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Protochlorophyll Biosynthesis in Cucumber (*Cucumis sativus*, L.) Cotyledons[1]

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ABSTRACT

The formation of protochlorophyllide and protochlorophyllide phytyl ester was investigated during etioplast biogenesis in order to study the biosynthetic relation of these two compounds. Protochlorophyllide accumulates slowly during the first 2 days of germination, its rate of formation increases sharply during the 3rd day, and then it decreases. Protochlorophyllide phytyl ester starts accumulating a day later; its formation coincides with the initiation of xanthophyll biosynthesis. Kinetic analysis of specific radioactivities after ^{14}C labeling of the protochlorophyll pools does not support the currently accepted conversion of protochlorophyllide into protochlorophyllide phytyl ester, but suggests that both compounds originate simultaneously from a common precursor pool.

Recent studies (16, 17) indicated a substantial production and accumulation of protochlorophyll[3] and carotenoids in cucumber seed, germinating in complete darkness. The protochlorophyll fraction consisted of protochlorophyllide and protochlorophyllide ester (18). Since protochlorophyll and carotenoids are located in the prolamellar body of etioplasts (3, 14), the accumulation of these pigments during dark germination was interpreted as an expression of etioplast biogenesis (17, 20).

It is currently assumed that protochlorophyllide ester originates from protochlorophyllide by phytylation (4, 5) although this deduction is not supported by experimental evidence (23). This report investigates the precursor-product relationship of these two compounds during etioplast biogenesis by kinetic analysis of specific radioactivities. A preliminary communication based on this research was presented to the Eleventh International Botanical Congress, Seattle, Washington, in August, 1969 (20).

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[3] The terminology proposed by Kirk and Tilney-Basset (14) is followed in this paper: "protochlorophyll" denotes the mixture of protochlorophyllide and protochlorophyllide phytyl ester which accumulates in etiolated tissues; the term "protochlorophyllide ester" is used to denote the protochlorophyllide phytyl ester component of protochlorophyll.

MATERIALS AND METHODS

Plant Material. Cucumber seed (*Cucumis sativus*, L. variety Alpha green) were germinated in white sand at 28 C in complete darkness (16).

Chemicals and Radiochemicals. 4-^{14}C-δ-Aminolevulinic acid was purchased from N. V. Phillips-Duphar, Amsterdam, Holland.

Preparation of Thin Layers. Thin layers of Silica Gel H (Merck 7736) were prepared by mixing 20 g of silica gel and 60 ml of distilled H_2O with a fast magnetic stirrer for 90 sec. The slurry was applied with a Desaga spreader as a 500-μ layer to 10 glass plates 5 × 20 cm. The plates were activated before use for 30 min at 105 C.

Thin layers of cellulose were prepared by homogenizing at high speed in a Waring Blendor 13 g of cellulose powder (Macherey, Nagel, MN 300) with 78 ml of distilled H_2O for 4 min. The slurry was applied as a 500-μ layer to grease-free glass plates 5 × 20 cm. Just before use the plates were activated at 105 C for 45 min.

Determination of Protochlorophyllide and Protochlorophyllide Ester Content. All manipulations on the live tissue were performed either in complete darkness or under a 2-amp green safelight, transmitting at 508 nm. The half band width of the transmitted light did not exceed 20 nm and was without noticeable photochemical effect on the tissue (16). Chemical manipulations were performed at less than 1 ft-c of light at bench level, and whenever possible at 1 to 4 C.

Four grams of etiolated cucumber cotyledons were homogenized in 80 ml of cold acetone containing a few milligrams of solid $MgCO_3$. This operation was performed in an ice-jacketed Ten Broeck homogenizer driven at 1450 rpm. The brei was centrifuged, the supernatant was decanted, and the pellet was washed twice with 10 ml of acetone–ether (1:1, v/v). The pooled acetone extract and washings were diluted with 60 ml of ether, and most of the acetone was removed by washing twice with 800 ml of cold distilled H_2O containing solid $MgCO_3$. The ether extract was centrifuged briefly, the residual H_2O layer was discarded, and the volume was adjusted to 100 ml with ether for spectrophotometric determination of the protochlorophyll content. The protochlorophyll extract was subsequently dried under nitrogen, and the residue was transferred quantitatively with a minimum amount of benzene to 10 plates of Silica Gel H. The chromatograms were immediately developed to 18 cm in the dark at 1 C, in benzene–ethyl acetate–absolute ethanol (8:2:0.5, v/v) (18). Immediately after development, while the chromatogram was still wet and cold, the protochlorophyllide ester was scraped off and placed in a beaker containing ice-cold ether. The silica gel was centrifuged, and the pellet was washed with several portions of ice-cold ether until free of pigment. The volume of the combined ether extracts was adjusted to 35 ml

with ether for the spectrophotometric determination. The amount of protochlorophyllide was determined by subtracting the protochlorophyllide ester from that of protochlorophyll. Protochlorophyllide ester was recovered with 70% yield.

