REGENERATION OF PROTOCHLOROPHYLL IN DARK GROWN SEEDLINGS FOLLOWING ILLUMINATION WITH RED AND FAR RED LIGHT

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SUMMARY

The rates of regeneration of protochlorophyll (Pchl) in leaves of dark grown seedlings of bean, pea and maize were studied, following a saturating dose of either red alone, or red followed by far red. In neither material have we found evidence for red – far red control of the initial rates of pigment regeneration. In older maize leaves, an effect was observed but only upon the final level of Pchl, reached in prolonged darkness, indicating that in this plant the rate of pigment production decreases less rapidly during the last stages of regeneration after terminal red than after far red. There is considerable pigment photobleaching in red light. It is concluded that the effect of a red pre-illumination upon the kinetics of the greening process in subsequent continuous light, is not due to a red – far red control of Pchl regeneration as such, but to a latent formative process, becoming manifest during the earlier part of a subsequent prolonged light period.

1. INTRODUCTION

Chlorophyll a (Chl a) in plants is supposed to be formed via delta-aminolevulinic acid and a series of tetrapyrrole precursors (*e.g.* BOGORAD 1960). In Angiosperms, light is supposed to be required for the transformation of the precursor protochlorophyllide to chlorophyllide a. All other steps appear to be light independent and seedlings reared in the dark accumulate protochlorophyllide as the temporary end product. The concentration of this pigment in dark grown plants remains far below the level of Chl a, ultimately reached under continuous illumination. Build-up of a relatively low level of protochlorophyllide in the plant evidently has a repressing effect upon an early step in the biosynthesis of this compound, since no other intermediates have been reported to accumulate in normal plants. A brief illumination of dark grown material results in transformation of the protochlorophyllide to chlorophyllide a and subsequent transfer to darkness is followed by formation of additional protochlorophyllide. Several reports in the literature indicate that the initial rate of Chl a formation during a second illumination is strongly dependent upon the light quality, given during the first light treatment of the dark grown material. (PRICE & KLEIN 1961; VIRGIN 1961; MITRAKOS 1961; HENSHALL & GOODWIN 1964). Usually, in these experiments the dark grown plants were pretreated either

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with red alone, or with red followed by far red. After a dark period, the rate of Chl a formation in continuous light was measured. In a number of cases, far red given after red was reported to slow down the initial rate of Chl a formation during an illumination, given some hours after the first (WITHROW *et al.* 1956; VIRGIN 1958; see also above). This manifests itself mainly in the duration of the lag phase in the formation of Chl a in continuous illumination: the rate of production of this pigment shows an induction phase, the length of which depends upon pretreatment. The action spectrum for the elimination of the induction phase by a preceding short illumination has a peak at about 660 nm (VIRGIN 1961). At the completion of the lag phase, the subsequent steady state rate of pigment formation appears to be independent of the pretreatment. These observations might suggest that phytochrome is involved in regeneration of Pchl. During a study of phytochrome dark reactions after illumination of etiolated pea seedlings (SPRUIT 1967), we became interested in the rates of Pchl regeneration in this material after a saturating irradiation with either red or red followed by far red. Both direct spectroscopic evidence and the results of some preliminary *in vitro* Pchl estimations indicated that in this material the rates of Pchl regeneration were not significantly different for these two light treatments. Since at first sight this seems to form a contrast with the findings, quoted above, we undertook a more extensive study. Since pea, because of the small size of the leaves of seedlings, grown in darkness, as well as the rather low pigment content per unit weight, is not a particularly suitable material, we have included in our study the leaves of dark grown bean seedlings, which are considerably larger and especially rich in Pchl. Maize was chosen as a representative from a more distant taxonomic group.

In this article, no distinction will be made between phytylated and non-phytylated forms of the pigments, since this did not appear to be relevant in the present context. Therefore, the terms protochlorophyll and protochlorophyllide have been used promiscuously for the pigment(s) having an absorption maximum at about 628 nm in 80% acetone. Likewise, "chlorophyll a" denotes the pigment mixture, absorbing at 665 nm. We intend to discuss the fate of these various phytylated and non-phytylated pigment forms, under the conditions of our present experiments, in a separate paper. It may be stated here, however, that we have invariably found a fraction of our protochlorophyll to be non-transformable by light (SHIBATA 1957), and that this fraction appears to represent a phytylated form of the pigment (SIRONVAL *et al.* 1965).

