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CHLOROPLAST CULTURE: THE CHLOROPHYLL REPAIR POTENTIAL OF MATURE CHLOROPLASTS INCUBATED IN A SIMPLE MEDIUM

MAARIB B. BAZZAZ and CONSTANTIN A. REBEIZ *

Department of Horticulture, University of Illnois, Urbana, Ill. 61801 (U.S.A.)

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Summary

The chlorophyll repair potential of mature *Cucumis* chloroplasts incubated in a simple Tris-HCl/sucrose medium is described. The chloroplasts were isolated from green, fully expanded *Cucumis* cotyledons which were capable of chlorophyll repair. This was evidenced by a functional chlorophyll biosynthetic pathway in the mature tissue. The biosynthesis of protochlorophyllide from exogenous δ-aminolevulinic acid was used as a marker for the operation of the chlorophyll biosynthetic chain between δ-aminolevulinic acid and protochlorophyllide. The conversion of exogenous protochlorophyllide into chlorophyll *a* was used as a marker for the operation of the chlorophyll pathway beyond protochlorophyllide. It appeared from these studies that contrary to published reports, unfortified fully developed *Cucumis* chloroplasts incubated in Tris-HCl/sucrose without the addition of cofactors exhibited a partial and limited chlorophyll repair capability. Their net tetrapyrrole biosynthetic competence from δ-aminolevulinic acid was confined to the accumulation of coproporphyrin. No net tetrapyrrole biosynthesis beyond coproporphyrin was observed. However, the plastids were capable of incorporating small amounts of δ-amino-[4-^{14}C]levulinic acid into [^{14}C]protochlorophyllide but were incapable of converting exogenous protochlorophyllide into chlorophyll. After prolonged incubation of the unfortified chloroplasts in the dark, a fluorescent protochlorophyllide-like compound accumulated. This compound [Cp (E_{430}-F_{631})] exhibited a soret excitation maximum at 430 nm (E_{430}) and a fluorescence emission maximum at 631 nm (F_{631}) in methanol/acetone (4 : 1, v/v). Cp(E_{430}-F_{631}) was shown to be neither protochlorophyllide nor zinc-protochlorophyllide but an enzymatic degradation product of chlorophyll. The exact chemical identity of this compound has not yet been determined.

* To whom correspondence and reprint requests should be directed: Department of Horticulture, University of Illinois, 101 Vegetable Crops, Urbana, Ill. 61801, U.S.A.

Abbreviations: Pchlide, Protochlorophyllide; chl, chlorophyll; Pchl, Protochlorophyll.

Introduction

Although various aspects of chloroplast biogenesis have been studied intensively during the past four decades [1,2,3], the metabolic requirements of mature chloroplasts have remained largely unknown. In other words, little is known about the metabolic demands which are imposed on higher plant cells in order to maintain the mature chloroplasts in a functional state. Consequently, we have undertaken a long range effort to assess the extent of the metabolic dependence of fully differentiated chloroplasts on other subcellular components.

In this work, we assess the chlorophyll (chl) repair potential of mature chloroplasts incubated in a simple Tris-HCl/sucrose medium devoid of added cofactors. The biosynthesis and accumulation of protochlorophyllide * (Pchlide) from exogenous δ-aminolevulinic acid was used as a marker for the operation of the chl biosynthetic chain between δ-aminolevulinic acid and Pchlide. The conversion of exogenous Pchlide into chl *a* was used as a marker for the operation of the Chl pathway beyond Pchlide. It appeared from these studies that contrary to published reports [4], mature chloroplasts incubated in Tris-HCl/sucrose, without the addition of cofactors, had limited tetrapyrrole biosynthetic capabilities. During in vitro incubation of the chloroplasts, the accumulation of a fluorescent Pchlide-like compound was observed. It is shown that this hitherto unreported compound is a product of degradation of the endogenous chl.

Materials and Methods

Radiochemicals. δ-Amino[4-^{14}C]levulinic acid was purchased from New England Nuclear, Boston, Mass.

Plant materials and growth conditions. Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were purchased from the Niagara-Chemical Division, FMC Corporation, Modesto, Calif. The seeds were germinated under a light (14 h) and dark (10 h) photoperiodic regime at 28°C as previously described [5]. Illumination was generated by 14 banks of cool white fluorescent light and six 60 W tungsten bulbs. The light intensity as measured with a calibrated Isco spectroradiometer Model SR and a Weston light meter Model 756 was 3.5 mW · cm^{-2} (900 ft-candles).

Isolation of the chloroplasts. Unfortified chloroplasts were isolated from expanded 8—10-day-old cotyledons essentially as described elsewhere but without the addition of cofactors [6]. 5 g tissue were ground gently (25—30 strokes) at 0—5°C in 7.5 ml 0.5 M sucrose/0.2 M Tris-HCl buffer adjusted to pH 8.0 at room temperature. 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 2.5 ml 0.5 M sucrose/0.2 M Tris-HCl adjusted to pH

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* Protochlorophyllide is the immediate precursor of chlorophyllide; the latter is converted to chl *a* upon esterification. Pchlide ester differs from Pchlide by the esterification of the propionic residue at position 7 of the macrocycle with a long chain alcohol.

