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

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ABSTRACT

A crude homogenate obtained from greening cucumber (*Cucumis sativus*, L.) cotyledons in tris-sucrose, pH 7.7, containing coenzyme A, glutathione, potassium phosphate, pH 7.7, methyl alcohol, magnesium, nicotinamide adenine dinucleotide, and adenosine triphosphate, is able to incorporate 4-^{14}C-δ-aminolevulinic acid into chlorophyll *a* and *b* in the presence of oxygen. If the homogenates are prepared from etiolated cotyledons which have been exposed to light for two and one-half hours, ^{14}C-chlorophyll *a* is synthesized. However, when the homogenates are prepared from cotyledons illuminated for four and one-half hours, both ^{14}C-chlorophyll *a* and *b* are produced.

Recently Rebeiz and Castelfranco (10) reported the biosynthesis of ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester from 4-^{14}C-δ-aminolevulinic acid by a cell-free system prepared from etiolated cucumber cotyledons. They were able to show that the activity is associated with etioplasts and has a pH optimum of 7.7. For the formation of ^{14}C-protochlorophyllide ester, oxygen, GSH, methyl alchol, Mg^{2+}, P_i, and NAD are required (10). For the formation of ^{14}C-protochlorophyllide, CoA, ATP, and possibly K^+ are needed in addition to the above cofactors (10).

Previous work with etiolated cucumber cotyledons has shown that it takes 1 to 1.5 hr of exposure to white light before chlorophyll *a* begins to accumulate and about 3.0 hr before the appearance of chlorophyll *b* (7). Therefore, we have chosen to investigate the chlorophyll-biosynthetic ability of cell-free homogenates derived from three types of plant tissues: (*a*) etiolated cucumber cotyledons, (*b*) etiolated cotyledons after 2.5 hr of light treatment, (*c*) etiolated cotyledons after 4.5 hr of light treatment. When the cell-free homogenates derived from greening cotyledon tissues were incubated in the presence of ^{14}C-δ-aminolevulinic acid, O_2, and the other cofactors needed for *in vitro* protochlorophyll biosynthesis (10), ^{14}C-chlorophylls were obtained.

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 germinated in vermiculite (Terra Lite) at 24 C in complete darkness (3, 10).

Chemicals and Radiochemicals. ^{14}C-ALA[2] (33.8 μc/μmole) was purchased from Tracerlab, Waltham, Massachusetts. Lead-manganese activated calcium silicate short wave phosphor was purchased from Kensington Scientific Corporation. The commercial source of other materials was described elsewhere (10). Chloroform was purified by washing twice with H_2O and drying over anhydrous sodium sulfate just before use (8).

Irradiation of Etiolated Cucumber Cotyledons. Etiolated cucumber cotyledons, 4.5 days old, were harvested with full hypocotyl hook (3). They were placed in beakers with enough distilled H_2O to keep them moist and irradiated with 240 ft-c of white fluorescent light at 28 C, for 2.5, 4.5, or 12 hr.

Preparation of Crude Homogenates. Five grams of 4.5-day-old etiolated or greening cotyledons were gently macerated with mortar and pestle in 7.5 ml of 0.5 M sucrose, 0.2 M tris-HCl, pH 7.7, containing 37.5 μmoles of GSH, 2.25 μmoles of CoA, 3.75 μmoles of $MgCl_2$, 375 μmoles of potassium phosphate, pH 7.7, 180 μmoles of methyl alcohol, 3 μmoles of ATP, and 0.45 μmole of NAD. The slurry was filtered through four layers of cheese cloth. The resulting filtrate is called the crude homogenate; it contains no detectable intact cells as evidenced by light and phase contrast microscopy (9, 10). Total proteins were determined by biuret as described previously (11).

Incubation of the Crude Homogenates with ^{14}C-ALA. Two milliliters of crude homogenates prepared from etiolated or greening cotyledons were incubated on a metabolic shaker in glass tubes, with 2 μc (59 mμmoles) of ^{14}C-ALA and an additional 1.02 mmole of methyl alcohol; the final volumes were adjusted to 2.5 ml with H_2O. The incubations were carried out in the dark or under 10 ft-c of white fluorescent light at 28 C with moderate shaking speed for 16 hr.

