Studies on the Regeneration of Protochlorophyllide after Brief Illumination of Etiolated Bean Leaves

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Summary. The effects of various inhibitors of nucleic acid and protein synthesis on protochlorophyllide synthesis in dark-grown Phaseolus vulgaris var. Red Kidney have been studied. Actinomycin D, chloramphenicol, and puromycin inhibit the regeneration of protochlorophyllide holochrome (detected as a 650 mμ absorption peak) in vivo in darkness after photoconversion of endogenous protochlorophyllide a to chlorophyllide a; this inhibition does not occur in similarly treated leaves supplied with δ-aminolevulinic acid.

These data suggest that the regeneration of protochlorophyllide results from the synthesis of RNA and enzymes required for the production of δ-aminolevulinate.

The protochlorophyllide content of etiolated leaves becomes constant with increasing age of tissue (5) despite the fact that such tissues can produce additional pigment when provided with ALA (2). Is the cessation of protochlorophyllide production a consequence of feed-back inhibition of an enzyme of ALA synthesis by, for example, protochlorophyllide (6), or is such an enzyme lacking?

Soon after the protochlorophyllide of an etiolated leaf is photoconverted to chlorophyllide, a small amount of additional pigment is formed (fig 1a). If plants are illuminated only briefly, the maximum amount of new pigment subsequently produced in darkness is about equal to the protochlorophyllide present before irradiation. This phenomenon has been studied most thoroughly by Madsen (4). He reports rapid accumulation to the initial level of protochlorophyllide within 30 minutes following exposure of etiolated wheat to a 1/1000 second light flash; synthesis begins after a 5 minute lag and the rate declines to 0 within an hour. These results plus those of Virgin (8,9) suggest that chlorophyll formation proceeds through protochlorophyllide synthesis during continuous illumination and the availability of precursors of protochlorophyllide is rate limiting. In turn, the production of protochlorophyllide is restricted by the level of ALA (2).

Gassman and Bogorad (1) report that the inhibition of chlorophyll synthesis in excised bean leaves by chloramphenicol can be partially overcome if 10 mμ δ-aminolevulinic acid (ALA) is supplied under dim red illumination. They suggest that enzyme(s) of ALA synthesis are labile and require light for continuous production and maintenance at an effective level. The experiments reported here examine the effect of actinomycin D, chloramphenicol, and puromycin on the resynthesis of transformable protochlorophyllide in etiolated bean leaves and the effect of ALA on pigment formation in the presence of these inhibitors.

Materials and Methods

Plant materials were grown and prepared as described previously (1). Illumination was provided by a tungsten lamp; light intensity was measured with a Weston illumination meter. Inhibitors and ALA were prepared as described (1).

In vivo spectrophotometry was performed on 10 leaf halves in a Cary Recording Spectrophotometer Model 11 using the opal glass method of Shibata (7).

Results

Leaf-halves of 7-day old etiolated red kidney bean plants placed in petri plates with or without additions were illuminated for 1 minute at 1000 ft-c and returned to darkness for 4 hours at 25° (fig 1a). The initial restoration of protochlorophyllide, seen in vivo as increased absorption at 650 mμ, does not occur or is severely inhibited in leaves treated with 5 mμ chloramphenicol (CAM).
or 1 mM puromycin for 4 hours or with actinomycin D (90 μg/ml) for 17 hours prior to illumination (fig 1b, c, d). These drugs affect neither the spectral shift from 650 to 684 mμ following a 1 minute illumination of etiolated bean leaves, i.e. the conversion of protochlorophyllide to chlorophyllide, nor the subsequent dark shift of the absorption maximum from 684 to 673 mμ. Thus,

Fig. 1. In vivo determination of protochlorophyllide regeneration in etiolated leaves. Leaves irradiated for 1 minute, returned to darkness for 4 hours, and the spectra determined. a) control b) 5 mM CAM added 4 hours prior to the irradiation c) 1 mM puromycin added 4 hours before the irradiation d) 90 μg/ml actinomycin D added 16 hours prior to the irradiation e) 10 mM ALA added immediately after the irradiation f) as in b) except 10 mM ALA added after the irradiation.

Fig. 2. As in figure 1 but following a 1 minute irradiation; i.e. 1' lt. → 4 hours dark → 1' lt.

these inhibitors appear to block the first observable restoration of protochlorophyllide but do not interfere with the normal light-triggered changes in the protochlorophyllide already present.

Preincubation of leaves with 50 μg/ml of cycloheximide (Actidione) for 16 hours had no effect upon the regeneration of the peak at 650 mμ in vivo. This antibiotic will, however, inhibit massive chlorophyll accumulation in these leaves during continuous illumination at concentrations as low as 1 μg/ml (cf. ref. 3 with Euglena).

Etiolated leaves treated with CAM produce protochlorophyllide if supplied 10 mM ALA (fig 1f). Upon illumination (fig 2f) some of this
pigment is transformed to chlorophyllide. Similar results were obtained with actinomycin D and with puromycin. If these inhibitors are preventing synthesis of the protein moiety of protochlorophyllide holochrome, these data suggest the holochrome can be reused, or is in excess, and acts as an enzyme for chlorophyllide synthesis. Large amounts of inactive protochlorophyllide (632 m\(\mu\) peak) are also evident in figure 1e.

**Discussion**

When etiolated leaves are illuminated, their protochlorophyllide is converted to chlorophyllide and a series of spectral changes occur. These phenomena are not affected by treating etiolated leaves with chloramphenicol. On the other hand, the rapid but limited regeneration of protochlorophyllide in briefly illuminated leaves fails to occur in bean leaves treated with this antibiotic. This observation, plus Madsen's report (4) that pigment renewal in barley leaves begins only after a delay of 5 minutes, argues against the possibility (e.g.) that protochlorophyllide holochrome is a masked ALA synthetase whose action is regulated via feed-back inhibition by protochlorophyllide; de novo synthesis of an enzyme required for some stage of ALA-synthesis appears to be required. In view of the observations (1) on the relatively rapid cessation of pigment production observed when bean leaves are placed in darkness or exposed to various inhibitors of protein and RNA synthesis during rapid greening (Stage III, ref. 1), the arrest of the protochlorophyllide resynthesis which occurs shortly after brief illumination is easy to rationalize. The difficulty of explaining the lag phase in continuously illuminated leaves remains but may be related to endogenous pool sizes.

If the effect of inhibitors of protein synthesis on greening were primarily or solely on the production of holochrome protein as proposed by Kirk and Allen (3), the accumulation in darkness of inactive protochlorophyllide (absorbing at about 632 m\(\mu\) in vivo) by CAM or puromycin-treated leaves should have been observed; it was not (fig 1b, c). Therefore, it appears that the limitation in the production of protochlorophyllide by etiolated leaves is due to a lack of precursor, specifically ALA, and not to a lack of deposition or transformation sites.

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**Literature Cited**


