Plant Physiol. (1973) 51, 660–666

Biosynthesis and Accumulation of Microgram Quantities of Chlorophyll by Developing Chloroplasts *in Vitro*[1]

Received for publication July 31, 1972

CONSTANTIN A. REBEIZ,[2] JULIAN C. CRANE, CHIC NISHIJIMA, AND CAROLE C. REBEIZ
Department of Pomology, University of California, Davis, California 95616

ABSTRACT

Developing chloroplasts were incubated under conditions previously shown to induce protochlorophyll and chlorophyll biosynthesis, as well as chloroplast maintenance and partial differentiation *in vitro*. In the presence of air, δ-aminolevulinic acid, coenzyme A, glutathione, potassium phosphate, methyl alcohol, magnesium, nicotinamide adenine dinucleotide, and adenosine triphosphate, microgram quantities of chlorophyll accumulated after 1 hour of incubation. Part of the chlorophyll was not extractable in organic solvents; it is referred to as bound chlorophyll. The amount of bound chlorophyll depended on the degree of cotyledon greening at the time of plastid isolation. Etioplasts with or without a lag phase of chlorophyll biosynthesis synthesized nonphototransformable protochlorophyll and smaller amounts of extractable chlorophyll. As the greening of excised cotyledons progressed, more of the chlorophyll became bound before and after *in vitro* incubation. It is suggested that this increase in the fraction of bound chlorophyll reflects the biosynthesis of membrane-bound chlorophyll receptor sites. In the absence of cofactors, chlorophyll biosynthesis was blocked and porphyrins accumulated, indicating damage of the chlorophyll biosynthetic chain. It is concluded that chlorophyll accumulation constitutes a potentially convenient tool for the study of thylakoid membrane biogenesis *in vitro*.

Rebeiz *et al.* (12, 13, 18) have shown that cell-free homogenates and etioplasts isolated from cucumber cotyledons and incubated with ^{14}C-ALA[3] and cofactors, were capable of synthesizing ^{14}C-Mg protoporphyrin monoester, ^{14}C-Protochlorophyll and ^{14}C-chlorophyll *a* and *b*. These findings led to the conclusion that etioplasts and developing chloroplasts contained the biosynthetic machinery required for chlorophyll biosynthesis from ALA (12, 13). The foregoing results were subsequently confirmed by Wellburn and Wellburn (22), who demonstrated the incorporation of the label from ^{14}C-glycine, succinate, and ALA into ^{14}C-chlorophyll *a* by isolated etioplasts. However, the net synthesis of microgram quantities of chlorophyll from ALA *in vitro* has not been reported. Abundant evidence strongly suggests that *in situ* chloroplast development during greening is obligatorily coupled to chlorophyll accumulation (2, 6, 19). Grana appear to be the main site of this accumulation (21). These observations suggest that chlorophyll biosynthesis and accumulation *in vitro* and thylakoid membrane biogenesis might also be related. The study of chloroplast differentiation *in vitro* might, therefore, improve our understanding of the relationship of chlorophyll biosynthesis and accumulation to thylakoid membrane biogenesis. It might also provide direct insight into the influence of nuclear cytoplasmic operators on the assembly of a functional photosynthetic apparatus.

A systematic study of chloroplast differentiation *in vitro* will eventually require a reliable marker of chloroplast development. The unique spectroscopic properties of chlorophyll make it a convenient candidate for these studies. On the other hand, a cell-free system capable of accumulating microgram quantities of chlorophyll, as well as intermediates of the chlorophyll biosynthetic chain, is well suited for the step by step study of the chlorophyll biosynthetic pathway.

Rebeiz *et al.* (19) have shown recently that, when developing chloroplasts were incubated for 16 hr in subdued light with the cofactors previously shown to be essential for photochlorophyll and chlorophyll biosynthesis, the structure of some of the plastids was remarkably well preserved. In addition, membranous constituents appeared to be assembled into elongated grana *in vitro*. In this communication, we describe the accumulation of microgram quantities of chlorophyll *in vitro* under similar incubation conditions. Two preliminary communications based on this research were presented to the annual meetings of the American Society of Plant Physiologists, Asilomar, California in August 1971, and to the VI International Congress on Photobiology, Bochum, Germany in August 1972 (11, 15).

MATERIALS AND METHODS

Germinating Cucumber Seeds. Cucumber seeds (*Cucumis sativus* L. cv. Alpha Green) were germinated in vermiculite (Terra Lite) at 24 C in complete darkness for 4 days (12).

Chemicals. The commercial source of chemicals was reported elsewhere (12).

Light Pretreatment of Etiolated Cucumber Cotyledons. Etiolated cotyledons were harvested with full hypocotyl hook under a green safelight (7). They were placed in culture dishes with enough distilled H_2O to keep them moist. The excised cotyledons were illuminated with 240 ft-c of white fluorescent light at 28 C for 1 min or for 1, 2, and 4.5 hr. Other light pretreatments were aimed at removing the lag phase by a short preillumination followed by dark incubation (8). This was achieved

[1] This work was supported in part by Research Grant GB 8546 from the National Science Foundation and by funds from the Illinois Agricultural Experiment Station.

[2] Communications concerning this paper should be addressed to: Constantin A. Rebeiz, Department of Horticulture, University of Illinois at Urbana-Champaign, Urbana, Ill. 61801.