7.7 at room temperature. Total proteins were determined by biuret [7]. 2 ml of the chloroplast suspension contained 2.5—4.0 mg of proteins.

Incubation of the isolated chloroplasts. In experiments where the conversion of δ-aminolevulinic acid into Pchlide was investigated, 2 ml of the chloroplast suspension, in a total reaction mixture of 3 ml, were incubated in 50 ml polypropylene tubes in the dark at 28°C on a metabolic shaker operated at 10 strokes per min. 3 ml of the reaction mixture contained 400 μmol Tris-HCl (pH 7.7), 1 mmol sucrose and 100 nmol δ-aminolevulinic acid unless otherwise indicated.

Extraction of porphyrins and phorbins. The extraction of porphyrins and phorbins was as previously described [6]. After termination of the incubation by addition of 15 ml acetone/0.1 M NH_4OH (9 : 1, v/v) and centrifugation at 39 000 × *g* for 10 min, two fractions were obtained: a lipoprotein pellet that contained bound porphyrins [uroporphyrin, coproporhyrin and their zinc-analogs as well as traces of bound chl] and a 75% acetone extract that usually contained chl *a* and *b*, chlorophyllide, Pchlide, and Mg-protoporphyrin monoester plus longer wavelength metalloporphyrins [8]. At this stage of greening, the tissue lacked any Pchlide ester [15]. Chlorophyll *a* and *b* were transferred to hexane by extraction with 1 vol hexane, followed by an additional $\frac{1}{3}$ volume of hexane. The other tetrapyrroles remained in the hexane extracted acetone fraction.

Spectrophotometry. Spectrophotometric measurements were performed with an Aminco dual wavelength spectrophotometer, Model DW-2, operated in the split beam mode.

Spectrofluorimetry. Corrected fluorescence emission and excitation spectra were recorded at room temperature on a Perkin-Elmer spectrofluorimeter Model MPF-3, equipped with a corrected spectra accessory [6]. All emission spectra were recorded at an excitation bandwidth of 6 nm and an emission bandwidth of 3 nm. Excitation spectra were recorded at an excitation bandwidth of 3 nm and an emission bandwidth of 6 nm.

Detection of bound porphyrins and metalloporphyrins. The acetone-precipitated lipoprotein pellet was suspended in 3 ml 0.5 M sucrose/0.2 M Tris-HCl (pH 7.7), with a Ten Broeck homogenizer. The bound porphyrins and metalloporphyrins were detected by their fluorescence emission spectra. The latter were elicited by excitation at 400 nm [8]. To eliminate stray excitation light, a yellow sharp cut-off filter that excluded light below 520 nm was interposed between the sample and the emission monochromator. A blue filter, Pyrex No. 5543, transparent in the 350 to 500 nm region, was placed between the excitation monochromator and the sample.

Determination of protoporphyrin and protochlorophyllide. The amount of these two tetrapyrroles in the hexane-extracted acetone fraction (vide supra) was determined from their fluorescence emission spectra with equations that were published elsewhere [8].

Determination of Mg-protoporphyrin monoester. Mg-protoporphyrin monoester, whenever present, remained in the hexane-extracted acetone fraction and was determined by spectrofluorimetry as described elsewhere [9].

Chromatography. Protochlorophyllide, metalloporphyrins and protoporphyrin were transferred from the hexane-extracted acetone fraction to peroxide-

free ether as described in [10]. This essentially involved adding to the hexane-extracted acetone fraction, 1/17 of its volume of saturated NaCl and 1/70 of its volume of 0.5 M KH_2PO_4. This was followed by two successive extractions with 10 and 5 ml ether, respectively. The combined ether extracts were concentrated under N_2 gas and chromatographed on thin-layers of silica gel H which were developed in benzene/ethyl acetate/ethanol (8 : 2 : 2, v/v) at 4°C in darkness [10]. Protochlorophyllide and metalloporphyrins were eluted in methanol/acetone (4 : 1, v/v) [10] or in acetone/H_2O/0.1 M NH_4OH (9 : 2 : 1, v/v) that was previously extracted with hexane [6].

Determination of ^{14}C incorporations. 0.1—0.5-ml aliquots of the ^{14}C-labelled tetrapyrroles eluted from silica gel H were counted in 10 ml toluene scintillation solution (0.6 g 1,4-bis[2-(5-phenyloxazolyl]]benzene and 7.0 g 2,5-diphenyloxazole in 1 l toluene). The counting was done in a Packard Tri-Carb scintillation counter Model 3375. Quenching was determined by using the instrument internal standard. Samples were counted to a standard deviation of ±5% or less.

Synthesis of Zn-protochlorophyllide. Zn-protochlorophyllide was synthesized by an adaptation of the method recommended by Falk [11]. 2-ml aliquots of Pchlide dissolved in chloroform, were reacted with 0.1 ml glacial acetic acid saturated with zinc acetate at 100°C. The reaction was continued until the emission maximum of Pchlide in chloroform at 637 nm shifted to 627—630 nm. The reaction was usually completed in 60 s.

