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INDUCTION OF RAPID CHLOROPHYLL ACCUMULATION IN DARK GROWN SEEDLINGS. I. ACTION SPECTRUM FOR PEA

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SUMMARY

A short pre-irradiation with red light some hours before the start of continuous illumination shortens the lag phase in chlorophyll-*a* (Chl-*a*) accumulation. This induction of rapid Chl-*a* accumulation was studied in leaves of dark grown seedlings of pea, bean, and maize. Considerable differences in sensitivity to red light were observed; correlation with total spectrophotometrically demonstrable phytochrome was not found. In pea leaf, induction proved extremely light sensitive. The action maximum about 660 nm points to phytochrome as a photoreceptor. It is concluded that phytochrome has a dual effect upon the kinetics of the greening process. P_{fr} increases the capacity of the biosynthetic system forming protochlorophyllide (Pchlide) and initiates the development of a mechanism which protects chlorophyllous pigments from photodestruction.

1. INTRODUCTION

Upon illumination of dark grown leaves the Pchlide already present is rapidly converted to chlorophyllide-*a* (Chlide-*a*). Following this initial Pchlide-Chlide-*a* photoconversion, usually a lag phase is observed before the onset of rapid greening. The duration of this lag phase may vary considerably, depending upon factors such as leaf age (SISLER & KLEIN 1963) and light intensity and temperature (VIRGIN 1955). The phytochrome system also seems to be involved, since a short pre-illumination with red light at the absorption maximum of phytochrome shortens the lag period, and the effect of this pretreatment is reported to be reversed partially or completely by far red light (WITHROW *et al.* 1956).

However, during a study of the rates of regeneration of Pchlide following a saturating dose of either red alone, or red followed by far red, we have previously found no evidence for phytochrome control of the initial rates of pigment regeneration (SPRUIT & RAVEN 1970). Since at first sight this seems to contrast with the findings quoted above, we have studied the induction of rapid Chl-*a* accumulation. Simultaneously, we have given attention to the time course of dry weight following a red inductive irradiation and under continuous illumination, since there might be a connection between both phenomena. As we have invariably found very low reversals of red induction by far red light (Raven, in preparation) the rôle of phytochrome in these reactions was questionable. We attempted, therefore, to determine the action spectrum of the inductive effect of pre-irradiation upon rapid Chl-*a* accumulation in subsequent continuous light.

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Pea was chosen as plant material for several reasons: Firstly, the leaves are not covered with seedcoats or cotyledons (as, e.g., in the case of young bean seedlings), so that they are exposed more evenly to the light. Secondly, this plant material proved extremely sensitive towards inductive light. Thirdly, in contrast with monocots, no action spectrum for induction of rapid Chl-*a* accumulation in dicots was available as yet.

The light sensitivity of pea will be compared with that of other plant species in relation to their phytochrome content, as determined spectrophotometrically.

2. MATERIALS AND METHODS

2.1. Plant material

For the construction of the action spectrum for induction of rapid Chl-*a* accumulation, 7 days old, dark grown seedlings of *Pisum sativum* L. cv. 'Krombek' were used. In seedlings of *Pisum sativum* cv. 'Alaska', *Phaseolus vulgaris* L. cv. 'Widusa' and *Zea mays* L. cv. 'Caldera' red light sensitivity was also tested. In all experiments whole seedlings grown in flower pots at 20°C in absolute darkness were used.

2.2. Irradiation

Induction of rapid Chl-*a* accumulation with relatively high intensity light was effected with spectral bands isolated from a number of Leitz 'Prado' 500 W slide projectors by means of interference filters (Balzers, Liechtenstein, type Filtraflex B 40), the transmission characteristics of which were checked in a Cary model 14 spectrophotometer. For the determination of the action spectrum a Bausch & Lomb 'high intensity' monochromator, type 33-86-25, with a near-infrared (#1) grating, type 33-86-03, for the region of 700–1600 nm was used.

