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CHLOROPHYLL FORMATION AND PHYTOCHROME

C. W. RAVEN

Laboratory of Plant Physiological Research, Agricultural University, Wageningen, The Netherlands

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1. GENERAL INTRODUCTION

It has been known for a long time that visible light drastically influences the appearance of a plant (RAY, 1686). As an example, plate 1 shows seedlings of higher plants that were either raised in the dark or in continuous white fluorescent light. It illustrates the differences between seedlings grown in the absence of light and light-grown ones of the same age.

In dark-grown seedlings of the dicotyledonous plants pea (*Pisum sativum* L.) and bean (*Phaseolus vulgaris* L.), leaf unfolding and expansion hardly occurs, whereas stem or hypocotyl strongly elongate. Also the number of leaves is greatly depressed. Just below the leaves, a characteristic curvature in the stem or the hypocotyl can be observed, the so-called plumular hook. The monocotyledonous plant maize (*Zea mays* L.) under the same conditions produces long, relatively narrow and still fairly rolled leaves. Also, the root system of dark-grown seedlings is poorly developed.

For the present investigation the observation is important that the colour of the leaves of the dark-grown seedlings of plate 1 is yellow instead of green. Analysis shows that pigments absorbing in the blue wavelength region of the spectrum, such as carotenoids, are the coloured compounds, predominating in dark-grown seedlings, and that chlorophylls are present only in minute quantities. The main pigment, absorbing in the red region is protochlorophyll. It accumulates, however, only in an insignificant amount as compared with that of chlorophyllous pigments present in a mature green leaf.

Generally, plants grown in darkness are called 'etiolated' (étioler = to bleach). CHARLES BONNET may have been the first to use this term in 1754 (WASSINK *et al.*, 1957). The word implies that it should only be a useful term for dark-grown seedlings of Angiosperms, since several more primitive plants are able to synthesize appreciable amounts of chlorophylls in the dark. Also light-grown plants that have remained in darkness over a long period, are often designated as etiolated as well (e.g. HARRIS and NAYLOR, 1967). In plant ecology, the term etiolation is often used in cases of growth of plants in low light intensities. In this paper we will call plants raised in complete darkness: 'dark-grown'; the term 'de-etiolated' will be used for plant material, raised in complete darkness but thereafter exposed to radiation for a time so that they have undergone a photobiological change of some sort.

Generally, when dark-grown seedlings are brought into the light, they approach a 'normal' appearance within a few days. One of the first changes, visible to the naked eye is the greening of their leaves owing to chlorophyll formation. The plants also rather quickly unfold and expand their leaves and open their plumular hooks.

It is obvious that these ultimate photomorphogenic effects are the result of numerous biophysical and biochemical processes. Of special interest are the photoreceptor pigments involved and the chain of events between the pigment

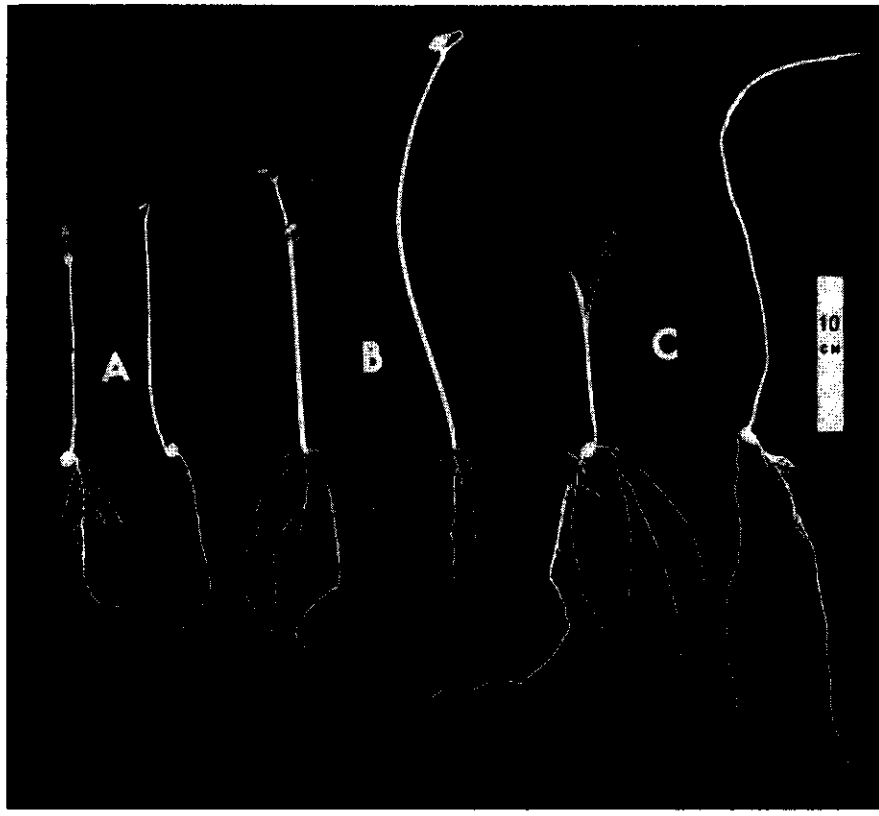

PLATE 1. Seven-day old seedlings of higher plants that were either reared in the dark (right) or in continuous white fluorescent light (1500 ergs/cm² sec) (left) at 25 °C; A: pea cv. Krombek, B: bean cv. Widuco, C: maize cv. Caldera.

and the ultimate photoresponse(s). This aspect of plant photomorphogenesis has been studied extensively, and a variety of photoreceptor systems and possible reaction mechanisms are known (see reviews as written by: PARKER and BORTHWICK (1950), WASSINK and STOLWIJK (1956), MOHR (1962), and HILLMAN (1967)). Much attention has been focussed on phytochrome, that appears to trigger many formative effects such as seed germination, development of seedlings, movements of plant parts, flowering, etc. In most cases, a very small amount of light energy is required to trigger the photoresponse (e.g. WITHROW, 1959). Many photoresponses, however, including some involved in the de-etiolation process, still await for the elucidation of their photoreceptor systems.

An important aspect of the process of de-etiolation is the development of the photosynthetic apparatus. The products of light energy conversion in photosynthesis are indispensable for the development of the normal plant and, ultimately, determine its quality as a source of energy for animal and man. Aside of this, the action of morphogenic pigment systems is another basic functional aspect of higher plants. In mutual interaction and cooperation productive (light energy fixing) and morphogenic (stimulation sensitive) processes determine the ultimate appearance of a (higher) plant.

Against this background, the present study, concerned with the greening of dark-grown seedlings transferred to light can provide information about an important pathway in the rôle of light in the life of plants.

2. SURVEY OF THE PIGMENTS INVOLVED

Understanding of the subsequent experimental data may be facilitated by the following brief enumeration of some general properties of the main pigments involved. Certainly, these surveys will be far from complete. Therefore, also reference will be made to some recent books and reviews.

2.1. PROTOCHLOROPHYLL

Seedlings of Angiosperms, reared in the dark, accumulate protochlorophyll as a temporary end product, since light is required for its transformation into chlorophyll-*a*. The chemical structure of a protochlorophyll, isolated from pumpkin seed coats has been elucidated by FISCHER and coworkers (1939), who identified it as magnesium vinyl pheoporphyrin-a_5 phytyl (methyl) ester. Upon extraction in organic solvents, the pigment shows a characteristic absorption band in the red wavelength region and a very pronounced peak in the blue, their positions depending upon the solvent used (fig. 1).

More recent investigations, however, showed that the main protochlorophyll type pigment in leaves of dark-grown seedlings of higher plants is not protochlo-

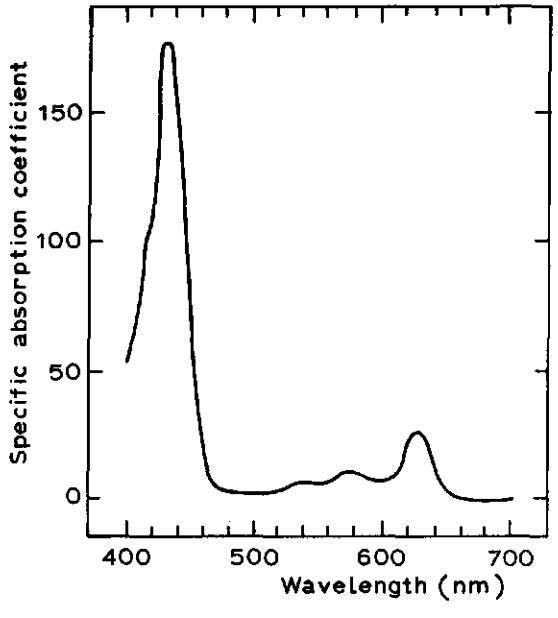

FIG. 1. Absorption spectrum of protochlorophyll in methanol (From KOSKI *et al.*, 1951).

rophyll, but the non-phytylated compound, protochlorophyllide, or magnesium vinyl pheoporphyrin-a_5 (LOEFFLER, 1955; WOLFF and PRICE, 1957). Protochlorophyll is also present, but in much smaller amounts (about 20%, depending upon age and species of the plant). These findings raised certain problems in nomenclature, since the word 'protochlorophyll' was generally used in the literature to refer to both pigments, irrespective of whether they are esterified or not. According to a proposal of KIRK (1967), we will use in this paper, from now on, the words protochlorophyllide ester (Pchlide ester) and protochlorophyllide (Pchlide) when their distinction is needed. Protochlorophyll (Pchl) will be used as a collective term, or when the nature of the pigment is not completely established. There is experimental evidence, indicating that Pchlide ester is not phototransformed at all, or at a much slower rate than Pchlide (WOLFF and PRICE, 1957; VIRGIN, 1960). *In vivo*, Pchl has its absorption peak in the red wavelength region at 650 nm with a shoulder around 636 nm (SHIBATA, 1957). The absorption around 636 nm may be attributed to Pchlide ester. Upon extraction with organic solvents, Pchl immediately loses its phototransformability. However, it is possible to obtain Pchl in cell-free extracts that does retain the ability to undergo phototransformation (KRASNOVSKY and KOSOBUTSKAYA, 1952; SMITH, 1952). This active form of Pchl – called Pchl holochrome by SMITH and collaborators (1957) – appears to be a complex of Pchl chromophore and protein.

Pchlide phototransformation *in vivo* or as purified holochrome, is a very fast process. The photoact can be completed within a few milliseconds (e.g. MADSEN, 1963). Phototransformation is not observed at liquid nitrogen temperature, but there is a fairly rapid conversion at –70 °C (SMITH and BENITEZ, 1954). Action spectra for Pchl phototransformation with peaks in the blue (445 nm) and red (650 nm) wavelength regions (FRANK, 1946; KOSKI *et al.*, 1951), clearly point to Pchl as photoreceptor for its own conversion.

2.2. CHLOROPHYLL

A comparison of the empirical formulae for chlorophyll-*a* (Chl-*a*) and Pchl shows that the latter differs from Chl-*a* in having 2 hydrogen atoms less. This implies that its phototransformation is accompanied by a reduction. The nature of the hydrogen donor is still unknown, but it seems to form an intrinsic part of the holochrome, since Pchlide phototransformation occurs even after extensive purification.

The first more or less stable product of the phototransformation absorbs around 684 nm (SHIBATA, 1957). Generally, this absorption maximum is ascribed to chlorophyllide-*a* (Chlide-*a*). During the next 30–60 minutes at room temperature, a shift to 673 nm occurs (SHIBATA shift). This shift may be supposed to be the result of phytolization of Chlide-*a*, yielding chlorophyllide-*a* ester (Chlide-*a* ester). Finally, the 673 nm-peak shifts back very slowly to 677 nm which is about the position of the main absorption peak of Chl-*a* in the red wavelength region in a mature green leaf (KRASNOVSKY, 1960).

Generally, this shift is ascribed to aggregation of Chl molecules in the thylakoids.

Chl-formation during the greening process is accompanied by drastic changes in the internal structure of the etioplast (ROSINSKY and ROSEN, 1972). Pchl is assumed to be located in the so-called prolamellar body, which appears to be a complex network of tubular membranes having the appearance of a crystal lattice. Upon Pchl phototransformation the prolamellar body loses its crystalline appearance and lamellar membranes extrude from the disrupted prolamellar body, giving rise to thylakoids. After prolonged irradiation the primary lamellae, over certain regions, become double, which is the initial stage of granum formation.

Recent books and reviews on Pchl and Chl-*a*: VERNON and SEELY (1966); KIRK (1967, 1970); VIRGIN (1972).

2.3. PHYTOCHROME

Phytochrome is a blue-green pigment, probably belonging to the open-chain tetrapyrroles, such as the bile pigments (e.g. RÜDIGER and CORRELL, 1969; RÜDIGER, 1972). Until now, it has only been detected spectrophotometrically in higher plants and in very few lower plants. Angiosperm phytochrome exists in two interconvertible forms, one with maximum absorption in the red region of the spectrum at around 660 nm (P_r), the other in the far red around 730 nm, (P_{fr}). Red light is the most effective in forming P_{fr} from P_r, whereas for the back-reaction far red light is most suitable. This is schematically represented in the following way:

$$P_r \underset{730\text{ nm}}{\overset{660\text{ nm}}{\rightleftharpoons}} P_{fr}$$

It is generally assumed that only phytochrome in the P_{fr}-form is physiologically active.

The concentration of phytochrome in plants is extremely low, and especially sensitive spectrophotometric equipment is required for its detection (BUTLER *et al.*, 1959; SPRUIT, 1970). In principle, the techniques involved are based upon the photochromic behaviour of the pigment. Those tissues that contain substantial amounts of chlorophyllous pigments, however, are inaccessible to spectrophotometric estimation of phytochrome. In such cases, only physiological evidence reveals the presence of the pigment, and generally, the criterion of red-far red photoreversibility of the physiological photoresponse(s) is used as such (e.g. MOHR, 1957). In the course of the present study, however, some doubts are raised against the general validity of the criterion.

P_{fr} is known to undergo a number of dark reactions. In dark-grown monocotyledonous tissue, P_{fr} formation is followed by a gradual decrease in photoreversibility during continued darkness. This process depends upon temperature and requires oxygen (BUTLER *et al.*, 1963; DE LINT and SPRUIT, 1963); it seems

to involve a complete disappearance of P_{fr} without any concomitant formation of other pigments, and is called phytochrome decay.

