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Rapid Regeneration of Protochlorophyllide$_{650}$[1]

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S. Granick and Merrill Gassman[2]
The Rockefeller University, New York, New York 10021, and Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT

The rate of regeneration of protochlorophyllide$_{650}$ was examined spectrophotometrically after a saturating light flash using 8- to 9-day-old dark-grown bean leaves. The regeneration occurred to the extent of 15% with a half rise time of about 20 seconds. Feeding δ-aminolevulinic acid to the excised leaves in the dark increased protochlorophyllides$_{635}$ but not the absorption at 650 nanometers, suggesting that the holochrome was normally saturated with protochlorophyllide and that the holochrome protein was not controlled by the level of protochlorophyllide. After a light flash, the excess protochlorophyllide, formed from exogenous δ-aminolevulinic acid, readily combined to regenerate the 650 nanometer absorbing species; the regeneration occurred to the extent of 60 to 80% with a half rise time of about 50 seconds. Regeneration was blocked at 0°, suggesting that there was some enzymic process required for regeneration, possibly the formation of a reductant component of the protochlorophyllides$_{650}$ holochrome.

The protochlorophyllide holochrome obtained from dark-grown bean leaves by Smith (13) is a protein containing 2 protochlorophyllide molecules per 550,000 mol wt (9) and a source of reducing power. In the dark-grown bean leaf this complex has an absorbance maximum at 650 nm and is apparently localized in the prolamellar body. Light causes the photoreduction of protochlorophyllide to chlorophyllide in a step which probably represents a one-quantum event (1); thus, photoreduction is accompanied by a decrease in the optical density at 650 nm and the appearance of a new band at 678 nm (4). After a flash of light, the protein may be used to regenerate the 650 nm absorbing complex (3). This regeneration might go by the following reaction in which the apophotoenzyme (E)[3] combines with a reductant (R) and protochlorophyllide (PChld) to form the regenerated PChld$_{650}$ complex,

$$\text{PChld} + \text{R} + \text{E} \rightarrow \text{PChld}_{650}$$

There are several changes in the absorption spectra which occur after the light flash. The photoreduction of PChld$_{650}$ produces Chld$_{678}$ (4); this may represent Chld while still on the photoenzyme. Within the next 30 sec a shift to Chld$_{683}$ occurs concomitant with a decrease in crystallinity of the prolamellar body (8), the dissociation of Chld from the photoenzyme, and its transport to some region where phytylation may occur. During the next 15 to 60 min (12) phytylation occurs accompanied by a shift to Chl$_{673}$ (2, 11), and within several hours the maximum shifts to about 680 nm, this latter shift possibly representing some chlorophyll aggregates.

In this paper we examine primarily the rapid regeneration of the PChld-R-E 650 nm absorbing complex in the dark-grown 8- to 9-day germinated primary bean leaves.

METHODS

Red kidney beans were germinated in vermiculite in the dark at 22° for 8 to 9 days. The two primary leaves, folded together as they are in the seedling, make a unit of four leaf thicknesses. This unit of leaves was mounted inside a cuvette, one side of the leaf unit being against the cuvette wall and held snugly in place by a glass strip and spring clip that pressed against the other side of the leaf unit. A mask with opening of 3 × 6 mm length was mounted against the cuvette between the leaf unit and photomultiplier so that only light that had passed through the leaf unit and mask opening could reach the photomultiplier.

A Cary 14 spectrophotometer was used to scan the leaf unit from 500 to 740 nm and to monitor changes in optical density at 650 nm. In order to prevent PChld to Chld conversion by the 650 nm monitoring beam, the following modifications were made in the apparatus. The standard phototube was replaced by a red-sensitive phototube, Hamamatzu-R136. The voltage used for the tungsten iodine lamp was 30 v; the slit at 650 nm was 0.7 mm with a dispersion of 3 nm; and the dynode was at position 3. The cuvette was mounted as close as possible to the phototube compartment to widen the impinging light beam and to capture more scattered light onto the phototube. This light intensity impinging on an etiolated leaf unit steadily for 30 min did not cause any detectable decrease at 650 nm or increase at 680 nm.