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

by irradiating the excised cotyledons for 4 min with 250 ft-c of white fluorescent light. The irradiated cotyledons were returned to darkness for 3 hr at 28 C, then illuminated for 4 min to phototransform the protochlorophyll formed during the dark incubation.

Isolation of Greening Etioplasts. "Fortified" and "unfortified" plastid preparations refer to isolated greening etioplasts or developing chloroplasts prepared with or without cofactors in the homogenization buffer. The cofactors (GSH, CoA, methyl alcohol, Mg, Pi, NAD, ATP, and K) were shown previously to be necessary for protochlorophyll and chlorophyll biosynthesis (12, 13). Unfortified plastids were prepared by removing the hooks and grinding gently 5 g of greening cotyledons with mortar and pestle without sand. The grinding was accomplished in 7.5 ml of 0.5 M sucrose, 0.2 M tris-HCl, pH 8.0, at 0 to 5 C. The brei was filtered through four layers of cheesecloth, and the resulting homogenate was centrifuged at 0 C for 3 min at 200*g*. The supernatant was centrifuged for 7 min at 1500*g*, and the plastid pellet was suspended in 0.5 M sucrose, 0.2 M tris-HCl, pH 7.7. Fortified plastids were prepared by grinding 5 g of greening cotyledons in 7.5 ml of fortified 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0, containing 37.5 μmoles of GSH, 2.25 μmoles of CoA, 3.75 μmoles of $MgCl_2$, 375 μmoles of potassium phosphate, 180 μmoles of methyl alcohol, 3 μmoles of ATP, and 0.56 μmole of NAD. The plastids were sedimented as described above, and the pellet was suspended in the fortified tris-sucrose buffer at pH 7.7. The plastids extractable from 5 g of cotyledon were generally used for a single assay. This represented 4 to 7 mg of proteins per assay. Total proteins were determined by biuret as described previously (14).

Incubation of Isolated Plastids and Excised Cotyledons. Two ml of fortified and unfortified plastid suspensions were incubated in cylindrical flat bottom glass tubes (2 × 10 cm) on a metabolic shaker operated at about 50 shakes per min. The incubations were performed at 28 C under 10 ft-c of white fluorescent light for 1 hr. Two and a half ml of unfortified reaction mixture at pH 7.7 contained 400 μmoles of tris-HCl, 1 mmole of sucrose, and 1 μmole of ALA. The fortified reaction mixture of 2.5 ml at pH 7.7 contained, in addition to the above components, 100 μmoles of potassium phosphate, 1 μmole of $MgCl_2$, 10 μmoles of GSH, 0.6 μmole of CoA, 1.2 mmole of methyl alcohol, 0.8 μmole of ATP, and 0.15 μmole of NAD.

For reference purposes 10 pairs of preirradiated cotyledons excised with full hypocotyl hook were simultaneously incubated in a minimum volume of distilled H_2O at 28 C under 250 ft-c of white fluorescent light. Ten pairs of preirradiated cotyledons served as a zero hr control.

Separation of the Products of Incubation. At the beginning or end of incubation, the 2.5-ml reaction mixtures were transferred to 50-ml centrifuge tubes with an eye dropper. The reaction was stopped by the addition of 10 ml of acetone. The incubation vessels were washed free of pigments with 6 to 8 drops of 80% aqueous acetone (v/v) followed by 3 drops of H_2O. The washes were added to the centrifuge tube. After centrifugation at 39,000*g* for 10 min, the 80% acetone extracts were decanted and adjusted to a known volume. If the 80% acetone extracts were slightly turbid, the turbidity was eliminated by the addition of 2 to 5 drops of H_2O. The 80% acetone extracts were used for the spectrophotometric determination of extractable chlorophyll and protochlorophyll.[4] It was also used for recording absorption and difference spectra. The green pellets were uniformly suspended in 12 or 24 ml of 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0, for spectrofluorometric determination of precipitated chlorophyll.

Extraction of Excised Incubated Cotyledons. Ten preirradiated cotyledon pairs incubated in H_2O were homogenized for 3 min at 0 C in a VirTis 45 homogenizer in 10 ml of acetone-0.1 N NH_4OH (9:1 v/v). The homogenate was centrifuged at 39,000*g* for 10 min. The supernatant was decanted, and the pellet was washed with a few ml of 80% aqueous acetone (v/v). The acetone extract and wash were combined for spectrophotometric determinations. The pellet was suspended in 10 ml of H_2O, and aliquots were used for the determination of the total cotyledonary proteins.

Spectrophotometric Determinations. Matched cells of 1 cm internal length and a Beckman DU spectrophotometer were used for the determination of chlorophyll and protochlorophyll in the 80% acetone extract. All absorbancies were corrected for slight light scattering by referring to wavelength-dependent scatter calibration curves as described previously (17). Absorption and difference spectra were recorded with a Beckman double beam spectrophotometer Model DK-2A. The amounts of extractable chlorophyll *a*, chlorophyll *b*, and protochlorophyll were determined according to Anderson and Boardman (1).

Spectrofluorometric Determinations. The chlorophyll that precipitated with the proteins when fortified reaction mixtures were stopped by the addition of acetone was not extractable in organic solvents; it was estimated spectrofluorometrically.