Preparation of the protochlorophyll substrates. Protochlorophyllide and Pchlide ester were extracted from freshly harvested 4-day-old etiolated *Cucumis* cotyledons and were purified on thin-layers of silica gel H as described [8]. [^{14}C]protochlorophyllide was prepared as described elsewhere [12].

Electron microscopy. The isolated chloroplasts were prepared for electron microscopy essentially as described elsewhere [13].

Results

Biosynthesis and accumulation of phototransformable protochlorophyllide by green, fully expanded Cucumis cotyledons

It is reasonably well established that some mature green tissues are still capable of chl biosynthesis [14,15]. In these mature tissues, the capacity to synthesize chl is probably a form of chl repair. Before evaluating the chl repair potential of fully developed *Cucumis* chloroplasts, it was therefore desirable to evaluate the chl biosynthetic competence of the green, fully expanded *Cucumis* cotyledons from which the chloroplasts were to be isolated.

It was first determined that in the mature cotyledons the chl biosynthetic pathway between δ-aminolevulinic acid and Pchlide was still functional. The cotyledons were excised and incubated with δ-aminolevulinic acid in the dark. Under these conditions, a functional chl biosynthetic pathway is usually expressed by the accumulation of Pchlide [1]. As shown in Table I, a considerable amount of Pchlide accumulated in the dark-incubated cotyledons. This in turn indicated that in this green mature tissue, the reactions between δ-aminolevulinic acid and Pchlide were still functional. The small amount of Pchlide reported in Table I and which was detectable before incubation with δ-amino-

TABLE I

δ-AMINOLEVULINIC ACID-INDUCED PCHLIDE ACCUMULATION IN GREEN MATURE COTYLEDONS

Five 10-day-old *Cucumis* cotyledons (1.5 g) were excised in the light and were incubated in a Pyrex (100 × 80 mm) petri dish, in 6 ml H_2O containing 10 μmol δ-aminolevulinic acid for the indicated times in darkness. Incubation was terminated by homogenizing the tissue in 10 ml acetone/0.1 M NH_4OH (9 : 1, v/v) for 2 min. The extraction and the determination of Pchlide was as described in Methods. The probable error (P_x) of the measured quantities was calculated from standard deviation values (S_x) which had been previously reported [8].

Time of dark incubation (h)	nmol Pchlide/100 mg protein ± P_x *
0	0.51 ± 0.02
2	37.5 ± 1.5
5	47.1 ± 1.9
21	72.5 ± 2.9

* $P_x = 0.67\ S_x$.

levulinic acid, represented the endogenous level of the Pchlide which is usually observed in this mature tissue during a typical light cycle [5].

Next the phototransformability of the endogenous Pchlide that accumulated in the mature tissue during the dark cycles was examined. Since only a functional (phototransformable) Pchlide can contribute positively to chl repair in green tissues, this determination was as essential to the evaluation of the chl repair potential as the net synthesis of Pchlide in the dark. Thus at the end of the 10th dark cyclic, the mature seedlings were illuminated with 3.5 mW · cm^{-2} of white fluorescent light for various periods of time in order to phototransform the endogenous Pchlide that accumulated during the dark cycle. The phototransformability of Pchlide was determined by the decrease of its fluorescence amplitude at its emission maximum as described earlier [16,17]. The Pchlide formed during the preceding dark cycle was obviously partially phototransformable as evidenced by the decrease in the Pchlide pool size in the light (Table II). Under these illumination conditions, Pchlide photodestruction was probably negligible [16,17]. The foregoing results were therefore similar to those reported by others [14,15].

Altogether the forementioned results indicated that in mature *Cucumis* cotyledons the chl biosynthetic pathway was apparently still functional.

Partial operation of the chlorophyll biosynthetic pathway in unfortified mature chloroplasts

In order to monitor the operation of the chl biosynthetic chain in unfortified chloroplasts prepared from the mature tissue, the chloroplasts were isolated from green, fully expanded cotyledons in a simple Tris/sucrose medium devoid of added cofactors. Electron micrographs of the isolated chloroplasts showed all the structural characteristics of mature plastids. The chloroplasts had usually 15 or more grana per chloroplast; most of the grana had 10 or more compartments. There was no evidence of prolamellar body membranes or stroma tubules as was observed in developing cotyledons greening under a photoperiodic dark-light regime (Rebeiz, C.C. and Rebeiz, C.A. unpublished).

TABLE II

TIME COURSE OF ENDOGENOUS PCHLIDE PHOTOTRANSFORMATION IN MATURE COTYLEDONS

At the end of the 10th dark cycle, 3 g of cotyledonary tissue were homogenized with 20 ml acetone/0.1 M NH_4OH (9 : 1, v/v) before and after the periods of illumination indicated. The Pchlide content in the hexane-extracted acetone fraction was determined as described in the Methods. The Δ-change refers to the amount of phototransformed Pchlide and is the difference between the amounts of Pchlide detected before and after illumination.