Determination of ^{14}C-Incorporations into ^{14}C-Mg Protoporphyrin Monoester, ^{14}C-Protochlorophyllide or ^{14}C-Chlorophyllide, and ^{14}C-Protochlorophyllide Ester or ^{14}C-Chlorophyll. The extraction, chromatography, and determination of ^{14}C-incorporations into these pools were described earlier for ^{14}C-Mg protoporphyrin monoester, ^{14}C-protochlorophyllide and, ^{14}C-protochlorophyllide ester (10).

Spectrophotometric Determinations. Absorption spectra were recorded with a Perkin-Elmer double beam spectrophotometer Model 202. The wave length calibration was regularly checked against the visible spectrum of a standard holmium oxide filter. A Zeiss PMQII spectrophotometer was used for all quantitative spectrophotometric determinations. Absorbancies were corrected for light scattering as described previously (12). The amounts of chlorophyll *a* and *b* in ether were calculated from

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

corrected absorbancies using simultaneous equations derived from the specific absorption coefficients of Zscheile and Comar (4, 17). The amount of pheophytin *a* in ether was calculated with a molar extinction coefficient of 10.98×10^4 at 408 nm, calculated from the specific absorption coefficient for pheophytin *a* reported by Zscheile and Comar (17).

Extraction of Crude ^{14}C-Chlorophyll *a* and ^{14}C-Chlorophyll *b* for Purification to Constant Specific Radioactivity. The reaction was stopped by the addition of 12.5 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 2.5 ml of the acetone:NH_4OH mixture. The combined supernatant was extracted twice with 5-ml portions of hexane. The combined hexane extracts containing the chlorophylls were washed with about 40 ml of cold H_2O saturated with $MgCO_3$. To the washed hexane extracts, 20 ml of methanol:H_2O (84:16, v/v) were added. Two phases were obtained; a deep green upper phase containing ^{14}C-chlorophyll *a* and β-carotene (crude ^{14}C-chlorophyll *a* fraction) and a pale yellow-green phase containing most of the ^{14}C-chlorophyll *b* and the xanthophylls (crude ^{14}C-chlorophyll *b* fraction).

Purification of ^{14}C-Chlorophyll *b* to Constant Specific Radioactivity. The crude ^{14}C-chlorophyll *a* fraction in hexane was dried under N_2 and redissolved in ether; its absorption spectrum was recorded and its specific radioactivity in dpm per mμmole of chlorophyll *a* was determined. The ether extract was subsequently concentrated under N_2 and transferred to thin layers of Silica Gel H, 500 μ thick. The chromatograms were developed in the dark at 1 to 4 C in benzene:ethyl acetate:ethanol (8:2:2, v/v) (10). The broad ^{14}C-chlorophyll band was eluted in ether, its absorption spectrum was recorded, and its specific radioactivity was determined. The ^{14}C-chlorophyll eluate was subsequently concentrated under N_2 and chromatographed on thin layers of cellulose MN 300, 500 μ thick, in petroleum ether (63–75 C):acetone:*n*-propanol (90:10:0.45, v/v) at room temperature in the dark. The ^{14}C-chlorophyll *a* band was eluted in ether, and again its absorption spectrum was recorded, and its specific radioactivity was determined. At this stage the ^{14}C-chlorophyll *a* in ether was pheophytinized by shaking with 2 ml of cold 6.85 N HCl (25% w/v). The acid phase was neutralized with solid sodium acetate and pheophytin *a* in ether was thoroughly washed with H_2O. It was concentrated under N_2 and chromatographed on thin layers of cellulose MN 300, 500 μ thick, in petroleum ether (60–90 C):acetone:acetic acid (70:30:0.1, v/v) in the dark at room temperature. The ^{14}C-pheophytin *a* was eluted in ether, its absorption spectrum was recorded, and its specific radioactivity in dpm per mμmole of pheophytin *a* was determined.