The flash tube for the conversion of PChld$_{650}$ to Chld was a Zeiss Ukatron microflash Xenon arc tube that delivered 60 w sec of energy per flash with a discharge time of about 0.5 msec. One flash of the flash tube, when held adjacent to the cuvette containing the leaf unit, was of sufficient intensity to convert all of the PChld$_{650}$ to Chld. With the aid of an assistant it was possible to flash and then begin reading the absorbance 1.5 to 2.5 sec later; this represents the delay time. The time constant of the spectrophotometer is about 1 sec. The measured absorbancy changes were extrapolated back to flash or zero time. The chart speed was 1 mm/sec.

To obtain leaves with larger amounts of PChld (5), the leaf units were wetted thoroughly with ALA-HCl (1 mg/ml) in 0.01 M phosphate buffer, pH 6.8, and placed in Petri dishes on

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[2] Present address: Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680.

[3] Abbreviations: ALA: δ-aminolevulinic acid; Chl: chlorophyll $_a$; Chld: chlorophyllide $_a$ (*i.e.*, devoid of phytol); PChld: protochlorophyllide $_a$; E: apoprotein, of the holochrome or photoenzyme; R: unknown reductant on the photoenzyme.

filter paper wetted with a slight excess of the solution. All manipulations with the leaves were carried out in low intensity green light insufficient to photoreduce PChld. Absorbancy measurements were made at 22 to 24° unless otherwise noted.

RESULTS

All of $PChld_{650}$ Photoreduced in Leaf by a Flash of Sufficient Intensity. An etiolated leaf unit was flashed three times with intervals of 9 min between the first and second, and 7 min between the second and third flashes (Fig. 1). The decrease of absorption at 650 nm immediately after each flash attained the same low OD value. A flash was of sufficient intensity to bring about the complete photoreduction of $PChld_{650}$. Analyses of the absorption spectra of the leaf unit scanned before flashing (Fig. 2A) and after the three flashes (Fig. 2B) also indicated complete photoreduction of $PChld_{650}$.

The areas under the absorption curves were analyzed approximately into their components by curve fitting, assuming that the components are bell-shaped Gaussian curves. The absorption curves were first corrected for the base line which increased toward shorter wave lengths, by assuming zero absorbancy at 540 and 730 nm. Then the main absorption band at the red end was divided longitudinally at its maximum. Assuming that the band was symmetrical, the long wave length half was mirror-imaged to form the opposite half. To approximate the component bands of $PChld_{650}$ and $PChld_{635}$, it was assumed that the component absorption bands in PChld in ether were displaced to the red equally with the main PChld band as had been done by Shibata (11). In general the absorbancy values of the smaller component bands were found to be higher than expected from the ratios in ether solution.

In Figure 2A the absorption bands of $PChld_{650}$ in the leaves were recognizable as maxima 649, 635, and 595 nm. $PChld_{635}$ components had maxima at 630, 615, and 577 nm. In addition there was a 554 band, possibly a cytochrome. The band width for PChld at 649 nm at half-maximum height was 18 nm.

In Figure 2B the absorption bands of Chl_{672} were recognizable as maxima at 672, 637, 622, and 590 nm. The 637 band may represent both Chl_a and some $PChld_{635}$. The 560 band may belong to a cytochrome. The band width for Chl_{672} at half-maximum height was 40 nm. This is approximately 4 times the band width for Chl_{660} in organic solvents (10). The shoulder represented by the two component bands at 637 and 622 nm was especially prominent and was reminiscent of the two shallow bands in this region for Chl_a in pyridine; this shoulder may also contain contributions from small amounts of $PChld_{635}$. In the leaf, the prominence of the shoulder and width of the Chl_{673} band is compatible with an interpretation that the plane of the Chl molecule may interact with substances lying parallel to its plane (10).