2. MATERIALS AND METHODS

2.1. Plant material

Seedlings of *Pisum sativum* cv. "Krombek", *Phaseolus vulgaris* cv. "Widusa" and *Zea mays* cv. "Caldera" were reared in total darkness at 20°C and about 85% rel. hum. Seeds were sown in pasteurized soil. As far as necessary, water was added in the dark during the growth period. The leaf material used in the experiments consisted of the "plumules" of the pea (third and fourth unex-

panded internodes with the attached leaf material), the primary leaf pair of the bean and the primary leaves or the whole coleoptiles of maize. All harvesting was done in absolute darkness.

2.2. Irradiation

The phototransformation of Pchl was effected with spectral bands isolated from the light of a Leitz "Prado" 500 W slide projector by means of interference filters (Balzers, Liechtenstein, type Filtraflex B 40). The intensity at the samples was about 1.4×10^4 erg/cm^2 sec at 529 nm, 2.2×10^4 erg/cm^2 sec at 650 nm and 2.9×10^4 erg/cm^2 sec at 735 nm. All radiations were given for 5 minutes.

2.3. Safelights

During subsequent handling of the samples, a little light was found unavoidable. We used a weak green safelight, consisting of a green monophosphor fluorescent tube (Philips TL 40, colour 17), wrapped in a layer of blue "Cinemoid" nr. 62 (The Strand Electric Corp.) and a layer of orange-yellow "Cinemoid" nr. 46. Alternatively, a safelight was used, consisting of a 25 W incandescent lamp, the light of which was filtered through 3 mm blue "Plexiglass" (Röhm und Haas) nr. 0248 plus one layer of "Cinemoid" nr. 46. Precautions were taken that the light from these sources did not reach the samples directly.

2.4. Protochlorophyll regeneration

The irradiated samples were placed in petri dishes the lids of which were lined with 4 layers of moist filter paper. These were put in light-tight tins in a thermostat at 25 °C for the required periods.

2.5. Pigment estimations

One gram samples of the leaves were weighed as quickly as possible on a "Centogram" balance (Ohaus Scale Corp. model 311) to the nearest 0.01 gram. After the appropriate treatment, samples were extracted by grinding in a small mortar together with 12 ml pure acetone, a little washed sand, and a small amount of calcium carbonate. The liquid was removed by filtration over a glass filter with suction. The residue on the filter was again extracted with about 7 ml 80% acetone-water. Vessels and filter were washed with a few milliliters of the same solvent. The combined extracts were then centrifuged for 30 minutes at 60.000 g and 2 °C, and the clear supernatant transferred to a 25 ml volumetric flask and made up to volume with 80% acetone. For storage periods up to a few hours, the flasks were kept in the dark at 2 °C.

The optical density of the extracts was measured throughout the region 600 to 700 nm as well as at 800 nm in a Beckman spectrophotometer model DU, using 5 cm path length cells. If necessary, the optical density readings at 800 nm were subtracted from the readings at the other wavelengths as a correction for a trace of scatter. This correction rarely amounted to more than 0.01 O.D. units.

2.6. Calculation of pigment concentrations

We have adopted the molar absorption coefficients used by BOARDMAN (1962). The wavelength calibration of the spectrophotometer was checked during each measurement. This is a necessary precaution, since the calculated values are rather sensitive to small wavelength errors, and the spectrometer calibrations proved somewhat temperature dependent.

3. RESULTS

3.1. Protochlorophyll regeneration

Fig. 1 gives the concentrations of total Pchl in bean leaves as a function of the dark period following a saturating red or red - far red irradiation. The rate of Pchl regeneration proved strongly dependent upon the age of the material, confirming the observations of AKOYUNOGLOU & SIEGELMAN (1968). In young seedlings, the plateau ultimately reached appears to approach the level before illumination. During regeneration periods up to 24 hours, no significant effect of a terminal far red irradiation upon either the initial rate or the final level of Pchl was found.