Purification of ^{14}C-Chlorophyll *b* to Constant Specific Radioactivity. The crude ^{14}C-chlorophyll *b* fraction was mixed with an equal volume of diethyl ether and transferred to the ether by washing with 500 ml of cold H_2O saturated with $MgCO_3$. At this stage the ^{14}C-chlorophyll *b* fraction in ether was mixed with carrier chlorophyll *b* in ether prepared as described above from greening cucumber cotyledons. The absorption spectrum was recorded, and the specific radioactivity in dpm per mμmole of chlorophyll *b* was determined. The ^{14}C-chlorophyll *b* plus carrier chlorophyll *b* was subsequently chromatographed on thin layers of cellulose MN 300, 500 μ thick, in petroleum ether (63–75 C):acetone:*n*-propanol (90:10:0.45, v/v) in the dark at room temperature. The ^{14}C-chlorophyll *b* was eluted in ether, its absorption spectrum was recorded, and its specific radioactivity was determined. After concentration under N_2, it was rechromatographed exactly as above, and again the absorption spectrum of the ^{14}C-chlorophyll *b* fraction in ether was recorded, and its specific radioactivity was determined.

Degradation of ^{14}C-Pheophytin *a* to ^{14}C-Pheophorbide *a*. ^{14}C-Pheophytin *a* purified as described above was degraded to pheophorbide *a* according to Perkins and Roberts (6).

Degradation of ^{14}C-Pheophorbide *a* to ^{14}C-Maleimides. The ^{14}C-pheophorbide *a* fraction was degraded to maleimides (1, 2) essentially as described by Rüdiger *et al* (13): About 2 μg of the ^{14}C-pheophorbide *a* fraction were reacted with 1 ml of 2% (w/v) CrO_3 in 2 N H_2SO_4 at 100 C for 2 hr. The mixture was extracted seven consecutive times with 1.5-ml portions of diethyl ether. The combined ether extracts were washed with H_2O, concentrated under N_2, and chromatographed on one plate of Silica Gel H, 250 μ thick, in petroleum ether (60–90 C):ethyl acetate:*n*-propanol (8.8:1.0:0.2, v/v) in the dark at room temperature (1). The Silica Gel H plates contained lead-manganese activated calcium silicate short wave phosphor.

Chromatography. The preparation of thin layers of Silica Gel H and cellulose MN 300 was described elsewhere (8). For the preparation of short wavelength fluorescent Silica Gel H plates, 100 mg of lead-manganese activated calcium silicate short wave phosphor was added to 70 ml of H_2O and 30 g of Silica Gel H; the plates were prepared as usual (8).

^{14}C-Chlorophyll *a* was separated from ^{14}C-protochlorophyllide ester by ascending chromatography on Whatman No. 1 paper in the dark at room temperature in petroleum ether (60–90 C):acetone:acetic acid (7:3:0.01, v/v). ^{14}C-Chlorophyll *a* and ^{14}C-chlorophyll *b* purified to constant specific radioactivity were chromatographed on Whatman No. 1 paper in petroleum ether (63–75 C):acetone:*n*-propanol (90:10:0.45, v/v). In order to determine the contamination of ^{14}C-chlorophyll *a* by ^{14}C-chlorophyll *b* at various stages of purification, the fractions were monitored by two-way paper chromatography on Whatman No. 1 paper as described in an earlier communication (8): the chromatogram was developed in the first direction in purified chloroform:petroleum ether (60–90 C) (25:75, v/v) and in the second direction in petroleum ether (60–90 C):acetone:*n*-propanol (90:10:0.45, v/v). The chromatogram was viewed under ultraviolet and visible light, the colored and fluorescent areas were marked, and the chromatogram was subsequently cut in strips 4-cm wide parallel to the second solvent front. Radioactive areas on all the paper strips were localized with a Nuclear Chicago radiochromatogram scanner Model 1025. Thin layer plates were scanned with a Packard radiochromatogram scanner Model 7201.

Preparation of Porphyrin and Phorbin Standards. The preparation of various porphyrin and phorbin standards was reported elsewhere (9, 10, 12). Crude standard chlorophyll *a* and *b* fractions were prepared from etiolated cucumber cotyledons excised with full hook (3) irradiated for 12 hr at 28 C with 240 ft-c of white fluorescent light. After the light treatment the hooks were removed. Three grams of greening cotyledons were homogenized in 20 ml of acetone:0.1 N NH_4OH (9:1, v/v). After centrifugation, the acetone:NH_4OH extract was processed as described for the extraction of crude ^{14}C-chlorophyll *a* and *b* for specific radioactivity determinations.