Protochlorophyll Labeling. Five cotyledons were incubated in the dark in 1 ml of distilled H_2O containing 1 μc (20 mμmoles) of 4-^{14}C-ALA.[4] Dry seed were soaked for 30 min in distilled H_2O and then carefully shelled before incubation. All seeds had their seed coat removed before incubation. After 24 hr the cotyledons were washed with distilled H_2O, and the protochlorophyll was extracted.

Extraction of ^{14}C-Protochlorophyllide and ^{14}C-Protochlorophyllide Ester. After incubation the five cotyledons were homogenized in 10 ml of cold acetone containing $MgCO_3$, as described above. The brei was centrifuged, the supernatant was decanted, and the pellet was washed three times with 2 ml of acetone–ether (1:1, v/v). The pooled extract and washings were diluted with 10 ml of ether, and most of the acetone was removed by washing once with 800 ml of H_2O. The ether extract was centrifuged briefly, the residual H_2O layer was discarded, and the ether extract was dried at room temperature in a stream of N_2. The residue was quantitatively transferred with benzene to two plates of Silica Gel H, and the chromatogram was developed to 18 cm in the dark at 1 C in 12 ml of benzene–ethyl acetate–ethanol (8:2:2) (22). Immediately after development and while the chromatograms were still wet and cold, the protochlorophyllide and protochlorophyllide ester zones were scraped off into two separate beakers containing methanol and ether, respectively. After centrifugation the Silica Gel H pellet was washed three times with small quantities of solvent. The volume of extract and washings was adjusted to 5 ml with ether or methanol, and an aliquot was used for the determination of the radioactivity incorporated.

Spectrophotometric Determinations. Matched cells of 10 cm internal length and a Unicam Sp 500 spectrophotometer were utilized for all spectrophotometric determinations. The wave length of the instrument was checked before use on the 653.3 nm mercury line obtained from the ultraviolet source of the instrument. All absorbance readings were corrected for light scattering by referring to wave length-dependent scatter calibration curves. These were prepared from 0.1 M KCl solutions of bovine serum albumin mixed with equal volumes of 5% trichloroacetic acid. The quantity of protochlorophyll and protochlorophyllide ester in mμmoles were computed from the corrected absorbance reading at 624 nm with molar extinction coefficients of 3.56×10^4 and 3.29×10^4, respectively. These values were calculated from the specific absorption coefficients in ether of unprecipitated protochlorophyll and precipitated and dried protochlorophyll, respectively (15).

The amount of protopheophytin in 0.01 N methanolic HCl extracts was calculated from the corrected absorbance reading at 567 or 421 nm with the respective extinction coefficients of 15.6×10^3 and 19.3×10^4. These were calculated from absorbance readings in methanol of known quantities of protochlorophyllide before and after acidification to 0.01 N HCl.

Determination of Radioactive Incorporations. Aliquots of pigment extract were added to a scintillation vial containing 10 ml of Beckman's scintillation fluor, Fluoralloy formula TLA No. 161233. These were counted for 10 min in the auto quench mode with a Beckman DPM-100 liquid scintillation counter at ambient temperature. The quench-corrected dpm computation was better than $\pm 5\%$ of the true dpm values.

Radioactive spots were localized on the chromatograms with a Packard radiochromatogram scanner, model 7201.

Purification of ^{14}C-Protochlorophyllide to Constant Specific Radioactivity. Sixty cotyledons were incubated in 5 ml of distilled H_2O containing 8 μc of 4-^{14}C-ALA. After preparation of the protochlorophyll ether extract, the specific radioactivity of the latter in dpm per mμmole of protochlorophyll was determined. This was followed by separation of ^{14}C-protochlorophyllide from ^{14}C-protochlorophyllide ester, as outlined above, and determination of its specific radioactivity. The ^{14}C-protochlorophyllide in methanol was made 0.01 N with HCl, the methanol was evaporated under N_2, and the residue was transferred to thin layers of cellulose. The chromatograms were developed in toluene at room temperature, and the protopheophytin was scraped into a beaker of methanol. The specific radioactivity of the protopheophytin in dpm per mμmole was also determined.

Determination of Specific Radioactivities for Kinetic Computations. Four grams of cotyledons and five cotyledons were simultaneously incubated with 240 mμmoles of ALA and 1 μc (20 mμmoles) of 4-^{14}C-ALA, respectively. The large batch incubation was utilized for the spectrophotometric determination of protochlorophyllide and protochlorophyllide ester. The radioactive incubation was utilized for the determination of radioactivity incorporated into ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester as indicated above. Specific radioactivities were then determined on the basis of dpm incorporated per mμmole of protochlorophyllide or protochlorophyllide ester.

