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Changing Ratios of Phototransformable Protochlorophyll and Protochlorophyllide of Bean Seedlings Developing in the Dark[1]

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ABSTRACT

Protochlorophyll (Pchl) and protochlorophyllide (Pchlide) are at comparable levels in 2-day-old (young) etiolated bean leaves (*Phaseolus vulgaris* L. var. Red Kidney). During subsequent development in the dark, both pigments increase, but the rate of Pchlide increase is greater than that of Pchl, leading to the commonly observed predominance of Pchlide beyond 7 days (old leaves). Both protopigments are phototransformable to their respective chlorophyll(ide) photoproducts throughout dark development. The rate of protopigment regeneration in young leaves after illumination is rapid and displays no lag, whereas this process in old leaves begins slowly and achieves only about one-fifth the rate of younger leaves. The rate of chlorophyllide esterification is also faster in the younger tissue. Since the proplastid-related properties of young bean leaves are quite similar to those of *Euglena*, young leaves and *Euglena* may represent an evolutionarily primitive case compared with older bean leaves which contain etioplasts. Since *Euglena* and young beans green perfectly well when exposed to light, the extensive modifications associated with prolonged dark growth do not seem to be obligatory for plastid development. The properties of older beans are viewed as being the consequence of prolonged etiolation which may provide a faster rate of plastid development and appearance of photosynthesis as the plant nears the limits of its stored reserves.

Throughout this paper, protochlorophyll (Pchl) will be used to designate the epiphasic pigment on petroleum ether-alkaline acetone partition, assumed to be an esterified form of Mg vinylpheoporphyrin a_5 which moves on chromatography in a similar manner to chlorophyll *a* (Chl). Protochlorophyllide (Pchlide) designates the hypophasic pigment assumed to be unesterified at the propionic acid residue of ring IV, which moves in a similar manner to Pchlide extracted from etiolated barley leaves. Pchl(ide) will be used when the exact nature of the protopigment has not been determined or when a mixture of the two species is present. Chlide designates chlorophyllide *a*.

It has recently been demonstrated that both Pchl and Pchlide are present in dark-grown cells of *Euglena* and that both protopigments are transformed to Chl-like pigments upon illumination (9, 10). Klein and Schiff (19) investigated proplastid development in 2- to 9-day-old etiolated bean leaves and observed that (*a*) the 3-day-old bean proplastids are about the same size as those of *Euglena;* (*b*) both contain similar amounts of protopigments; (*c*) when prolamellar bodies are present, they are noncrystalline; (*d*) both show a predominant Pchl(ide) absorption peak *in vivo* at about 635 nm; (*e*) upon illumination, Chl(ide) absorbing at 672 nm is formed directly, without an intervening Shibata shift.

Because of the marked similarities between plastid precursors in young dark-grown bean leaves and those of *Euglena,* we undertook a study of the forms of protopigments present and their phototransformation at various stages in the development of etiolated bean seedlings, particularly at times preceeding 7 to 9 days, the material commonly employed in such studies. In the usual material, Pchlide is the predominant protopigment which is phototransformed to Chlide *a* and then finally esterified to yield Chl *a*. Pchl photoconversion is extremely low and is not regarded as a significant route of Chl formation (8, 12, 16, 31, 32, 35, 37, 39). In this work we show that Pchl conversion in young etiolated bean seedlings represents a highly significant fraction of the convertible protopigments, and results in the direct formation of Chl. A brief abstract of this work has appeared (22).

MATERIALS AND METHODS

Seeds of *Phaseolus vulgaris* L. var. Red Kidney were germinated in wet vermiculite at 26 C in total darkness. The vermiculite was kept wet at all times by uniformly watering the trays, each containing 200 beans, with 1 liter of water each day. After time periods ranging from 2 to 17 days, a specified number of seeds, depending on the age, were removed, washed with water, and the cotyledons separated to expose the primary leaf pair. All manipulations were performed under dim green safelights (27) or in complete darkness. Roughly 1000 leaf pairs were required for each analysis of 3-day-old material. The number of leaf pairs required could be reduced to 35 at 5 days, 20 at 9 days, 15 at 13 days, and 10 by the 17th day.