In dicotyledons, disappearance of P_{fr} mostly is accompanied by dark transformation of P_{fr} to P_r ('dark reversal'), which does not require oxygen (BUTLER and LANE, 1965). In light-grown tissue such as cauliflower heads and white parts of variegated leaves, only dark reversal can be detected (BUTLER and LANE, 1965; SPRUIT, 1970). The same holds true for purified phytochrome extracts of both monocotyledons and dicotyledons (BRIGGS and RICE, 1972).

Initially, it was hoped that profound study of phytochrome dark reactions would lead to an understanding of its morphogenic action. However, clear correlations between the amount of P_{fr} as determined spectrophotometrically and its associated physiological response(s) are only rarely established (HILLMAN, 1967, 1972).

In contrast to the chlorophylls that, in higher plants, are localized exclusively in the plastids, nothing is known about the exact intracellular distribution of phytochrome. PRATT and COLEMAN (1971) reported that in dark-grown maize, phytochrome did not appear to be restricted to any special cell organelle or structure.

Recent books and reviews: HILLMAN (1967), BRIGGS and RICE (1972), MITRAKOS and SHROPSHIRE (1972), MOHR (1972).

3. REVIEW OF THE LITERATURE ON CHLOROPHYLL FORMATION AND STATEMENT OF THE PROBLEM

3.1. REVIEW OF THE LITERATURE

The quantity of Chl-*a* formed upon transformation of the Pchl, initially present in a dark-grown leaf, is only a minute fraction of the total amount of chlorophylls present in a mature green leaf. Obviously, considerable pigment accumulation has to occur if dark-grown seedlings of higher plants are submitted to a prolonged irradiation with light of suitable wavelengths and intensity. The time course of this greening process has first been described by LIRO (1908), and many reports on this topic have since been published (e.g. BLAAUW-JANSEN *et al.*, 1950; KOSKI, 1950; VIRGIN, 1955; GOEDHEER, 1961; ANDERSON and BOARDMAN, 1964; AKOYUNOGLOU *et al.*, 1966; GASSMAN and BOGORAD, 1967a). Generally, three different phases in the greening process can clearly be distinguished (see also fig. 20): after the initial Pchl-Chl-*a* phototransformation which is very rapid at the usual light intensities, there is a rather slow phase in the accumulation of Chl-*a*. after a certain period, this is followed by a rapid increase in the rate of Chl-*a* synthesis. This slow phase before the onset of rapid greening is one of the most intriguing features of Chl accumulation.

The duration of this so-called 'lag phase' may vary considerably depending upon temperature (VIRGIN, 1955) and light intensity (VIRGIN, 1955; RAVEN, 1972b). Also the age (SISLER and KLEIN, 1963; AKOYUNOGLOU and ARGYROUDI-AKOYUNOGLOU, 1969), the species (GASSMAN and BOGORAD, 1967a), and the degree of starvation (SISLER and KLEIN, 1963) of the seedlings may influence the lag. In 1956, WITHROW and coworkers reported that the lag phase in Chl-*a* accumulation in dark-grown bean leaves could be eliminated if the leaves were pre-irradiated with a low dosage of red light ('induction') followed by a dark period of 5–15 hours (see also fig. 37). They also observed that far red reversed this effect of the red pretreatment, and that maximum effectiveness for induction was found near 660 nm. These observations have since been confirmed by a number of investigators MITRAKOS, 1961; (PRICE and KLEIN, 1961; SISLER and KLEIN, 1963; AUGUSTINUSSEN, 1964; HENSHALL and GOODWIN, 1964; JAQCUES, 1968). They suggest that the phytochrome pigment system (section 2.3.) is involved in the greening process of higher plants. However, VIRGIN (1961) observed only a very weak far red reversal of Chl-*a* induction in wheat seedlings. His action spectrum for induction, nevertheless, pointed to phytochrome as the photoreceptor.

According to many reports in the literature, Pchl can be considered as the precursor of Chl-*a* throughout the greening process (e.g. GASSMAN and BOGORAD, 1967a). This appears also to be true for the slow but distinct turnover-process of chlorophylls in mature green leaves (SHLYK *et al.*, 1969). Transfer of seedlings from the light to prolonged darkness, results in reaccumulation of

Pchl in the so-called 'Pchl regeneration process' (e.g. LIRO, 1908; SCHARFNAGEL, 1931; VIRGIN, 1955; AUGUSTINUSSEN and MADSEN, 1965; GASSMAN and BOGORAD, 1967b). Similar to the greening process, Pchl regeneration proved to be strongly dependent on the age of the seedlings (AKOYUNOGLOU and SIEGELMAN, 1968) and on temperature (VIRGIN, 1955). In 1965, it was reported by AUGUSTINUSSEN and MADSEN and by RUDOLPH, that the phytochrome pigment system was also involved in regulating rate and capacity of Pchl regeneration.

VIRGIN (1958) found that the ability to regenerate Pchl in the dark was greatly stimulated if the seedlings were pre-irradiated with red light. Thus, the inductive effect of a red pre-exposure on the elimination of the lag phase in greening might be due to an effect on Pchl biosynthesis (see also KIRK, 1967).

However, SPRUIT (1967) reported that in pea leaves the levels of Pchl, reached in 4 hours darkness following illumination with a saturating dose of either red alone or red followed by far red, were not significantly different. This was confirmed by JACQUES (1968) for oat seedlings and by MEIJER (personal communication) for gherkin seedlings. MEGO and JAGENDORF (1961) found no difference in the amounts of Chl-*a* accumulated in bean leaves as a result of 4 short exposures at 24-hour intervals to red or to red followed by far red. Since the Chl-*a* formed under these conditions depends almost completely upon the quantity of Pchl, formed during the dark intervals, those results are another indication that Pchl regeneration is not under red-far red control.

3.2. STATEMENT OF THE PROBLEM

In the previous section we have discussed a number of literature reports pointing to phytochrome regulation of the rate of Chl formation during the greening process. However, it was also shown that phytochrome action on Pchl formation as such was questionable. Therefore, assuming that Pchl acts as the precursor of Chl-*a* throughout the greening process, the findings quoted above appeared contradictory. Since these investigations had been made with a wide variety of plants and, for that reason, may be difficult to compare, we decided to study the possible rôle of phytochrome on Pchl regeneration in seedlings of various species, and in relation to their age and pretreatment. Moreover, we have tested the involvement of phytochrome in Chl formation in prolonged illumination in order to obtain information about the mechanism of the action of the photomorphogenic pigment system in the greening process. To that end, the nature of the lag phase in Chl formation was studied. For purposes of comparison, we also paid attention to accumulation of Chl-*b*, carotenoid pigments, and to the development of the fresh weight of the leaves.

4. MATERIALS AND METHODS

4.1. PLANT MATERIAL

Seeds of *Phaseolus vulgaris* L. cv. Widusa and of *Pisum sativum* L. cv. Krombek were obtained from NUNHEM's Zaden N.V. (Haelen, Holland). Seeds of *Phaseolus vulgaris* L. cv. Widuco were obtained from RUITER's Zaden (Andijk, Holland). Seeds of *Zea mays* L. cv. Caldera were obtained from VAN DER HAVE N.V. (Kapelle-Biezelinge, Holland). Seeds of *Phaseolus vulgaris* L. cv. Red Kidney, cv. Resistant ASGROW Valentine, cv. BURPEE's Stringless Green-Pod, and of *Pisum sativum* L. cv. Alaska were obtained from W. Atlee BURPEE Co. (Philadelphia, Pa, U.S.A.).

4.2. CULTIVATION OF SEEDLINGS

Seeds of pea and maize were soaked in water for a few hours under dim daylight conditions. They were then densily sown in pasteurized soil. Bean seeds were sown without previous soaking. The seedlings were grown either in plastic flower pots (14 cm diameter, 11 cm height) or in square earthenware seed-pans (23 × 23 × 5 cm), depending upon the type of experiment. The seeds of pea and bean were lightly dusted by hand with a blower containing the fungicide TMTD (tetramethylthiuramdisulfide). Finally, all seeds were covered with a thin layer of coarse sand, and the pots and seed-pans were abundantly watered. The seeds were raised in complete darkness at 20 °C and about 85 % rel. hum. As far as necessary, water was added in the dark during the growth period. This had to be done very carefully and moderately when the seedlings were still young. Pots and seed-pans were surrounded by a metal gauze enclosure in cases that the growth of the seedlings should be extended over a prolonged period; this prevented the stems from early collapse. In this way seedlings of acceptable quality could be obtained over a period of about 3 weeks.

Most experiments were made with the seedlings left intact in their containers. However, sometimes only the leaves were used. They were detached in absolute darkness prior to the experiments. The leaf material, generally used in the experiments, consisted of the primary leaf pair of the bean, the 'plumules' of the pea (= third and fourth unexpanded internode with the attached leaf material), and the primary leaves or whole coleoptiles of maize. Before starting an experiment, the seedlings were again supplied with water.

4.3.1. *Equipment for irradiation with monochromatic light*

In a number of experiments, narrow spectral bands were isolated from the light of a LEITZ 'Prado' slide projector, with a 220 V, 500 W, incandescent lamp, by means of interference filters (BALZER's, Liechtenstein, type Filtraflex B40). The coloured light beam was deflected by a mirror so as to irradiate samples of

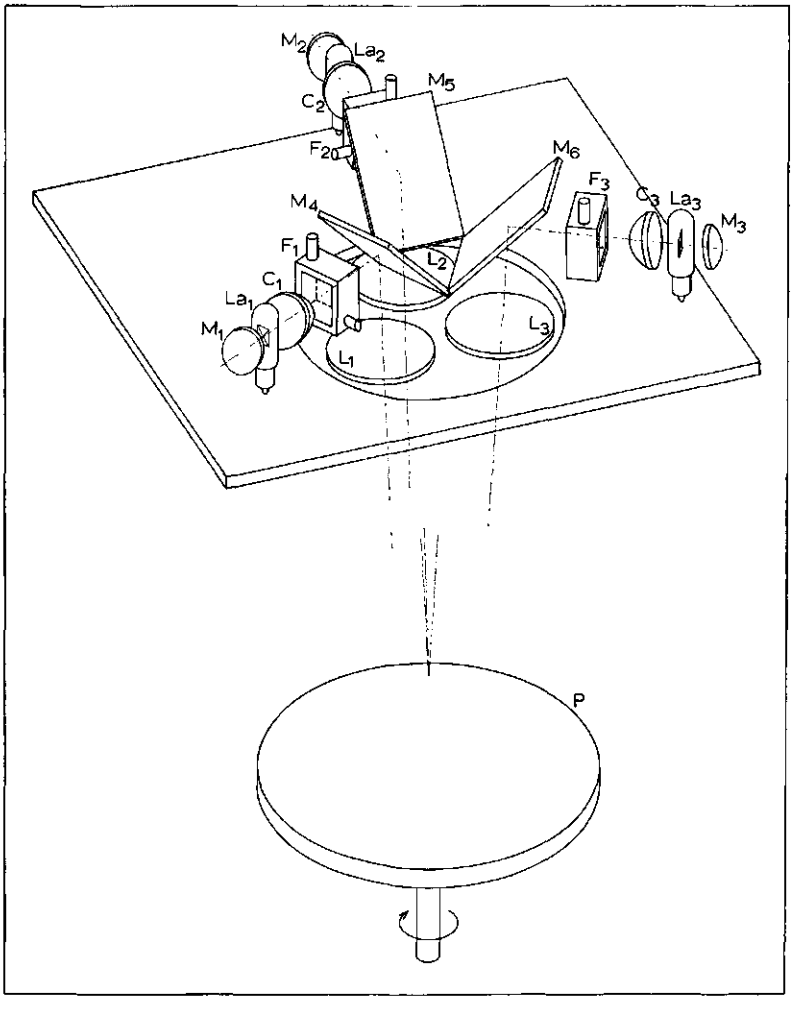

FIG. 2. Cabinet for irradiation of intact seedlings with monochromatic light. M_1, M_2, M_3: concave mirrors; La_1, La_2, La_3: 500 W slide projector lamps; C_1, C_2, C_3: aspheric condensers; F_1, F_2, F_3: filter cuvettes; M_4, M_5, M_6: plane mirrors; L_1, L_2, L_3: focussing lenses, forming an image of the filter aperture in the plane of the leaves; P: rotating platform. Parts M_1, M_2, M_3, La_1, La_2, La_3, and C_1, C_2, C_3 form part of three slide projectors.

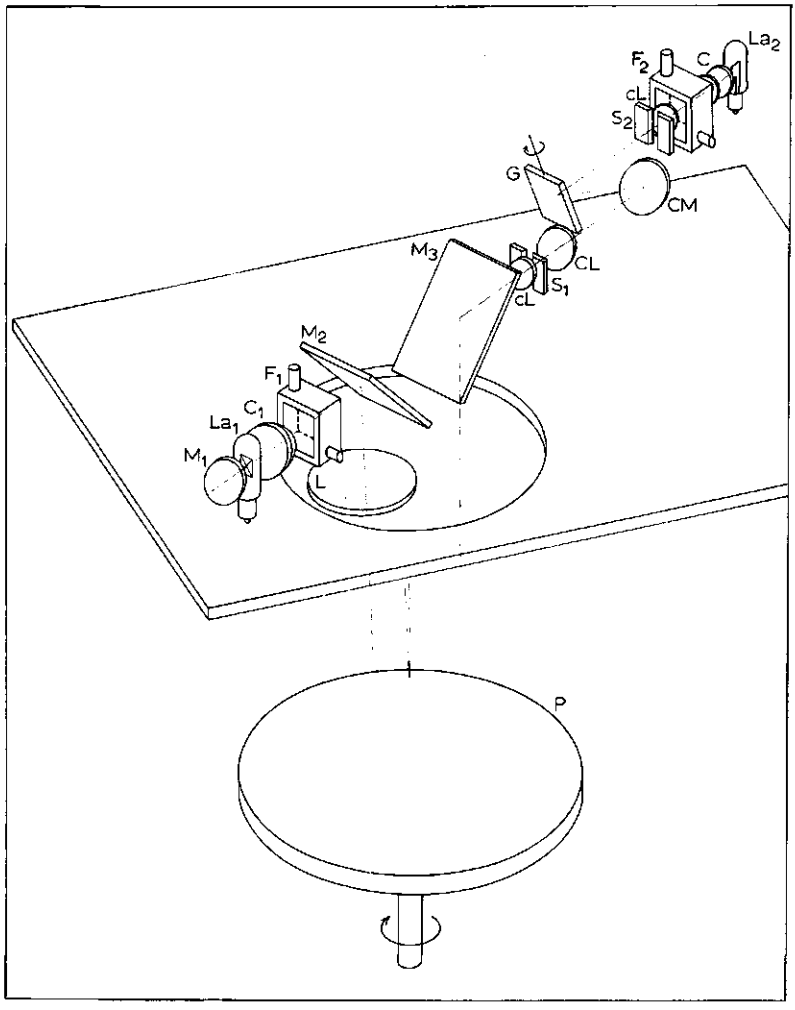

FIG. 3. Cabinet for irradiation of intact seedlings with monochromatic light. M_1: concave mirror; M_2, M_3: plane mirrors; La_1: 500 W slide projector lamp; La_2: 32 W straight filament lamp; C: condenser lenses; C_1: aspheric condenser; F_1, F_2: filter cuvettes; L: focussing lens; S_1, S_2: monochromator slits (adjustable); cL: collective lenses; CL: corrector lens; G: grating; CM: concave mirror; P: rotating platform.

detached leaves from above. A wooden construction and two sheets of black cloth protected the sample against straylight from the projector. The intensity of the light at the level of the sample was about 1.4×10^4 ergs/cm^2 sec at 529 nm, 2.2×10^4 ergs/cm^2 sec at 650 nm and 2.9×10^4 ergs/cm^2 sec at 735 nm. Variations in light dose were obtained by changing the duration of irradiation up to a maximum of 15 minutes. In this set-up the interference filters were insufficiently cooled to stand much longer exposures.