Determination of the Phototransformation of ^{14}C-Protochlorophyllide. After incubation with 4-^{14}C-ALA, five cotyledons were washed with distilled H_2O and irradiated at 28 C for 30 min with 40 ft-c of white fluorescent light. The ^{14}C-chlorophyll was extracted as outlined for ^{14}C-protochlorophyll and separated from other pigments on thin layers of Silica Gel H in benzene–ethyl acetate–absolute ethanol (8:2:2, v/v) in the dark at 1 C. Chlorophyll was detected by its blue-green color; radioactivity was located with the radiochromatogram scanner.

Saponification of ^{14}C-Protochlorophyll and Extraction of Phytol. These operations were performed as described by Bacon (2).

RESULTS

Accumulation of Protochlorophyllide and Protochlorophyllide Ester during Etioplast Biogenesis. Protochlorophyllide becomes spectrophotometrically detectable after 24 hr of germination (Fig. 1). However, the first detectable indication of protochlorophyllide biosynthesis as evidenced by ^{14}C-ALA incorporation is obtained after 12 hr of germination (Table I). Protochlorophyllide accumulates slowly during the 2nd day of germination, its rate increases sharply during the 3rd day, and then it decreases (Fig. 1). The first detectable indication of protochlorophyllide ester biosynthesis as evidenced by ^{14}C-incorporation is obtained after 1 day of germination (Table I); after 2 days protochlorophyllide ester is detectable spectrophotometrically (Fig. 1). It accumulates linearly during the next 3 days of germination (Fig. 1). Table II presents the ratio of protochlorophyllide to protochlorophyllide ester during the first 5 days of germination. This ratio decreases on the 2nd day, increases on the 3rd day, and then decreases again on the 4th and 5th days.

Therefore, it appears that protochlorophyllide biosynthesis is initiated about 12 hr before protochlorophyllide ester biosynthesis. Moreover, the initiation of protochlorophyllide ester biosynthesis coincides with xanthophyll biosynthesis (17).

Incorporation of ^{14}C-ALA into Protochlorophyll. Since our interpretations are based on a kinetic analysis of the specific radioactivities, it was necessary to find out whether the ^{14}C was incorporated into radiochemically pure ^{14}C-protochlorophyll components or not. Figure 2A indicates that ^{14}C-ALA is not incorporated into carotenoids while protochlorophyllide and protochlorophyllide ester are substantially labeled. Figure 2B indicates that, beside protochlorophyllide and protochlorophyllide

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

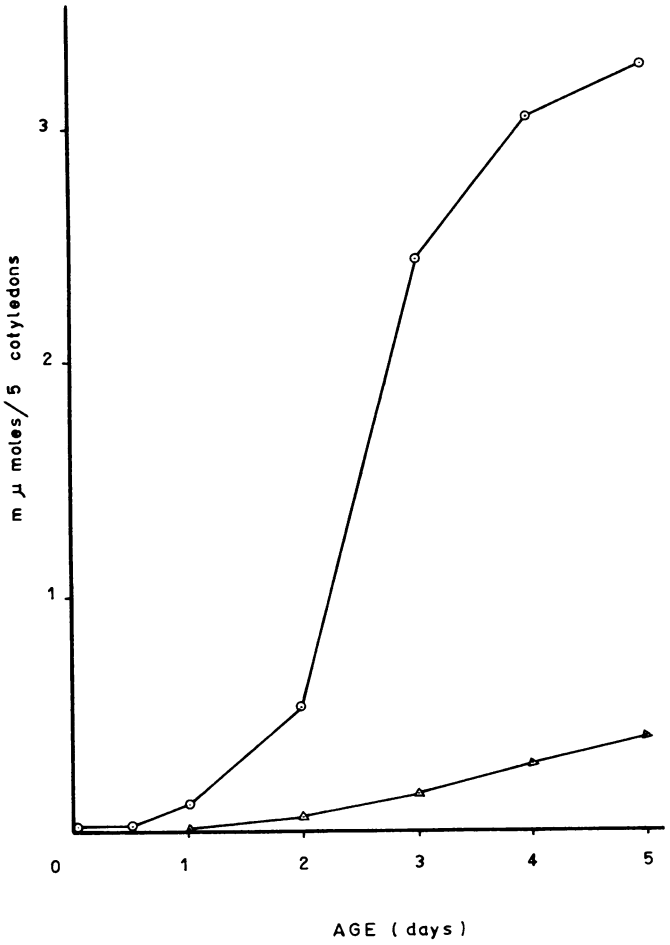

FIG. 1. Protochlorophyllide (○) and protochlorophyllide ester (△) content of excised cotyledons at various stages of germination.