The methods for pigment extraction were modified from Shlyk (31). Before pigment extraction, the leaves were either (*a*) frozen in approximately 25 ml of liquid N_2, (*b*) steamed for 10 min, or (*c*) extracted directly. All solvents used were reagent grade (Fisher).

For experiments in which the leaves were heated, the specified number of leaf pairs were harvested, weighed, placed in a tight mesh wire basket, and steamed for 10 min. The leaves were then ground in a chilled mortar containing a small amount of $MgCO_3$ and 50 ml of cold 80% (v/v) acetone. All procedures up to this point were carried out under dim green safelights. After the initial extraction of the leaves, all manipulations were done at room temperature, under dim laboratory lighting (less than 5 ft c). The homogenates were centrifuged in a Clay-Adams table top centrifuge at top speed for 5 min. Extraction was repeated three more times, and the combined supernatants were quantitatively transferred to a 500-ml separatory funnel. Extraction was complete since the pellets, when scanned in a Biospect spectro-

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photometer coupled to a Fabritek Computer (see below), indicated the absence of Pchl(ide) absorption peaks. Pigments were quantitatively transferred to fresh, peroxide-free diethyl ether by adding 30-ml portions to the acetone solution and gently mixing. Extractions were repeated three times to ensure maximal extraction from the acetone hypophase. The ether fractions were combined and reduced to dryness *in vacuo* at no more than 25 C. The residue was resuspended in a known volume of anhydrous diethyl ether and the total pigment concentration was determined by measurement of the absorbance from 400 to 700 nm in a Cary Model 14 recording spectrophotometer. The hypophase was always viewed under a low intensity, long wavelength UV lamp (GE F15T8-BLB) to ensure complete extraction of pigments as evidenced by the absence of red fluorescence. After spectrophotometry, the sample was again taken to dryness (under a stream of N_2 gas), and was resuspended in 100 ml of cold acetone:0.1 M NH_4OH (9:1, v/v).

This solution was then transferred to a separatory funnel and extracted three times with 30-ml portions of cold, mid-range petroleum ether (boiling range 30–50 C). The petroleum ether fractions were combined, placed in a separatory funnel, and washed with 50 ml of cold distilled H_2O (which was then added back to the acetone-NH_4OH hypophase). Each fraction (epiphase and hypophase), after complete separation, was then treated separately.

The petroleum ether fraction was evaporated to dryness *in vacuo* and the residue was resuspended in 1.5 to 5 ml of anhydrous diethyl ether. This constituted the "epiphase" or "petroleum ether fraction."

The pH of the aqueous layer was adjusted to 5.5 with a saturated solution of NaH_2PO_4 and was then extracted three times with 30-ml portions of anhydrous diethyl ether. The total ether extract was then washed with 25 to 30 ml of cold distilled H_2O. For spectrophotometric analysis, the ether phase was reduced to dryness as above and then resuspended in approximately 5 ml of diethyl ether ("hypophase" fraction). Pigment concentrations in both fractions were determined from the Cary absorption spectra using the equations of Koski (21), and converted to moles as suggested by Boardman (6) and Sestak (30).

When the sum of the Pchl and Pchlide fractions was compared to the initial total protopigment concentration obtained prior to the aqueous acetone-petroleum ether partition, it was observed that recovery was always at least 85 to 90% in all experiments.

Chromatographic analysis was performed in all experiments to further verify the identification of the pigments. The samples were spotted from diethyl ether onto cellulose MN 300 TLC plates and developed with methyl alcohol-methylene chloride-water (100:18:20, v/v/v) in darkness (29). In some cases, Chl *a* from spinach (Sigma Chemical Co.) was used as a standard for comparison of R_F values. When development was complete, the plates were dried under a stream of N_2 and then examined under long wavelength UV light to detect the red-fluorescing areas.

For experiments in which the leaves were extracted directly, the specified number of leaf pairs were harvested, weighed, and extracted as for the heated material. For experiments in which the leaves were frozen in liquid N_2, the specified number of leaf pairs were harvested, weighed, and dumped into a chilled mortar containing 25 to 30 ml of liquid N_2. Once the material was frozen and the major portion of the liquid N_2 had evaporated, the leaves were extracted as before, except that the solvent used was cold acetone:0.1 M NH_4OH (9:1, v/v), all steps were performed in a 4 C room, and the combined extracts were immediately subjected to petroleum ether partitioning in the cold. From this point on, all manipulations were done as before.