Monochromatic light in the wavelength region of 380–700 nm was isolated from the second order of the spectrum. Filters were used to eliminate first-order wavelengths. This method yielded higher light intensities than the standard 350–800 nm grating in the first order, at the same bandwidths. As first-order blocking filters, a BG 38 filter (Schott & Gen.) for the wavelength region 380–600 nm and interference filters for the wavelength region 550–700 nm were used. Above 700 nm the first order of the spectrum was used in combination with interference filters. Between the light source and the filters a cuvette of 1 cm depth was placed, through which tap water was passed.

Bandwidth was 5 nm at 550–700 nm, and 10 nm at shorter and longer wavelengths.

While the experiments were in progress, it proved desirable to replace the tungsten (quartz-iodine) light source of the monochromator by a straight filament lamp (Philips, type 13305N, 8.5V–4A), operated from a stabilized power supply, since this gave both a higher light output and better stability. Light energy was measured with a thermopile in connection with a mirror galvanometer or with a calibrated photomultiplier tube (EMI 9558 B) and amplifier.

The photomultiplier-amplifier combination was calibrated for spectral sensitivity against the thermopile at high light intensities, and the linearity was checked over a broad light intensity range.

In this way intensities down to 0.3–0.6 erg/cm² sec could be measured.

Inductive irradiations were administered to the plants from above for periods of 60 seconds (except in a few cases, as indicated elsewhere), while the pots with the seedlings rotated on a turntable at a constant speed of 40 r.p.m. This minimizes intensity differences due to mutual shading of leaves. Variations in light energy were obtained by inserting neutral filters (Schott & Gen., type NG) in the light beam or by changing the voltage of the lamp supply. During the induction procedure rigorous precautions were taken to prevent straylight from reaching the seedlings. After an inductive exposure the plants were kept in darkness at 20°C for a 16-hour period, which was then followed by 5 hours of continuous illumination with white light at 25°C to allow greening of the leaves. White fluorescent light filtered through a dense wire screen was used to avoid photobleaching of pigments. The intensity of this white light at the level of the leaves was about 1500 erg/cm² sec.

2.3. Safelights

During the weighing and extraction procedure at the end of the 5-hour continuous illumination period, the harvested leaf samples were exposed to weak green so-called safelight.

We used a safelight, consisting of a green monophosphor fluorescent tube (Philips TL 40, colour 17), the light of which was filtered through one layer of orange-yellow 'Cinemoid' nr. 46 plus 3 mm blue 'Plexiglass' (Röhm und Haas) nr. 0248.

The intensity at the level of the leaves was about 10 erg/cm² sec. All previous handling was done in absolute darkness.

2.4. Pigment estimations and calculation of pigment concentrations

Samples of pea leaves of one half gram, 25 primary leaves (one of each pair) of bean seedlings, or one gram of maize leaves were weighed and extracted in acetone.

The optical density of 80% acetone extracts was measured at 473, 647, 664, and 800 nm in a Zeiss spectrophotometer, model PMQ II, equipped with a grating monochromator M 20.

We have adopted the molar absorption coefficients given by MACKINNEY (1941) for the calculation of Chl-*a* and Chl-*b* contents.

The optical density at 473 nm gives some indication of the total of carotenoids present in the sample (GOODWIN 1955).

Measurements of the relative phytochrome content of dark grown seedlings were performed in a dual wavelength spectrophotometer (SPRUIT 1970).

2.5. Dry weight determinations

The dry weight of pea plumules was determined by drying samples of 50 plumules to constant weight at 110°C.

3. RESULTS

3.1. Effect of duration of dark incubation period in pea

As indicated in the introduction, a short pre-irradiation with red light some hours before the start of continuous illumination shortens the lag phase in Chl-*a* accumulation. In *fig. 1* the Chl-*a* content, as measured after 5 hours of continuous light, is plotted against the length of the dark incubation period following 5 minutes red light. According to experiments of VIRGIN (1957), MITRAKOS (1961), and AKOYUNOGLOU (1970), the potential capacity of plants for Chl formation should reach a maximum after 4–6 hours darkness. In pea plumules no evident maximum in this period could be found. Extension of the dark period to 48 hours still gave rise to a marked increase in subsequent Chl-*a* formation, especially when the Chl-*a* content is based upon the number of pea plumules. Only in very young (4 days old) bean seedlings AKOYUNOGLOU (1970) could find a similar increase in stimulatory effect of a short pre-exposure over a 24-hour dark incubation period.