Table I. *Incorporation of ^{14}C-ALA into Protochlorophyllide and Protochlorophyllide Ester during the First 24 hr of Germination*

Five seeds were shelled and incubated in the dark in 1 ml of H_2O containing 1 μc (20 mμmoles) of 4-^{14}C-ALA for the indicated time. The ether extract was chromatographed ascendingly on thin layers of Silica Gel H in benzene–ethyl acetate–ethanol (8:2:2, v/v). Incorporations of ^{14}C into protochlorophyllide and protochlorophyllide ester were detected with a radiochromatogram scanner. Minus sign: No ^{14}C incorporation; plus sign: positive ^{14}C incorporation.

Incubation	^{14}C Incorporation	
	Into protochlorophyllide	Into protochlorophyllide ester
hr		
6	−	−
12	+	−
15	+	−
16	+	−
17	+	−
24	+	+

ester, substances remaining at the origin are also labeled. These were shown to consist of protoporphyrin, coproporphyrin, and traces of uroporphyrin (unpublished work). In order to find out whether the protochlorophyllide isolated from Silica Gel H was contaminated with colorless radioactive substances having the same chromatographic mobility, it was converted into protopheophytin and chromatographed ascendingly in toluene on thin layers of cellulose. In this solvent protopheophytin HCl remains at the

Table II. *Rates of Protochlorophyllide and Protochlorophyllide Ester Formation per 24-hr Incubation and Corresponding Ratios of Protochlorophyllide to Protochlorophyllide Ester during the First 5 Days of Germination*

Four grams of cotyledons were incubated with 240 mμmoles of ALA in the dark at 28 C.

Age of tissue	ΔA^1	ΔB^2	Phyllide/Phyllide Ester
days	*mμmoles*		
1	0.09	Trace	Large
2	0.43	0.05	10.6
3	1.93	0.10	16.9
4	0.63	0.13	11.0
5	0.22	0.11	8.4

[1] Increment of protochlorophyllide in 24 hr/5 cotyledons.
[2] Increment of protochlorophyllide ester in 24 hr/5 cotyledons.

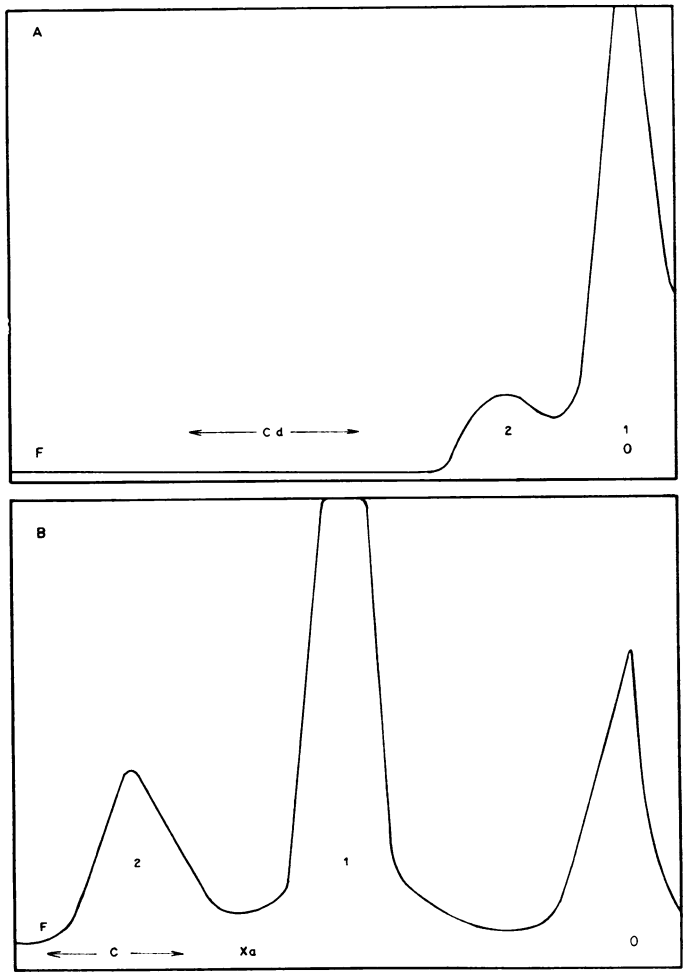

FIG. 2. Scannings of paper (A) and Silica Gel H thin layer (B) chromatograms of cotyledonary pigments. Two-day-old etiolated cotyledons were dark-incubated with 1 μc of 4-^{14}C-ALA for 24 hr before extraction. The symbols denote the following areas: O: origin; F: front; Cd: unseparated carotenoids; C: carotene; Xa: xanthophylls; 1: protochlorophyllide; 2: protochlorophyllide ester.