RESULTS

Radioactive Products of ^{14}C-ALA Incubation with Homogenates Prepared from Etiolated and Greening Cotyledons. Three types of cotyledons were used for the preparation of homogenates: etiolated cotyledons still subject to a lag phase of chlorophyll *a* and *b* biosynthesis in the light, cotyledons irradiated for 2.5 hr and capable of chlorophyll *a* biosynthesis *in vivo*, and, finally, cotyledons irradiated for 4.5 hr and capable of both chlorophyll *a* and *b* biosynthesis *in vivo* (7).

When the homogenates prepared from 2.5-hr irradiated cotyledons (green homogenate) or etiolated cotyledons (etiolated homogenate) were incubated with ^{14}C-ALA for 16 hr at 28 C

in the presence and absence of light, the ^{14}C-Mg protoporphyrin monoester, ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester pools became radioactive (Table I). In both cases the ratios of ^{14}C-protochlorophyllide ester to ^{14}C-protochlorophyllide were 2- to 3-fold higher when the incubations were carried out in the light than in the dark (Table I). Although chromatography on thin layers of Silica Gel H in benzene:ethyl acetate:ethanol (8:2:2, v/v) did not separate the ^{14}C-protochlorphyllide from ^{14}C-chlorophyllide and the ^{14}C-protochlorophyllide ester from ^{14}C-chlorophyll *a*, these results suggested that in the light ^{14}C-chlorophyll *a* might be the end product rather than the usual mixture of ^{14}C-protochlorophyllide ester and ^{14}C-protochlorophyllide (10 [Table I]). Therefore, the phytyl ester bands from the green reaction mixtures and the ones from the etiolated reaction mixtures were eluted from Silica Gel H and chromatographed on Whatman No. 1 paper in petroleum ether (60–90 C): acetone:acetic acid (7:3:0.01, v/v). The radioactivity from the light incubation of the green homogenate moved with standard chlorophyll *a* (Fig. 1, A and B), while the radioactivity from the dark incubation of the etiolated homogenate moved with standard ^{14}C-protochlorophyllide ester (Fig. 1, A and C). When the two phytyl ester bands derived from the light and dark incubations of the green homogenate were eluted, mixed and rechromatographed on paper, two bands appeared corresponding to standard chlorophyll *a* and standard protochlorophyllide ester (Fig. 1D).

No efforts were made to separate ^{14}C-protochlorophyllide from ^{14}C-chlorophyllide by chromatography. The ^{14}C-chlorophyll *a* from the light incubation of the green homogenate was further submitted to two-way paper chromatography in chloroform: petroleum ether (60–90 C) (25:75, v/v) in the first direction followed by chromatography in petroleum ether (60–90 C):acetone:*n*-propanol (90:10:0.45, v/v) in the second direction. ^{14}C-Chlorophyll *a* cochromatographed with standard chlorophyll *a* in both solvents. It moved with an R_F of 0.78 in the first solvent and an R_F of 0.63 in the second solvent. No ^{14}C-chlorophyll *b* could be detected on these chromatograms.

The purity of the ^{14}C-chlorophyll *a* fraction was tested further: a hexane extract from the light incubation of the green homogenate (2.5-hr irradiated cotyledons) was mixed with carrier standard chlorophyll *a*, and the specific radioactivity was determined at several stages of purification (Table II, experiment A) It appears from Table II, experiment A, that the ^{14}C-chlorophyll *a* had reached a constant specific radioactivity after the Silica Gel H purification.

Table I. *Biosynthetic Activity of Crude Homogenates Prepared from Etiolated and Greening Cotyledons, and Incubated in the Presence and Absence of Light*

In a total volume of 2.5 ml the reaction mixture contained: 2 μc (59 mμmoles) of ^{14}C-ALA, 400 μmoles of tris-HCl, pH 7.7, 1 mmole of sucrose, 100 μmoles of potassium phosphate buffer, pH 7.7, 1 μmole of $MgCl_2$, 10 μmoles of GSH, 0.6 μmole of CoA, 1.2 mmole of methyl alcohol, 1 μmole of ATP, 0.15 μmole of NAD, and about 100 mg of protein. Time of incubation was 16 hr in the dark or under 10 ft-c of white fluorescent light at 28 C. The homogenates were prepared either from 2.5-hr irradiated cotyledons (green) or from etiolated cotyledons (etiolated).