Spectrofluorometric determinations were made with a Beckman spectrophotometer Model DK-2A supplied with a Beckman Model 73500 fluorescence attachment. The latter accommodated a Beckman UG-11 ultraviolet filter. The exciting light had a maximum emission at about 350 nm and a half intensity band width of about 40 nm. The instrument settings for all determinations were as follows: slit 0.9 mm, sensitivity 300, photomultiplier 20 ×, and time constant 0.1. The green pellet was suspended in a total volume of 12 or 24 ml of 0.2 M tris HCl, 0.5 M sucrose, pH 8.0. All pellets of the same experiment were adjusted to the same volume. The apparent emission between 750 and 550 nm was recorded on a 3-ml aliquot. Fluorescence Vycor tubes were used. The same tube was used for all samples of the same experiment. The spectral region between 750 and 550 nm was scanned in about 1.1 min at 2 to 5 C. Before every recording, the response of the apparatus was checked by measuring the fluorescence output from a red Plexiglas filter, with an apparent emission maximum at 690 nm. Corrections in sensitivity were made accordingly. However, the stability of the light source was fairly high and corrections were infrequently made during an experiment. The apparent emission spectra thus obtained were not corrected for the wavelength-dependent photomultiplier sensitivity or monochromator efficiency. Since the quantitative data is represented as a difference between two identical samples which were scanned through the same wavelength region before and after incubation, the correction is essentially a constant. It does not, therefore, interfere with the quantitation of the apparent differences in fluorescence intensity. The relative fluorescence intensity at the apparent emission maximum was converted to nanomoles of chlorophyll by referring to a standard curve. The curve was calibrated in nanomoles of chlorophyll $a + b$ per 3 ml of tris-sucrose suspension. The construction of the calibration curve is described below.

Preparation of the Standard Chlorophyll Fluorescence Curve. Etiolated cotyledons were harvested with full hypocotyl hooks and irradiated for 24 hr as described above. The cotyle-

[4] Protochlorophyll refers to the mixture of protochlorophyllide and protochlorophyllide phytyl ester which accumulates in etiolated tissues.

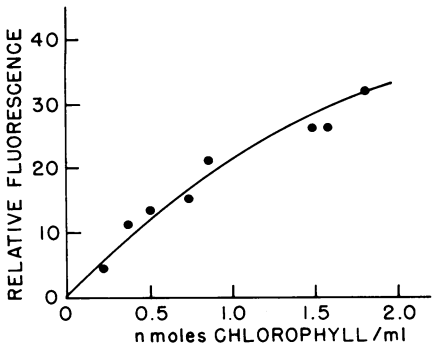

FIG. 1. Apparent fluorescence intensity of chlorophyll-protein pellets precipitated with acetone and suspended in 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0, at various concentrations.

dons were homogenized in 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0, and the unfortified plastids were prepared as already described. They were suspended in 0.2 M tris-HCl, 0.5 M sucrose, pH 7.7. Aliquots in duplicate were brought to a total volume of 4.0 ml with the unfortified suspension buffer at pH 7.7. One ml of H_2O was added, and the proteins were precipitated with 20 ml of acetone. After centrifugation at 39,000*g* for 10 min, the supernatant was decanted. One of the two identical green pellets was suspended in 3 ml of 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0, and its apparent emission spectrum recorded as already described. The duplicate green pellet was extracted with 4 ml of 80% acetone. After centrifugation the amount of chlorophyll *a* and *b* in the 80% acetone extract were determined as already described. In the absence of cofactors, the second 80% acetone extraction freed the pellet from nearly all the chlorophyll pigments. The amounts of chlorophyll *a* + *b* were plotted against the corresponding relative emission intensity at the apparent fluorescence maximum. Within the concentration range used, the fluorescence of 3 ml aliquots of the bound chlorophyll fractions suspended on tris-sucrose varied linearly with the amount of chlorophyll determined spectrophotometrically (Fig. 1).

Preparation of Pigment Standards. Chlorophyll *a* + *b* was prepared from greening cucumber cotyledons and purified by thin layer chromatography as previously described (10). A subchloroplastic soluble enzyme system (S_3) capable of uro, copro, and protoporphyrin biosynthesis and accumulation was prepared from developing chloroplasts (16). It was incubated with 25 μmoles of ALA and cofactors for 16 hr, as described in a previous communication (16).

RESULTS

Biosynthetic Activity of Developing Chloroplasts Prepared from 4.5 hr Preirradiated Cotyledons and Incubated with Cofactors for 1 hr. Extraction of the fortified reaction mixtures before and after 1 hr of incubation yielded a yellow-green 80% acetone extract and a green pellet. Chlorophyll in the acetone extract is referred to as "extractable chlorophyll." Chlorophyll in the green pellet is referred to as "bound chlorophyll." Pellets precipitated with acetone from unfortified and fortified reaction mixtures are referred to as unfortified and fortified pellets respectively. The absorption spectra of the 80% acetone extracts obtained before and after 1 hr of incubation with cofactors were qualitatively identical. There was no evidence of porphyrin accumulation (Fig. 2). Chromatographic analysis (10) failed to detect chlorophyll and carotenoid degradation. However, after 2 hr of incubation with cofactors minor amounts of porphyrins started to accumulate (Fig. 2). This was evidenced in the difference spectrum "2 hr–0 hr" by the appearance of the fourth absorption band of free porphyrins around 500 nm and a Soret band around 400 nm (Fig. 2B).