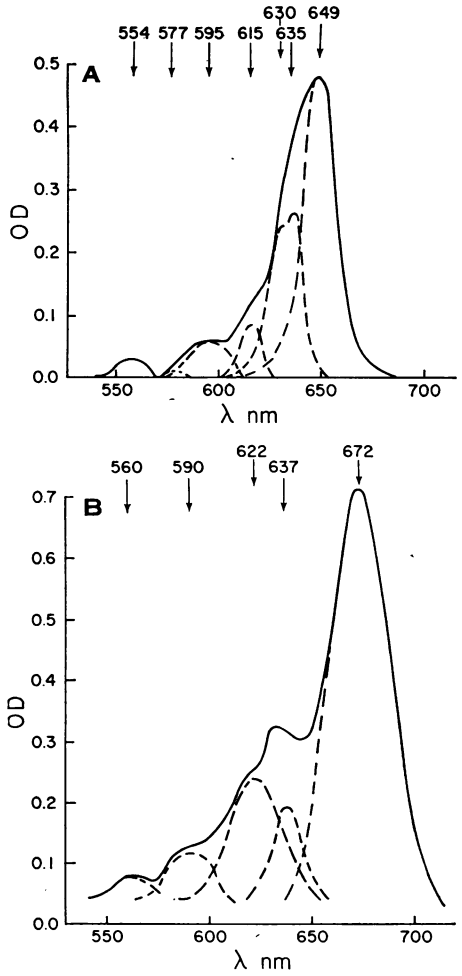

FIG. 2. A: Absorption curve through a control 9-day-old etiolated leaf unit approximately 20 mm in length corrected for scatter and analyzed for Gaussian component curves. According to this analysis, $PChld_{650}$ components have maxima at 649, 635, and 595 nm. $PChld_{635}$ components have maxima at 630, 615, and 577 nm. The 650 nm band width at half-maximum height is 180A. B: Absorption curve of the leaf unit of part A after three saturating light flashes with intervals of 9 and 7 min between the second and third flashes. It is assumed that the shift from 683 to 672 was completed before the leaf absorption was scanned. The component curves of Chl *a* are represented by maxima at 672, 637, 622, and 590 nm. Possibly there is a small contribution of $PChld_{635}$ hidden under the 637 nm band. The 672 band width at half-maximum height is ~400A.

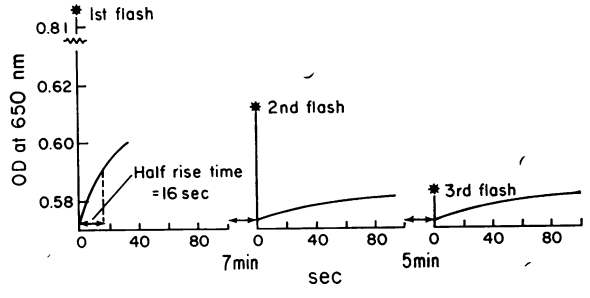

FIG. 1. Decrease in OD at 650 nm after flashes, and recovery profiles in a control leaf. The leaf unit in the cuvette was flashed with a saturating light to convert all $PChld_{650}$ to Chld. The absorbancy at 650 nm after each flash reached the same low OD value. After the first flash the slope at the origin was 0.001 OD unit/sec, and the half rise time was 16 sec. There was a 13% recovery by 100 sec after the flash.

A previous hypothesis that there was a $PChld_{650}$ species which lacked a reductant is incorrect (6). It was based on experiments which did not use a sufficiently intense light flash for photoreduction.

Supporting Evidence That Photoconversion of $PChld_{650}$ is Stoichiometric with Chld Formation. To compare quantitatively the content of PChld pigment before the photoreduction with that of the Chld after the flash, the following procedure was used for extraction and determination. Sixty leaf units of 8-day-old germinated seedlings were extracted in the dark and cold with 100 ml of a solution of acetone-0.1 N $NaHCO_3$ (9:1, v/v) in a blender and filtered. The acetone was removed in vacuum; and with the aid of NaCl the pigments were extracted several times into a total of 100 ml of ether. Sixty other leaf units were flashed once and treated similarly. The absorptions were deter

mined in 5-cm long cells using a 0.1 OD full scale slide wire. The wet weight per leaf unit was 0.101 g and the content of Chld found was 3.84 μg/g, fresh wt. The ratio of the OD values for Chld at 663 nm to that for PChld at 623 nm was 2.5. The theoretical ratio using reported values (1) for EmM of Chl_a to PChl in ether solution, 90.1 at 662 nm to 35.6 at 623 nm is 2.53.