Since the size of the equipment described above did not permit the irradiation of intact seedlings, another type of light cabinet was constructed as shown in figs. 2 and 3. On top of one of them (fig. 2), 3 LEITZ 'Prado' slide projectors were arranged in a circle, 3 mirrors directing the light downwards through a hole upon the plants placed inside the cabinet on a turntable. On top of the other (fig. 3), a similar slide projector was mounted together with a BAUSCH & LOMB 'high intensity' monochromator, type 33–86–25; the other provisions were the same. Planoconvex lenses of 9 cm diameter, mounted in a suitable position in the light beams, formed images of the interference filters some 15 cm above the level of the turntables. The latter were kept rotating at a constant speed of 40 r.p.m. by means of variable speed motors. Since the beams from the different light sources do not come from exactly the same directions, this method minimizes intensity differences due to mutual shading of leaves. Spectral bands were isolated from the light of the slide projectors by means of BALZER's interference filters, the transmission characteristics of which were checked in a CARY model 14 spectrophotometer (e.g.: fig. 4, curve a). The interference filters were mounted to form one side of a glass box of 1 cm width, through which tap water was passed (fig. 5). In this way, long term irradiations at high intensities could be administered without damaging the filters.

The 'high intensity' monochromator was equipped with a near-infrared grating (nr. 1, type 33–86–03) for the wavelength region of 700–1600 nm. For irradiations above 700 nm the first order of its spectrum was used in combination

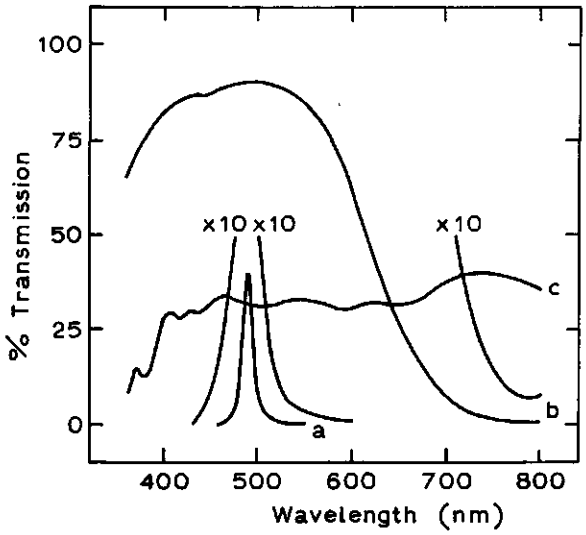

FIG. 4. Transmission spectra of different types of filters: a. Interference filter for 491 nm light b. Blue-glass filter BG 38, 2 mm thickness; c. Neutral-glass filter NG 5, 2 mm thickness

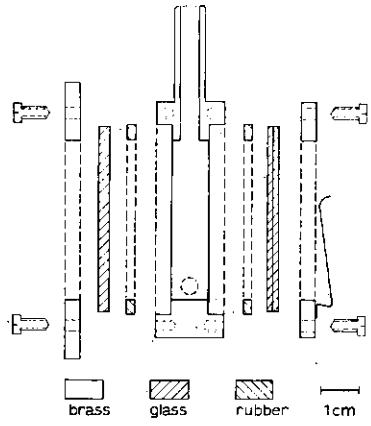

FIG. 5. Glass box for water cooled interference filters. Additional filters can be inserted under the spring (right).

with interference filters of the desired wavelengths to block stray light and higher orders of the grating spectrum.

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 and stray light. This method yielded higher light intensities than the standard 350–800 nm 'visible' grating in the first order, at the same bandwidths. As first-order blocking filters, a BG 38 filter (SCHOTT & Gen.) (fig. 4, curve b) for the wavelength region 380–600 nm, and interference filters for the wavelength region 550–700 nm were used. Between the light source of the monochromator and the blocking filters a glass box of 1 cm width was placed, through which tap water was passed. Bandwidths obtained in this way were about 5 nm in the region of 550 to 700 nm, and 10 nm for wavelengths outside this range.

While the experiments were in progress, it proved desirable to replace the tungsten (quartz-iodine) light source supplied with the monochromator by a straight filament incandescent lamp (PHILIPS, type 13305 N, 8.5 V-4 A), operated from a stabilized power supply, since this gave both a higher light output and a better stability. Mostly the inductive irradiations were administered to the seedlings for periods of 60 seconds. The duration of these short inductive irradiations was regulated via an electronic timer. If otherwise, it will be indicated in the text.

Normally, the greening experiments in coloured light were extended over a 5 hour period.

Variations in light energy were obtained by inserting one or more neutral filters (type NG, SCHOTT & Gen.) (e.g.: fig. 4, curve c) in the light beam; changing the voltage of the lamp supply was an alternative possibility. To obtain relatively high light intensities, two or three slide projectors were sometimes used in cooperation, equipped with interference filters of identical wavelengths. It was also possible to irradiate the seedlings simultaneously with monochromatic light of two or three different wavelengths.

During all types of irradiations, rigourous precautions were taken to prevent stray light from reaching the seedlings.

4.3.2. *White light equipment*

To avoid photobleaching of pigments, white light of rather low intensity was used in most cases. This was obtained from 8 fluorescent tubes (PHILIPS TL 33/40 W), placed at a mutual distance of about 20 cm. They were mounted behind a dense metal wire screen at a distance of about 100 cm from the leaves of the seedlings. The intensity of this white light source was about 1500 ergs/cm^2 sec at the level of the leaves.

High intensity white light (about 60,000 ergs/cm^2 sec) was supplied by the standard white fluorescent light fields in the controlled climate rooms of this laboratory. The tubes were mounted at a distance of 5 cm from each other and at 65 cm above the surface of the leaves.

4.3.3. *Safelights*

The use of a small amount of light was found unavoidable during some procedures. Weighing and extraction of the detached leaves in the experiments described in Chapters 5 and 6 was made under weak green safelight, obtained from a green monophosphor fluorescent tube (PHILIPS TL 40, colour 17), wrapped in a layer of blue 'Cinemoid' nr. 62 and a layer of orange-yellow 'Cinemoid' nr. 46 (The Strand Electric Corp.).

Alternatively, a safelight was used for some experiments, consisting of a 25 W incandescent lamp, the light of which was filtered through 3 mm blue 'Plexiglass' (RÖHM und HAAS) nr. 0248 and one layer of 'Cinemoid' nr. 46. Precau-

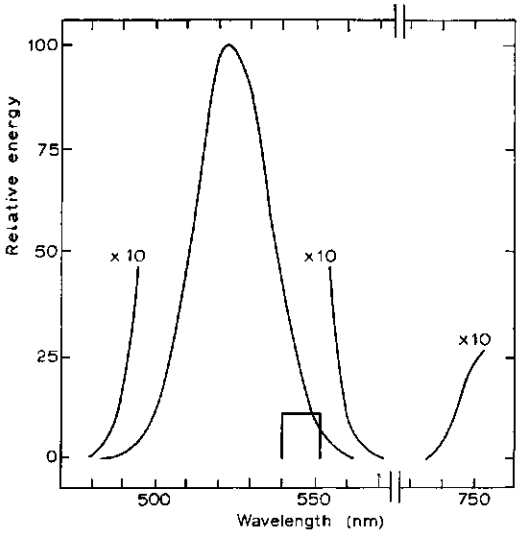

FIG. 6. Relative spectral energy distribution of the green safelight.

tions were taken that the light from these sources did not reach the samples directly.

The weighing and extraction procedure for most other experiments took place in dim green safelight, provided by a green monophosphor fluorescent tube, mounted behind one layer of orange-yellow 'Cinemoid' nr. 46 plus 3 mm blue 'Plexiglass' (RÖHM und HAAS) nr. 0248. In a few experiments, this darkroom green safelight was also used as an inductive light source for rapid Chl accumulation. In these cases, the intensity at the level of the leaves was about 10 ergs/cm^2 sec. The spectral energy distribution of this light source is shown in fig. 6. It was obtained by multiplying the emission spectrum of the green fluorescent tube by the transmission spectra of the filters, as measured in a CARY-14 recording spectrophotometer. The spectral energy distribution of the tube was obtained from PHILIPS Lampworks, Eindhoven, The Netherlands, through the courtesy of Dr. G. MEIJER.

4.4. MEASUREMENT OF LIGHT INTENSITY

Light intensities were measured with a thermopile (Radiatiometer E11, KIPP en Zonen, Delft, Holland) connected to a mirror galvanometer. In the lower intensity range, measurements were made with a calibrated photomultiplier tube (EMI 9558 B) and d.c. amplifier. The photomultiplier-amplifier combination was calibrated for spectral sensitivity against a thermopile at high intensities (fig. 7). Its linearity over a broad light intensity range was checked. In this way, it proved possible to measure intensities down to 0.3–0.6 ergs/cm^2 sec, depending upon the wavelength.

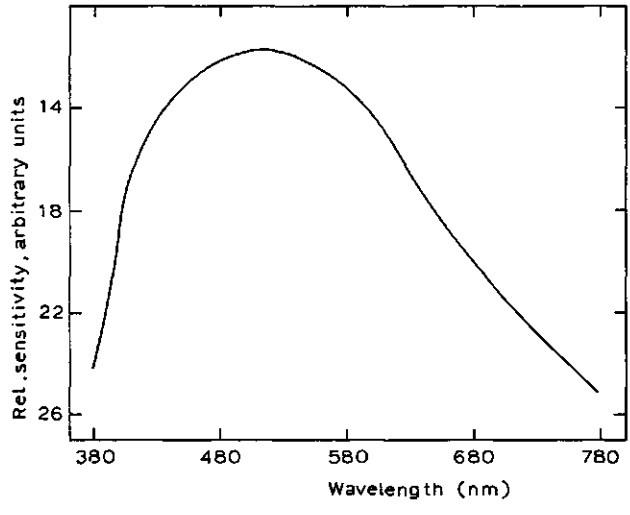

FIG. 7. Relative spectral sensitivity of the EMI 9558 B photomultiplier tube.

4.5. Application of chemicals

Most chemicals were administered as solutions in phosphate buffer, pH about 6.5. The basal ends of 25 sections, cut from stems plus plumules of pea, 3 cm in length, were placed in such solutions. They were held upright by means of a sheet of perforated plastic (diameter 5 cm, 0.4 cm diameter of the holes), mounted at 1 cm above the bottom of a 100 ml glass beaker containing about 20 ml solution.

Detached bean leaves were floated in petri dishes on 15 ml of a solution of the antibiotic chloramphenicol (1.6 mg/ml in distilled water).

4.6. Pigment estimation

Except for phytochrome, all pigment estimations were made in organic extracts from the leaves. A standard procedure was developed in which 1- or ½-gram samples of leaves were weighed on a 'Centrogram' balance (Ohaus Scale Corp. model 311) to the nearest 0.01 gram. These samples were taken from a predetermined number of leaves, out of which 1 or ½ gram fresh weight was collected. The remaining leaves were counted so that the actual number of leaves in the sample was also known. The samples were extracted by grinding with 12 ml pure acetone and a little washed sea sand (Merck, Darmstadt, Germany) in a small pre-cooled mortar. A small amount of calcium carbonate was added to prevent pheophytin formation during extraction. The liquid was removed by filtration with suction on a porous glass filter. The residue on the filter was again extracted with about 7 ml of a mixture of 8 volumes acetone and 2 volumes of distilled water. Vessels and filters were washed with a few milliliters of the same solvent. The combined extracts were then centrifuged for 30 minutes at 60,000 g (at the top of the tube) and 2 °C. The clear supernatant was removed quantitatively, 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 at several wavelengths in the region 473–800 nm in a Beckman spectrophotometer model DU (some experiments in Chapters 5 and 6) or in a Zeiss model PMQ II spectrophotometer, equipped with a grating monochromator M 20. Occasionally, absorption spectra of the extracts were run on a Cary model 14 spectrophotometer. The extracts were transferred to the spectrophotometer as quickly as possible in dim daylight, to avoid photobleaching of pigments (Bruinsma, 1963).

Phytochrome was estimated spectrophotometrically *in vivo* in leaves of pea, bean, and maize, using a dual wavelength spectrophotometer (Spruit, 1970).

4.7. Calculation of pigment concentrations

We have adopted the molar absorption coefficients used by Boardman (1962)

for the calculation of Pchl and Chl-*a* concentrations. This results in the use of the following equations to obtain the individual pigment concentrations in μg/ml in terms of the extinctions (E) at the various wavelengths:

$$[\text{Pchl}] = 28.7\ E_{628} - 5.2\ E_{664-666}$$

$$[\text{Chl-}a] = 12.2\ E_{664-666} - 0.1\ E_{628}$$

For reasons to be discussed in more detail later on (section 5.3.) a slight deviation from BOARDMAN's procedure was adopted. The maximum extinction in the wavelength region 664–666 nm was used for calculation of Pchl and Chl-*a* concentrations, the exact situation of the peak showing slight shifts dependent on the time of extraction after Pchl photoconversion.