TABLE III. *Specific Radioactivities of ^{14}C-Protochlorophylls at Various Stages of Purification*

Pigment	Specific Radioactivity
	dpm/mμmole of pigment × 10^{-3}
Protochlorophyll, crude ether extract	38.9
Protochlorophyllide purified on Silica Gel H	29.0
Vinyl pheoporphyrin a_5[1] purified on cellulose Mn 300	29.3
Protochlorophyllide ester purified on Silica Gel H	38.1

[1] Vinyl pheoporphyrin a_5 designates the Mg-free derivative of protochlorophyllide.

origin while protochlorophyllide and protochlorophyllide ester move with approximate R_F values of 0.10 and 0.48, respectively. Table III presents the specific radioactivity of ^{14}C-protochlorophyllide at various stages of purification. It is obvious that ^{14}C-protochlorophyllide isolated after thin layer chromatography on Silica Gel H has already reached constant specific radioactivity which is not altered by conversion into the corresponding pheophytin. Upon alkaline hydrolysis (2) of ^{14}C-protochlorophyll isolated after Silica Gel H chromatography, no radioactivity could be detected in the phytol fraction. These results confirm the advantage of using 4-^{14}C-ALA as a specific precursor of tetrapyrroles (8). They also suggested that ALA is incorporated as such into the tetrapyrroles by whole cotyledons without undergoing prior catabolic reactions.

Phototransformation of ^{14}C-Protochlorophylls. When etiolated barley leaves are supplied with ALA, they form large amounts of protochlorophyllide. However, little if any of this protochlorophyllide is converted to chlorophyllide (9, 14), if the tissue is subsequently illuminated. On the other hand, the photoconversion of ^{14}C-protochlorophyllide synthesized during a 30-min dark incubation of etiolated cucumber cotyledons with 4-^{14}C-ALA under our experimental conditions is complete (Fig. 3). Similar results are obtained when the cotyledons are incubated for 24 hr with ^{14}C-ALA. The quantity of ^{14}C-ALA added (20 mμmoles) is about 500,000 times smaller than that used by other workers in analogous experiments (9) and evidently results in the production of phototransformable ^{14}C-protochlorophyllide holochrome. If the quantity of holochrome protein is limiting, as might well be the case here, while active enzymes for the production of protochlorophyllide from ALA are not (4, 12), this would explain the formation of large quantities of 632 nm-absorbing protochlorophyllide (4) obtained with other tissues. In that case the protochlorophyllide, lacking a specific association with a protein component, is incapable of undergoing photoconversion to chlorophyllide.

Precursor-Product Relationship of Protochlorophyllide and Protochlorophyllide Ester during Etioplast Biogenesis. Four possibilities can be stated for the biosynthetic relationship of protochlorophyllide and protochlorophyllide ester during protochlorophyll biosynthesis: (*a*) protochlorophyllide ester arises from protochlorophyllide; (*b*) protochlorophyllide and protochlorophyllide ester are formed from a common precursor; (*c*) protochlorophyllide arises from protochlorophyllide ester; (*d*) protochlorophyllide and protochlorophyllide ester are formed by independent paths from essentially different precursors. This last alternative is improbable in view of the close structural resemblance of the pigments and the incorporation of ^{14}C-ALA into both tetrapyrrole rings (Table II, Fig. 2). Alternative *c* is also improbable as protochlorophyllide biosynthesis is initiated 12 hr prior to protochlorophyllide ester (Fig. 1). Therefore we will be mainly concerned with differentiating between alternatives *a* and *b*.

Let throughout our discussion:

$t_2 - t_1$ = time interval under consideration
γ = specific radioactivity
γ_A = specific radioactivity of protochlorophyllide
γ_B = specific radioactivity of protochlorophyllide ester
γ_P = specific radioactivity of the common precursor
p = content of a common precursor of protochlorophyllide and protochlorophyllide ester
A = protochlorophyllide content, mμmoles/5 cotyledons
$\Delta A = A_2 - A_1$ = increment in protochlorophyllide content during time interval $t_2 - t_1$
B = protochlorophyllide ester content, mμmoles/5 cotyledons
$\Delta B = B_2 - B_1$ = increment in the protochlorophyllide ester content during $t_2 - t_1$.

It is possible to investigate the contribution of alternative *a* during protochlorophyll biosynthesis by comparing the experimental ^{14}C incorporation into protochlorophyllide ester with the theoretical incorporation which would be expected on the basis of this model.