Pea leaves present a somewhat different picture (*fig. 2*). Regeneration never reached more than 50% of the original Pchl level. Because of the small amounts of pigment formed, the values scatter considerably. Nevertheless, within the limits of accuracy, we observed no difference in the rate of Pchl regeneration

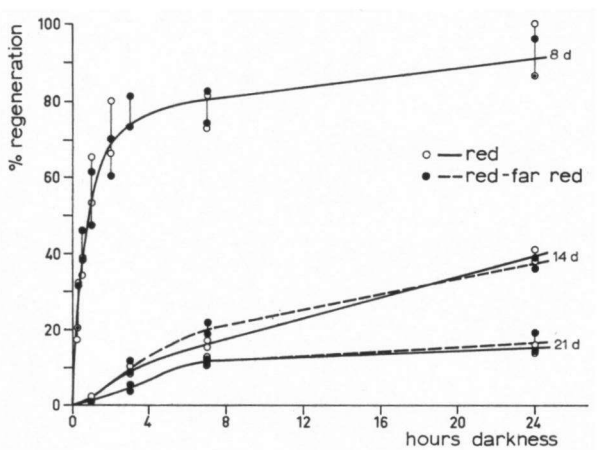

Fig. 1. Regeneration of protochlorophyll in bean leaves of varying age, during a dark period following 5 min. red or 5 min. red plus 5 min. far red. Results expressed as per cent of the amounts of phototransformed protochlorophyll. The 100% level represents the following absolute amounts of pigment per gram fresh weight: 8 day old seedlings, ± 12 μg; 14 days, ± 23 μg; 21 days, ± 23 μg.

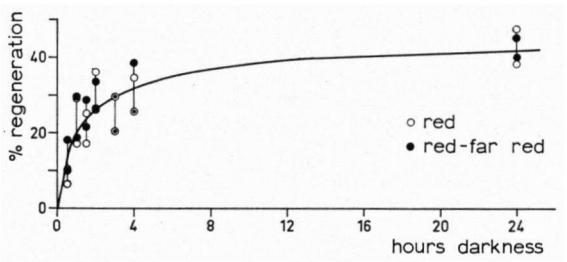

Fig. 2. Regeneration of protochlorophyll in leaves of 7 day old pea seedlings. See also legend to fig. 1. The 100% level represents a pigment regeneration of ± 3.5 μg per gram fresh weight.

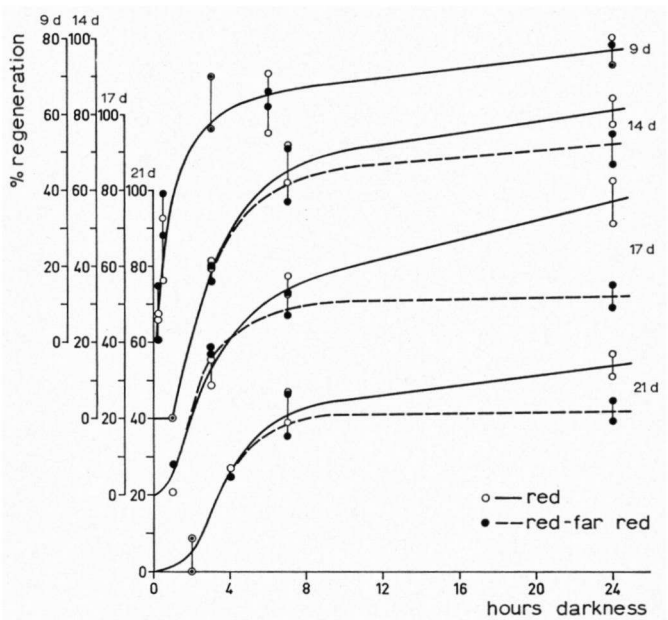

Fig. 3. Regeneration of protochlorophyll in 9 day old maize coleoptiles and in leaves of various ages. To facilitate the reading, the curves for the successive ages are shifted along the vertical axis. See further legend to fig. 1.
The 100% level represents the following pigment regenerations per gram fresh weight: 9 days, ± 3.5 μg; 14 days, ± 8 μg; 17 days, ± 8 μg; 21 days, ± 7 μg.

after red or red followed by far red in pea also. In seedlings between 5 and 14 days old, regeneration uniformly stopped at a low level.

In maize of various ages (*fig. 3*), regeneration was fairly rapid, leading to a final level of about 80% of the original value in 9 day old coleoptiles. During the period of rapid increase in Pchl, no significant differences between red and red-far red could be found. Upon longer dark incubation, a difference between