Illumination (min)	Pchlide concentration before illumination *	Pchlide concentration after illumination * (nmol/100 mg protein)	Δ-Change **
0	1.1 ± 0.1	—	—
5	1.1 ± 0.1	0.69 ± 0.03	0.41 ± 0.1
20	1.1 ± 0.1	0.45 ± 0.02	0.65 ± 0.1

* The probable error was calculated as in Table I.

** The absolute uncertainty of the Δ-change was calculated as the square root of the sum of squares of the probable error of the quantities measured before and after the times of illumination indicated [18].

The operation of the reactions between δ-aminolevulinic acid and Pchlide in the isolated chloroplasts was monitored by determining the dark-incorporation of δ-amino[4-^{14}C]levulinic acid into [^{14}C]Pchlide. Mature chloroplasts were isolated from fully expanded green cotyledons and were incubated in the dark at 28°C for 1 h. The complete reaction mixture contained in a total volume of 3 ml: 2.5 μCi (100 nmol) of δ-amino[4-^{14}C]levulinic acid; 400 μmol Tris-HCl, pH 7.7; 1 mmol sucrose and 2 ml plastids (3.4 mg protein). The net ^{14}C incorporation into Pchlide was determined by calculating the difference between the dpm recovered in the Pchlide band of the unheated and heat-inactivated plastids after 1 h of dark incubation. Heat inactivation was achieved by heating the incubation mixture containing the plastids for 5 min in a 100°C water bath just prior to adding the ^{14}C-labeled substrate. The results of such an experiment showed a slight (about $17 \cdot 10^3$ dpm/100 mg plastid protein) incorporation of δ-amino[4-^{14}C]levulinic acid into [^{14}C]Pchlide. This ^{14}C incorporation into Pchlide was completely inhibited by 50 μmol levulinic acid. The latter is a well documented inhibitor of Ala dehydratase (porphobilinogen synthetase; δ-aminolevulinate hydro-lyase (adding δ-aminolevulinate and cyclizing), EC 4.2.1.24) [19,20].

The operation of the reactions between Pchlide and chl was monitored by determining the conversion of exogenous [^{14}C]Pchlide to [^{14}C]chl as recently described by Mattheis and Rebeiz [12,17]. Essentially this assay involves the following sequential steps: (a) The dark incubation of exogenous [^{14}C]Pchlide with isolated plastids for 30 to 60 min, in order to allow the binding of the substrate to the appropriate apoprotein; (b) a short light treatment (15 s) to phototransform the [^{14}C]Pchlide-apoprotein complex formed in vitro into [^{14}C]Chlide; (c) an additional dark incubation of 30 min to allow the phytylation of the newly formed [^{14}C]Chlide to form [^{14}C]chl *a*.

The results of such an experiment are reported in Table III. It is evident that the unfortified chloroplasts did not possess the ability to convert exogenous [^{14}C]Pchlide into [^{14}C]chl *a*. This in turn indicated that mature chloroplasts

TABLE III

CONVERSION OF ADDED [^{14}C]PCHLIDE INTO [^{14}C]CHL BY ISOLATED CHLOROPLASTS

5 ml chloroplast suspension, in a total reaction mixture of 7.5 ml were incubated in a 50-ml Erlenmeyer flask in the dark at 28°C with [^{14}C]Pchlide having a specific radioactivity of 0.169 dpm/nmol. The added [^{14}C]Pchlide was dissolved in 0.075 ml methanol. After 1 h of incubation, the sample was divided into two equal portions. One portion was incubated for an additional 0.5 h in the dark while the other portion was illuminated with 320 $\mu W \cdot cm^{-2}$ of white fluorescent light for 15 s at 0°C followed by an additional 0.5 h incubation in the dark. At the end of the second dark incubation, the samples were extracted with 75% acetone. All pigments were transferred to ether as described in Methods. ^{14}C-labeled Pchlide and Chl were purified on thin-layers of silica gel H as described in [12].

Experiment	10^{-3} · dpm/100 mg plastid protein			
	^{14}C-labeled Pchlide added	^{14}C recovered in chl		
		After 90 min dark incubation	After 60 min dark incubation, 15 s illumination and 30 min dark incubation	Net ^{14}C recovered in chl *
A	5 400	7.2 ± 5.1 **	7.7 ± 5.5	0.5 ± 7.5 ***
B	3 400	14.6 ± 18.4	14.1 ± 4.7	−0.5 ± 19

* Refers to the difference between the illuminated and non-illuminated samples.

** The standard error was calculated as the square root of the sum of the squares of the standard deviations of the background and sample counts per minute [12].

*** The uncertainties were calculated as the square root of the sum of squares of the standard errors of the illuminated and non-illuminated samples.

isolated in an unfortified Tris/sucrose medium were not capable of complete chl *a* repair in vitro.