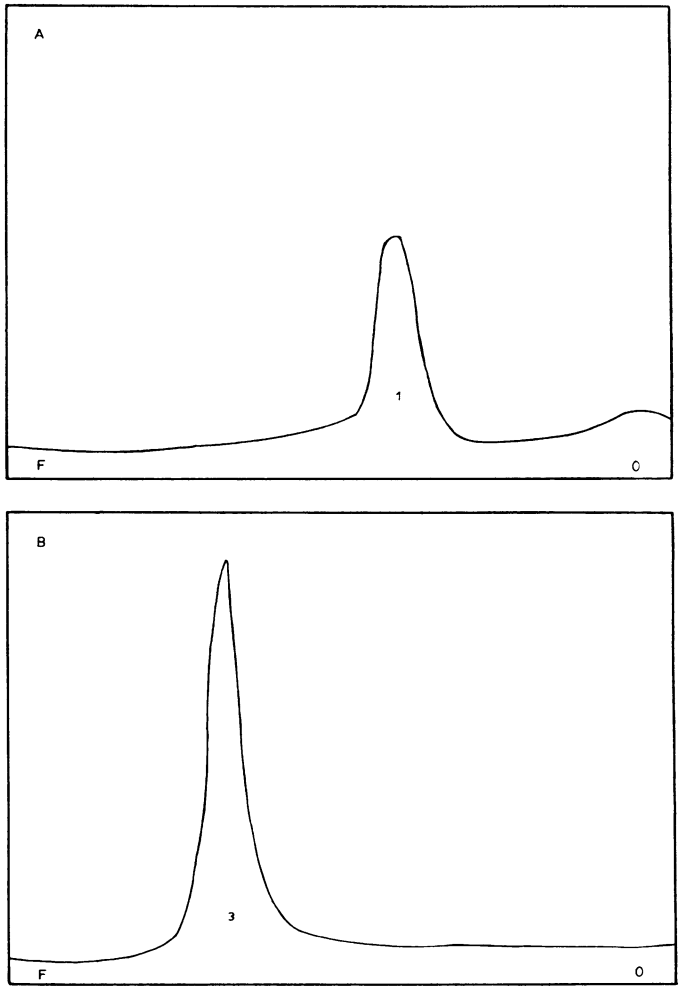

FIG. 3. Scannings of Silica Gel H thin layer chromatograms of the following cotyledonary extracts. A: 4-day-old etiolated cotyledons dark-incubated for 30 min with 1 μc of 4-^{14}C-ALA; B: same as above, but illuminated with 40 ft-c of white fluorescent light for 30 min at 28 C before extraction. The chromatograms were developed in benzene-ethyl acetate-ethanol (8:2:2, v/v) in the dark at 1 C. The symbols denote the following: 1: protochlorophyllide; 3: chlorophyll; the other symbols are as in Figure 2.

The accumulation of protochlorophyllide ester is linear with time between the 2nd and the 5th days of germination (Fig. 1). At a given time, t, it is described by the function

$$B = at - b \quad (1)$$

where a is the slope of the line and b its intercept on the ordinate. The rate of change of B with respect to time is then given by

$$a = \frac{dB}{dt} \quad (2)$$

If ^{14}C-protochlorophyllide ester is formed from ^{14}C-protochlorophyllide, the increase in ^{14}C-protochlorophyllide ester in a small interval of time (dt) is:

$$dB = a \cdot dt \quad (3)$$

The radioactivity (q_B) that accumulates in the ^{14}C-protochlorophyllide ester pool during dt is then given by

$$q_B = (\gamma_A)\, dB = (\gamma_A) a \cdot dt \quad (4)$$

For the time interval $t_2 - t_1$, the total radioactivity (Q_B) that accumulates in the ^{14}C-protochlorophyllide ester pool is given by

$$Q_B = \int_{t_1}^{t_2} (\gamma_A) a \cdot dt \quad (5)$$

During a ^{14}C incubation, γ_A increases from zero at t_1 to the values reported in Table IV at t_2. If γ_A is a linear function of time,

$$\gamma_A = ct \quad (6)$$

equation 5 takes on the form

$$Q_B = \int_{t_1}^{t_2} (ct) a dt$$

which is easily integrated:

$$Q_B = ac \int_{t_1}^{t_2} (t) dt = ac \left(\frac{1}{2} t^2\right)_{t_1}^{t_2} = \frac{1}{2} ac(t_2^2 - t_1^2)$$

Considering that $t_1 = 0$ in our case, we see that the incorporation of ^{14}C into the ester is proportional to the square of the incorporation period; that is:

$$Q_B = \tfrac{1}{2}\, act_2^2 \quad (7)$$

Furthermore, from equation 1 we have $at_2 = \Delta B$ and from equation 6 $ct_2 = \gamma_A$. Substituting these values into equation 7, we obtain

$$Q_B = \tfrac{1}{2}\, at_2 \cdot ct_2 = \tfrac{1}{2}\, \gamma_A \Delta B \quad (8)$$

This equation enables us to calculate theoretical values for Q_B, based on alternative a, which postulates that protochlorophyllide ester is formed from protochlorophyllide. The only assumption made in the derivation of equation 8 is that the increase in the specific radioactivity of protochlorophyllide is linear during the incubation: $\gamma_A = ct$. Table V compares the experimental values for the increase in radioactivity in the protochlorophyllide ester pool with the theoretical values computed according to equation 8. It is clear that the calculated theoretical values remain much lower than the experimental ones, and therefore alternative a is not consistent with our observations.