Unfortified pellets contained less chlorophyll than fortified ones (Table III). They were thoroughly re-extractable with 80% acetone before and after incubation; only trace amounts of chlorophyll remained in the unfortified pellet after a second acetone extraction (Fig. 3).

All attempts failed to re-extract the bound chlorophyll of the fortified pellets with 80% acetone, methanol, ethanol, ether, hexane, ethanol:ether (1:1 v/v), strong acids, or bases. The green pellet was sticky and difficult to manipulate. It was easily suspended in 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0. The suspension was homogeneous and lent itself to the spectrofluorometric determination of bound chlorophyll. Formation of bound chlorophyll depended on the continuous presence of cofactors in the fortified crude homogenate and in the reaction mixture prior to addition of acetone. A bound chlorophyll fraction was obtained from fortified reaction mixtures before and after incubation (Table I).

The apparent emission spectrum of bound chlorophyll in

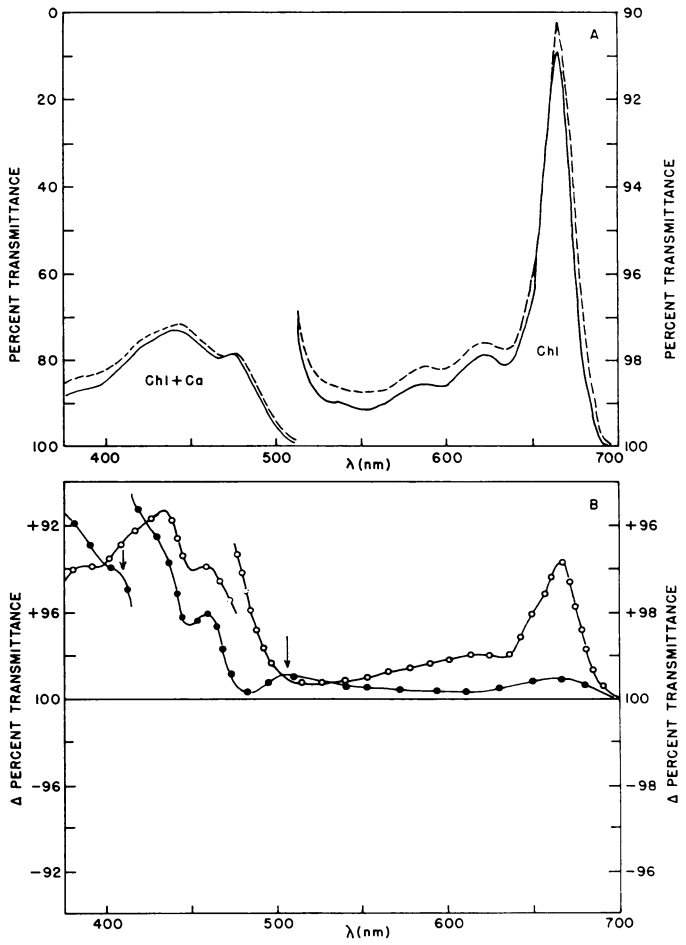

FIG. 2. Tracings of the absorption and difference spectra of the 80% acetone extract of fortified developing chloroplasts prepared from 4.5 hr preirradiated cotyledons. A: Absorption spectrum before incubation (——); after 2 hr of incubation (– – –); B: difference spectrum (2 hr–0 hr) (●); difference spectrum (1 hr–0 hr) after 1 hr of incubation (○). Chl, absorbance due to chlorophyll *a* + *b*; Ca, absorbance due to carotenoids. Arrows indicate absorption due to porphyrins. Breaks in the spectra indicate a shift to a less expanded ordinate scale.

tris-sucrose was similar to that of a 3 to 1 mixture of chlorophyll *a* + *b* dissolved in 80% acetone (Fig. 3). It exhibited an apparent emission peak at 673 to 676 nm. This corresponded to a blue shift of about 12 nm with respect to the emission maximum of fortified developing chloroplasts (Fig. 3). No evidence of porphyrin emission at 617 to 619 nm was detected

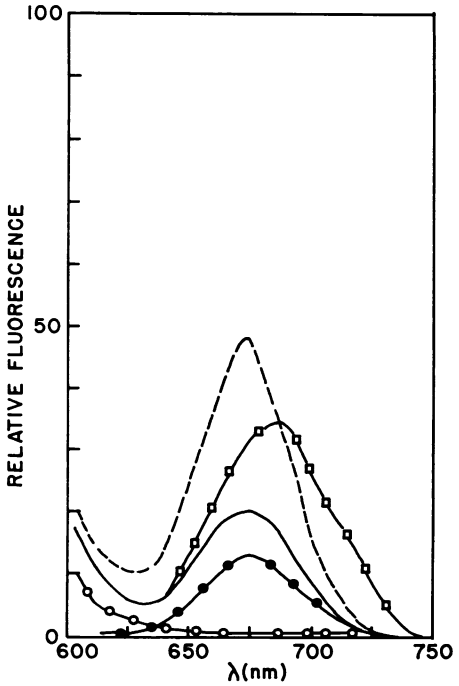

FIG. 3. Tracings of the apparent fluorescence spectra of various bound chlorophyll fractions. Fortified developing chloroplasts, prepared from 4.5-hr preirradiated cotyledons, at 0 hr before precipitation with acetone (□); standard chlorophyll *a* + *b* (3:1) in 80% acetone; (●); bound chlorophyll fraction precipitated from a fortified reaction mixture before incubation (——); bound chlorophyll fraction from the same reaction mixture after 1 hr of incubation (– – –); unfortified pellet precipitated from an unfortified reaction mixture at 0 hr, after a second acetone extraction (○). The bound chlorophyll fractions were suspended in equal volumes of 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0; the spectra were recorded on 3-ml aliquots.