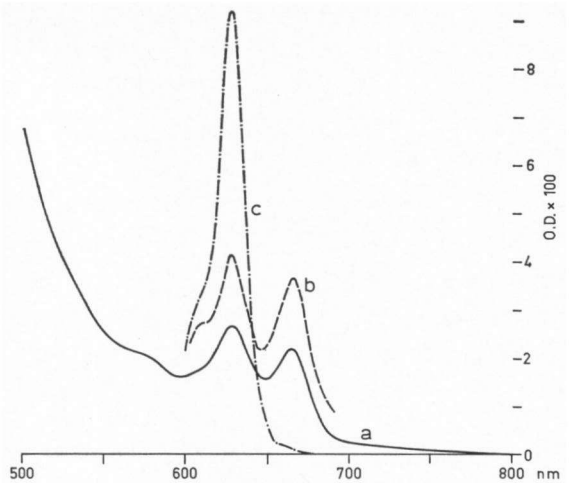

Fig. 4. Absorption spectra of extracts of dark grown pea and bean in 80% acetone.
a. About 100 g whole pea seedlings. Absorption cell 1 cm.
b. 1 g pea leaves. Absorption cell 5 cm.
c. 4.2 g bean leaves. Absorption cell 1 cm.

the two light treatments becomes apparent which is most marked in leaves of about 17 days. The difference in final level, reached in this way, is statistically significant, and amounts to about 25% under optimum conditions. It shows that far red following red decreases the final level of Pchl reached in darkness. In coleoptiles, this effect could not be demonstrated, whereas in leaves, older than 17 days, the magnitude of the effect decreased.

3.2. Chlorophyll in dark grown leaves

We have confirmed the earlier observation (SPRUIT 1966) that leaves of pea, grown in complete darkness, contain a substance spectroscopically similar to Chl a in significant amounts, *fig. 4*. The concentration amounts to roughly 25% of the Pchl present, but there are considerable variations between individual batches.

In most experiments, peas were soaked in water for a few hours before sowing. During this treatment as well as during sowing, they were exposed to diffuse daylight. To check whether this treatment was responsible for the "chlorophyll" found in the seedlings, control batches were imbibed in absolute darkness and sown under our green safelight. The leaf material likewise contained the pigment in about the same proportion as in the material treated in the usual way, *fig. 4*. Exposure of the seeds to light during the imbibition period therefore does not appear responsible for the "chlorophyll" found in the dark grown seedlings.

Trace amounts of a chlorophyll-like substance could be observed in the dark controls of our bean and maize experiments, but the quantities were very small

and of doubtfull significance. Evidently, pea occupies a different position in this respect. It appears at least possible, that the pigment in the leaves of this plant originates in the green cotyledons and is transported to the embryo during the early stages of development.

3.3. Yield of protochlorophyll photoconversion

Obviously, one mole of Pchl converted by light should give one mole of Chl a. We have always estimated both the quantity of Chl a formed and of Pchl converted. After red irradiation, the ratio of these two quantities is lower than one, *fig. 5*. As each of the points in this figure is derived from a separate sample, the points show some scatter. It is evident that a fraction of the pigment is lost during illumination with red light, less so with green. At 0° a relatively higher pigment loss was observed than at 25°. Most likely, this temperature effect is

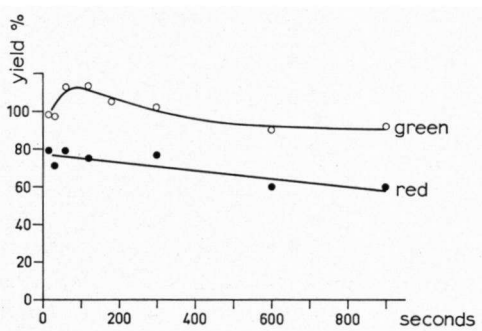

Fig. 5. Yield of protochlorophyll photoconversion in primary leaves of 8 day old bean leaves at 0° in green and red light as a function of the duration of irradiation.

due to Pchl regeneration during the illumination at 25°. Similar yields for the photoconversion of Pchl were reported by SMITH (1948). GOEDHEER (1961) also observed photobleaching of initially formed Chl a. We have attempted to measure this pigment loss by spectrophotometry *in vivo*. *Fig. 6* gives an example of a difference spectrum for bean leaves at 0°. The leaves were preirradiated at this temperature with green light for 2 min. followed by one min. red. The spectrum gives the changes in optical density, caused by an additional dose of 9 min. red. It must be stated here that the absorption changes can be very complex and it appears that several pigments or pigment forms may be involved. At any rate, it is likely that the pigment loss we have observed is due to photobleaching of a form of Chl a absorbing at about 685 nm.

4. DISCUSSION

Our results have failed to show significant effects of the phytochrome system upon Pchl regeneration during the first hours of darkness, confirming earlier observations (SPRUIT 1967). A similar result was obtained by JACQUES (1968) with oat seedlings.