Experiment	Source	Incubation	^{14}C-Incorporated			Esterified phorbin/non-esterified phorbin
			Mg Protoporphrin monoester	Protochlorophyllide or chlorophyllide	Protochlorophyllide ester or chlorophyll *a*	
			dpm per 100 mg protein			
A	Green	Dark	29700	5900	7700	1.3
	Green	Light	11400	3500	8400	2.4
B	Etiolated	Dark	24700	5800	2100	0.4
	Etiolated	Light	11200	3500	4200	1.2

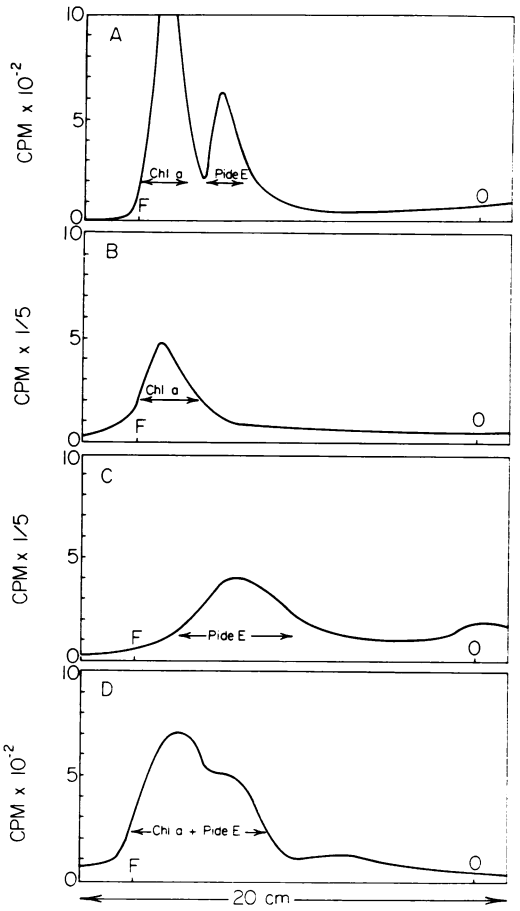

FIG. 1. Radiochromatogram tracings of ^{14}C-chlorophyll *a* and ^{14}C-protochlorophyllide ester from various sources chromatographed on Whatman No. 1 paper in acetone:petroleum ether (60–90 C):acetic acid (3:7:0.01, v/v). A: ^{14}C-Chlorophyll *a* and ^{14}C-protochlorophyllide ester standards; B: ^{14}C-phytyl ester band from the light incubation of the green homogenate eluted from Silica Gel H; C: ^{14}C-phytyl ester band from the dark incubation of the etiolated homogenate eluted from Silica Gel H; D: mixture of the ^{14}C-phytyl ester band from the light and dark incubations of the green homogenate eluted from Silica Gel H O: Origin; F: front; Chl a: standard chlorophyll *a*; Pide E: standard protochlorophyllide ester.

These results strongly suggest that the green homogenate prepared from cotyledons exposed to light for 2.5 hr is able to synthesize ^{14}C-chlorophyll *a* but not ^{14}C-chlorophyll *b*.

Biosynthesis of ^{14}C-Chlorophyll *a* and *b* by Green Homogenates Prepared from Cotyledons Irradiated for 4.5 hr. When etiolated, excised cotyledons are irradiated with white fluorescent light for 4.5 hr, they become capable of substantial chlorophyll *b* biosynthesis in addition to chlorophyll *a* (7). In order to find out whether homogenates prepared from such greening cotyledons are capable of chlorophyll *b* biosynthesis, they were incubated in the light with ^{14}C-ALA. The crude chlorophyll *a* and chlorophyll *b* fractions were both highly radioactive.

The ^{14}C-chlorophyll *a* fraction was subsequently purified to constant specific radioactivity (Table II, experiment B). Chromatography on Silica Gel H purified the ^{14}C-chlorophyll *a* from other ^{14}C-porphyrins (9, 10). This was shown by a strong

Table II. *Specific Radioactivities of ^{14}C-Chlorophyll a and ^{14}C-Chlorophyll b from Green Homogenates at Various Stages of Purification*

Experiment A: ^{14}C-Chlorophyll *a* from the light incubation of the green homogenate was derived from 2.5-hr irradiated cotyledons and mixed with extra amounts of carrier standard chlorophyll *a*; experiment B: ^{14}C-chlorophyll *a* from the light incubation of the green homogenate was derived from 4.5-hr irradiated cotyledons; experiment C: ^{14}C-chlorophyll *b* from the same homogenate as B was mixed with extra amounts of carrier standard chlorophyll *b*.