For the determination of cotyledon dry weight, five cotyledon pairs were harvested, weighed, and then placed in a Precision Thelco Model 4 oven set at 43 C for a period of 5 days. Extended drying beyond this period brought about no significant change in weight.

Phototransformation Experiments. Primary leaf pairs were harvested under a dim green safelight and spread open in order to reduce self-absorption during illumination. The red light source was a General Electric 30 v locomotive head lamp filtered by Edmund Scientific color filter sheets No. 809 "straw" and no. 821 "light red," and 18 cm of distilled H_2O. Energy as measured with a Yellow Springs Radiometer was either 55 or 340 w/m^2, both of which were saturating for the phototransformation.

Postillumination Measurements. Following illumination at 120 w/m^2 with the same light source as above, the leaves were quickly transferred to wet filter paper in a Petri dish for the specified dark period. The dish was covered, wrapped with aluminum, foil, and shielded in a light-tight box at 25 C. At various times the leaves were removed and steamed and extracted as above. To obtain points for time zero, however, the leaves were illuminated and immediately dumped into 25 ml of liquid N_2 and extracted appropriately.

Pchl(ide) Regeneration. The kinetics of regeneration *in vivo* of Pchl(ide) in 3- and 7-day-old etiolated bean leaves was determined using 25 or 2 leaf pairs, respectively, in a Biospect Model 61 Spectrophotometer coupled to a Nicolet 1072 Computer. The absorption of the appropriate number of leaves was scanned into one quarter of the memory 16 times (D). The sample was then illuminated with red light from a tungsten bulb filtered by a Corning No. 2404 cutoff filter, at an intenstiy of 125 w/m^2, for 60 sec. The absorption (L) was then immediately remeasured in the same way as for the dark (D) sample, and stored in another quarter of the memory. The difference spectrum, D-L, was computed and plotted, yielding the value for the transformable Pchl(ide), determined as the difference between the relative optical density at 635 nm in the case of the 3-day-old leaves, and 650 nm in the case of the 7-day-old leaves, as compared to the absorption difference at 715 nm which was taken to be zero. The leaves were then allowed to incubate in darkness at 25 C and were rescanned at intervals. The L absorption spectrum was subtracted from each of these dark spectra, and the difference in absorbance at 650 nm compared with that at 715 nm was used as the measure of regeneration of Pchl(ide).

RESULTS AND DISCUSSION

Figure 1 shows that the partition methods employed in this study are effective in separating Pchl and Pchlide, since the petroleum ether epiphase shows only Pchl on chromatography while the hypophase contains only Pchlide. Mixtures of epiphasic and hypophasic pigments also show the expected chromatographic separation, indicating that their R_F values are not influenced by other materials in the extracts.

Figure 2 shows that Pchl and Pchlide are at the comparable levels in 2-day-old etiolated leaves. During subsequent development in the dark, both pigments increase, but the rate of Pchlide increase is greater than that of Pchl, leading to a predominance of Pchlide at later stages, in agreement with the observations of others who have found that 6 day and older etiolated leaves, the material usually employed for studies of greening, contain predominantly Pchlide (23, 31, 32, 37, 39). Since the data are comparable for leaves extracted directly or heated before extraction, the distribution found is probably not an artifact of enzymic modification of the pigments during extraction or subsequent manipulations. When computed on a fresh weight basis, the same results are obtained (calculated results not shown), as might be expected from the comparable kinetics of increase in leaf fresh weight (Fig. 1). It might be inferred that the embryonic or juvenile form of protopigment in the bean is Pchl and as leaf development proceeds in the dark, Pchlide accumulation increases while Pchl accumulation is attenuated and eventually ceases before total protopigment accumulation is achieved. Pchlide becomes the predominant leaf pigment under conditions of prolonged etiolation.

Correlated with these changes is the decrease in cotyledonary dry weight (Fig. 3) as might be expected if seed storage materials are being used for the formation of leaf constituents, including Pchl and Pchlide. The ratio of Pchl to Pchlide decreases with age, reflecting the differential rates of synthesis of the two pigments noted in Figure 2.