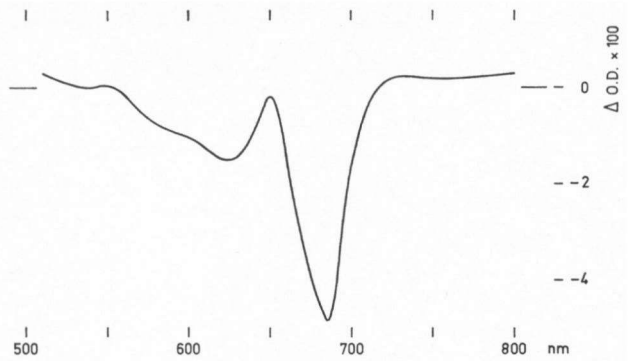

Fig. 6. Difference spectrum of primary leaves of dark grown bean. The material was preilluminated for 2 min. at 529 nm plus 1 min. at 653 nm. The difference spectrum represent the changes during an additional illumination for 9 min. at 653 nm. All treatments at 0°.

In young material, there is little or no evidence for an induction period in the regeneration and the rate of Pchl formation is essentially constant during the first hour following the irradiation. In older leaves of maize and possibly also of bean, there seems to be an induction in Pchl regeneration lasting a few hours.

In maize only, a red-far red effect could be observed after longer dark periods. This led to a final pigment level after 24 hours darkness, that is depressed by a preceding terminal far red illumination, *fig. 3*. We must conclude that the initial rate of Pchl regeneration following a brief photoperiod is not itself controlled by the red-far red mechanism.

It is not immediately clear how these findings fit in with those, demonstrating red-far red control of Chl a formation during a subsequent continuous illumination period. This aspect of the problem falls somewhat outside the scope of the present article. However, we want to make a few remarks that may aid in defining the nature of the problem.

Several authors have reported that the shortening of the lag period in the accumulation of Chl a in continuous light by a pre-exposure to red, can be eliminated by far red given immediately after red (WITHROW *et al.* 1956; MITRAKOS 1961; PRICE & KLEIN 1961; SISLER & KLEIN 1963; HENSHALL & GOODWIN 1964; AKOYUNOGLOU 1968). According to VIRGIN (1961), in wheat, far red is only weakly antagonistic to red. We can confirm the red-far red antagonism for pea and maize leaves. With bean, far red reversal of the red effect was doubtful, however. A detailed report of our findings in this matter will appear in due course.

If we assume that the rate of Pchl formation as such is not influenced by light, the rate of Chl a accumulation should equal the initial rate of dark regeneration of Pchl, which condition clearly is not satisfied. In many experiments the initial rate of Chl a accumulation during illumination is much lower than the initial

rate of Pchl regeneration in the dark, whereas the Chl a accumulation rate after several hours of illumination as a rule surpasses severalfold the initial rate of Pchl regeneration. We are inclined to attribute the kinetics of Chl a accumulation to a combination of an initial photobleaching of newly formed pigment with light stimulation of Pchl biosynthesis by continuous illumination. Upon irradiation of dark grown material, the Pchl already present, is rapidly converted to Chl a. From then on, further production of Chl a follows the rate of production of Pchl, at least at a light intensity sufficient to saturate Pchl photoconversion. At the same time, part of the initial photoproducts are destroyed by light and, depending upon light intensity, the overall rate of Chl a accumulation is, therefore, less than the rate of Pchl formation. In continuous light, the capacity of the biosynthetic system forming Pchl increases, concomitant with the first stages of chloroplast development. Ultimately, a net rate of Chl a accumulation is reached, surpassing the original rate of Pchl formation. This mechanism may account for the kinetics of Chl a accumulation, observed during the lag phase and afterwards.

Given the failure of a short pre-illumination to influence the rate of Pchl regeneration, pigment photodestruction and development of the biosynthetic apparatus during a further irradiation, are possible points of attack for a red -far red effect. The provision is, that they are prepared in some way by the pre-illumination and become manifest only during the subsequent prolonged irradiation period. We may, therefore, suppose that these effects represent either a photoreversible mechanism, protecting pigments from photodestruction during the lag phase in the accumulation of Chl a, or of a formative effect, regulating the capacity of the pigment forming system during the subsequent prolonged irradiation. An experimental approach to these problems may be the study of the influence of light intensity and wavelength upon Chl a accumulation. We have started work along this line.

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