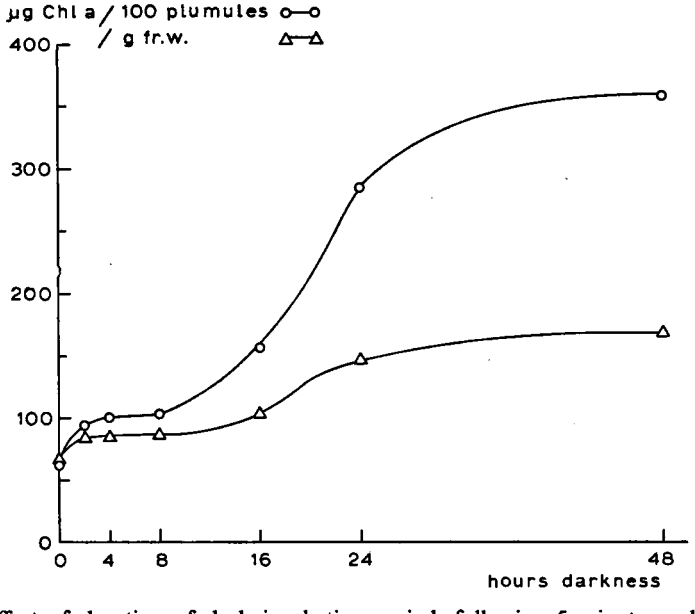

Fig. 1. Effect of duration of dark incubation period, following 5 minutes red (651 nm, 3000 erg/cm² sec) inductive light upon Chl-*a* accumulation in continuous light in pea cv. 'Krombek'. Chl-*a* content was measured after 5 hours of continuous white light.

We have studied the time course of the dry weight of pea leaves following this short pre-exposure to red light in an attempt to find an explanation for this discrepancy.

3.2. Dry weight accumulation

Fig. 2 gives the time course of dry weight of pea plumules during darkness following a short red light impulse and in continuous light. At the points indicated by arrows the whole pea seedlings were irradiated with 5 minutes red light; they were then kept in darkness until the start of continuous illumination with white light. The small dose of red light resulted in a considerable increase in dry weight of plumules in darkness, starting after a lag phase of about 4 hours. This rapid dry weight accumulation lasted for about 24 hours, thereafter it slowed down. Upon transfer to continuous light the increase in dry weight accelerated again. After long (> 16 hours) dark incubation periods no lag phase

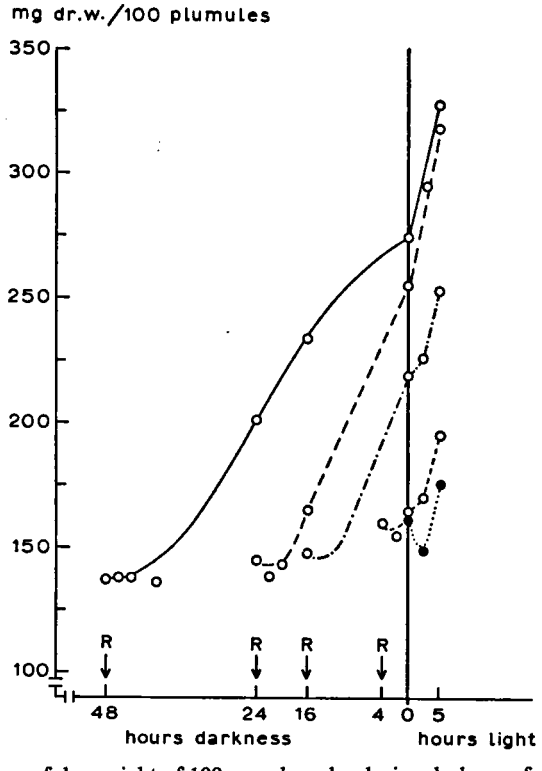

Fig. 2. Time course of dry weight of 100 pea plumules during darkness, following an irradiation with 5 minutes red (651 nm, 3000 erg/cm² sec) light and under continuous illumination with white light. The short red light impulse was administered to the whole pea seedlings at the points indicated by arrows.