Chl-*a* and Chl-*b* contents were calculated in an analogous way, using MACKINNEY's (1941) specific absorption coefficients in the following equations:

$$[\text{Chl-}a] = 12.7\ E_{664} - 2.7\ E_{646}$$

$$[\text{Chl-}b] = 22.9\ E_{646} - 4.7\ E_{664}$$

Optical density readings at 800 nm were subtracted from the readings at the other wavelengths as a correction for scatter. This correction rarely amounted to more than 0.01 O.D. units.

In order to obtain some indication of the total quantity of carotenoids in the sample, the extinction at 473 nm was also determined (BOTTOMLEY, 1970).

4.8. DRY WEIGHT DETERMINATION

The dry weight of pea plumules was determined after drying weighed, freshly picked samples of 50 plumules to constant weight at 110 °C.

4.9. ELECTRON MICROSCOPY

After the appropriate treatment, leaf samples of pea were cut into small pieces (about 1 mm^2) in green safelight and fixed in a 4 % solution of distilled glutaraldehyde (FAHIMI and DROCHMANS, 1965). Thereafter they were rinsed, post-fixed with 1 % osmium tetroxide, rinsed, dehydrated with alcohol and embedded in Epon-Araldite. All aqueous solutions were made up in 0.1 molar phosphate buffer, pH 7.0.

After cutting, sections were stained with uranyl acetate and lead citrate and examined with a PHILIPS EM 300 electron microscope. All handling after the glutaraldehyde fixation was performed by the Technical and Physical Engineering Research Service at Wageningen.

5. PROTOCHLOROPHYLL PHOTOTRANSFORMATION

5.1. INTRODUCTION

Upon illumination of dark-grown leaves of higher plants the Pchl already present is rapidly converted to Chl-*a*. This phototransformation can be considered as the first phase in the greening process of dark-grown leaves (VIRGIN, 1958). It is now generally assumed that Pchl functions as the immediate precursor of Chl-*a* not only in the greening process (SMITH, 1960) but also in normally developed, mature green leaves during Chl-turnover (SHLYK *et al.*, 1969). As expounded in section 2.1., two Pchl type pigments have been observed in plants. Therefore, we have attempted to determine for our plant material the contributions of Pchlide and Pchlide ester respectively in the photoconversion process and the nature of the products resulting from their phototransformation.

It also appeared important to know the exact Pchl phototransformation rates as affected by wavelength, intensity of light, and temperature under our experimental conditions.

5.2. RESULTS

In fig. 8, the absorption spectra for Pchl and two Chl type pigments in the orange-red wavelength region of the spectrum are shown. The pigments were extracted from the plants with 80% acetone (v/v) and measured in this solvent.

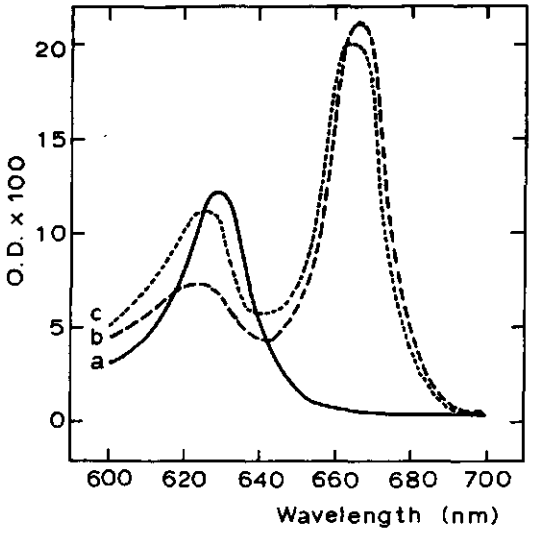

FIG. 8. Absorption spectra of extracts of 10-day old bean leaves of cv. Widusa in 80% acetone: a. Dark-grown (———); b. Immediately following 5 minutes red light (650 nm, 2.2 × 10^4 ergs/cm² sec) (– – –); c. After an additional 60 minutes darkness at 25 °C (· · · · · ·).

The spectra were run in a CARY-14 recording spectrophotometer. Only the absorption spectra above 550 nm are presented, since carotenoids and other yellow compounds, present in the crude pigment extracts, strongly distort the spectra in the blue wavelength region. For the same reason, only the red absorption bands of Pchl and Chl are normally used for routine quantitative spectrophotometric estimation.

The Pchl absorption band of acetone-extracted leaves of completely dark-grown bean seedlings is situated around 628 nm (fig. 8, curve a). Only trace amounts of Chl appeared to be present in dark-grown leaves of bean and maize. In pea leaves, however, significant amounts of Chl-*a* and Chl-*b* were detected, that possibly originated in their embryonic stage of development (RAVEN, 1972a).

Brief irradiation with light of suitable wavelengths and intensity followed by immediate extraction with acetone yielded a Chl type pigment absorbing around 666 nm (fig. 8, curve b). An absorption band was observed at 664 nm, however, when a dark interval was inserted between Pchl photoconversion and acetone-extraction procedure (fig. 8, curve c), and it appears likely that different compounds were present in these two cases. We have attempted to analyze these photoproducts of Pchl conversion on the basis of their distribution between 80% acetone and petroleum ether. If the extracted pigments were partitioned between the two organic layers immediately following irradiation of the plants, most of the Chl was found in the acetone phase. Intercalation of a dark period of 60 minutes between Pchl photoconversion and pigment extraction and partitioning, yielded a product, mainly moving into the hydrocarbon phase. These observations form an indication that the initial product from the Pchl photoconversion is more polar than the product formed after a dark period. This can be most readily interpreted as indicating that the dark reaction is the conversion of a short chain type Chlide-*a* into the long chain Chlide-*a* phytyl ester (see also section 2.2.). In contrast to what is generally assumed (SHIBATA, 1957), one may conclude that the visible absorption spectra, in 80% acetone, of Chlide-*a* and Chlide-*a* ester are significantly different, the red peak of the latter being shifted downward by about 2 nm. However, we have observed that esterification of Chlide-*a* appeared to take about 60 minutes for completion at 25 °C in 8-day old bean leaves. Most of the '2 nm *in vitro* shift' had already occurred within about 15 minutes at this temperature. Obviously, isolation and purification of the products is required to establish their identity.

It is of interest to note in fig. 8, curve c, the increase in absorption around 628 nm, which is due to Pchl regeneration during the 1-hour dark incubation at 25 °C. This process will be discussed in more detail in Chapter 6.

The reappearance of this Pchl absorption band cannot be responsible for the *in vitro* shift in the Chl peak, for it was possible to demonstrate the latter under conditions of no Pchl regeneration. To this end, either very old bean leaves were used, or regeneration was inhibited by chloramphenicol or by low (0 °C) temperature.

In the experiment, illustrated in fig. 9, the photoconversion of Pchl was

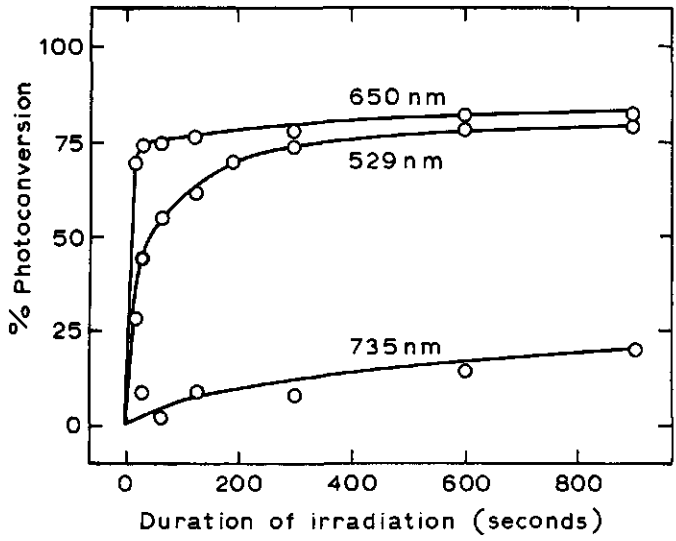

FIG. 9. Pchl photoconversion percentage in leaves of 8-day old bean seedlings of cv. Widusa at different wavelengths at 0°C as a function of the duration of irradiation.
Light intensities: 1.4×10^4 ergs/cm² sec at 529 nm, 2.2×10^4 ergs/cm² sec at 650 nm, 2.9×10^4 ergs/cm² sec at 735 nm.

measured as a function of the duration of irradiation with light of different wavelengths. The experiment was carried out at 0°C to inhibit Pchl regeneration. Separate samples of detached bean leaves were irradiated for the indicated periods of time. Immediately thereafter, the pigments were extracted with 80% acetone and estimated in a BECKMAN spectrophotometer. Both the quantities of Chl-*a* formed and of Pchl converted were determined. The results show that irradiations even as short as a few seconds with red (650 nm, 7.2×10^{15} quanta/cm² sec) light seem to saturate the photoreaction. Green (529 nm, 3.8×10^{15} quanta/cm² sec) light proved much less effective, although in this figure this effect is overestimated because of the lower quantum flux density of the green light. Far red light (735 nm, 1.1×10^{16} quanta/cm² sec) was highly though not completely ineffective for Pchl phototransformation.

We have invariably found a fraction of total Pchl non-transformable by light. The preferential solubility of this fraction in petroleum ether suggests that it represents the phytylated form of the pigment. Whereas the absolute amount of this non-photoconvertible Pchl remained rather constant with increasing age of the seedlings, it decreased when expressed as percentage of total Pchl present.

Obviously, one mole of Pchl converted by light should give one mole of Chl-*a*. After red irradiation, however, the ratio of the quantities of both pigments was lower than one (fig. 10). Apparently, a fraction of the pigment is lost during irradiation with red light, and less so with green. At 0°C, a relatively

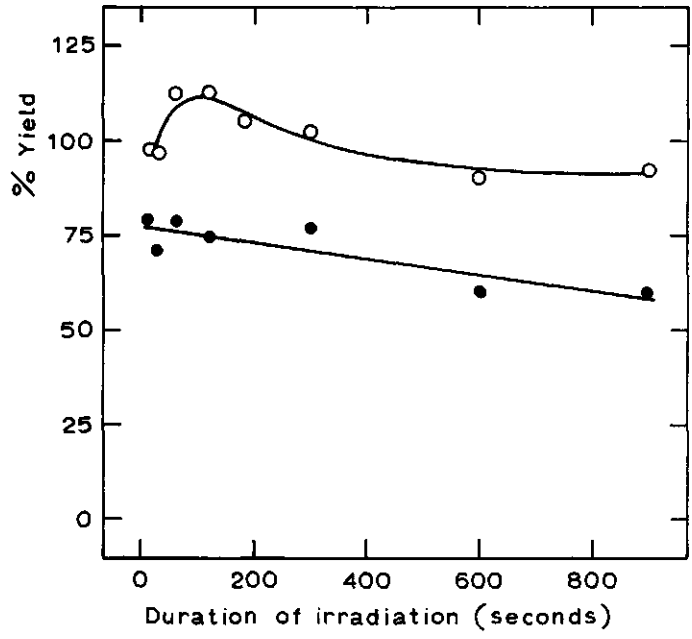

FIG. 10. Yield of Pchl photoconversion in leaves of 8-day old bean seedlings of cv. Widusa at 0 °C in green (529 nm, ○) and red (650 nm, ●) light as a function of the duration of irradiation. Light intensities: see legend to fig. 9.

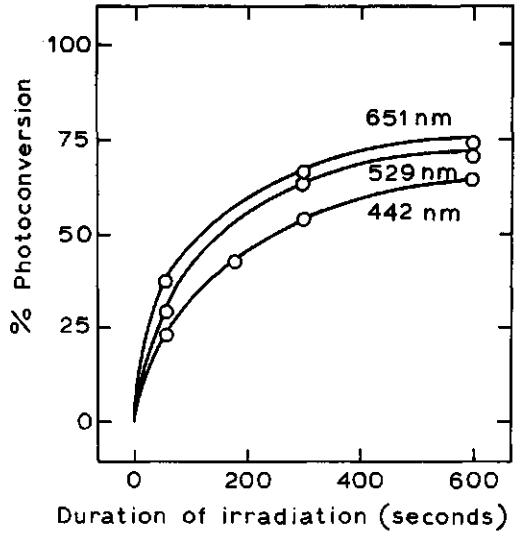

FIG. 11. Pchl photoconversion percentage in leaves of 7-day old pea seedlings of cv. Krombek at different wavelengths at 25 °C as a function of the duration of irradiation. The 100% level represents a photoconversion of 6.43 ± 0.13 μg Pchl/g fr. w. Light intensities: 335 ergs/cm² sec at 442 nm, 1200 ergs/cm² sec at 529 nm, 230 ergs/cm² sec at 651 nm.

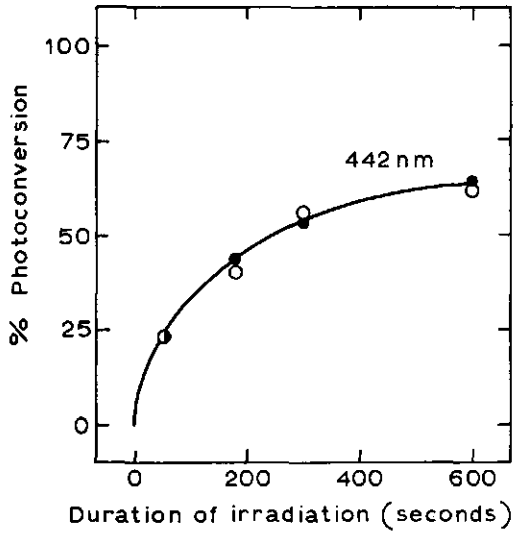

FIG. 12. Pchl photoconversion percentage in leaves of 7-day old pea seedlings of cv. Krombek provided with widely divergent carotenoid contents in blue (442 nm) light at 25 °C (see text); ● = seedlings not pretreated with red light, ○ = seedlings pretreated with 1 minute red at 16 hours prior to the irradiation with blue light. The 100% level for red light pretreated leaves represents a photoconversion of 5.58 ± 0.20 μg Pchl/g fr. w.

higher pigment loss was observed than at 25 °C. Most likely, this temperature effect is due to Pchl regeneration during illumination at 25 °C. High pigment losses were also observed in leaves of bean, pea, and maize, when briefly irradiated with high intensity (60,000 ergs/cm² sec) white fluorescent light.