This experiment indicates that the photoconversion was stoichiometric and that a single flash of the flash lamp through four leaf thicknesses was light-saturating. Sundqvist (14) also found, by a pigment extraction procedure, the almost stoichiometric conversion of PChld to Chl in wheat leaves after light flashes and appropriate dark intervals.

To determine spectrophotometrically, on the intact leaves, the absorbance ratio of the Chl generated to the PChld that disappeared by flashing illumination, the following experiment was conducted. An etiolated leaf unit (Fig. 1) was flashed three times with intervals of 9 and 7 min between the flashes. In the leaf unit the OD ratio of the 672 nm band that appeared after flashing (Fig. 2B) to that of the 649 nm band that disappeared (Fig. 2A) was 1.5. This ratio is low compared to that of 2.5 obtained by pigment extraction into ether. In the leaf the height of the Chl band was only 60% of that which might have been expected if the ratio were 2.5. The low ratio of 1.5 and the 40 nm band spread of the Chl_{672} at half-maximum height may represent chlorophylls in physically different states with respect to their immediate surrounds. After a flash, not only is $PChld_{650}$ reduced to Chld but this photostep is accompanied by a distortion of the prolamellar body (8) in which the photoreaction occurs, followed more slowly by phytylation of Chld to Chl_{672}. The low OD ratio and broad spread of the Chl_{672} band may represent chlorophyll molecules in various stages of becoming organized in the thylakoid membranes. In the flashed leaf, even though the concentration of Chl is only $\frac{1}{100}$ that of the adult green leaf, it is conceivable that Chl aggregations into monolayer islands can already occur.

When experiments were made at 0 to 4° to block conversion of $Chld_{683}$ to $Chld_{672}$, it was found that the band widths at half-maximum height were 160A for $PChld_{650}$ and 240A for the $Chld_{683}$ which arose on photoreduction. These spreads of band width are much narrower than the 400A spread found for Chl_{672}.

Photoenzyme Content of Leaf Not Affected by Feeding ALA. When an etiolated control leaf unit was irradiated with a flash of light sufficient to convert all $PChld_{650}$ to Chld, the decrease in absorbance varied between 0.15 and 0.25 OD unit with an average of 0.17 ± 0.02 unit (Table I).

When etiolated leaves were treated with ALA for 18 hr at 22°,

Table I. *Characteristics of Regeneration of $PChld_{650}$ in 8- to 9-Day Etiolated Leaf Units*

Values are means for eight experiments. For details about treatment, see "Methods."

Treatment	Temp	Decrease in OD at 650 nm after First Flash	Slope at Origin per 10^4 sec	Half Rise Time	Regeneration after First Flash
		OD units	*OD units*	*sec*	%
Control	22–24°	0.17 ± 0.02	9 ± 3	22 ± 6	17 ± 6
	0–2°	0.20	5	12	3
	6–8°	0.18	7	17	9
ALA, 5 hr	22–24°	0.19	13	49	64
	22–24°	0.16	13	52	60
ALA, 18 hr	22–24°	0.12	16	52	83
	0°	0.19	0	∞	0
	4–6°	0.16	5	18	24