in dry weight accumulation in continuous white light could be observed. When no short irradiation preceded the continuous light treatment, a considerable initial decrease in dry weight was observed. This decrease in dry weight apparently coincides with the lag phase in Chl-*a* accumulation, and we may consider the possibility that there is a connection between these two phenomena. Even after a short exposure to red light an initial decrease in dry weight of the leaves in darkness is sometimes found. In our opinion the dry weight accumulation of the leaves in darkness as well as in the relatively short period of continuous light results from translocation processes from the cotyledons or other organs to the leaves since photosynthesis can be excluded (BRADBEER 1969). In view of the effect of the red pre-illumination, these translocation processes are triggered by relatively small light doses.

3.3. Dose-response curves

Fig. 3 shows some dose-response curves for induction of rapid Chl-*a* accumulation as observed in different plants. The degree of induction was calculated from the following equation:

$$\% \text{ Induction} = \frac{C_T - C_D}{C_R - C_D} \times 100,$$

where C_D = Chl-*a* content in μg per g fresh weight (maize) or μg per constant

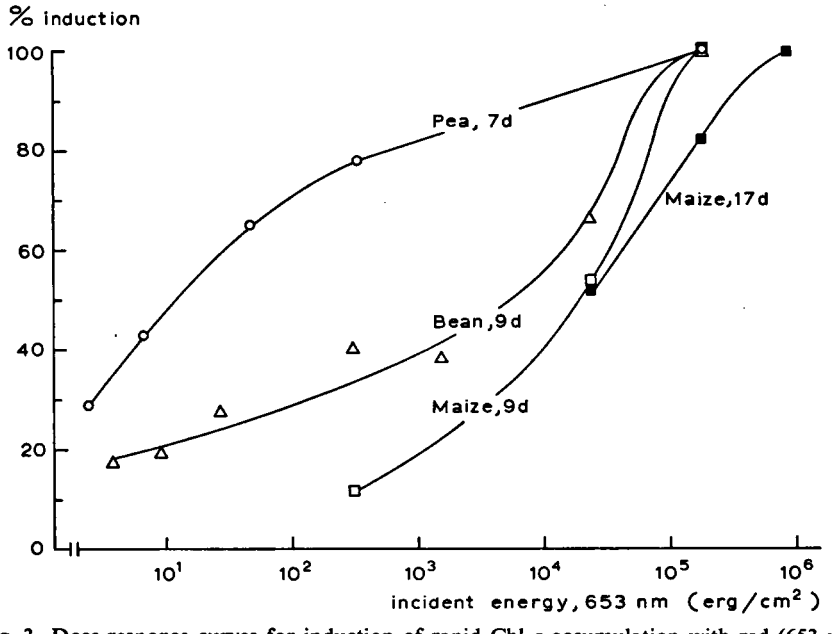

Fig. 3. Dose-response curves for induction of rapid Chl-*a* accumulation with red (653 nm) light in previously dark grown seedlings of varying age.

number of leaves (pea and bean), measured after 5 hours of white light without any inductive light treatment preceding this continuous illumination.

C_R = Chl-*a* content resulting from a pretreatment with a saturating standard dose of red (651 nm) light, eliciting a maximum response, followed by a 16-hour dark incubation period and 5 hours of continuous light.

C_T = Chl-*a* content resulting from a pretreatment with a light dose of given wavelength and energy, followed by a 16-hour dark incubation period and 5 hours of continuous light.

As shown in this figure, it is clear that a widely divergent range in spectral sensitivity exists with respect to the induction of rapid Chl-*a* accumulation with red light. But also marked differences in the shape of the dose-response curves can be observed.

Obviously, they do not fit straight lines and may be composed of sections with different slopes (PARKER *et al.* 1949; BLAAUW *et al.* 1968).