Further insight into the question of which pathway is operating can be gained by using the equations initially developed by Duranton (8) to investigate the precursor-product relationship of chlorophylls a and b.

If protochlorophyllide and protochlorophyllide ester are formed simultaneously from a common precursor (alternative b), the specific radioactivity of protochlorophyllide at the end of incubation is given by $\gamma p \cdot \Delta A/(A + \Delta A)$. Similarly, the specific radioactivity of protochlorophyllide ester is given by $\gamma p \cdot \Delta B/(B + \Delta B)$. Therefore

$$R = \frac{\gamma_A}{\gamma_B} = \frac{\Delta A}{\Delta B} \cdot \frac{B + \Delta B}{A + \Delta A}$$

Whenever during the incubation the ratio of protochlorophyllide to protochlorophyllide ester decreases (Tables II and VI),

$$\frac{A}{B} > \frac{A + \Delta A}{B + \Delta B} > \frac{\Delta A}{\Delta B}$$

Under these conditions

$$R = \frac{\Delta A}{\Delta B} \cdot \frac{B + \Delta B}{A + \Delta A} > 1$$

TABLE IV. *Specific Radioactivity of the ^{14}C-Protochlorophyllide Pool after 24 hr of Incubation with ^{14}C-ALA, at Various Stages of Germination*

Initial Age of Tissue	Specific Radioactivity of ^{14}C-Protochlorophyllide
days	*dpm/mμmole* × 10^{-3}
1	35.0
2	14.9
3	12.8
4	14.4
5	15.5

Table V. *Comparison of Theoretical ($\frac{1}{2}\, \gamma_A \cdot \Delta B$) and Experimental Values for the Incorporation of ^{14}C-ALA into Protochlorophyllide Ester by Germinating Cucumber Cotyledons*

Incubation with ^{14}C-ALA was carried out for 24 hr.

Initial Age of Tissue	$\frac{1}{2}\, \gamma_A \cdot \Delta B$/5 Cotyledons	^{14}C-Incorporation into Protochlorophyllide Ester/5 Cotyledons
days	*dpm* × 10^{-3}	
1	...	1.62
2	0.44	5.77
3	0.78	11.22
4	0.96	11.26
5	0.84	12.77

Table VI. *Specific Radioactivity Ratios of Protochlorophyllide and Protochlorophyllide Ester at Various Periods of Protochlorophyll Biosynthesis*

Initial Age of Tissue	$\frac{A}{B}$[1]	γ_A[2]	γ_B[3]	$R = \frac{\gamma_A}{\gamma_B}$
days		*dpm/mμmole* × 10^{-3}		
1	Large	35.1	Large	...
2	10.6	15.0	119.3	0.13
3	16.9	12.8	77.3	0.16
4	11.0	14.4	40.5	0.36
5	8.4	15.4	33.0	0.47

[1] A/B = protochlorophyllide content/protochlorophyllide ester content.

[2] γ_A = specific radioactivity of protochlorophyllide.

[3] γ_B = specific radioactivity of protochlorophyllide ester.

On the other hand, if protochlorophyllide ester is formed from protochlorophyllide (alternative *a*) and γ_A is the specific radioactivity of protochlorophyllide, the specific radioactivity of protochlorophyllide ester is similarly given by $\gamma_A \cdot \Delta B/(B + \Delta B)$: in that case

$$R = \frac{\gamma_A}{\gamma_B} = \frac{\gamma_{A(B+\Delta B)}}{\gamma_{A(\Delta B)}} = \frac{B + \Delta B}{\Delta B}$$

when $B = 0$, $R = 1$; when $B > 0$, $R > 1$. Thus, according to alternative *a*, R cannot be less than 1. In other words, experimental values of R greater than unity denote the operation of pathway *a*, while R values smaller than unity denote the operation of pathway *b*. The experimental values of R obtained at various stages of protochlorophyll biosynthesis are presented in Table VI. Obviously, R remains less than unity and increases from 0.13 to 0.47, indicating that during protochlorophyll biosynthesis protochlorophyllide and protochlorophyllide ester are probably formed from a common precursor.