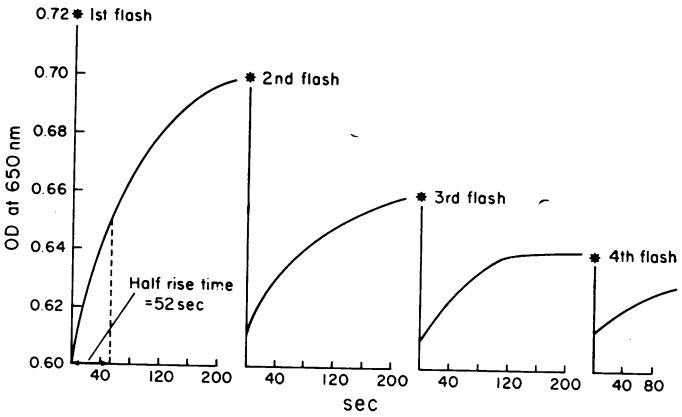

FIG. 3. Decreases in OD at 650 nm after flashes and recovery profiles in a leaf fed ALA. An etiolated leaf unit from an 8-day seedling was fed ALA for 18 hr in the dark. The leaf unit was flashed with a saturating light that converted all $PChld_{650}$ to Chld. The absorbancy at 650 nm after each flash reached approximately the same low OD value. After the first flash the slope at the origin was 0.0015 OD unit/sec, the half rise time 52 sec, and the regeneration was 83% during the next 4 min. There were 5-min intervals between the successive flashes.

there was as much as a 10-fold increase in PChld, as indicated by the height of the *in vivo* absorption band at 635 nm, and the leaf was distinctly pale green due to its high content of $PChld_{635}$. After a flash of light the decrease in OD at 650 nm was not greater than that found for control leaf units (Table I and Fig. 3). Similarly, leaves treated with ALA for 5 hr did not have more photoconvertible $PChld_{650}$ than control leaves.

The decrease in OD at 650 nm on illumination is a measure of the concentration of complex PChld-R-E and therefore of the photoenzyme E. In the presence of excess PChld, generated by feeding ALA, there was no increase in the photoconvertible complex. It is inferred from this experiment that in the control leaf, the photoenzyme is already saturated with PChld because the addition of more PChld does not increase the photoconvertible 650 complex. It may also be inferred that an increase in unbound PChld does not induce an increase in the concentration of the photoenzyme. That is, the formation of photoenzyme is not controlled by the level of unbound PChld. In contrast, in hemoglobin formation it has been shown that heme controls the formation of globin and ALA controls the formation of heme (7).

Source of PChld for $PChld_{650}$ Regeneration. In the etiolated leaf fed ALA for 6 to 20 hr a large excess of $PChld_{635}$ is formed. After 6 hr, Sundqvist (14) showed by extraction of the leaf pigments that about two-thirds of the total PChld could be converted almost stoichiometrically to Chld during an hour by a series of flashes at 14-min intervals. Because most of the PChld in an ALA-fed leaf was in the form of $PChld_{635}$, the Chld must have originated from this stored $PChld_{635}$.

We have observed spectrophotometrically that in ALA-fed leaves exposed to a saturating light flash, all the $PChld_{650}$ was converted to Chld. Within a period of several minutes in the dark, $PChld_{650}$ was regenerated. However, no concomitant decrease in $PChld_{635}$ was readily apparent spectrophotometrically.

How may one explain this lack of correlation between changes determined by pigment isolation from the leaf and spectrophotometric determinations directly on the leaf? One explanation may be that in addition to compensatory absorption changes, $PChld_{635}$ in the leaf is in a polymeric state; a small loss of pigment from the polymer may then not be detectable. If Sundqvist's data are not considered, one would have to postulate that during regeneration of $PChld_{650}$ in the dark, the PChld moiety would have to arise *de novo*.

Sundqvist (14) observed that the regeneration of $PChld_{650}$

could occur with a half rise time of about 3 min; the determinations were made on the extracted pigments. We have studied the rate of regeneration of the 650 nm absorbing species in the intact leaf unit after a saturating light flash, by observing the changes with time in the absorbancy at 650 nm. After a saturating light flash, there was an instantaneous decrease in OD at 650 nm, and a rapid increase, *i.e.*, regeneration, over a period of 200 to 250 sec (Fig. 3). The half rise time for regeneration was about 50 sec and the slope at the origin was about 0.0015 OD unit/sec. The amount of $PChld_{650}$ regenerated was 83% after the first flash, 42% after the second flash, and 26% after the third flash; there were 5-min intervals between the flashes.