Fig. 4 shows that 'Alaska' peas were even more sensitive to red (660 nm) light than 'Krombek'. The incident energy at 660 nm required for 50% induction in pea cv. 'Krombek' is of the same order of magnitude as the threshold red light dosage in BRIGGS & CHON's (1966) experiments on the alteration of the phototropic sensitivity of corn coleoptiles.

However, in contrast with the findings of CHON & BRIGGS (1966), our pea material showed also a relatively high positive sensitivity in the far red region

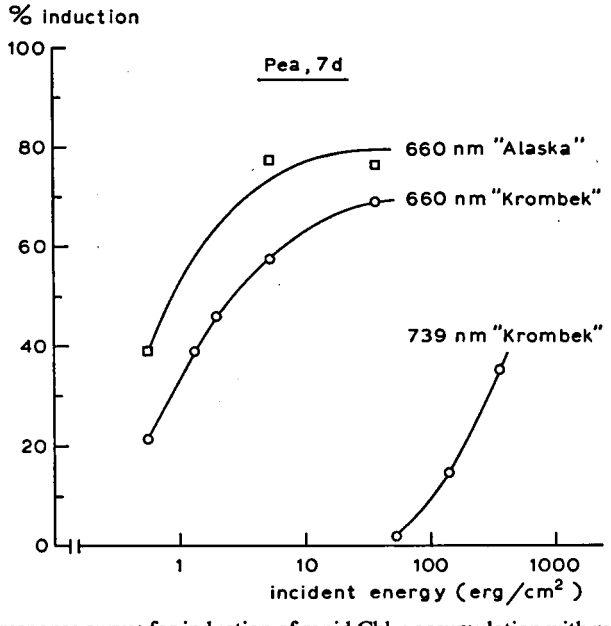

Fig. 4. Dose-response curves for induction of rapid Chl-*a* accumulation with red (660 nm) and far red (739 nm) light in 7 days old pea seedlings.

of the spectrum. From this high sensitivity to short irradiations with far red light can be concluded that a virtually stationary concentration of the active phytochrome (P_{fr}) maintained over a considerable period of time seems not to be required to get this photomorphogenic effect (KASEMIR & MOHR 1967). At any rate it points to an extremely low P_{fr} requirement of the induction reaction. After making these observations we were not surprised to find that even exposures as short as 6 seconds to our so-called darkroom safelight were able to induce rapid Chl-*a* accumulation in pea up to about 50% of the maximum. Accumulation of Chl-*b* and carotenoids, as well as gain in fresh weight of the leaves, followed the same pattern in their response to light.

3.4. Action spectrum

Dose-response curves or parts of dose-response curves, as shown in *fig. 3* and *4*, were used for the construction of an action spectrum for induction of rapid