Table I. *Biosynthetic Activity of Fortified Developing Chloroplasts and Excised Cotyledons*

Fortified developing chloroplasts were prepared from greening cotyledons preirradiated for 4.5 hr. The fortified reaction mixture was incubated for 1 hr at 28 C under 10 ft-c of white fluorescent light. The excised preirradiated cotyledons were incubated in H_2O under 250 ft-c of the same light.

Experiment	Pigment	Amount		Change after Incubation
		Before incubation	After incubation	
		nmoles/100 mg protein		Δ %
Fortified reaction mixture	Protochl	22.8	30.1	+32.0
	Extractable chl	255.8	250.0	−2.3
	Bound chl	123.7	290.3	+134.7
	Total chl	379.5	540.3	+42.4
		nmoles/100 mg cotyledonary proteins		
Excised cotyledons	Total chl	111.8	144.8	+29.5

Table II. *Survey of 38 Consecutive Experiments Monitoring the Change in Total Chlorophyll and Protochlorophyll of Fortified Developing Chloroplasts*

	Change in Pigments after 1 Hr of Incubation							
	Δ %							
	−44 to −20	−19 to −10	−9 to −1	0.0	+1 to +9	+10 to +19	+20 to +44	>44
	No. of experiments							
Total chl[1]		1	9	3	7	12	6	
Protochl	3	3	5	3	5	4	6	9

[1] Extractable chlorophyll + bound chlorophyll.

after 1 hr of incubation (Figs. 3 and 4). Increases in chlorophyll and protochlorophyll content of 42 and 32%, respectively, occurred after 1 hr of incubation (Table I). In this particular experiment, the increase in chlorophyll was confined to bound chlorophyll. The apparent emission of the bound chlorophyll is depicted in Figure 3. For reference purposes, the chlorophyll accumulation *in situ* of excised greening cotyledons incubated under 240 ft-c of white fluorescent light is also shown (Table I).

Changes in extractable chlorophyll, bound chlorophyll, and protochlorophyll after 1 hr of incubation were monitored in 38 consecutive experiments. Twenty-five experiments exhibited chlorophyll increases, ten showed losses, and three exhibited no change (Table II). Synthesized chlorophyll was either confined to the extractable fraction, to the bound fraction, or to both, but most frequently it was bound. Eleven experiments exhibited protochlorophyll decreases, three indicated no change, and 24 showed increases after 1 hr of incubation (Table II). Changes in protochlorophyll did not necessarily parallel changes in chlorophyll content.

Biosynthetic Activity of Unfortified Developing Chloroplasts Prepared from 4.5-hr Preirradiated Cotyledons and Incubated without Cofactors for 1 hr. After 1 hr of incubation in the absence of cofactors, extractable chlorophyll decreased or remained unchanged (Table III). The bound chlorophyll fraction exhibited a strong apparent emission at 617 to 619 nm (Fig. 4). The net chlorophyll emission at 673 to 676 nm decreased; it appeared as a weak shoulder on the long wavelength tail of the 617 to 619 nm band (Fig. 4, Table III). The apparent emission at 617 to 619 nm was identical to the apparent emission of a fortified acetone pellet precipitated from a standard fortified S_3 incubation containing uro, copro, and protoporphyrin (Fig. 4). It was not possible to determine the amount of protochlorophyll formed after 1 hr of incubation, in the presence of unknown quantities of porphyrins, since the simultaneous equations used here did not correct for porphyrin absorption at 620 to 630 nm.

After 1 hr of incubation in the presence of cofactors, no porphyrin emission was detected in the bound chlorophyll fraction. Instead it exhibited a substantial increase in chlorophyll content (Fig. 4, Table III). The extractable chlorophyll either decreased or remained unchanged. Protochlorophyll either decreased or accumulated slightly (Table III).

Various experiments were designed to test whether trace amounts of added porphyrins would enhance the fluorescence yield of bound chlorophyll. They failed to show any net increase in chlorophyll emission.

These results indicated that after 1 hr of incubation in the absence of cofactors total chlorophyll decreased slightly and porphyrins accumulated. The presence of cofactors prevented

Table III. *Biosynthetic Activity of Unfortified and Fortified Developing Chloroplasts*

The developing chloroplasts were prepared from greening cotyledons preirradiated for 4.5 hr.

	Pigment	Amount		Change after Incubation
		Before incubation	After 1 hr of incubation	
		nmoles/100 mg proteins		Δ %
Experiment A				
−Cofactors	Porphyrins[1]	none	+	
	Protochl	22.4		
	Extractable chl	236.4	227.1	
	Bound chl	10.8	3.3	
	Total chl	247.2	230.4	−6.8
+Cofactors	Porphyrins[1]	none	none	
	Protochl	18.0	21.3	
	Extractable chl	200.0	169.2	
	Bound chl	134.2	259.3	
	Total chl	334.2	428.5	+28.2
Experiment B				
−Cofactors	Porphyrins[1]	none	+	
	Protochl	trace		
	Extractable chl	402.0	402.3	
	Bound chl	22.9	10.3	
	Total chl	424.9	412.6	−2.9
+Cofactors	Porphyrins[1]	none	none	
	Protochl	19.7	18.0	
	Extractable chl	180.1	180.1	
	Bound chl	51.6	134.2	
	Total chl	231.7	314.3	+35.6

[1] The plus sign denotes porphyrin accumulation.

the accumulation of free porphyrins and enhanced the accumulation of chlorophyll (Fig. 4, Table III).