One interpretation of this experiment is that $PChld_{635}$, formed from exogenous ALA, may be used to regenerate the photoactive $PChld_{650}$ species. The percentage regeneration of the 650 species decreased after successive flashes, because either $PChld_{635}$ or reductant became limiting in the neighborhood of the photoenzyme. The concentration of the photoenzyme (*i.e.*, the apoenzyme) does not appear to be rate-limiting in Chld formation.

It might have been expected that leaves given exogenous ALA to form excess PChld would produce Chl in the light more rapidly and in great excess compared to control leaves; this does not happen. In constant illumination greater than a few foot-candles, excess PChld tends to be irreversibly bleached (6, 14). An additional reason may be that excess PChld may act as a feedback inhibitor to prevent the leaf from making more ALA. Another possibility that cannot as yet be ruled out is that the reductant might limit Chld formation.

Slight Excess of PChld in Etiolated Leaf, Which May Be Used for Regeneration. In the etiolated leaf, there was a decrease in OD at 650 nm after a flash, but within 100 sec there was a regeneration of 12% (Fig. 1). The half rise time was 16 sec for the regeneration, and the slope at the origin was about 0.0013 OD unit/sec. During the next 7 min in the dark the OD had risen slightly, so that by 9 min after the first flash the regeneration was 16%. After the second and third flashes, regenerations were 3% each. Immediately after each flash the OD value came to the same low value.

These results indicate that the etiolated leaf from a seedling germinated for 9 days contains sufficient PChld so that after a flash, some 12% of photoactive $PChld_{650}$ can be regenerated within 100 sec, probably from some reserve $PChld_{635}$. The presence of this reserve PChld was suggested by the absorption curve of the etiolated leaf (Fig. 2A) with component curves at 630, 615, and 577 nm which may represent the absorption bands of $PChld_{635}$.

Regeneration of $PChld_{650}$ Requires Several Minutes. After the first flash and decrease in OD at 650 nm, there occurred an increase which in untreated leaves was approximately 15% and which took place with a half rise time of ~20 sec. In leaves treated with ALA for 5 to 18 hr the increase in 650 nm absorbancy was 60 to 83% and occurred with a half rise time of ~50 sec.

These increases at 650 nm represent regeneration of $PChl_{650}$ and are not caused by other changes. For example, the spectral shift of Chl from 678 to 683 nm occurs in 30 sec at room temperature (4); if it had affected the 650 nm absorbancy region, the low OD readings at 650 nm immediately after each flash would not have been identical (Figs. 1, 3). Another shift in absorption is that of $Chld_{683}$ to Chl_{673}; this is a relatively slow shift requiring 15 to 60 min and would be too slow to affect the more rapid changes in OD involved in $PChld_{650}$ regeneration studied here.

After a flash, one of the slower steps in the regeneration process is probably the release of Chld from the photoenzyme. This reasoning is analogous to that for other enzyme reactions in which diffusion of substrate to enzyme is very rapid compared to the release of product. In ALA-treated leaves, *i.e.*, containing excess PChld, the regeneration was only slightly more rapid (slope at origin, 13 to 16 $\times 10^{-4}$ OD unit/sec) than in untreated leaves (slope at origin, $9 \pm 3 \times 10^{-4}$ OD unit/sec), suggesting that the rate of regeneration was not controlled primarily by the rate of movement of $PChld_{635}$ to the photoenzyme.

For regeneration of the photoactive 650 absorbing species, in addition to $PChld_{635}$, an unknown reductant is required. This reductant may attach directly to the photoenzyme, or it may act to reduce some group on the photoenzyme. In ALA-treated leaves the greater percentage regeneration, *i.e.*, 60 to 80% compared to 17 ± 6% for control leaves, and the longer rise time, *i.e.*, 50 sec as compared to 22 ± 6 sec for control leaves, suggest that the reductant may be rate-limiting, at least in the presence of a large excess of PChld.