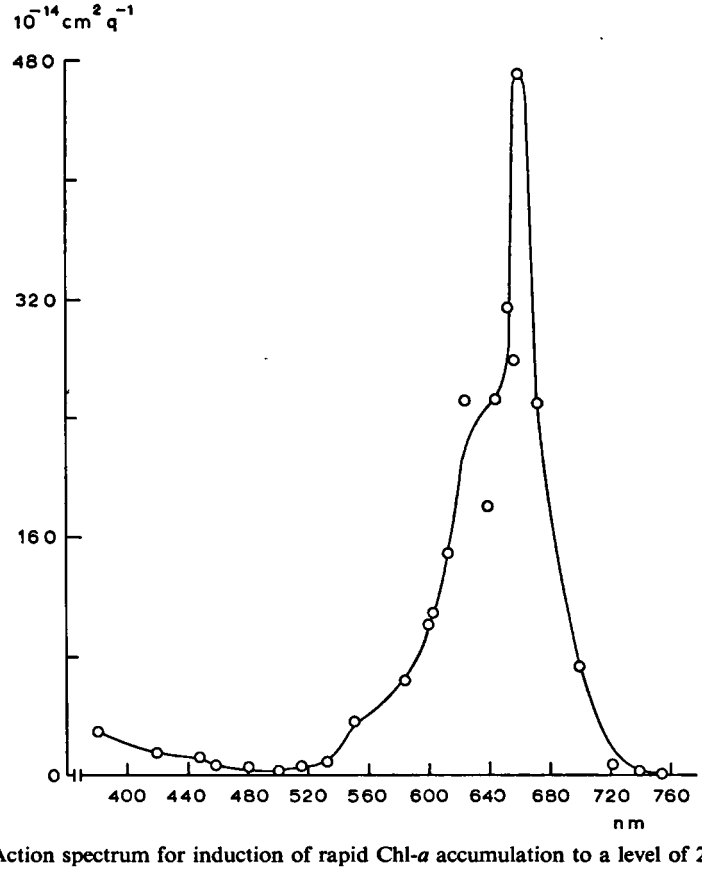

Fig. 5. Action spectrum for induction of rapid Chl-*a* accumulation to a level of 25% of the maximum in 7 day old pea seedlings. Vertical axis: reciprocal of quantum dose.

Chl-*a* accumulation in pea to a level of 25% of the maximum (*fig. 5*). The peak at around 660 nm suggests that the red absorbing form of phytochrome (P_r) acts as the photoreceptor, notwithstanding the almost complete lack of reversibility of the induction by far red light, as discussed in the introduction. Our action spectrum shows similarity to the one published by VIRGIN (1961) for wheat. The broader action maximum in wheat may in part be ascribed to a difference in bandwidth of the monochromatic light.

3.5. Phytochrome estimations

Fig. 6 gives the total amount of spectrophotometrically measurable phytochrome in leaves of pea, bean, and maize. It shows that pea leaves are quite rich in spectrophotometrically measurable phytochrome compared with leaves of maize. We have tried to correlate total 'spectrophotometric phytochrome' with sensitivity to induction (as shown in *fig. 3*). A positive correlation seems to exist, e.g. the particularly high △△O.D. values in pea leaves coincide with an

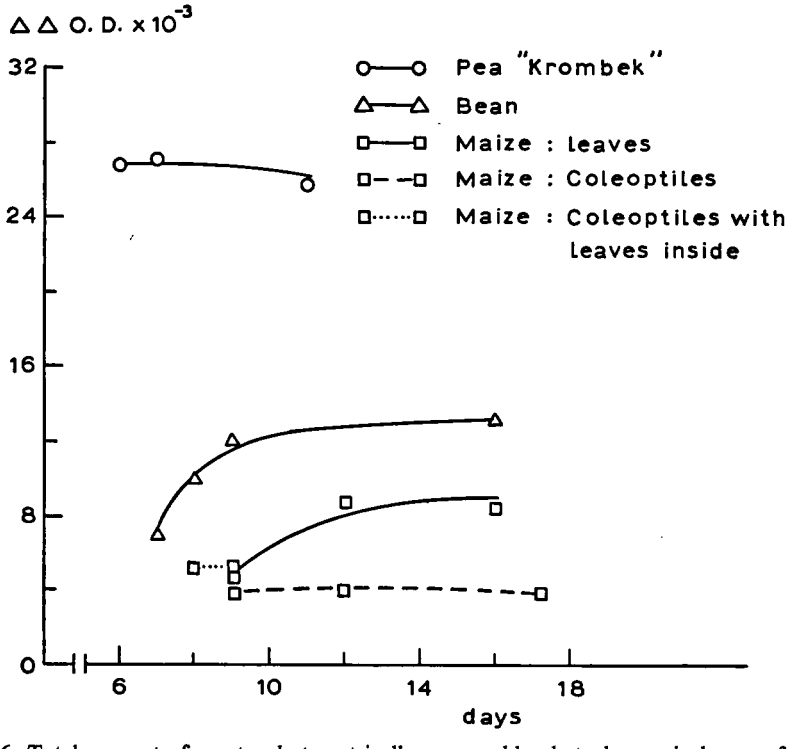

Fig. 6. Total amount of spectrophotometrically measurable phytochrome in leaves of dark grown seedlings of pea, bean, and maize of varying age. In case of maize different parts of seedlings were tested. Depth of samples 4 mm. Vertical axis: Optical density changes at 730 nm relative to 807 nm in response to actinic irradiations with red or far red light.

extremely high red light sensitivity. Closer examination of these data, however, makes the existence of a close correlation less plausible. The relative phytochrome content, as determined spectrophotometrically, of pea and bean leaves is not paralleled by comparable differences in light sensitivity.