Pchl phototransformation rates at rather low light intensities of different wavelengths were studied in 7-day old pea seedlings. Fig. 11 demonstrates that blue light (442 nm, 7.5×10^{13} quanta/cm² sec) is less effective than red (651 nm) of the same quantum flux density, in Pchl photoconversion. This is difficult to understand if Pchl really acts as the photoreceptor for its own photoconversion, since its blue absorption peak around 445 nm is much higher than its red absorption maximum around 650 nm (KOSKI *et al.*, 1951; see also fig. 1). The phenomenon is generally ascribed to substantial screening by carotenoids in the blue wavelength region. Green light (529 nm, 3.2×10^{14} quanta/cm² sec), again was less effective than red.

We have checked the possible interference of carotenoids in Pchl photoconversion in our material as follows: pretreatment with a saturating dose of red light followed by 16 hours darkness causes almost doubling of carotenoid pigments as compared with dark controls (see also section 8.2.1.). In such pretreated pea leaf material, the Pchl photoconversion rate in blue (442 nm) light was not significantly different from that of the non-pretreated control (fig. 12).

5.3. DISCUSSION

The experiments, summarized in connection with fig. 8 point to two Chl type products being formed from Pchl upon photoconversion. This observation is in

agreement with reports by WOLFF and PRICE (1957), VIRGIN (1960), and SIRONVAL and coworkers (1965), who detected Chlide-*a* as the main product, immediately following a brief irradiation, whereas after a dark interval only Chlide-*a* ester was observed.

Similarly, SIRONVAL and coworkers (1965) reported on a shift towards the blue of the red absorption band of the extracted Chl-*a*. They therefore concluded to a causal relation between these two sets of data. Since in our plant material the kinetics for these two reactions apparently were not completely identical, however, we feel that the matter should be studied in more detail before their conclusion can be accepted. Nevertheless, the observation has two important aspects with respect to the quantitative spectrophotometric estimation of the pigment concentrations concerned.

Firstly, this phenomenon makes it very difficult, in principle, to estimate accurately Pchl concentrations under conditions of a gradually changing background of Chl-*a* absorption. Especially, when the concentration of Chl-*a* is determined at a fixed wavelength, errors in Pchl estimation may be relatively serious, as is indicated schematically in fig. 13. For, the extinction value at the peak of Pchl absorption near 628 nm has to be corrected for concomitant absorption by Chl-*a*, which is normally measured at the peak of Chl-*a* absorption

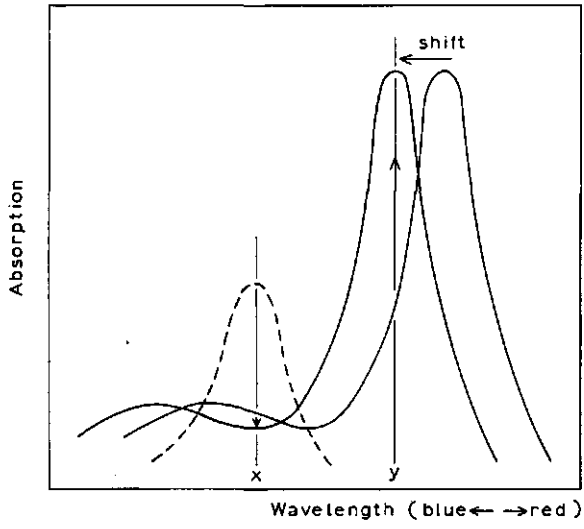

FIG. 13. Schematic representation of the shift towards the blue of the *in vitro* red absorption band of Chl-*a* (———) and of possible consequences of this phenomenon for the estimation of Pchl (– – – –). The molar absorption coefficients of Chl-*a* are assumed to be the same before and after the shift. Note the changing background of Chl-*a* absorption at measuring wavelength x, and the relatively large increase in absorption at measuring wavelength y. The absorption measurement at the peak of Chl-*a* is used as a basis for correcting for the contribution of Chl-*a* in the absorption at the peak of Pchl.

in the red wavelength region. We therefore always used the value of *maximum* absorption in the region 664–666 nm as a basis for correcting for the presence of Chl-*a*.

Secondly, whereas it is possible to determine in each separate extract the exact peak position of the red absorption band of Chl-*a*, it is as yet unknown whether the two Chl types have the same molar absorption coefficient. Since we have not observed large changes in apparent Chl-*a* content as determined by the standard spectrophotometric assay during darkness, following Pchl phototransformation, we have concluded that possible differences in the molar absorption coefficients between the two Chl-*a* forms at any rate must be small. For this reason, we feel assured that the molar absorption coefficients of BOARDMAN (1962) (section 4.7.) and the method for calculation of the quantities of Pchl remain useful. It may well be wise, however, not to attach much value to relatively small apparent changes in Pchl concentration.

Differences in Pchl phototransformation rates due to the wavelength and/or the intensity of the light are clearly shown in figs. 9 and 11. A very rough estimation, based upon the data of fig. 11, shows that, within reasonable limits, at the same quantum flux density red light appears to be 2 times more effective than blue in Pchl phototransformation. This figure is in rather good agreement with the results reported by KOSKI and coworkers (1951). An action spectrum, published by FRANK (1946), however, indicated a ratio of the effectivity in the red as compared to the blue of 0.8. Similarly, we have found that 651 nm light was about 5 times more effective than green (529 nm). From KOSKI's data a ratio of about 9, and from FRANK's data a factor of about 3 can be determined. In section 7.2.2. the relevance of these figures in connection with an action spectrum for greening will be discussed.

About 20% of total Pchl proved non-convertible by light. Similar results were reported by WOLFF and PRICE (1957) and GODNEV *et al.* (1968). They were interpreted to indicate that this fraction represents Pchlide (phytyl) ester. However, others have presented evidence that Pchlide ester in fact is partly and slowly phototransformable (LOEFFLER, 1955; SIRONVAL *et al.*, 1965; RUDOLPH and BUKATSCH, 1966). We are not aware of any chemical study, unequivocally identifying 'non-transformable Pchl' as the phytyl ester nor has it been demonstrated that the 'non-transformable Pchl' is a homogeneous fraction.

The observation that Chlide-*a* is one of the first products of Pchl phototransformation may have interesting implications for the explanation of the lag phase in Chl accumulation. The initial product of Pchl phototransformation has been reported to be sensitive to photobleaching (SMITH *et al.*, 1959; ANDERSON and ROBERTSON, 1961). Loss of pigment owing to photobleaching is also clearly demonstrated in fig. 10. SPRUIT and RAVEN (1970) reported that the Chl-*a* type absorbing *in vivo* at about 685 nm was the pigment being destroyed. SIRONVAL *et al.* had previously (1965) assumed that this is the *in vivo* form of the non-phytylated pigment.

KOSKI and coworkers (1951) observed that seedlings of a strain of albino corn, low in carotenoids, showed a blue/red peak ratio in the action spectrum

of Pchl phototransformation of 1.89. They compared this with a similar spectrum, made with seedlings of the wild type, where the ratio of the heights of the blue and red maxima was low, viz. 0.35, and concluded that in the wild type, carotenoids act as filters, decreasing the effectivity of blue light. As mentioned above (section 5.2.) we could not detect any significant difference in the Pchl phototransformation rate in blue (442 nm) light in pea seedlings containing twice as much carotenoids as the controls (fig. 12), confirming similar results obtained by FRANK (1946) with oat seedlings. At first sight these observations do not yield support for ideas about screening effects of carotenoids in the process of Pchl transformation. It must be borne in mind, however, that screening may already be near saturation at the initial carotenoid concentration.

6. REGENERATION OF PROTOCHLOROPHYLL IN DARKNESS FOLLOWING ILLUMINATION WITH RED AND FAR RED LIGHT

6.1. INTRODUCTION

A brief illumination of dark-grown plants results in the photoconversion of transformable Pchlide already present. Subsequent transfer of the plants to darkness is followed by formation of new Pchl molecules. We will call this process Pchl regeneration. According to many reports in the literature, phytochrome is involved in the greening of leaves of dark-grown seedlings. Pchl can be considered as the precursor of Chl-*a* throughout the greening process. Therefore, regulation of its biosynthesis could be a mechanism for phytochrome action on Chl formation. We have, therefore, studied regeneration of Pchl in leaves of dark-grown *Pisum sativum* L., *Phaseolus vulgaris* L., and *Zea mays* L. Pea was included since some earlier work had been performed with this plant, and its

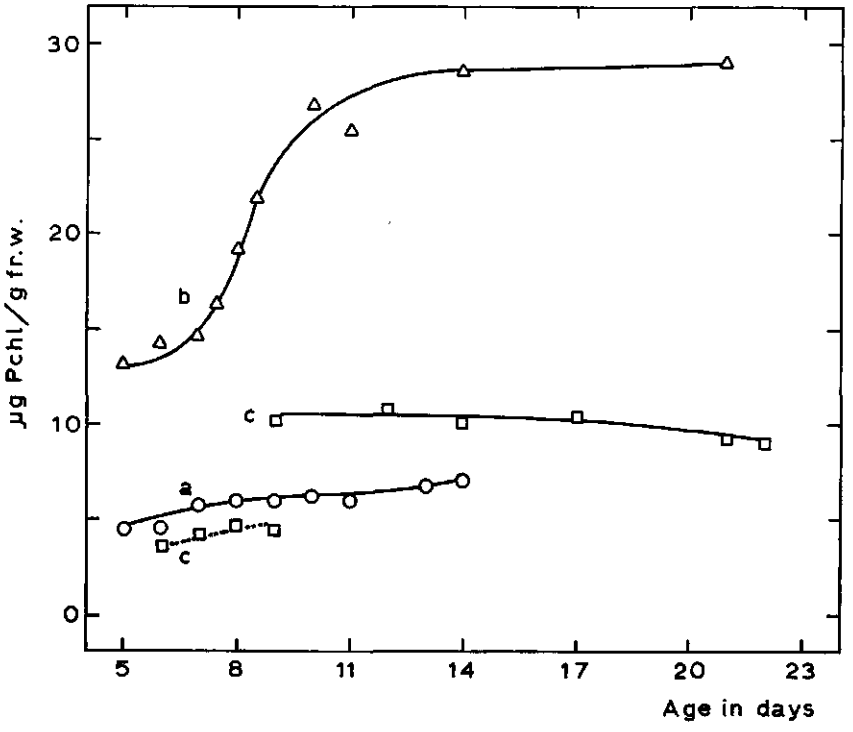

FIG. 14. Pchl content of leaves of dark-grown seedlings of varying age; ○ = pea cv. Krombek, △ = bean cv. Widusa, □···□ = maize (coleoptiles with leaves inside), □——□ = maize (leaves).

phytochrome content is particularly high. It proved a far from ideal plant because of the small size of the leaves of seedlings, grown in complete darkness, as well as their rather low pigment content per unit weight (fig. 14, curve a) and low regeneration capacity. Leaves of dark-grown bean are considerably larger and are especially rich in Pchl (fig. 14, curve b). Maize was chosen as a monocotyledon, a taxonomic group that e.g. differs from the dicotyledons in showing no phytochrome dark reversion *in vivo* (HILLMAN, 1967). Its Pchl content per unit weight is rather low, however (fig. 14, curves c).

6.2. RESULTS

Leaves of 8-day old bean seedlings were incubated in darkness at 25 °C during 4 hours while floating in petri dishes either on distilled water or on 5×10^{-3} molar chloramphenicol (CAM). Thereafter, the dishes with the leaves were irradiated with red light during 5 minutes, and replaced in the thermostat. Fig. 15 shows that regeneration of Pchl, as expressed in absolute amounts of pigment, started almost instantaneously following irradiation at 25 °C in the water control. After about 3 hours darkness the regeneration process reached its maximum level. However, in the leaves treated with CAM, regeneration of Pchl was strongly depressed during the first 2 hours at this temperature. The figure also suggests that incubation on this CAM concentration affected Pchlide phototransformability also. When the detached bean leaves were briefly irradiated at 0 °C, no Pchl regeneration could be demonstrated over a prolonged time.

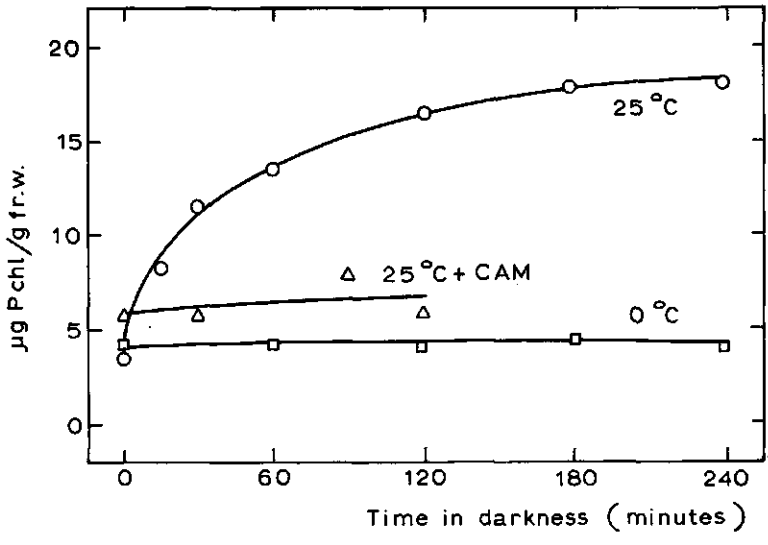

FIG. 15. Regeneration of Pchl in darkness following 5 minutes red (650 nm, 2.2×10^4 ergs/cm² sec) in 8-day old leaves of bean seedlings of cv. Widusa at different temperatures and after application of 5×10^{-3} M chloramphenicol (CAM) at 25 °C.