Net tetrapyrrole biosynthetic competence of the isolated chloroplasts

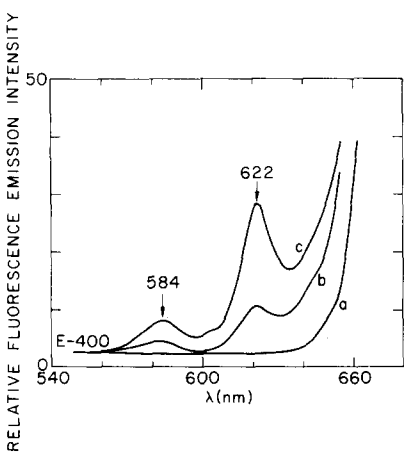
In order to assess the full measure of the Pchlide biosynthetic competence of the isolated plastids, the tetrapyrrole net biosynthetic activity of the mature chloroplasts was evaluated in vitro. Mature unfortified chloroplasts were isolated from fully expanded green cotyledons and were incubated with or without 100 nmol δ-aminolevulinic acid in 0.5 M sucrose, 0.2 M Tris-HCl, (pH 7.7) for various periods of time in darkness. After incubation, the reaction mixture was extracted with aqueous acetone and the reaction products were partitioned between the lipoprotein pellet, the hexane and the hexane-extracted acetone fractions as described in Methods.

A net synthesis of Zn-uroporphyrin and coproporphyrin was readily observed when the chloroplasts were incubated with δ-aminolevulinic acid (Fig. 1). With the exception of small amounts of chl, the freshly isolated lipoprotein pellets were devoid of any fluorescence that could be attributed to the forementioned porphyrins or to their Zn-analogs (Fig. 1a). However, after 2 and 5 h incubation respectively, broad fluorescence emission bands appeared in the lipoprotein pellet fraction at 560—600 nm and 600—640 nm (Fig. 1b and c). The short wavelength emission band exhibited a Zn-uroporphyrin emission maximum at about 584 nm, while the band between 600 and 640 nm exhibited a coproporphyrin emission maximum at about 622 nm [8]. The broadening of

these bands is probably due to small amounts of contamination of Zn-coproporphyrin with Zn-uroporphyrin and some uroporphyrin contamination of the coproporphyrin band [8].

Protoporphyrin, Mg-protoporphyrin monoester plus longer wavelength metalloporphyrins and Pchlide were monitored in the crude hexane-extracted acetone fractions [8]. The freshly isolated mature chloroplasts were devoid of either protoporphyrin or Mg-protoporphyrin monoester plus longer wavelength metalloporphyrins as evidenced by the absence of protoporphyrin fluorescence emission at 633 nm (400 nm excitation) (Fig. 2a) and Mg-protoporphyrin monoester plus longer wavelength metalloporphyrin emission at 600 nm (420 nm excitation) (Fig. 2c). The endogenous Pchlide pool (Fig. 2e) which was detected by its fluorescence emission at about 639 nm (excitation at 440 nm) [8] amounted to about 1.7 nmol per 100 mg plastid proteins.

During a 10-h incubation period with δ-aminolevulinic acid, no Mg-protoporphyrin monoester or longer wavelength metalloporphyrins were formed as evidenced by the lack of their emission at 600 nm (Fig. 2c and d). However, a fluorescent band appeared between 630 and 640 nm upon excitation at 400 or 440 nm (Fig. 2b and f). This suggested the accumulation of a protoporphyrin-



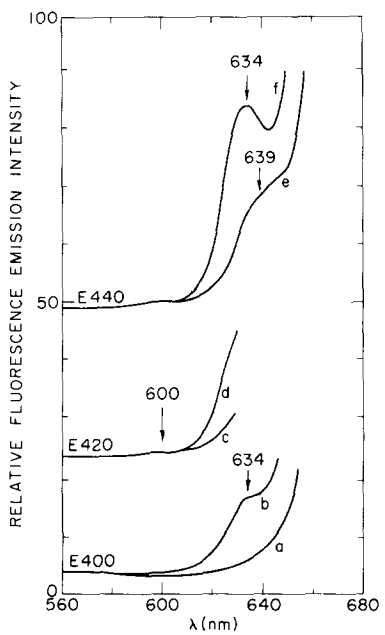


Fig. 1. Corrected fluorescence emission spectra of the lipoprotein pellets prepared from mature unfortified chloroplasts before and after incubation with δ-aminolevulinic acid. The emission spectra were recorded at an excitation wavelength of 400 nm; scale, 3.5 × ordinate. Before incubation (a); after 2 h of incubation (b); after 5 h of incubation (c). Arrows point to wavelengths of interest.