Moreover, the phytochrome content of maize leaves increases slightly over a long period of time, whereas light sensitivity decreases with increasing age of the seedlings.

However, a more serious problem hampers the comparisons as described above. Since we are dealing with different plant materials and different parts of the same plant (maize), such comparisons are only permitted as long as the scattering properties of the different samples are identical (BUTLER 1964; ROMBACH & SPRUIT 1968). In fact, nothing is known about the effective optical path lengths in these highly scattering samples. Also, differences in pigment distribution pattern may influence the optical density readings for total phytochrome content (Spruit, in preparation). For these reasons, data as shown in *fig. 6*, are to be interpreted with great caution. Until more data have been collected, the possibility cannot be excluded that the high sensitivity to inductive light of pea leaves and the high 'spectrophotometric phytochrome' in them, is a mere coincidence.

4. DISCUSSION

A pronounced effect accompanying the induction of rapid Chl-*a* accumulation by a short exposure to red light in pea (*fig. 1*) is the rise in dry weight and concomitant growth of the leaves (*fig. 2*). With respect to the induction of Chl-*a* formation the following conclusions may be drawn from this observation. Firstly the undisturbed prolonged increase in dry weight of the leaves of whole pea seedlings might explain the fact that the induction of rapid Chl-*a* accumulation is maintained over a very long dark incubation period (*fig. 1*). If translocation of metabolites is involved, this may not be the case in excised leaves, which would explain the findings of VIRGIN (1957) and AKOYUNOGLOU (1970). Secondly, the rise in dry weight of the leaves may also run parallel to an increase in capacity of the biosynthetic system forming Pchlide. Indeed, according to MEGO & JAGENDORF (1961) short irradiations of dark grown bean leaves induce an increase in plastid volume in darkness, although no progressive development in the internal structure seems to take place under these conditions, as no grana are formed.

A pretreatment with 30 seconds of light followed by a 4-hour dark incubation period, on the contrary, proved effective in speeding up the internal structural changes when dark grown leaves are exposed to continuous light (KLEIN *et al.* 1964).

Concomitantly with these phenomena, another response to a short irradiation can be clearly observed: The total amount of carotenoids rises considerably during subsequent darkness (COHEN & GOODWIN 1962). In pea we have observed that a saturating dose of red light followed by 16 hours of darkness causes

almost doubling of total carotenoid pigments as compared with dark controls. As certain carotenoids are known to protect chlorophylls both *in vitro* (CLAES & NAKAYAMA 1959) and *in vivo* (SMITH & KOSKI 1948) from photodestruction, accumulation of these pigments during the dark incubation period may benefit Chl-*a* accumulation under continuous illumination, especially when high intensities of white light are administered (Raven, in press). In this respect the phytolization process seems to be involved also, as especially Chlide-*a* has been reported to be sensitive to photobleaching (SMITH *et al.* 1959; ANDERSON & ROBERTSON 1961; SPRUIT & RAVEN 1970). Therefore, an enhancement of the rate of phytolization induced by red pre-irradiation (LILJENBERG 1966) could protect chlorophyllous pigments from subsequent photobleaching. As the rate of Pchlide regeneration as such seems not to be under phytochrome control (SPRUIT & RAVEN 1970), the remaining possible points of attack for a red-far red effect are, consequently, the rate of growth and development of plastids, and the induction of a mechanism which protects chlorophyllous pigments from photodestruction.

Another conclusion to be drawn from our observations is that the term 'safelight' can be quite misleading, since relatively short exposures to a good quality green 'safelight' induce rapid Chl-*a* accumulation in subsequent continuous white light. This demonstrates that such devices should be distrusted as long as rigorous tests have not shown them to be innocuous.

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