Under conditions where enzymic modifications are prevented by heating and/or cold extraction, it can be seen (Fig. 4) that both Pchl and Pchlide are phototransformable to Chl(ide). If they are transformable to the same extent, the data presented earlier would lead us to expect that comparable amounts of Pchl and Pchlide should be phototransformed at earlier stages of development, while Pchlide conversion should predominate at later stages, reflecting the actual amounts of the two pigments at various leaf ages. Figure 4 shows that this is so since the mole percent of the Pchl(ide) pool represented by Pchl and Pchlide are comparable in the dark-grown leaves at early stages of development, as are the mole percents of the Chl(ide) pool represented by Chl and Chlide after phototransformation. At later development stages, when Pchlide predominates in the dark, Chlide predominates after phototransformation. Pchl and Pchlide are a constant fraction of the Pchl(ide) pool after phototransformation with respect to the age of the leaves, and constitute the nontransformable Pchl(ide). Thus, Pchl and Pchlide appear to be equally photoconvertible to their respective Chl(ide)s throughout development, reflected in the strong correlation of the Pchl/Pchlide ratio in dark-grown leaves with the Chl/Chlide ratio after transformation throughout development, (Fig. 5). The predominance of the transformation of Pchlide to Chlide is well documented in studies with the older leaf material usually employed (8, 12, 16, 31, 32, 35, 37, 39). Reports do exist, however, of phototransformation of Pchl as well, usually in small amounts compared with the transformation of Pchlide (13, 16, 26, 32, 39). Pchl photoreduction has also been reported in roots (5) and in etiolated cucumber cotyledons (25), tissues which might be expected to have juvenile forms of protopigments predominating. If Pchl is the juvenile, and Pchlide the more mature intermediate in Chl(ide) formation in etiolated leaves, one may inquire into the situation in fully green leaves. Pchlide to Chlide phototransformation is the principal biosynthetic route of Chl formation in mature green leaves (summarized in 6, 18, 31). It would be interesting to determine whether Pchl, Pchlide, or both, are continuously phototransformed in younger leaves during greening.

As the leaves are incubated in the dark after phototransformation, the proportion of the Chl(ide) pool found in the petroleum ether epiphase increases with time (Fig. 6), as would be expected

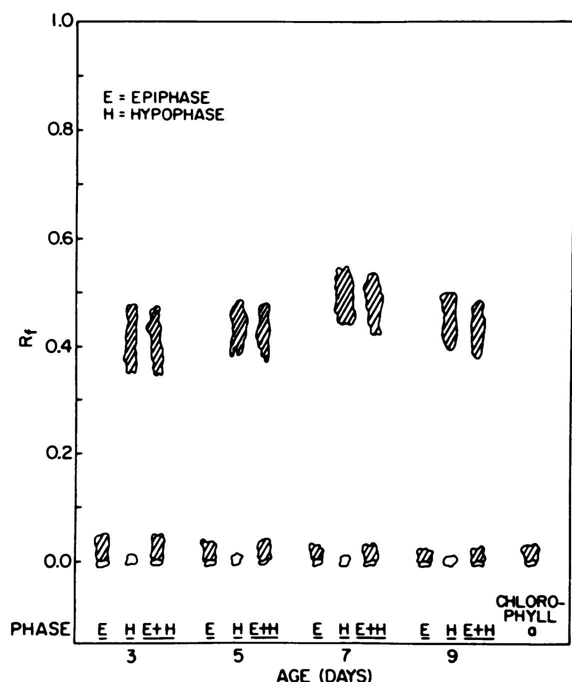

FIG. 1. Chromatography of pigments to verify their identities. A typical thin layer chromatograph of epiphase, hypophase, and mixtures of the two from extracts of etiolated leaves. Red fluorescent areas are denoted by cross-hatching. Spinach Chl *a* is present as a standard.