Biosynthetic Activity of Developing Chloroplasts Extracted from Etiolated Cotyledons Submitted to Short Light Pretreatments. The biosynthetic activity of greening etioplasts that were in the lag phase is presented in Table IV. The amount of bound chlorophyll detected at 0 hr depended on the length of preirradiation and little had accumulated at the end of incubation. Most of the increase in chlorophyll was confined to extractable chlorophyll. This was in contrast to developing chloroplasts obtained from rapidly greening cotyledons preirradiated for 4.5 hr (Table IV).

Etioplasts that were in the lag phase showed higher rates of nonphototransformable protochlorophyll biosynthesis than chlorophyll. This was illustrated by a high ratio of newly synthesized nonphototransformable protochlorophyll to chlorophyll in lag phase etioplasts after 1 hr of incubation. This ratio was very low in developing chloroplasts extracted from rapidly greening cotyledons that were preirradiated for 4.5 hr (Table IV).

In order to determine whether the above biosynthetic pattern was lag phase dependent or not, the lag phase was artificially removed by a short light pretreatment followed by a dark incubation (8). Such treatment removes the lag phase of chlorophyll biosynthesis without the need of continuous irradiation. Removal of the lag phase under these conditions did not change the biosynthetic pattern (Table IV). The bound chlorophyll fraction remained minimal. Most of the newly synthesized chlorophyll was confined to the extractable fraction. The plastids exhibited a typical high ratio of nonphototransformable protochlorophyll to chlorophyll biosynthesis (Table IV).

DISCUSSION

The biosynthesis and accumulation of microgram quantities of chlorophyll by isolated developing chloroplasts was achieved under the same incubation conditions that induced the incorporation of ^{14}C-ALA into protochlorophyll and chlorophyll (12, 13). These incubation conditions were previously shown to preserve the structure of incubated developing chloroplasts and appear to induce grana formation *in vitro* (19).

Nonphototransformable protochlorophyll was reported earlier at about 5% of the total chlorophyll that accumulated in greening cucumber cotyledons after 4 hr of irradiation (9). At zero hr the amount of protochlorophyll in developing chloroplasts prepared from cotyledons preirradiated for 4.5 hr was also within this range (Tables I, III, and IV). No detailed spectroscopic evidence was presented to ascertain the identity of the protochlorophyll estimated by the simultaneous equations. However, in the absence of porphyrin accumulation and chlorophyll and carotenoid degradation after 1 hr of incubation with cofactors (Fig. 2), we have no reason to doubt the identity of this protochlorophyll. Preliminary spectrofluorometric investigations with an instrument capable of recording true emission spectra at room temperature and 77 K confirmed our assertion. During incubation with cofactors, protochlorophyll formation was detected. At pH 7.7, it exhibited a true emission maximum at 636 to 637 nm at room temperature and at 632 to 633 nm at 77 K (C. A. Rebeiz, unpublished).

In the presence of the cofactors required for the biosynthesis of chlorophyll and protochlorophyll, part of the chlorophyll

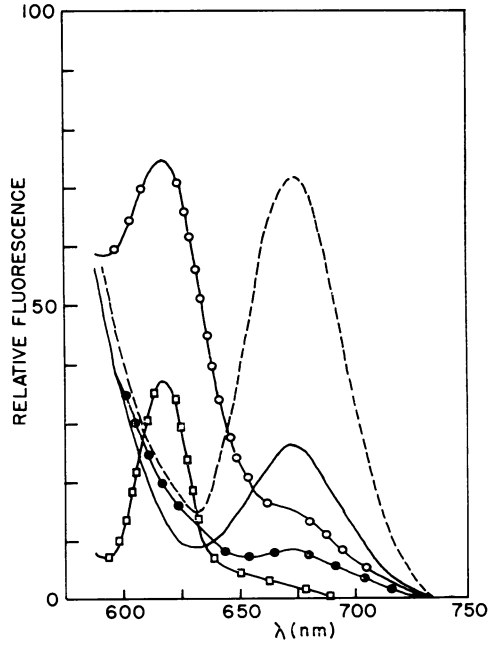

FIG. 4. Tracings of the apparent fluorescence spectra of bound chlorophyll fractions before and after 1 hr of incubation in the absence and presence of cofactors. Unfortified and fortified developing chloroplasts were prepared from 4.5-hr preirradiated cotyledons. Without cofactors, 0 hr (●); without cofactors, after 1 hr of incubation (○); with cofactors, 0 hr (——); with cofactors after 1 hr of incubation (– – –); pellet precipitated from a standard fortified S_3 incubation mixture that accumulated uro, copro, and protoporphyrin (□). The spectrum of the S_3 standard was determined on a small aliquot adjusted to 3 ml with tris-sucrose.