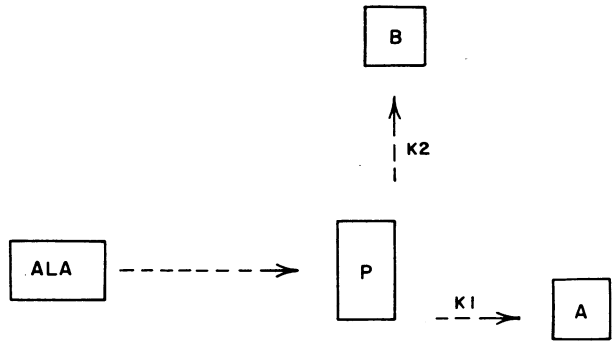

FIG. 4. Proposed model for the simultaneous production of protochlorophyllide and protochlorophyllide ester from a common precursor. The symbols denote the following: A: protochlorophyllide; B: protochlorophyllide ester; p: common precursor; K_1: rate of reactions leading to protochlorophyllide formation; K_2: rate of reactions leading to protochlorophyllide ester formation.

DISCUSSION

Etioplast biogenesis appears as a well organized process. During pigment biosynthesis protochlorophyllide is formed first, followed by the production of protochlorophyllide ester (Fig. 1) and xanthophylls (17). The coincidence of the initiation of protochlorophyllide ester and carotenoids biosynthesis during etioplast biogenesis may reflect the sharing of a common polyisoprenoid pool responsible for the production of carotenoids and phytol (6). The initiation of the polyisoprenoid biosynthetic activity might also act as a starter of protochlorophyllide ester biosynthesis.

Figure 4 suggests a model for the simultaneous formation of protochlorophyllide and protochlorophyllide ester from a common precursor. This model envisages a homogeneous precursor pool, *p*, where any molecule of *p* could form either protochlorophyllide or protochlorophyllide ester, at different rates (Fig. 4).

Of course, the preceding kinetic analysis does not preclude the possibility that the pathway leading from *P* to *B* might include a form of protochlorophyllide as a transitory intermediate. In view of the well known heterogeneity of chlorophyll *in situ*, this hypothesis, which calls for a dual pathway of protochlorophyll biosynthesis, is appealing.

Efforts to detect ^{14}C precursors of protochlorophyll under our incubation conditions revealed small quantities of ^{14}C-protoporphyrin IX and ^{14}C-coproporphyrin (1, 19). However, after 60 min in the presence of ^{14}C-ALA, radioactivity was detected in protochlorophyll as well as in a metal porphyrin identified as Mg-protoporphyrin monoester (1, 19); later on this compound disappeared (1, 19). This temporary accumulation of Mg protoporphyrin monoester can be explained by the flooding of the endogenous ALA pool by the sudden input of exogenous ^{14}C-ALA, bypassing the controls at the ALA synthetase level. Mg protoporphyrin monomethyl ester is presumably the last known intermediate before the formation of the cyclopentanone ring; hence, it could be conceived as the postulated precursor, *P*, which gives rise to protochlorophyllide and protochlorophyllide ester by two distinct pathways (Fig. 4). Irrespective of its identity, the precursor, *P*, is metabolized rapidly, as indicated by the lack of substantial accumulation of intermediates.

The concept of a single control of protochlorophyll biosynthesis at the ALA synthetase level (4, 5) fails to explain some of our results satisfactorily. Indeed, when the ALA synthetase checkpoint is bypassed by ^{14}C-ALA incubation, ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester are still formed at different rates, indicating the operation of other control mechanisms as well. Therefore, it seems probable that control of protochlorophyll biosynthesis is also exerted at the level of Mg protoporphyrin monoester, as suggested by the initial accumulation of the latter upon flooding the ALA pool.

The cytological implications of the simultaneous formation of ^{14}C-protochlorophyllide and ^{14}C-protochlorophyllide ester from a common precursor are not clear, and any suggestion concerning them at this stage is obviously speculative.

Since the etioplast contains protochlorophylls as integral components of the prolamellar body, the simultaneous biosynthesis of protochlorophyllide and protochlorophyllide ester from a common precursor would be advantageous if the soluble precursor of the two protochlorophylls was synthesized in the stroma and translocated to the different sites of utilization in the prolamellar body. Speaking teleologically, it appears that it would be easier for the cell to move Mg protoporphyrin monomethyl ester to two specific locations in the prolamellar body membrane system than to synthesize two phorbins in the stroma—one of which contains the bulky phytyl ester side chain—and then to translocate them into the prolamellar body.

The specific function of protochlorophyllide phytyl ester is not understood at present. If this compound proves to be completely nonphototransformable, it is still possible to suggest that it might serve to polarize selectively certain sites of the prolamellar body in conjunction with the future buildup of chlorophyll heterogeneity *in situ*. However, if it proves to be partially and slowly phototransformable, as claimed by Godnev *et al.* (10, 11) and by Rudolph and Bukatsch (21), it might be directly involved in the buildup of chlorophyll heterogeneity *in situ* during the greening process. In this connection it might be appropriate to remember Virgin's observation that the disappearance of protochlorophyllide phytyl ester in etiolated wheat seedlings which have been exposed to light coincides with the appearance of chlorophyll *b* (25).

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