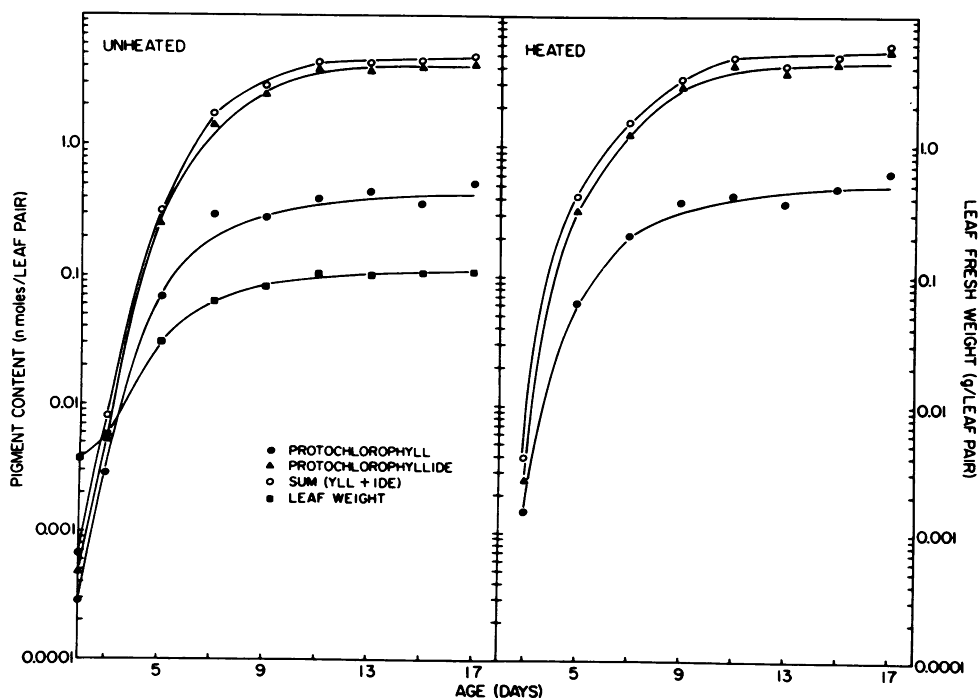

FIG. 2. Contents of protopigments during development of bean seedlings in the dark. The results are plotted semilogarithmically to allow the display of the wide range of concentrations on one graph. The two parts of the figure compare extraction in the cold of unheated leaves and leaves which had been steamed prior to extraction.

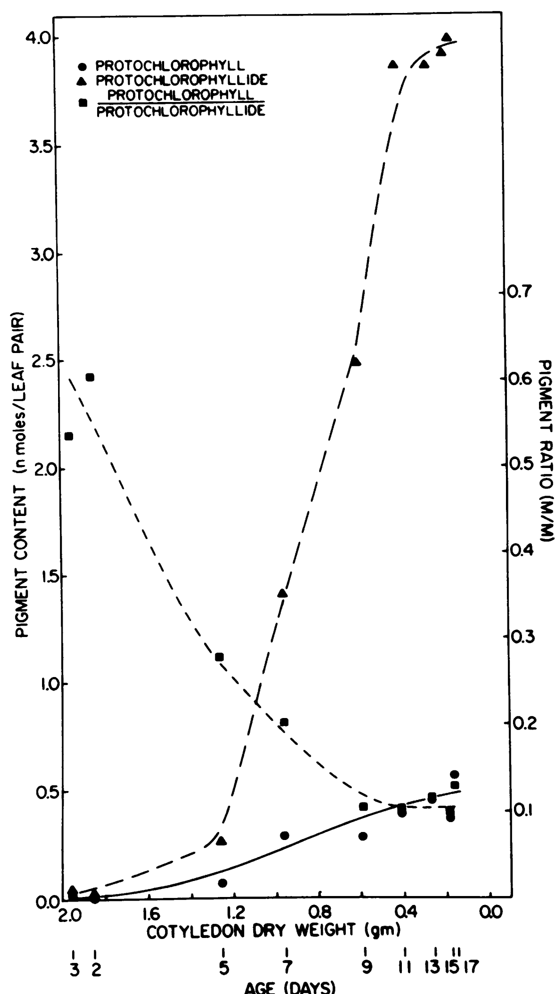

FIG. 3. Protopigment contents and ratios of leaves as a function of cotyledon dry weight during development of bean seedlings in the dark.