became tightly bound to the lipoprotein pellet. In the absence of cofactors this phenomenon failed to take place (Fig. 3). It is not clear whether this cofactor dependent binding preceded or followed the addition of organic solvents. The apparent emission of bound chlorophyll suspended in tris-sucrose was very similar to that of chlorophyll $a + b$ (3:1) dissolved in acetone. It differed significantly from the apparent emission of fortified developing chloroplasts (Fig. 3). This observation suggested that the environment of the chlorophyll molecules bound to the acetone pellet was very similar to the environment of chlorophyll in the acetone solution. Such an environment could be generated by the trapping of acetone by the chlorophyll-lipoprotein precipitate. The role of the cofactors in the formation of bound chlorophyll is not understood. Nonspecific chlorophyll-protein bindings have been reported (4), and ATP, NADH, and other anions have been shown to bind to structural mitochondrial proteins as the result of a nonspecific anion effect (5). The apparent nonphysiological properties of the chlorophyll-protein pellet obtained after acetone precipitation need not, however, detract from the possible physiological significance of this observation. This bound chlorophyll may differ from extractable chlorophyll in terms of its association with the plastid membrane proteins in their native condition.

Etioplasts prepared from cotyledons in the lag phase accumulated more nonphototransformable protochlorophyll than chlorophyll in the light (Table IV). Even when the lag phase was artificially removed by a brief light pretreatment, the isolated etioplasts exhibited the same protochlorophyll accumulation pattern as etioplasts prepared from dark-grown cotyledons (Table IV). The common feature of both types of etioplast is unquestionably the lack of massive chlorophyll accumulation by the inner membranes. Both preparations exhibited also a poor ability to form bound chlorophyll. As the greening of excised cotyledons progressed, more of the chlorophyll became bound before incubation, and the chlorophyll content of developing chloroplasts increased substantially following 1 hr of incubation *in vitro* (Tables I, III, and IV). Most of the increase was found in the bound chlorophyll fraction. Due to instrumental limitations, it was not possible to determine whether bound protochlorophyll was also formed. We have recently shown that rapidly greening chloroplast membranes were much more resistant to *in vitro*-induced degradation than etioplast membranes. This difference in the maintenance of the plastid membranes was ascribed to light-induced differences in their chemical composition (19). Furthermore, the need of continuous illumination over and beyond the removal of the lag phase for inducing a shift from a nonphototransformable protochlorophyll accumulation pattern to a chlorophyll accumulation pattern (Table IV) suggests that the biogenesis of the chlorophyll accumulation potential might be a high energy photoprocess. This observation and the foregoing results suggest that the increase in the fraction of bound chlorophyll during the progress of greening might reflect the biosynthesis of membrane-bound chlorophyll receptor sites *in situ* and *in vitro*. The possible relationship of the membrane-bound receptor sites to the reaction centers of chlorophyll metabolism described by Shlyk *et al.* (20) is not presently understood.

The early tetrapyrrole intermediates of the chlorophyll biosynthetic chain are reduced porphyrins (porphyrinogens) (3). During the greening of excised cucumber cotyledons incubated with H_2O, porphyrinogens and their oxidation products (porphyrins) do not normally accumulate (10, 18). Instead porphyrinogens are rapidly metabolized. It is significant that in the presence of cofactors and for the 1st hr of incubation, chlorophyll accumulation took place without the concomittant formation of oxidized porphyrins (Figs. 2–4, and Table III).

Table IV. *Biosynthetic Activity of Greening Etioplasts Extracted from Etiolated Cotyledons*

Cotyledons were submitted to various light-dark regimes at 250 ft-c of white fluorescent light and 28 C.

Experiment	Light-Dark Regime	Pigment	Amount		Change After Incubation	Δ[1] protochlorophyll/Δ chlorophyll
			Before incubation	After incubation		
			nmoles/100 mg protein		Δ %	
A	1 min light	Protochl	15.9	44.0	+177	
		Extractable chl	25.5	40.4		1.3
		Bound chl	0.0	7.3		
		Total chl	25.5	47.7	+87	
B	1 hr light	Protochl	8.3	26.3	+217	
		Extractable chl	21.3	26.6		2.6
		Bound chl	3.2	4.8		
		Total chl	24.5	31.4	+28	
C	2 hr light	Protochl	11.1	40.0	+260	
		Extractable chl	44.3	72.5		1.2
		Bound chl	15.1	10.4		
		Total chl	59.4	82.9	+40	
D	4.5 hr light	Protochl	21.0	23.1	+10	
		Extractable chl	165.6	189.9		0.04
		Bound chl	28.2	59.6		
		Total chl	193.8	249.5	+29	
E	5 min light + 3 hr dark + 5 min light	Protochl	2.6	31.1	+1096	
		Extractable chl	17.3	28.3		2.1
		Bound chl	4.1	6.8		
		Total chl	21.4	35.1	+64	

[1] Refers to the ratio of the increment in pigment after 1 hr of incubation.

These results suggest that during the initial stages of chlorophyll biosynthesis and accumulation *in vitro* and in the presence of cofactors, the chlorophyll biosynthetic chain remains vigorously intact.