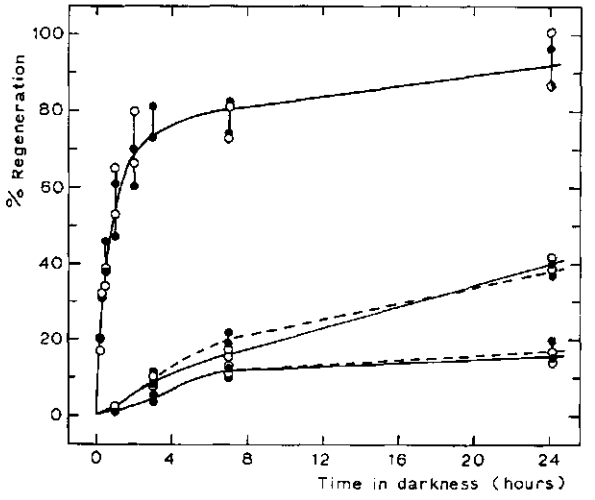

FIG. 16. Regeneration of Pchl in leaves of bean seedlings of cv. Widusa of varying age, during a dark period at 25 °C following 5 minutes red (650 nm, 2.2 × 10^4 ergs/cm² sec) (○——○) or 5 minutes red followed by 5 minutes far red (735 nm, 2.9 × 10^4 ergs/cm² sec) (●- - -●). Results expressed as per cent of the amounts of phototransformed Pchl. The 100% level represents the following absolute amounts of pigment per gram fresh weight: 8-day old seedlings, ca. 12 μg; 14 days, ca. 23 μg; 21 days, ca. 23 μg.

In the standard Pchl regeneration experiments leaf material was placed in petri dishes, the lids of which were lined with 4 layers of moist filter paper. After irradiation, these were put in light-tight tins in a thermostat at 25 °C for the required periods. Fig. 16 gives the percentage regeneration of Pchl for 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 plant material. In the 8-day old leaves regeneration started without a considerable lag phase and ultimately reached a plateau that appeared to approach closely the Pchl level before illumination. In the older leaves the regeneration capacity decreased to very low values. During regeneration periods up to 24 hours, no significant effects of a terminal far red irradiation upon either the initial rate or the plateau level of Pchl was found.

Pea leaves present a somewhat different picture (fig. 17). Regeneration never reached more than 50% of the original Pchl level. At any rate in leaves between 5 and 14 days old, regeneration uniformly stopped at this low level. In this plant, we neither observed any difference in the rates of Pchl regeneration after red or red followed by far red.

In maize of various ages (fig. 18), regeneration fairly rapidly leads to a plateau of about 80% of the original value in 9-day old coleoptiles. In older leaves – in contrast with bean – a high regeneration capacity was observed as well. The duration of the induction period increased with increasing age of the leaves.

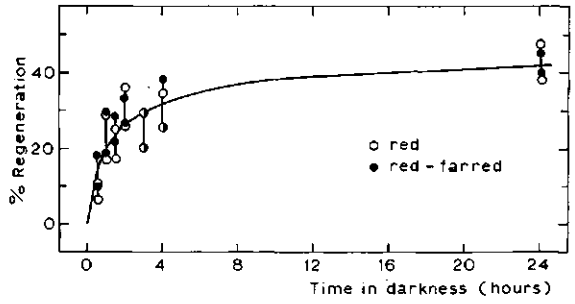

FIG. 17. Regeneration of Pchl in leaves of 7-day old pea seedlings of cv. Krombek. See also legend to fig. 16. The 100% level represents a pigment regeneration of ca. 3.5 μg/g fr. w.

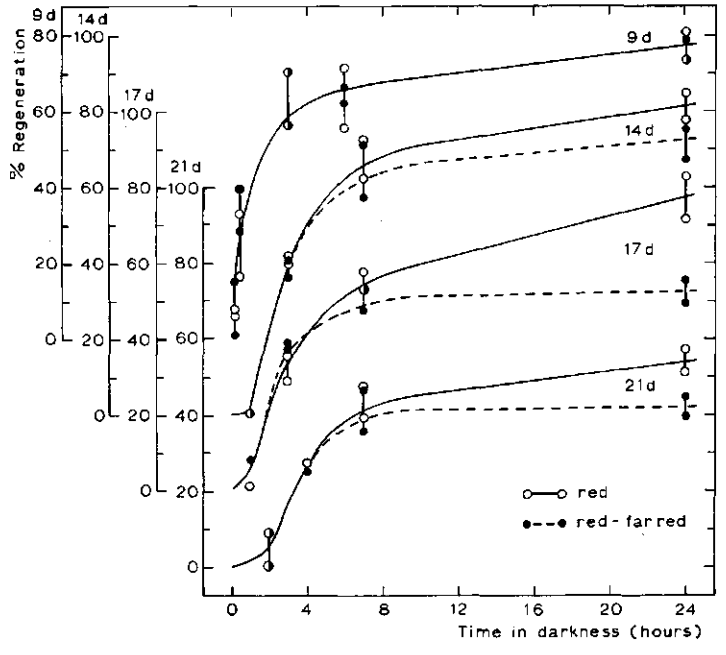

FIG. 18. Regeneration of Pchl in 9-day old maize coleoptiles with leaves inside and in leaves of various ages. To facilitate the reading, the curves for the successive ages are shifted along the vertical axis. See further legend to fig. 16. The 100% level represents the following pigment regenerations per gram fresh weight: 9 days, ca. 3.5 μg; 14 days, ca. 8 μg; 17 days, ca. 8 μg; 21 days, ca. 7 μg.

During rapid accumulation of Pchl, no significant differences between red and red-far red were observed. After longer dark incubation, a difference between the two light treatments became apparent, which was 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%. It shows that far red following red decreases the final level of Pchl reached in prolonged darkness. In coleoptiles, this effect could not be demonstrated, whereas in leaves, older than 17 days, the magnitude of the effect decreased again.

Since, at normal light intensities, the rate of Pchl phototransformation greatly exceeds the rate of dark regeneration, the steady state level of Pchl in the greening leaves should be very low. It is then the initial rate of Pchl regeneration rather than the final pigment level reached after prolonged dark periods that should govern the rate of Chl-*a* accumulation in continuous light. We decided to measure the initial Pchl accumulation rate again, but now following a second brief light treatment. A 1-hour dark interval was inserted between the two light treatments. Pigments were extracted after an additional 1-hour dark period at 25 °C. In table 1 the results are shown, obtained with detached bean leaves of various ages. In 9-day old leaves the initial increase in Pchl proved to be much lower than in younger leaves. The differences between the four irradiation experiments are not statistically significant, and there is no evidence that phytochrome is actively involved in the initial phase of Pchl regeneration. A similar type of experiment was performed with intact bean seedlings of cv. Widuco.

TABLE 1. Pchl regeneration in darkness at 25 °C following various light treatments in leaves of bean seedlings of cv. Widusa of varying age.

Treatment	Age (days)		
	7	8	9
Dark control	14.9	16.9	19.6
R, D, R, D	10.2	9.9	6.4
R/FR, D, R/FR, D	10.6	12.1	6.3
R/FR, D, R, D	9.9	9.3	6.5
R, D, R/FR, D	11.1	10.5	6.3

Pchl content in μg/g fr. w.; R = 2 minutes red (650 nm, 2.2×10^4 ergs/cm² sec), FR = 3 minutes far red (735 nm, 2.9×10^4 ergs/cm² sec), D = 1 hour darkness.

Repetitive exposures with either red only or red-far red were given at 2-hour intervals. The ultimate light-dark cycle was followed by an additional 14-hour dark period at 20 °C. Thereafter, leaf samples were harvested and their pigments were extracted in acetone. In fig. 19, the data are expressed as amounts of Pchl per constant number of leaves. In all cases, the level of Pchl at the end of the 14-hour dark period reached the level of the dark control and no clear cut indication for red-far red reversibility was found. Incidentally, this experiment also indicates that harvesting the leaves before performing Pchl regeneration experiments is not responsible for the relatively rapid cessation of Pchl regeneration.

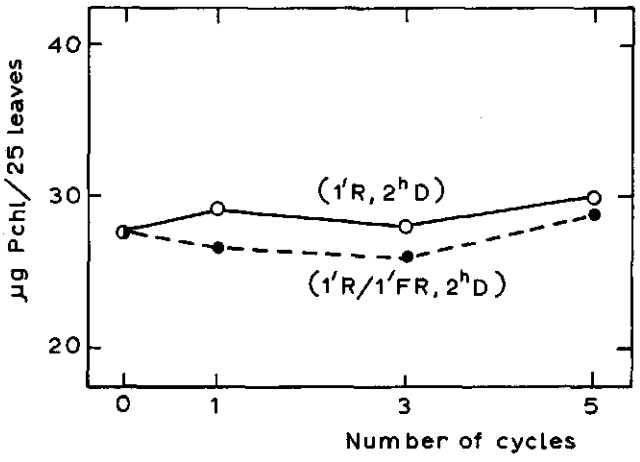

FIG. 19. Effect of different numbers of cycles, each consisting of a short light impulse followed by 2 hours darkness, upon the level of Pchl in leaves of 10-day old intact bean seedlings of cv. Widuco. Pchl was estimated after an additional 14-hour dark period at 20°C. R: 651 nm, 3000 ergs/cm² sec; FR: 739 nm, 4150 ergs/cm² sec.

6.3. DISCUSSION

Regeneration of Pchl was shown (fig. 15) to be temperature dependent as well as sensitive towards an inhibitor of protein synthesis. At 0°C, no Pchl regeneration was observed, which is in agreement with results of VIRGIN (1955) and GRANICK and GASSMAN (1970). For temperatures between 0° and 22°C, VIRGIN (1955) reported a Q_{10} of about 2, whereas in the temperature range 22° to 30°C, a Q_{10} of about 1 was observed. CAM at a concentration of 5×10^{-3} molar, applied 4 hours prior to the brief red exposure, strongly affected the initial rate of Pchl regeneration. It also slightly influenced Pchl photoconversion, as may be concluded from the increase in 'non-transformable' Pchl. On the other hand, we have found no indications of any effect of this agent on the rate of phytolization of Chlide-*a* formed, or on the *in vitro* spectral shift. GASSMAN and BOGORAD (1967b) obtained rather similar results, not only with CAM but also with puromycin and the inhibitor of RNA synthesis, actinomycin D. Interestingly, they observed no inhibition by these agents in leaves simultaneously supplied with δ-aminolevulinic acid and concluded that the regeneration of Pchl strongly depends on the synthesis of RNA and enzymes required for the production of δ-aminolevulinate.

In our experiments, regeneration of Pchl proved to be also strongly dependent on the age of the leaves. 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 irradiation. In older leaves of maize and possibly also of bean, there seems to be an induction in Pchl regeneration

lasting a few hours. Earlier reports on the age-dependency of the duration of this induction phase in Pchl regeneration were given by SHIBATA (1957), BUTLER (1965), and AKOYUNOGLOU and SIEGELMAN (1968). No induction period in the dark regeneration was found by SCHARFNAGEL (1931) and VIRGIN (1955), whereas GOEDHEER (1961), MADSEN (1962), AUGUSTINUSSEN and MADSEN (1965), and BOGORAD *et al.* (1968) always observed an induction period before the onset of rapid Pchl regeneration. We may assume, therefore, that contradictory observations concerning the kinetics of Pchl regeneration may be ascribed to differences in the age of the plant material. However, an explanation for the occurrence of an induction phase in older plant material is not available at present. A closer examination of the underlying biochemical mechanism would be required for the understanding of Pchl biosynthesis. Such a study, however, exceeds the scope of the present investigations.

In young leaves of bean and maize the plateau of Pchl regeneration ultimately reached, appears to approach closely the Pchl level before illumination. This has been observed repeatedly by others in similar experiments (e.g. AKOYUNOGLOU and SIEGELMAN, 1968). This favours the hypothesis that Pchl regeneration, at least initially, takes place in a constant number of generating centres (BOARDMAN, 1967; BOGORAD *et al.*, 1968; SUNDQVIST, 1969; SÜZER and SAUER, 1971; THORNE, 1971a, 1971b). However, in older leaves as well as in pea leaves of all ages studied, the regeneration capacity is depressed: figs. 16, 17 and 18. This observation is difficult to reconcile with the above hypothesis unless we suppose that depletion of precursors accounts for the low regeneration capacity in aging leaves. GRAHAM and coworkers (1968) found significantly less CALVIN cycle enzymes in pea leaves than in barley leaves. The latter have a full Pchl regenerating capacity. They suggested, therefore, that the ultrastructural development of pea etioplasts is primitive as compared with those of barley.

Our results have failed to show significant effects of the phytochrome system upon Pchl regeneration during the first hours of darkness, confirming earlier observations of SPRUIT (1967) with pea and of JACQUES (1968) with oat seedlings. In maize a red-far red effect could be observed after long dark periods only (fig. 18), showing that the final Pchl level after 24 hours darkness, is depressed by a preceding terminal far red illumination. Similar results were recently (1972) reported by MASONER *et al.* for 2-day old mustard seedlings. AKOYUNOGLOU (1970) observed a strongly diminished initial rate of regeneration in 9-day old bean leaves upon far red irradiation. AUGUSTINUSSEN and MADSEN (1965) reported that in barley both the initial rate and the capacity of Pchl regeneration following a white flash were depressed if the seedlings previously received a red-far red exposure. According to RUDOLPH (1965), in barley, far red was only antagonistic to red with respect to Pchl regeneration capacity when a repetitive irradiation scheme was used. We have not been able to confirm these observations after repeated irradiation of either detached bean leaves of various ages (table 1) or intact bean seedlings (fig. 19). We conclude, therefore, that under carefully controlled conditions, no indications can be found that the initial rate of Pchl regeneration is regulated by the red-far red morphogenic mechanism.

7. CHLOROPHYLL ACCUMULATION IN CONTINUOUS LIGHT

7.1. Introduction

This Chapter describes experiments on the influence of variables such as light intensity and wavelength upon the lag phase and the early development of the photosynthetic pigment apparatus in intact seedlings and in detached leaves. Several authors (Virgin, 1958; Miller and Zalik, 1965; Farineau, 1968; Jacques, 1968) have already reported on the effect of light colour upon Chl formation in higher plants. Their results, although not uniform, seem to point to continuous red light being about equally effective as blue light in the greening process. However, these conclusions have not been based upon a study of dose-response curves or action spectra for greening. In fact, an action spectrum exists for Chl-*a* formation during prolonged irradiation of oat seedlings (Frank, 1946), but it was measured at such low light intensities that Pchl photoconversion must have been the limiting factor in Chl-*a* formation (Virgin, 1955). We have, therefore, measured some action spectra involved in greening (section 7.2.2.). Considerable care was taken to use light intensities that did not cause photobleaching of pigments, while still saturating the Pchl phototransformation rate.

