biosynthesis of ^14C-protoporphyrinylide ester but depresses the biosynthesis of ^14C-Mg protoporphyrin monooester and ^14C-protoporphyrinylide (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 etioplast preparations is needed before a specific cofactor role can be assigned to K⁺ in protorhophyrinylide biogenesis. The concentration of Cl⁻ is without appreciable effect on the ^14C-ALA incorporation into Mg protorhophyrin monooester, protorhophyrinylide, or protorhophyrinylide 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 ^14C-methanol. Mg-protoporphyrin monooester as well as protorhophyrinylide and protorhophyrinylide 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 NH₄OH (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 ^14C-methanol into ^14C-protoporphyrinylide and ^14C-protoporphyrinylide 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 ^14C-protorhophyrinylide ester with ^14C-methanol. The possibility that methanol might be preferentially incorporated into the phytole of protorhophyrinylide 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 ^14C-methanol into ^14C-Mg protoporphyrin monooester and ^14C-protoporphyrinylide compared to the incorporation of ^14C-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 ^14C-methanol used was about 34 times lower than that of ^14C-ALA.

Effect of pH and Temperature on the Biosynthetic Activity of the Crude Homogenate. The multi-enzyme system appears to have a pH optimum of about 7.7 for the biosynthesis of Mg-protoporphyrin monooester, protorhophyrinylide, and protorhophyrinylide 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 ^14C-Mg protoporphyrin monooester accumulates than at 28 C whereas the incorporations into protorhophyrinylide and protorhophyrinylide ester are slightly depressed.

Intracellular Localization of Mg Protoporphyrin and Protorhophyrin 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 (P₁) and a supernatant (S₀), 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 (S₀). No recombination effect could be demonstrated (Table VI, experiment A). A crude homogenate (FC) was prepared from
etiolated cotyledons in a grinding buffer containing all the cofactors needed for maximal protoclorophyll activity. From this homogenate a fortified etioplast pellet (FP₂) 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 (FP₂) 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, protoclorophyllide, and protoclorophyllide 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 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 ¹⁴C-Mg protoporphyrin monoester is increased as evidenced by reduced levels of the latter and increased levels of ¹⁴C-protoporphyrin. Both ATP and NAD seem to be required for maximal accumulation of ¹⁴C-protoporphyrin by the washed fortified etioplasts (Table VII). The presence of ATP does not seem to stimulate ¹⁴C-protoporphyrin ester accumulation. On the other hand, NAD alone, in the absence of ATP, resulted in a marked increase of ¹⁴C-protoporphyrin ester (Table VII). These observations might indicate that although both ATP and NAD are required for ¹⁴C-protoporphyrin biosynthesis, only NAD might be needed for ¹⁴C-protoporphyrin ester biosynthesis.

**DISCUSSION**

The foregoing results clearly demonstrate the in vitro biosynthesis of ¹⁴C-protoporphyrin and ¹⁴C-protoporphyrin ester from added ¹⁴C-ALA, by cell-free preparations from etiolated cucumber cotyledons. These two compounds are chromatographically similar in every respect to protoclorophyllide and protoclorophyllide ester isolated from etiolated cucumber cotyledons. The two protoclorophylls 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 14C-Mg porphyrins and 14C-Mg phorbins synthesized from a specific tetrapyrole precursor such as 14C-ALA is discussed at length in another article (23). Were 14C-protoporphyrin and 14C-protodylporphyrin ester contaminated to any significant extent by 14C-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 monoster, protoclorophyllide, and protoporphyrin monoster 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 protoclorophyll 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 impermeable 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 protoclorophyll takes place in the etioplasts.

Electron microscopic observation and pigment analysis agree that in order to enhance protoclorophyll biosynthesis 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 14C ALA into Mg protoporphyrin monester and protoclorophyllide 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 protoclorophyll biogenesis or rather an indirect one mediated through organelle stabilization 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 O2 by the system (Table I, experiment C) might be attributed to the O2 requirement of coproporphyrinogen oxidase (29). Indeed, Sano and Granick (29) have clearly demonstrated the absolute requirement of molecular O2 for the decarboxylation of coproporphyrinogen by a beef liver enzyme prepared from acetone powders of mitochondria. That O2 is required in our system before Mg protoporphyrin monester biosynthesis is evidenced by the practically complete lack of 14C-ALA incorporation in this compound under anaerobic conditions (Table I, experiment C).

The requirement for Mg2+ (Table I, experiment A) is best justified by the structural role of Mg2+ as part of protoclorophyllide. 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 redundant 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, protoclorophyll 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 protoclorophyll 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 14C-methanol into Mg protoporphyrin monester and protoclorophyllide (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 protoclorophyllide and protoclorophyllide ester biosynthesis (Table VII) might be viewed as a requirement for a β-oxidation type dehydrogenase (analogous to hydroxacyl 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 monester. In this case, since the carbon undergoing oxidation is conjugated with the Mg tetrapyrole 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 protoclorophyllide, but not of Mg protoporphyrin monester or protoclorophyllide ester (Table II, VII). These findings suggest that the synthesis and accumulation of protoclorophyllide involves the activation of the free propionic residue at the seventh position of the macrocycle. In this case an activation of protoclorophyllide might be viewed as a prerequisite for positioning this molecule at a specific site on the membranes while the ester group in protoclorophyllide ester positions the latter at other sites, thus contributing to the membranes heterogeneity (22, 27).

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 monester and protoclorophyllide. Most intermediates existed in both the monovinyl and divinyl forms (6). These authors postulated a scheme for chlorophyll biosynthesis beginning at Mg protoporphyrin monester and leading to the formation in parallel of protoclorophyllide (Mg-vinyl pheophyrin a3) and vinyl protoclorophyllide (Mg-divinyl pheophyrin a3) (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). Extracellular enzymatic activity of plants is not novel, 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 14C-protoporphyrinylide fraction consisted of 14C-protoporphyrin chloride and closely related mono- and divinyl...
intermediates of the β-oxidation sequence. The same might be true for the 14C-Mg protoporphyrin monoester fraction.

The production of protochlorophyllide and protochlorophyll ester in parallel from Mg protoporphyrin monoester was suggested earlier by Rebeiz et al. (28). This hypothesis was based on the kinetics of 14C-protoporphyrillide and 14C-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 14C-protoporphyrinide is derived from one branch of the pathway (divinyl branch?) while the 14C-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 (22) 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).

Acknowledgments—The authors wish to thank Dr. C. R. Stocking for many helpful discussions.

LITERATURE CITED