Experiment	Pigment	Specific radioactivity
		dpm/mμmole of pigment $\times 10^{-3}$
A	^{14}C-Chlorophyll *a* + carrier chlorophyll *a* in ether	2.60
	Purified on Silica Gel H	0.80
	Purified on cellulose Mn 300	0.79
B	^{14}C-Chlorophyll *a* extract in ether	7.80
	Purified on Silica Gel H	4.70
	Purified on cellulose MN 300	3.60
	^{14}C-Pheophytin *a* purified on cellulose MN 300	3.40
C	^{14}C-Chlorophyll *b* + carrier chlorophyll *b* in ether	2.10
	Purified on cellulose MN 300	0.27
	Purified on cellulose MN 300	0.29

decrease in specific radioactivity (Table II, experiment B). Chromatography on cellulose MN 300 separated the ^{14}C-chlorophyll *a* efficiently from minor contamination by ^{14}C-chlorophyll *b* (Table II, experiment B). Spectrophotometric measurements indicated a negligible chlorophyll *b* contamination (about 2%). Upon conversion into ^{14}C-pheophytin *a* and rechromatography on cellulose MN 300, the specific radioactivity remained unchanged indicating that after the cellulose purification step, the ^{14}C-chlorophyll *a* was free of significant ^{14}C-porphyrin, phorbin, or colorless radioactive contaminants (5, 6, 16). Figure 2A represents a radiochromatogram tracing of ^{14}C-chlorophyll *a* purified to constant specific radioactivity as described above.

The ^{14}C-pheophytin *a* fraction was subsequently degraded to pheophorbide *a* according to Perkins and Roberts (6), and an aliquot was chromatographed on Silica Gel H in benzene:ethyl acetate:ethanol (8:2:5, v/v). As reported by Perkins and Roberts (6), this procedure degraded ^{14}C-pheophytin *a* extensively into ^{14}C-pheophorbide *a* and two slow moving red fluorescent radioactive products (Fig. 3A), one of which is probably ^{14}C-pyropheophorbide *a* (6, 16). The radiochromatogram tracing of this fraction chromatographed on Silica Gel H is given in Figure 3A. The mixture of ^{14}C-pheophorbide *a* and its ^{14}C-tetrapyrrole derivatives was degraded further to derivatives of the individual pyrroles, that is to maleimides. The technique utilized is supposed to preserve the vinyl side chains (13). Maleimides quench short wave length ultraviolet light (254 nm) and appear as blue spots on fluorescent thin layers viewed under ultraviolet light (1). Figure 3B represents the radiochromatogram tracing of the ^{14}C-pheophorbide *a* fraction after oxidation to maleimides with chromic acid. Although no efforts were made to identify the individual maleimides, the radioactive areas coincided with the blue quenching spots on the chromatogram (Fig. 3B).

The crude ^{14}C-chlorophyll *b* fraction was also purified to constant specific radioactivity (Table II, experiment C). The specific radioactivities listed in Table II, experiment C, do not represent authentic values as carrier chlorophyll *b* was added

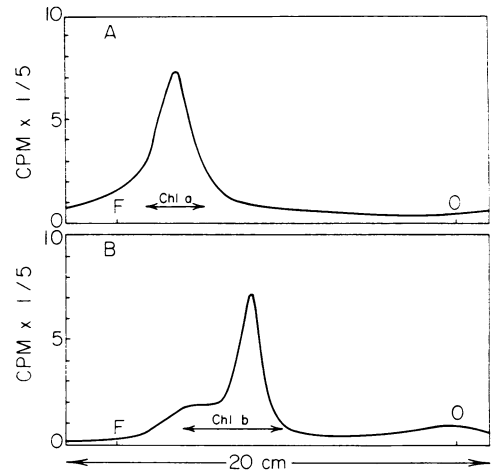

FIG. 2. Radiochromatogram tracings of ^{14}C-chlorophyll *a* and ^{14}C-chlorophyll *b* purified to constant specific radioactivity and chromatographed on Whatman No. 1 paper in petroleum ether (63–75 C):acetone:*n*-propanol (90:10:0.45, v/v). A: ^{14}C-Chlorophyll *a* after purification on cellulose MN 300; B: ^{14}C-chlorophyll *b* after the second purification on cellulose MN 300. Chl b: standard chlorophyll *b*; all other symbols are the same as in Figure 1.