7.2. Results

7.2.1. *Greening in white light*

There are a number of literature reports on greening experiments, performed with detached leaves. For reasons to be presented in more detail later on (section 7.3.), it appeared advisable to investigate whether or not this treatment affects the greening properties (section 7.2.1.2.). At the same time detached leaf material was used to study the effect of some chemicals upon the greening process.

7.2.1.1. Greening of intact seedlings. In fig. 20, the greening of 7-day old dark-grown pea plumules during a 48-hour period is shown. In the relatively low intensity (1500 ergs/cm^2 sec) of white fluorescent light, four distinct phases in the course of greening can be distinguished: first there is the rapid photoconversion of already existing Pchl to Chl-*a* which took about 5 minutes. This is followed by a lag phase, lasting about 1 hour, during which relatively little additional pigment accumulates. During the next 8-hour period, there is an accelerated formation of Chl-*a*. Subsequently, Chl accumulates at a more or less steady rate over a prolonged period, finally leveling off when the pigment content of the leaves approaches that of a mature green leaf (not shown in this figure).

During this 48-hour period, there is considerable development of the leaves.

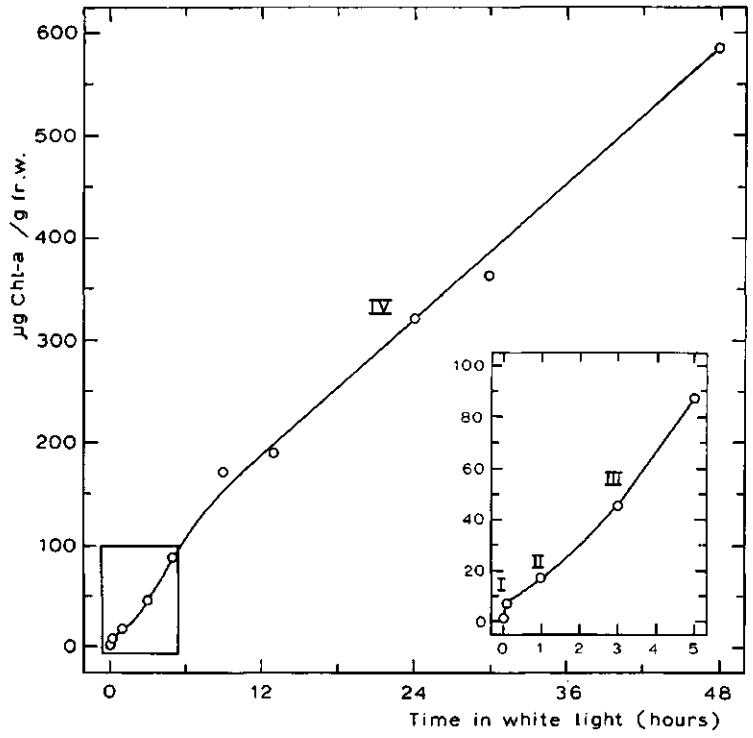

FIG. 20. Time course of Chl-*a* per g fr. w. in leaves of 7-day old, dark-grown pea seedlings of cv. Krombek in continuous white fluorescent light of low intensity (1500 ergs/cm² sec) at 25 °C.

Fig. 21 shows the kinetics of fresh weight accumulation of pea plumules, that show characteristic differences with the course of Chl-*a*. Before onset of the rapid plumule growth a marked initial decrease in fresh weight is observed, whereas upon prolonged illumination again an acceleration in gain in fresh weight becomes apparent. This has some consequences when the greening data are expressed as the amount of Chl-*a* per plumule (fig. 22). Plotted this way, the rate of Chl-*a* accumulation appears to increase almost continuously over a 24-hour period until it finally becomes constant.

In view of the observation of pigment photobleaching in red light (section 5.2.), it seemed of interest to study the influence of light intensity upon the greening process. The results are shown in fig. 23. Obviously, both the amount of early Chl accumulation, expressing itself as the lag phase, as well as the rapid phases of greening are affected by the intensity of the white light as obtained from fluorescent tubes. However, the marked difference between the accumulation rates in the two light intensities gradually disappears. We may also consider the possibility that the lower values at high light intensity may be due to differences in water supply; we can only say that this point always had our full attention.

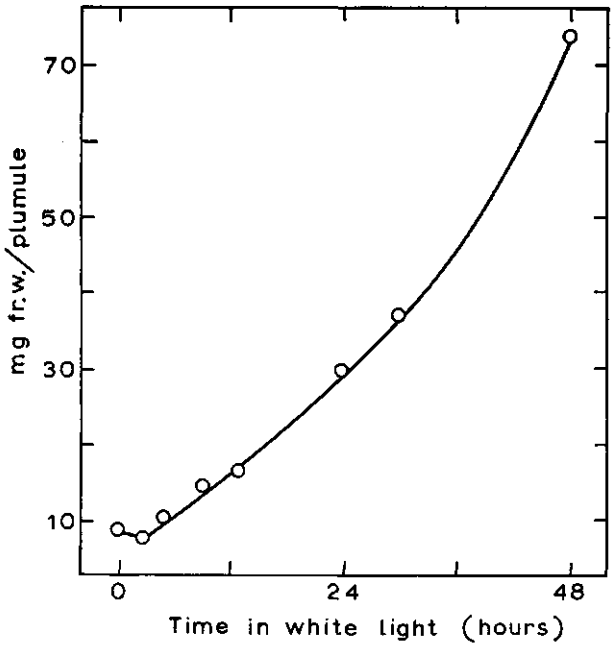

FIG. 21. Time course of fresh weight of plumules of 7-day old, dark-grown pea seedlings of cv. Krombek in continuous white fluorescent light of low intensity (1500 ergs/cm² sec) at 25 °C.

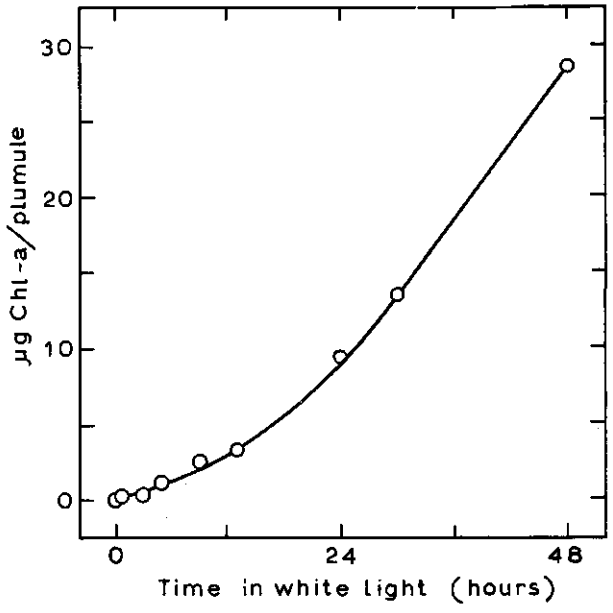

FIG. 22. Time course of Chl-*a* per plumule of 7-day old, dark-grown pea seedlings of cv. Krombek in continuous white fluorescent light of low intensity (1500 ergs/cm² sec) at 25 °C.

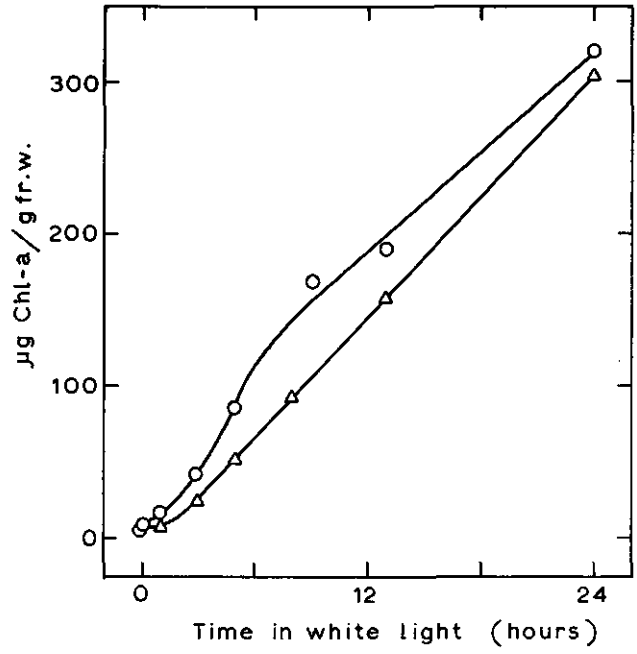

FIG. 23. Effect of the intensity of white fluorescent light upon the time course of Chl-*a* in leaves of 7-day old pea seedlings of cv. Krombek at 25 °C; ○ = 1500 ergs/cm² sec, △ = 60,000 ergs/cm² sec.

Initially, the main product of the greening process is Chl-*a*. However, formation of Chl-*b* appears to start soon after the photoconversion of the Pchl present (SHLYK *et al.*, 1970; THORNE and BOARDMAN, 1971), and gradually accelerates during prolonged irradiation. This means that the ratio Chl-*a*/Chl-*b* should drop from a high value, immediately after the start of irradiation to its ultimate value of 3–5 after prolonged irradiation.

We observed that during the early stages of greening Chl-*b* formation was even more sensitive towards the high light intensity, as shown by increased Chl-*a* to Chl-*b* ratios (fig. 24). In this figure, Chl-*a* to Chl-*b* ratios are not corrected for the small amount of Chl-*b* already present in the dark-grown pea leaves (RAVEN, 1972a), which explains their initial low values. The sudden decrease in the *a*/*b* ratio during the first hour of illumination may be due to preferential formation of a small amount of Chl-*b* during this period or to preferential Chl-*a* destruction. Thereafter, Chl-*b* formation apparently shows a lag period, which duration exceeds that of the lag phase in Chl-*a* accumulation, as may be judged from the second rise in *a*/*b* ratio. This differential duration of the respective lag phases in Chl-*a* and Chl-*b* formation becomes very pronounced at high light intensity. After prolonged irradiation both pigments, however, are formed in a rather constant ratio, which is nearly independent of the light intensity.

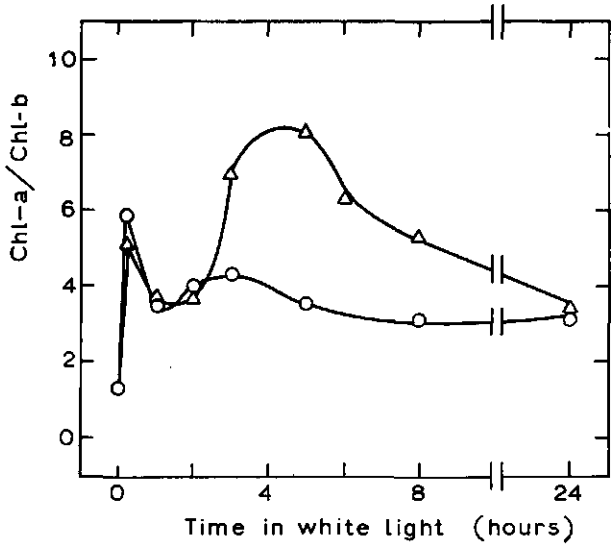

FIG. 24. Effect of the intensity of white fluorescent light upon Chl-*a* to Chl-*b* ratio during greening of 7-day old pea seedlings of cv. Krombek at 25 °C. The ratio is not corrected for the small amounts of Chl-*a* and Chl-*b* already present in the dark-grown leaves; ○ = 1500 ergs/cm² sec, △ = 60,000 ergs/cm² sec.

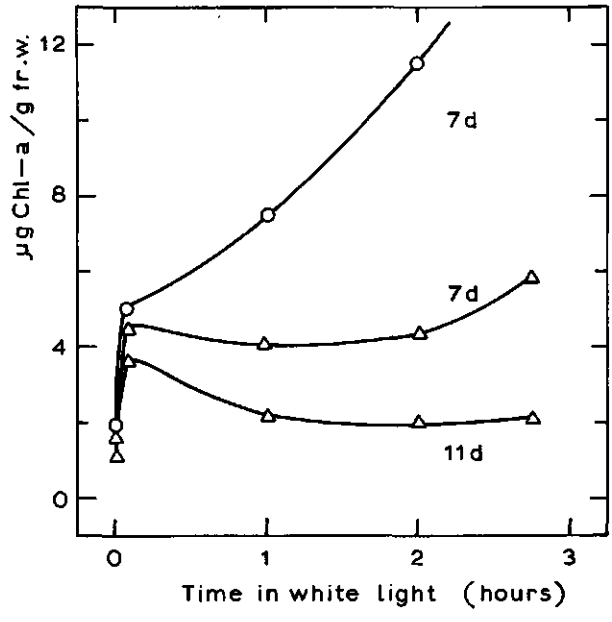

FIG. 25. Time course of Chl-*a* during the lag phase at 25 °C in dark-grown pea seedlings of cv. Krombek as influenced by the intensity of white fluorescent light and seedling age; ○ = 1500 ergs/cm² sec, △ = 60,000 ergs/cm² sec. For comparison: the initial rate of Pchl regeneration in darkness at 25 °C following 5 minutes red: 7-day old seedlings, 1.2 μg Pchl/g fr. w./h; 11 days, 0.5 μg Pchl/g fr. w./h.

We have attempted to compare the rate of Chl-*a* accumulation during the lag period with the initial rate of Pchl regeneration in darkness (fig. 25). For this experiment, seedlings were grown in shallow earthenware pans as described in section 4.2. This cultivation method gives less well-developed seedlings, compared with those, grown in flower pots, as judged from fresh weight of the leaves and their pigment content. This difference was not, however, reflected in the greening kinetics, and both cultivation methods yield comparable seedlings in this respect.

We must conclude from these experiments that pigment (most probably Chlide-*a*) photobleaching is an important factor in the greening process: at the highest light intensity, and especially in 11-day old pea plumules, the Chl-*a* content even decreases temporarily before rapid Chl accumulation sets in. The simultaneous data for Pchl regeneration (see legend to fig. 25) suggest that appreciable quantities of Pchl must have been formed within this period. If, on the other hand, we compare the rate of Chl accumulation during the lag phase in low intensity light with the rate of formation of Pchl in the dark, we note that the latter is slower. We conclude, therefore, that, if we avoid pigment (Chlide-*a*) photobleaching the rate of Pchl synthesis during the lag period starts to exceed the rate in the dark. This gradually becomes more marked during the rapid phases of greening.