Fig. 2. Fluorescence emission spectra of the hexane-extracted acetone fractions of the isolated chloroplasts before and after 10 h of dark incubation with δ-aminolevulinic acid. The spectra were recorded at the excitation wavelength (*E*) indicated in order to elicit the protoporphyrin (E_{400}), Mg-protoporphyrin monoester and longer wavelength metalloporphyrins (E_{420}) and Pchlide (E_{440}) pools [8]. Scale, 3.5 × ordinate. Before incubation (a,c,e); after 10 h of dark-incubation with δ-aminolevulinic acid (b,d,f). Other symbols are as in Fig. 1.

like or Pchlide-like compound during the incubation. However, upon further spectrofluorimetric examination of the crude hexane-extracted acetone fraction, it appeared that this compound was neither protoporphyrin IX nor Pchlide. Although it exhibited a protoporphyrin-like emission maximum at 633—634 nm (Fig. 2b and f), it had soret and red excitation maxima at about 432 and 625 nm, respectively. In hexane-extracted acetone, standard protoporphyrin exhibited a soret excitation maximum at 402 nm [8]. On the other hand, standard Pchlide in a crude Hexane-extracted acetone fraction of etiolated *Cucumis* cotyledons had an emission maximum at 637—639 nm [8] with soret and red excitation maxima at 437 and 628 nm, respectively. Since the accumulation of this unknown compound interfered with the quantitative determination of the protoporphyrin and Pchlide pools, it became necessary to segregate this compound from the other two tetrapyrroles prior to quantitative analysis.

Segregation of the unknown fluorescent compound from ptotoporphyrin and protochlorophyllide

Since the accumulation of the unknown fluorescent compound interfered with the determination of the protoporphyrin and Pchlide net biosynthetic competence of the unfortified plastids, it was attempted to segregate these two tetrapyrrole pools from the unknown compound prior to quantitative determinations. Mature unfortified chloroplasts were, therefore, incubated in the dark for 5 h with δ-aminolevulinic acid. By the end of 5 h incubation substantial amounts of the Pchlide-like unknown compound had accumulated. The hexane-extracted acetone fractions of the unfortified chloroplast preparations were prepared before and after dark-incubation as described in Methods. The tetrapyrroles in these fractions were transferred to ether. The ether fractions were concentrated under N_2 gas and chromatographed on thin-layer plates as described in Methods. The developed chromatograms were viewed under ultraviolet light (366 nm) and the segregated fluorescence bands were eluted in hexane-extracted acetone. The eluted fractions were monitored by spectrofluorimetry.

Protoporphyrin which remains close to the origin in this solvent system was not detectable either before or after dark incubation.

Protochlorophyllide was separated from the unknown fluorescent compound, and migrated with an R_F value of 0.38—0.45. The protochlorophyllide band eluted before incubation in hexane-extracted acetone exhibited a well-defined Pchlide emission maximum at about 639 nm and a soret excitation maximum at 438 nm. However, after 5 h of dark incubation with δ-aminolevulinic acid, the pool of Pchlide decreased by 31%, and its emission maximum at 639 nm appeared as a weak shoulder on the short wavelength emission tail of chlorophyllide. The latter was formed during the dark incubation from the endogenous chl (vide infra) and heavily contaminated the segregated Pchlide band.

The unknown fluorescent compound which had accumulated after 5 h of dark incubation with δ-aminolevulinic acid migrated more slowly than Pchlide and had an R_F value of 0.21—0.29. After elution in hexane-extracted acetone, it exhibited an emission maximum at 631 nm and a soret excitation maximum

at 430—431 nm. This compound (Cp) will, therefore, be referred to as Cp(E_{430}-F_{631}), where E and F refer to its soret excitation and fluorescence emission maxima, respectively. Compound(E_{430}-F_{631}) was not detectable before incubation.

Altogether the above results indicated that mature chloroplasts incubated with δ-aminolevulinic acid did not synthesize net amounts of protoporphyrin or of Pchlide. Instead an unknown Pchlide-like fluorescent compound was formed.

Relationship of Cp(E_{430}-F_{631}) formation to the addition of δ-aminolevulinic acid

If Cp(E_{430}-F_{631}) were a hitherto unknown intermediate of the Pchl biosynthetic pathway, its accumulation may then be an important component of the chl repair potential. It thus became necessary to determine whether or not Cp(E_{430}-F_{631}) was indeed an intermediate of Pchl biosynthesis. Since the biosynthesis in vitro of all the known intermediates of the chl biosynthetic pathway is dependent on the addition of exogenous δ-aminolevulinic acid [8,18, 21,22], the dependence of Cp(E_{430}-F_{631}) biosynthesis on exogenous δ-aminolevulinic acid was in turn investigated.

Mature chloroplasts were isolated from green, fully developed *Cucumis* cotyledons and were incubated in Tris-HCl/sucrose in the presence and absence of added δ-aminolevulinic acid. The tetrapyrrole pools were monitored spectrofluorimetrically. In the absence of added δ-aminolevulinic acid, no uroporphyrin or coproporphyrin accumulation was observed in the lipoprotein pellet as was just reported. However, Cp(E_{430}-F_{631}) accumulated in the absence as well as in the presence of exogenous δ-aminolevulinic acid. When the incubation was carried out in the presence of 2.5 μCi (100 nmol) of δ-amino[4-^{14}C]levulinic acid, and the pool of Cp(E_{430}-F_{631}) was segregated on silica gel, no ^{14}C label was found in the eluted compound. Altogether, these results indicated that the formation of Cp(E_{430}-F_{631}) was not dependent on the addition of exogenous δ-aminolevulinic acid. This in turn suggested that Cp(E_{430}-F_{631}) was not an intermediate of the Pchl bioxynthetic pathway, and therefore was probably not a component of the chl repair potential.