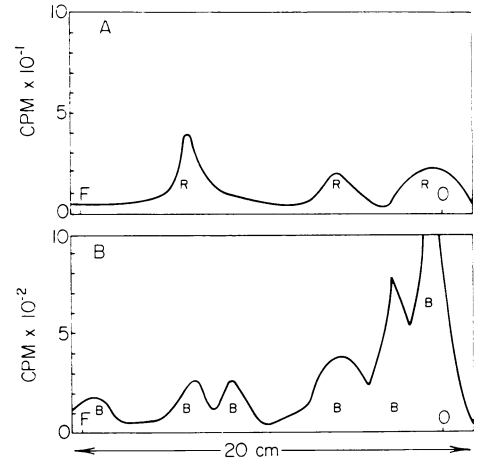

FIG. 3. Radiochromatogram tracings of the ^{14}C-pheophorbide *a* fraction and ^{14}C-maleimides from ^{14}C-pheophorbide *a* chromatographed on Silica Gel H. A: ^{14}C-Pheophorbide *a* fraction chromatographed in benzene:ethyl acetate:ethanol (8:2:5, v/v). B: ^{14}C-maleimides from the chromic acid oxidation of ^{14}C-pheophorbide *a* chromatographed in petroleum ether (60–90 C):ethyl acetate:*n*-propanol (8.8:1.0:0.2, v/v). R: Red fluorescent areas under ultraviolet light of 366 nm; B: blue quenching areas under ultraviolet light of 254 nm. All other symbols are the same as in Figure 1.

just before the first purification step. After the first purification on thin layers of cellulose, the specific radioactivity dropped sharply (Table II, experiment C). This was due to the separation of ^{14}C-chlorophyll *a*. Upon rechromatography of the ^{14}C-chlorophyll *b* on cellulose the specific radioactivity remained unchanged (Table II, experiment C), indicating that after the first cellulose purification the ^{14}C-chlorophyll *b* fraction was free of significant amounts of ^{14}C-porphyrins or ^{14}C-chlorophyll *a*. Spectrophotometric analysis indicated a negligible chlorophyll *a* contamination (about 1.5%) after the first cellulose purification. After the second cellulose purification no contaminating chlorophyll *a* could be detected spectrophotometrically. Figure 2B represents a radiochromatogram tracing of ^{14}C-chlorophyll *b* after the second purification step on thin layers of cellulose. The radioactive shoulder running ahead of the bulk of the ^{14}C-

chlorophyll *b* (Fig. 2B) probably represents ^{14}C-pheophytin *b* contamination and possibly traces of ^{14}C-chlorophyll *a*.

DISCUSSION

Isolated chlorophylls are notoriously contaminated by colorless lipids (5, 6, 14). These substances interfere with the nuclear magnetic and infrared spectra of chlorophylls (14) and above all with the purification of ^{14}C-chlorophylls to constant specific radioactivity (6, 16). This is especially true when ^{14}C-chlorophylls are prepared from nonspecific tetrapyrrole precursors such as 2-^{14}C-acetate, 2,3-^{14}C-succinate or $^{14}CO_2$; these substrates are then incorporated into the contaminating colorless substances as well (6, 16). Both Perkins and Robert (6) and Wickliff and Aronoff (16) report the purification of ^{14}C-chlorophyll *a* and *b* derived from nonspecific ^{14}C-precursors to ^{14}C-pheophytin *a* and *b* of constant specific radioactivity. Their purification consists of chromatographic separations of the chlorophylls from each other and from carotenoids, followed by pheophytinization, chromatography, and recovery of ^{14}C-pheophytins (6, 16). Our own experience with ^{14}C-porphyrin and phorbin derivatives biosynthesized both *in vivo* and *in vitro* from ^{14}C-ALA indicate that ^{14}C-ALA is a very specific precursor of tetrapyrroles and is not incorporated into carotenoids, phytol, or colorless lipids (9, 10, 12). In other words various ^{14}C-porphyrin and phorbin derivatives biosynthesized from ^{14}C-ALA are more likely to contaminate one another than to be contaminated by ^{14}C-carotenoids or ^{14}C-colorless lipids.