Regeneration of 650 nm Absorbancy Decreased at Temperatures Near 0°. To differentiate between reactions limited by diffusion and by enzymic reactions, in the regeneration of $PChld_{650}$, the leaves were exposed to lowered temperatures in the cell compartment, the temperature being monitored by a thermistor mounted in an adjacent cuvette.

When leaves were treated with ALA for 18 hr, brought to 0°, and given a first flash of light, there was no regeneration of the 650 nm absorbing species (Table I). At 4 to 6° the regeneration was 24%, and at 22° it was 83%. For control leaves the regeneration was 3% at 0 to 2° as compared to 17% at 22°; the latter result is in agreement with that of Virgin (15), who reported no PChld regeneration at 0°. Lowering the temperature to 0° did not affect the photoconversion of $PChld_{650}$ to Chld in the first flash, in agreement with the findings of Smith (13).

When the temperature is lowered, enzyme-limited reactions are decreased much more than diffusion-limited reactions. The marked decrease in regeneration of $PChld_{650}$ at lowered temperatures suggests that an enzyme reaction is involved. Perhaps the enzyme reaction which becomes rate-limiting is the one that forms the reductant. On the other hand, if one assumes that $PChld_{650}$ can only be regenerated from PChld that is being synthesized *de novo* rather than from stored $PChld_{635}$, then it is the enzyme reactions that form PChld that will become rate-limiting at low temperatures.

CONCLUSIONS

The events in rapid $PChld_{650}$ regeneration may be placed in context by summarizing the major steps and time periods in Chl synthesis in the primary bean leaves (Fig. 4).

By the 8th to 9th day of germination in the dark the primary

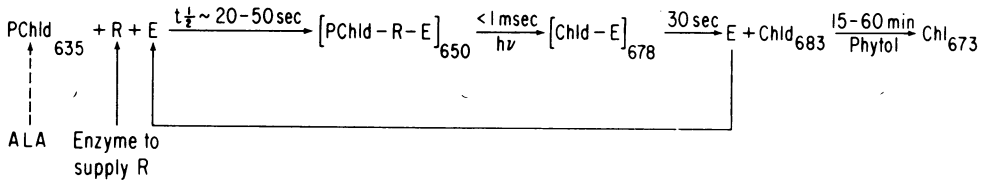

FIG. 4. Sequence of events and time periods in chlorophyll synthesis from protochlorophyllide. Abbreviations: ALA: δ-aminolevulinic acid; PChld: protochlorophyllide; R: reductant; E: photoenzyme; Chld: chlorophyllide; Chl: chlorophyll.

leaves have synthesized enough PChld to saturate the photoenzyme molecules completely and even to form a slight excess (15%) of PChld. When ALA is fed to the leaves, $PChld_{635}$ can accumulate in 18 hr to the extent of over 10 times that which is required to saturate the photoenzyme. Thus enzymes converting ALA to PChld remain active, but the endogenous formation of ALA is greatly diminished by the 8th day. Whether this diminution in ALA synthesis is by feedback inhibition of $PChld_{635}$ is conjectural.

The formation of $PChld_{650}$ requires that the apophotoenzyme combine with $PChld_{635}$ (either present in excess or newly formed) and reductant. After a saturating light flash, the absorption maximum shifts immediately to 678 nm; this shift is interpreted as a photoreduction of PChld of the complex, to Chld. During the next 30 sec the absorption maximum shifts to 683; this shift is accompanied by an incipient decrease in crystallinity of the prolamellar body, a dissociation of the Chld from the photoenzyme, and a migration of the Chld to some region where it will be phytylated. The phytylation occurs during the next 15 to 60 min, when the formation of Chl is accompanied by a shift in the absorption maximum to 673 nm.

After a flash of light, the photoenzyme is regenerated with a half-time of 20 sec to the extent of 15% in the normal leaf, and with a half-time of 50 sec to the extent of 60 to 80% in ALA-fed leaves. Studies of the regeneration of $PChld_{650}$ near 0° suggest that a slow step at this temperature may be an enzymic reaction, possibly one that provides the reductant.

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