In the absence of cofactors, chlorophyll biosynthesis and accumulation failed to take place; instead porphyrins accumulated after 1 hr of incubation (Fig. 4 and Table III). The appearance of oxidized porphyrins during incubations *in vitro* frequently coincided with the cessation of chlorophyll biosynthesis and accumulation (Fig. 4 and Table III). It invariably preceded an acceleration of subsequent degradative processes. The fundamental reason for porphyrin accumulation was not systematically investigated. Neither was the intimate effect, if any, of porphyrin accumulation on chlorophyll biosynthesis. It appears, however, that whenever the controls of the chlorophyll biosynthetic chain are bypassed by flooding them with exogenous ALA, porphyrins accumulate (3, 17, 18). The same appears to be true whenever the normal flow of the chlorophyll biosynthetic pathway is blocked or damaged by incubation in the absence of cofactors (Table III) or by lysis and fractionation of the developing chloroplasts (16).

So far it has been possible to show a net chlorophyll biosynthesis *in vitro* for limited periods of incubation. During this period, the use of chlorophyll accumulation as a marker of chloroplast differentiation is probably valid. The usefulness of

this specific marker would be much expanded if massive accumulation of chlorophyll *in vitro* were to be achieved for prolonged periods of incubation.

LITERATURE CITED

1. Anderson, J. M. and N. K. Boardman. 1964. Studies on the greening of dark-grown bean plants. II. Development of photochemical activity. Aust. J. Biol. Sci. 17: 93–101.
2. Boasson, R., W. M. Laetsch, and I. Price. 1972. The etioplast-chloroplast transformation in tobacco: correlation of ultrastructure, replication, and chlorophyll synthesis. Amer. J. Bot. 59: 217–223.
3. Bogorad, L. 1966. The biosynthesis of chlorophylls. *In:* L. P. Vernon and G. R. Seely, eds., The Chlorophylls. Academic Press, New York. pp. 481–510.
4. Criddle, R. S. 1966. Protein and lipoprotein organization in the chloroplast. *In:* T. W. Goodwin, ed., Biochemistry of Chloroplasts. Academic Press, New York. pp. 203–231.
5. Criddle, R. S. 1969. Structural proteins of chloroplasts and mitochondria. Annu. Rev. Plant. Physiol. 20: 239–252.
6. Eytan, G. and I. Ohad. 1970. Biogenesis of chloroplast membranes. VI. Cooperation between cytoplasmic and chloroplast ribosomes in the synthesis of photosynthetic lamellar proteins during the greening process in a mutant of *Chlamydomonas reinhardi y-1*. J. Biol. Chem. 245: 4297–4307.
7. 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.
8. Hardy, S. I., P. A. Castelfranco, and C. A. Rebeiz. 1971. Effect of the hypocotyl hook on chlorophyll accumulation in excised cotyledons of *Cucumis sativus* L. Plant Physiol. 47: 705–708.
9. Rebeiz, C. A. 1967. Studies on chlorophyll biosynthesis in etiolated excised cotyledons of germinating cucumber at different stages of seedling development. Magon Sér. Sci. 13: 1–21.
10. Rebeiz, C. A. 1968. The chloroplast pigments of etiolated and greening cucumber cotyledons. Magon, Sér. Scientifique 21: 1–25.
11. Rebeiz, C. A. 1972. Protochlorophyll and chlorophyll synthesis and accumulation during chloroplast differentiation *in vitro*. Proc. VI. Int. Congr. Photobiol., Bochum, Germany. p. 234.
12. Rebeiz, C. A. and P. A. Castelfranco. 1971. Protochlorophyll biosynthesis in a cell-free system from higher plants. Plant Physiol. 47: 24–32.
13. Rebeiz, C. A. and P. A. Castelfranco. 1971. Chlorophyll biosynthesis in a cell-free system from higher plants. Plant Physiol. 47: 33–37.
14. 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: 281–285.
15. Rebeiz, C. A., C. Nishijima, and J. C. Crane. 1971. The biosynthesis and accumulation of microgram quantities of protochlorophyll, chlorophyll *a* and chlorophyll *b* in a cell-free system from higher plants. Plant Physiol. 47: S267.
16. Rebeiz, C. A., J. C. Crane, and C. Nishijima. 1972. The biosynthesis of metal porphyrins by subchloroplastic fractions. Plant Physiol. 50: 185–186.
17. Rebeiz, C. A., M. Yaghi, M. Abou-Haidar, and P. Castelfranco. 1970. Protochlorophyll biosynthesis in cucumber (*Cucumis sativus* L.) cotyledons. Plant Physiol. 46: 57–63.
18. Rebeiz, C. A., M. Abou-Haidar, M. Yaghi, and P. A. Castelfranco. 1970. Porphyrin biosynthesis in cell-free homogenates from higher plants. Plant Physiol. 46: 543–549.
19. Rebeiz, C. A., S. Larson, T. E. Weier, and P. A. Castelfranco. 1973. Chloroplast maintenance and partial chloroplast differentiation *in vitro*. Plant Physiol. In Press.
20. Shlyk, A. A., L. I. Vlasenok, R. A. Chkanikova, L. I. Fradkin, S. A. Mikhailova, G. E. Savchenko, and L. K. Sukhover. 1967. Conception of reaction centers of chlorophyll metabolism. Studia Biophys. 5: 17–24.
21. Weier, T. E. and A. A. Benson. 1966. *In:* T. W. Goodwin, ed., The Biochemistry of Chloroplasts. Academic Press, New York. pp. 92–113.
22. Wellburn, F. A. M. and A. R. Wellburn. 1971. Chlorophyll synthesis by isolated intact etioplasts. Biochem. Biophys. Res. Comm. 45: 747–750.