Relationship of Cp(E_{430}-F_{631}) to Zn-vinylphaeoporphyrin a_5

It was reported earlier that the disappearance of endogenous Pchlide was accompanied by the appearance of Cp(E_{430}-F_{631}). Since Cp(E_{430}-F_{631}) exhibited the soret excitation properties of a metalphorbin (430—450 nm) rather than those of a free or metalporphyrin (400—420 nm) it stood to reason that Cp(E_{430}-F_{631}) may be a metal degradation product of endogenous Pchlide. This was also suggested by the observation that the most ubiquitous metalated derivatives of the free base porphyrins which are formed during in vitro incubations are Zn-porphyrins [8,9,22,23]. Thus, it seemed possible that Cp(E_{430}-F_{631}) was a Zn-vinylphaeoporphyrin a_5, i.e., a Zn-Pchlide which was formed by substitution of Zn for Mg in the endogenous Pchlide.

In order to test the above hypothesis, Zn-Pchlide was synthesized as described in Methods and its fluorescence properties were compared to those of Cp(E_{430}-F_{631}). Except for the similarity in their short wavelength emission

maximum at 630—631 nm in methanol/acetone (4 : 1, v/v), synthetic Zn-Pchlide and Cp(E_{430}-F_{631}) exhibited different emission and excitation maxima. In methanol/acetone (4 : 1, v/v) and in hexane-extracted acetone, synthetic Zn-Pchlide exhibited a soret excitation maximum at 435 nm as compared to 430—431 for Cp(E_{430}-F_{631}). Furthermore, the short wavelength emission maximum of Zn-Pchlide in hexane-extracted acetone was at 633—634 nm as compared to 630 for Cp(E_{430}-F_{631}).

Altogether, the above results indicated that Cp(E_{430}-F_{631}) was not identical to Zn-Pchlide.

Relationship of Cp(E_{430}-F_{631}) formation to endogenous chlorophyll

Since Cp(E_{430}-F_{631}) did not appear to be either an intermediate of the chl biosynthetic pathway or a Zn-analog of protochlorophyllide, the possibility remained that it was a degradation product of the endogenous chl. This hypothesis was tested as follows: Mature chloroplasts were isolated from fully expanded *Cucumis* cotyledons and chl was extracted in 15 ml acetone/0.1 M H_4OH (9 : 1, v/v) as described in Methods. The resulting lipoprotein pellet was washed twice with 80% aqueous acetone and was resuspended in 3 ml 0.5 M sucrose/0.2 M Tris-HCl (pH 7.7). Such a preparation was free of bound porphyrins and phorbins except for traces of chl *a* and *b*. It retained the ability, however, to convert very efficiently added δ-aminolevulinic acid to uroporphyrin, coproporphyrin and their Zn-analogs (Rebeiz, C.A., unpublished). The Chl *a* and *b* in the 75% acetone extract was transferred to hexane as described in Methods. Aliquots of the hexane extract were evaporated to dryness under N_2 gas and the chl residue was dissolved in diethyl ether. An aliquot of the chl *a* plus *b* substrate in ether was added to 2 ml of the lipoprotein pellet suspension in Tris/sucrose. The total volume of the reaction mixture was adjusted to 3 ml with H_2O. The chl substrate in ether did not separate into a distinct phase but became uniformly bound to the lipoprotein suspension. Incubation was carried out at 28° C, in the dark at 10 shakes per min. In a separate reaction Pchl (Pchlide plus Pchlide ester (10 : 1, mol/mol) which was dissolved in ether, was also used as a substrate. In addition, two controls were included. One control consisted of the lipoprotein suspension without added chl substrate and the other control contained chl *a* plus *b* substrate and a lipoprotein suspension that had been preheated for 15 min in a boiling water bath before incubation. The reactions were stopped by precipitation with 15 ml acetone/0.1 M NH_4OH (9 : 1, v/v) and the reaction products were partitioned into a hexane fraction containing the unreacted chl *a* and *b* substrate and a hexane-extracted acetone fraction that contained the more polar chlorophyllides and phaeophorbides that were formed by the degradation of the added chl.

After 10-h dark incubation, a considerable degradation of the chl substrate was observed. This was evidenced by a significant decrease in the chl fluorescence amplitude of the apolar hexane fraction after 10 h of incubation as compared to the same fraction before incubation (Fig. 3 Aa and Ab). In the heat-treated control, the degradation of the chl substrate was significantly less pronounced (Fig. 3 Ac). The degradation of the chl substrate was accompanied by a substantial increase in the chlorophyllide/phaeophorbide pool fluorescence of the hexane-extracted fraction (Fig. 3 Bb). The mixed nature of the