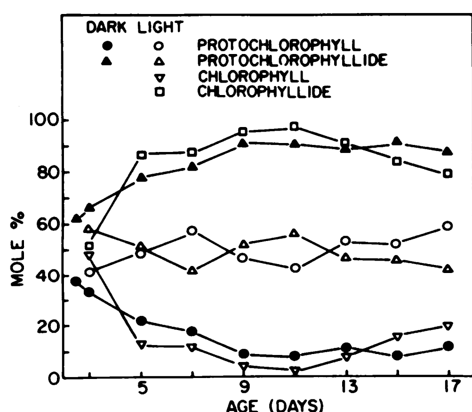

FIG. 4. Pigments in etiolated bean leaves before (dark) and after (light) phototransformation as a function of seedling age. Each point represents the mean of two experiments. In one experiment illumination was for 30 seconds at 55 w/m^2; in the other 30 sec at 340 w/m^2. The two treatments did not produce significantly different results. At each age, Pchl + Pchlide in light or dark was taken as 100% for those protopigments while in light Chl + Chlide was taken as 100% for chlorophyll pigments. At 3 days, the conversion of Pchl to Chl and Pchlide to Chlide were both about 40%. From 5 days onwards, the conversion of Pchl to Chl remained at 40% or less, while conversion of Pchlide to Chlide rose to 80 to 90%.

if the unesterified Chlide was being esterified to form Chl. The rate of esterification is faster in the 3-day-old than in the 7-day-old tissue, and the 3-day-old material does not display a lag characteristic of the 7-day-old leaves. These results are in agreement with the findings of others in studies of 5- to 12-day-old etiolated bean leaves (2, 7, 39).

Figure 7 shows the regeneration of Pchl(ide) measured in the

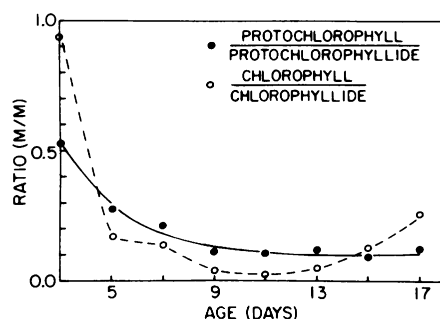

FIG. 5. Ratios of protopigments before phototransformation compared with ratios of Chl pigments after transformation as a function of age of etiolated bean seedlings. Illumination conditions were the same as in Fig. 4.

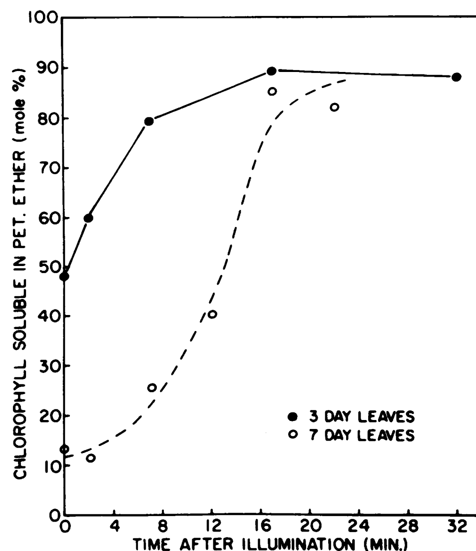

FIG. 6. Kinetics of Chlide *a* esterification after phototransformation in 3-day-old and 7-day-old etiolated bean leaves.

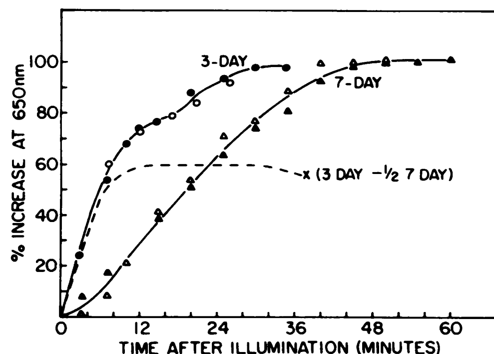

FIG. 7. Kinetics of protopigment regeneration in intact 3-day-old and 7-day-old etiolated bean leaves after phototransformation of initial protopigments. The dashed curve was obtained by subtracting one-half of the value for the 7-day-old leaves from the value for the 3-day-old leaves at each time point.