That ^{14}C-chlorophyll *a* biosynthesized *in vitro* was radiochemically free of ^{14}C-colorless contaminants is shown by its constant specific radioactivity after pheophytinization and chromatography (Table II, experiment B). In this solvent pheophytin *a* moves to the front leaving colorless substances behind at the original emplacement of chlorophyll *a*. That purified ^{14}C-chlorophyll *a* was free of appreciable amounts of ^{14}C-chlorophyll *b* is evidenced by spectrophotometry and chromatography (Fig. 2, A and B).

Due to the lack of sufficient amounts of ^{14}C-chlorophyll *b*, it was not degraded further to pheophytin *b* and pheophorbide *b*. However, due to the specificity of ^{14}C-ALA as a tetrapyrrole precursor and the absence of ^{14}C-colorless contaminants we feel confident that the radioactivity of the ^{14}C-chlorophyll *b* fraction purified on cellulose is authentic and not due to other ^{14}C-contaminating substances. As mentioned earlier, cross-contamination by chlorophyll *a* was nil after the second cellulose purification.

Although the phototransformation of ^{14}C-protochlorophyllide is unanimously acknowledged, that of ^{14}C-protochlorophyllide ester is not (12). However, some of our data do lend support to the phototransformability of protochlorophyllide ester into chlorophyll *a*. When the green homogenate was incubated with ^{14}C-ALA in the light, and the ^{14}C-phytylated pools were analyzed, ^{14}C-chlorophyll *a* accumulated (Fig. 1B). When it was incubated in the dark, ^{14}C-protochlorophyllide ester accumulated instead (Fig. 1C). Although these results do not show the direct phototransformation of dark biosynthesized ^{14}C-protochlorophyllide ester into ^{14}C-chlorophyll *a* upon exposure to light, they do suggest that in the light chlorophyll biosynthesis proceeds via both protochlorophyllide and protochlorophyllide ester. Present research is under way to investigate the direct phototransformation of ^{14}C-protochlorophyllide ester, biosynthesized in the dark, into ^{14}C-chlorophyll.

In contrast to chlorophyll *a*, chlorophyll *b* (in excised etiolated cotyledons illuminated with continuous white light) becomes noticeable during the 3rd hour of irradiation; and thereafter it becomes substantial (7). The lack of significant ^{14}C-chlorophyll *b* biosynthesis by crude homogenates prepared from 2.5-hr irradiated cotyledons is in sharp contrast to the substantial ^{14}C-chlorophyll *b* biosynthesis by homogenates prepared from 4.5 hr irradiated cotyledons. This is readily explained by the prolonged lag of chlorophyll *b* biosynthesis *in vivo* as mentioned above (7). It seems that once the cell-free system is prepared, the subsequent 16 hr illumination is not capable of inducing the biogenesis of chlorophyll *b* as would be the case *in vivo* (7). Whether or not this induction involves *de novo* enzyme biogenesis, biosynthesis of activators or derepressors, and whether it is under nuclear control is not known at present. However, it seems that once the chlorophyll *b* biosynthetic capacity has been acquired after prolonged illumination *in vivo* (7), it is preserved in the cell-free system. This is seen by the significant chlorophyll *b* biosynthesis upon subsequent light incubation *in vitro*. The biosynthesis of ^{14}C-chlorophyll *a* and *b* appears feasible with the same cofactors needed for ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester formation (10).

Electron microscopic observations indicate that the structure of differentiating plastids capable of chlorophyll *a* and *b* biosynthesis was remarkably well preserved after the 16 hr light incubation *in vitro* (C. A. Rebeiz, P. A. Castelfranco, S. Larson, and T. E. Weier, in preparation). A most important phase of future research will be studies *in vitro* on the relationships between chlorophyll biosynthesis, the biogenesis of thylakoid membranes, and the buildup of chlorophyll heterogeneity. Hopefully, this research will eventually lead to the complete differentiation *in vitro* of etioplasts into chloroplasts. If this goal were accomplished, it would afford a tremendous insight into the various nuclear and cytoplasmic factors that affect the course of organelle differentiation.

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