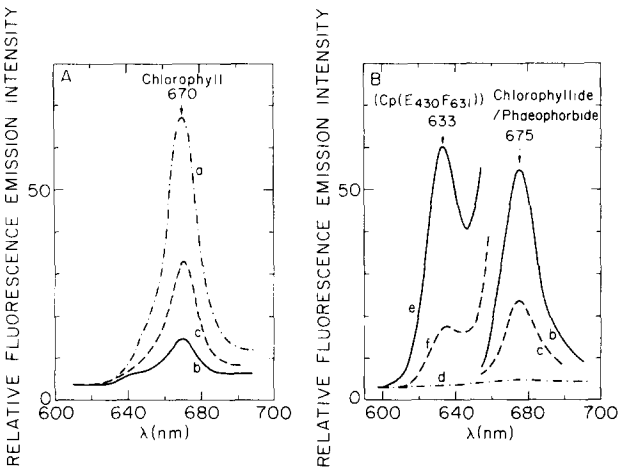


Fig. 3. Formation of Cp(E_{430}-F_{631}) from exogenous chl *a* and *b* incubated for 10 h in the dark with acetone-extracted chloroplasts. All spectra were recorded at the excitation wavelength (*E*) indicated; (A) fluorescence emission spectra of the hexane fractions containing the unreacted chl *a* and *b* substrate before and after incubation; scale, 1 × ordinate; before incubation (a); after incubation with an unheated chloroplast preparation (b); after incubation with heat inactivated preparation (c); (B) fluorescence emission spectra of the hexane-extracted acetone fraction containing chlorophyllides, phaeophorbides and Cp(E_{430}-F_{631}) before and after incubation. Chlorophyllide/phaeophorbide pool; scale 1/10 × ordinate; before incubation (d); after incubation with an unheated chloroplast preparation (b); after incubation with a heat inactivated preparation(c). The pool of Cp(E_{430}-F_{631}); scale 1 × ordinate; before incubation (d) after 10 h of dark incubation with an unheated chloroplast preparation (e); after 10 h of dark incubation with a heat-inactivated preparation (f).

pool was evidenced by its chlorophyllide *a* and phaeophorbide *a* excitation maxima at 432 and 412 nm, respectively. The formation of chlorophyllide and phaeophorbide was considerably less pronounced in the heat inactivated control than in the unheated treatment (Fig. 3Bc and Bb).

In addition to an increase in chlorophyllide and phaeophorbide formation, the degradation of the chl *a* and *b* substrate was also accompanied by a substantial accumulation of Cp(E_{430}-F_{631}). This was evidenced by the appearance of a distinct fluorescence emission maximum at 633 nm in the hexane-extracted acetone fraction (Fig. 3Be). In the heat-treated control, considerably less Cp(E_{430}-F_{631}) was formed (Fig. 3Bf).

In the absence of chl as well as in the presence of Pchl substrates, the accumulation of Cp(E_{430}-F_{631}) was not observed. Altogether these results indicated that Cp(E_{430}-F_{631}) was most probably an enzymatic degradation product of chl *a* and/or *b* or of their respective chlorophyllides and phaeophorbides. The specific contribution of these compounds to the formation of Cp(E_{430}-F_{631}) and the exact nature of this product will have to await further investigations.

Discussion

In this work, it was shown unambiguously that mature unfortified chloroplasts isolated from fully developed *Cucumis* cotyledons and incubated in a simple Tris-HCl/sucrose medium, devoid of exogenous cofactors, were incapable of significant chl repair. They were capable, however, of a limited incor-

poration of δ-amino[4-^{14}C]levulinic acid into [^{14}C]Pchlide but lacked the competence to convert exogenous [^{14}C]Pchlide to [^{14}C]chl (Table III). The net tetrapyrrole biosynthetic capacity from added δ-aminolevulinic acid was confined to the net synthesis of Zn-uroporphyrin and of coproporphyrin (Fig. 1). No evidence was found for a net tetrapyrrole biosynthetic capacity beyond coproporphyrin. These results are in sharp contrast to those of Shlyk et al. [4]. These workers reported that crude homogenates of green barley leaves incubated in 60% sucrose, in the absence of added substrates and cofactors, were capable of synthesizing significant amounts of Pchlide. Since no spectroscopic evidence of the putative Pchlide that accumulated in their system was presented, it was impossible to compare their data to the observations reported in this work. It is quite possible, however, that the formation and accumulation of Cp(E_{430}-F_{631}) in their system may have been interpreted as evidence of Pchlide net synthesis in vitro.

The biosynthetic-capabilities of the isolated plastids which were described in this work were those of mature, fully developed chloroplasts. The tissue from which the plastids were isolated had ceased to expand [5], and no evidence of further chloroplast division and development was apparent from electron microscopic examinations. Therefore, it is justifiable to consider that the limited pigment biosynthetic activity of the isolated plastids was just a manifestation of a potential for chl repair in isolated chloroplasts. Efforts aimed at improving the chl repair capability of isolated chloroplasts are presently in progress.

During the incubation of the fully developed chloroplasts in vitro, we observed the accumulation of a hitherto unreported compound [Cp(E_{430}-F_{631})] (Fig. 2), which appeared to be an enzymatic degradation product of chl (Fig. 3). Although the structure of Cp(E_{430}-F_{631}) remains to be determined, the preliminary evidence presented in this paper suggests that it may be a hitherto unreported product of chl catabolism.

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