Table I. *Comparison of Properties of Dark-Grown Euglena 2-3-Day-Old and 7-9-Day-Old Bean Leaves*

Property	Dark-grown *Euglena*	2 to 3-Day Etiolated Bean	7 to 9-Day Etiolated Bean
Structure	Proplastid	Proplastid	Etioplast
Size (μm)	1-2	2-3	~ 4
Prolamellar Body	Small, noncrystalline	Absent, or small noncrystalline	Large, crystalline
Predominant absorption *in vivo*	Pchl(ide)$_{635}$	Pchl(ide)$_{635}$	Pchl(ide)$_{650}$
Total Protopigment (pg/plastid)	~ 1×10^{-4}	~ 0.5×10^{-4}	~ 7-10 $\times 10^{-4}$
Protopigments present	Pchlide and Pchl	Pchlide and Pchl	Predominantly Pchlide
Ratio: moles -ide/moles -yll	~ 3	~ 1	~ 6
First stable photoproduct *in vivo*	Chl(ide)$_{675}$ directly	Chl(ide)$_{675}$ directly	Chlide$_{685}$ Shibata shifting to 675 nm
Pigments produced directly on illumination	Chlide and Chl	Chlide and Chl	Chlide (predominantly)
% Conversion Pchl(ide) to Chl(ide)	~ 10-50%	~ 40%	~ 80-90%
Rate of Chlide esterification	Fast (no lag)	Fast (no lag)	Slow (lag)
Rate of protopigment regeneration	Fast (short lag)	Fast (no lag)	Slow (lag)

intact leaves. The regeneration in the 7-day-old material exhibits a lag followed by increase, while the 3-day-old leaves regenerate protopigment(s) much more rapidly, without a lag. Since Pchl and Pchlide are present in comparable amounts in 3-day-old leaves (Fig. 2) and Pchlide predominates at later developmental stages, it is possible that Pchl regeneration is faster than Pchlide regeneration. If this were so, the curve for regeneration in the 7-day-old leaves should represent almost exclusively the regeneration of Pchlide, while the curve for the 3-day-old leaves should contain the kinetics for regeneration of approximately equal amounts of Pchl and Pchlide. On this assumption, a curve for 7-day-old leaves adjusted to yield 50% of total pigments at each time point was subtracted from the 3-day-old leaf regeneration curve (Fig. 7), yielding a new difference curve. This difference curve shows that what remains after subtracting a Pchlide regeneration curve from the kinetics at 3 days is a curve representing a rapidly regenerating component, presumably Pchl. A study of the rapid regeneration of Pchlide$_{650}$ has appeared (17).

CONCLUSIONS

Our work on the development of bean leaves in the dark was prompted by certain discrepancies between the properties of the Chl-forming system and its structure in *Euglena* and in higher plant material represented by the well studied older etiolated leaves of beans. Earlier work (19) indicated that these discrepancies existed only for the older bean leaves; young bean leaves in the dark resembled dark-grown *Euglena* cells very closely (11, 19, 20, 24, 28). The results in this paper confirm and extend these observations and comparisons. Table I compares all of the properties of young and old bean leaves and *Euglena* cells that we have been able to measure.

The properties of young bean leaves are similar to those of *Euglena* cells, whereas those of 7-day-old leaves are very much different. It has been known for some time, and confirmed in our own work, that the simpler proplastids of *Euglena* and of young leaves are capable of light-induced Chl formation and normal plastid development (1, 4, 14, 15, 33, 34, 36). Thus, a highly elaborate etioplast with a crystalline prolamellar body is not an obligatory intermediate in normal light-induced Chl synthesis or chloroplast development. It might be questioned whether seedlings (or leaf buds) in nature ever develop such an advanced condition of etiolation under normal conditions. Seeds that are not planted too deeply emerge rapidly and green at a stage comparable to the young leaves used in this study (F. Rickson, personal communication). If seeds are planted deeply or find themselves in situations where they cannot emerge into the light for some time, etiolation is carried much further, with concomitant membrane elaboration and storage, resulting in the formation of crystalline prolamellar bodies in enlarged etioplasts containing increased amounts of protopigments . Presumably, these increased reserves permit faster development when light is finally available to permit a rapid development of photosynthesis and, hence, survival when a large amount of the seed reserves may have been depleted. From this point of view, one might consider the properties of *Euglena* and young bean leaves as representative of the more usual or normal mode of development, and those of older etiolated bean leaves as part of the pathology of prolonged etiolation (3, 6, 19).

Note Added in Proof. Robertson and Laetsch (Plant Physiol. 1974, 54: 148-159) also note the normal development of proplastids (rather than etioplasts) to chloroplasts as does von Wettstein (Brookhaven Symp. 1959, 11:138-159).

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