TABLE 2. Effect of damaging of 10-day old bean seedlings of cv. Widuco upon Chl-*a* accumulation during 5 hours white light (1500 ergs/cm^2 sec) at 25 °C.

Number of cotyledons present	Isolated leaves	Isolated hook tissue + leaves	'Intact' seedlings
0	87.5	74.5	184.5
1	–	125.0	229.3
2	–	138.0	276.0

Chl-*a* content in μg/25 leaves.

7.2.1.2. Greening of isolated parts of seedlings. In fig. 26 results are shown of greening experiments with pea cuttings, 3 cm in length, placed on distilled water or various solutions in beakers in low intensity white fluorescent light. A comparison with fig. 22 shows that the rates as well as the total capacity for Chl formation are strongly depressed as compared with leaves of intact plants. Application of 2% sucrose appears to give some improvement, although not during the lag phase in which energy supply still may be non-limiting. Surprisingly, 5×10^{-3} molar ALA in 35×10^{-3} molar phosphate buffer, pH 6.5, tends to depress Chl accumulation even further. These data suggested a rôle for reserve substances as e.g. stored in the cotyledons, upon the rate of Pchl synthesis. Table 2 shows that greening in isolated bean leaves also is very

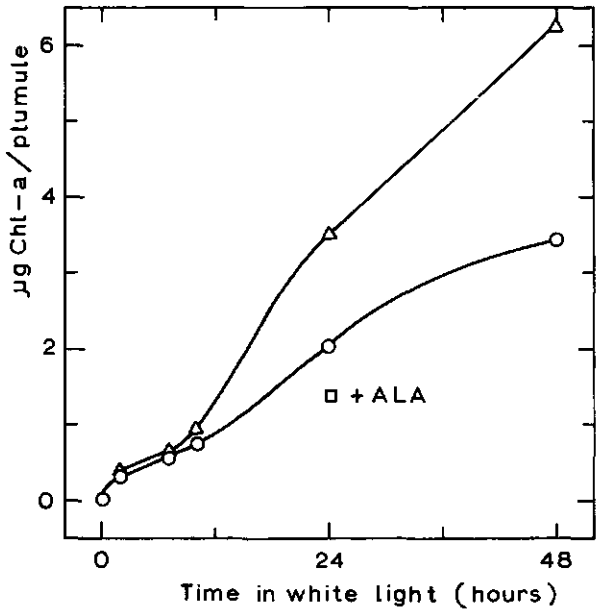

FIG. 26. Time course of Chl-*a* per plumule in cuttings of 8-day old pea seedlings of cv. Krombek in continuous white fluorescent light of low intensity (1500 ergs/cm² sec) at 25 °C as influenced by various chemicals; ○ = distilled water, △ = 2% sucrose in distilled water, □ = 5×10^{-3} M aminolevulinic acid (ALA) in 35×10^{-3} M phosphate buffer, pH 6.5.

poor as compared with leaves of bean left on the seedling in the simultaneous presence of both cotyledons. The removal of one or both cotyledons from seedlings otherwise left intact, already strongly affects Chl-*a* formation. The remaining difference in Chl-*a* content between isolated leaves and leaves on seedlings, deprived of their cotyledons, indicates that the cotyledons are not the only sources of energy and additional factors during the early stages of greening. The greening data for leaves left on isolated hooks point to a similar conclusion.

Fig. 27 shows the effect of concentration of some antibiotics in the presence or absence of added sucrose on Chl accumulation in isolated pea plumules. In the absence of sucrose both cycloheximide and CAM strongly inhibit Chl accumulation. Sucrose has no counteracting effect with cycloheximide, whereas it clearly counteracts the inhibition at low doses of CAM. This may indicate that CAM is a more specific inhibitor of greening than cycloheximide. It is possible that the latter affects the synthesis of structural proteins, required for binding of newly formed Chl molecules, which is suggested by its lack of effect on Pchl regeneration (BOGORAD *et al.*, 1968). ALA has no effect at all in the simultaneous presence of 10 µg cycloheximide per ml.

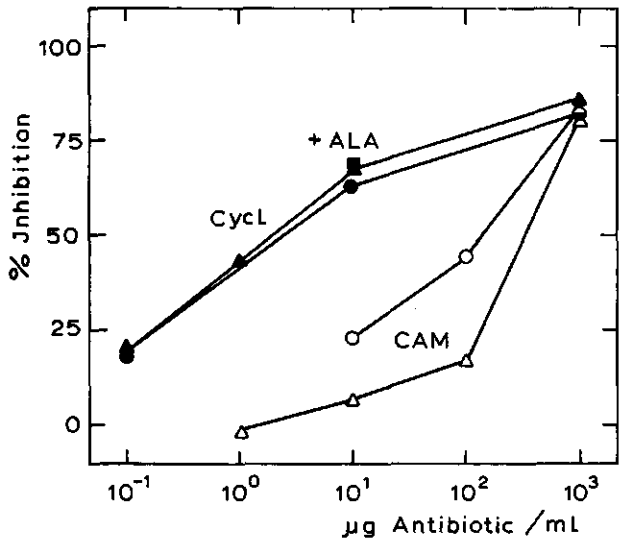

FIG. 27. Effect of the concentration of chloramphenicol (CAM) and cycloheximide (Cycl) with or without added sucrose (2%) and aminolevulinic acid (ALA) (5×10^{-3} M) upon the accumulation of Chl-*a* in cuttings of 8-day old pea seedlings of cv. Krombek during 24 hours white light of low intensity (1500 ergs/cm² sec) at 25 °C. All chemicals in 35×10^{-3} M phosphate buffer, pH 6.5; ○ = CAM, △ = CAM + sucrose, ● = Cycl, ▲ = Cycl + sucrose, ■ = Cycl + ALA.

7.2.2. *Greening in monochromatic light*

In this section attention will be given to the photoreceptor(s) involved in greening of 7-day old dark-grown pea leaves. Of special interest in this connection is the problem of phytochrome action. As a first experimental approach, greening was studied either in red light only or in mixed red-far red irradiation. These light regimes bring about large differences in phytochrome decay rate (KENDRICK and HILLMAN, 1972) due to the maintenance of different photostationary states of phytochrome. Fig. 28 illustrates that the Chl-*a* accumulation rates under these experimental conditions are not affected by the total amount of phytochrome present or by the $P_{fr}/P_{tot.}$ ratio, as they are indistinguishable except for a slight but significant difference with respect to the duration of the lag period in Chl-*b* formation.

Fig. 29 gives the effect of the intensity of monochromatic light of different wavelengths on the amounts of Chl-*a* accumulated per gram fresh weight of the leaves during 5 hours irradiation. In red (651 nm) light an optimum in Chl-*a* accumulation rate is reached at relatively low light intensities. The results discussed in section 7.2.1.1. indicate that at light intensities approaching this maximum, pigment photobleaching in this wavelength region may begin to influence Chl-*a* accumulation. This is also in agreement with our observation (fig. 10) on differences in yield of Pchl photoconversion in green (529 nm) and red (650 nm) monochromatic light. Based upon dose-response curves such as those of fig. 29,

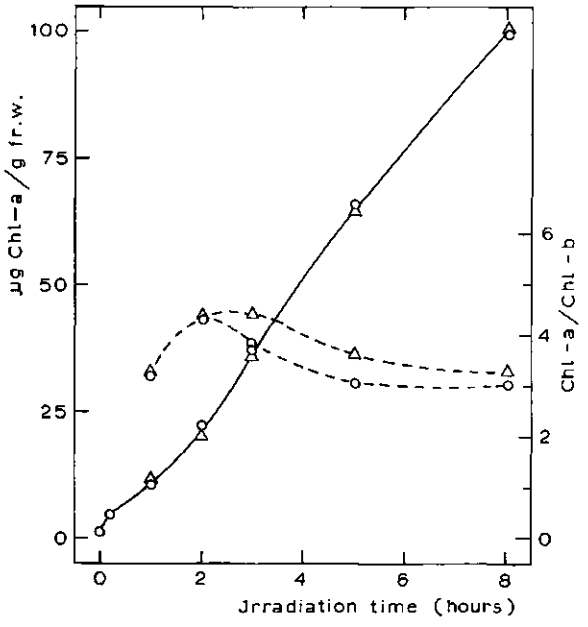

FIG. 28. Time course of Chl-*a* per g fr. w. (———) and of Chl-*a* to Chl-*b* ratio (– – –) in leaves of 7-day old pea seedlings of cv. Krombek in continuous red (651 nm, 350 ergs/cm² sec) and mixed red and far red (739 nm, 1450 ergs/cm² sec) light at 25 °C; ○ = red, △ = mixed red and far red.

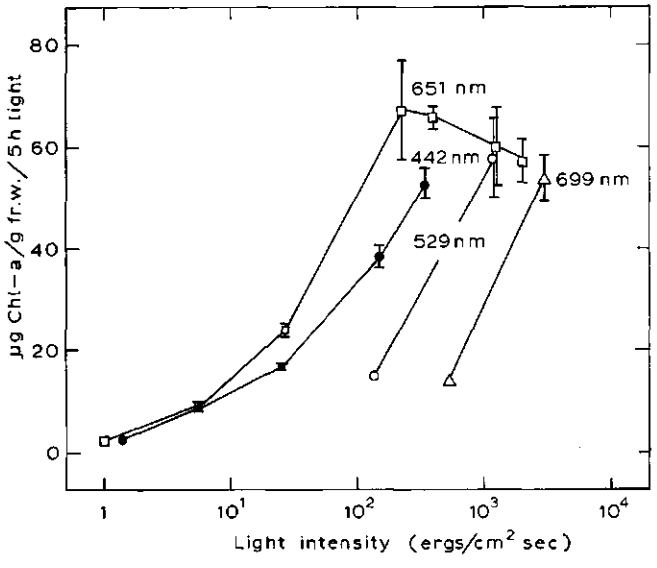

FIG. 29. Effect of the intensity of monochromatic light of different wavelengths upon the amounts of Chl-*a* accumulated per g fr. w. in leaves of 7-day old pea seedlings of cv. Krombek during 5 hours irradiation at 25 °C.

a standard slope was calculated and an action spectrum for greening could be constructed. To that end the reciprocal of the quantum dose for accumulation of 50 μg Chl-*a* per g fr. w. during a 5-hour irradiation period was plotted against wavelength. For wavelengths other than those of fig. 29, only one point of the dose-response curve was determined, the assumption being made that the curves for all wavelengths should be parallel. We have attempted to select a level of Chl-*a* accumulation compatible with the somewhat conflicting requirements that the photobleaching of Chl-*a* should be negligible, and the rate of Pchl photoconversion should not be the limiting step. For this reason, we have chosen the highest accumulation rate at which the dose-response curves are still reasonably linear (fig. 29).

At first sight the spectrum of fig. 30 seems to suggest that Pchl, absorbing around 650 nm *in vivo*, is the photoreceptor in the greening process of pea seedlings. This conclusion, however, appeared somewhat doubtful since, as explained above, we had taken precautions to avoid Pchl photoconversion to act as the rate limiting step in pigment accumulation. It should be emphasized that

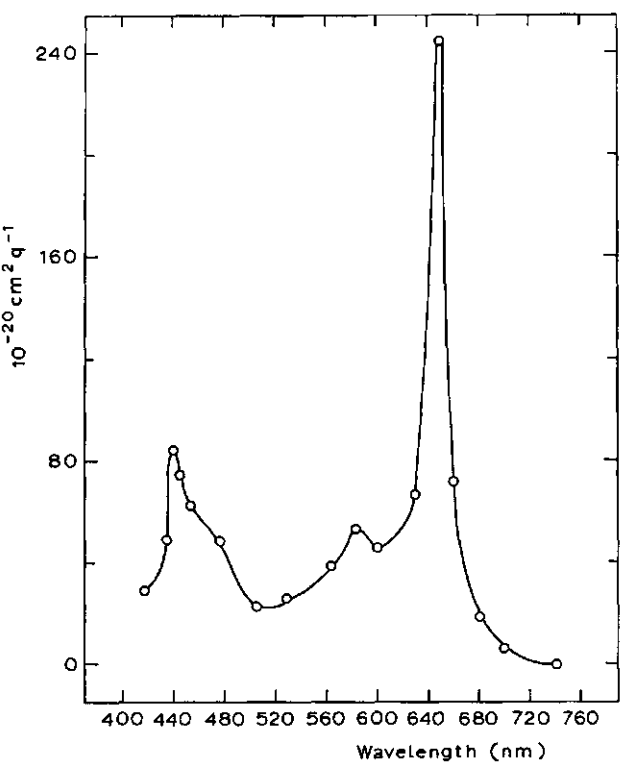

FIG. 30. Action spectrum for Chl-*a* accumulation during continuous illumination in leaves of 7-day old pea seedlings of cv. Krombek. Ordinate: reciprocal of quantum dose.

our spectrum of fig. 30 cannot be directly compared with those, published by FRANK (1946) and KOSKI *et al.* (1951), which were obtained at light doses where Pchl photoconversion indeed may be assumed to be rate limiting. Under our experimental conditions there seems no good reason to assume that Pchl-650 should act as the photoreceptor for Chl accumulation. There is still another reason to make a rôle of Pchl-650 as the principal photoreceptor and regulating mechanism in the greening process difficult to understand. Fig. 31 compares the action spectra for carotenoid accumulation and Chl-*a* formation. It shows that the positions of the principal effectiveness bands are the same for both carotenoid and Chl-*a* accumulation. The same phenomenon was described by WOLKEN and MELLON (1956) with respect to Chl-*a* and carotenoid synthesis in etiolated cells of *Euglena gracilis* during a 96-hours illumination period. It would be most remarkable if Pchl-650 should prove to be directly involved in carotenoid biosynthesis. The data of fig. 31 suggest, at least, that synthesis of both Chl and carotenoids is under control of the same basic regulating mechanism. Since the irradiation was applied over a period of 5 hours, the concomitant increase in rate of growth and development of plastids may be a factor. Since growth and development of etioplasts to a large extent appear to be under control of phytochrome (MEGO and JAGENDORF, 1961; SCHNARRENBERGER and MOHR, 1969; HOLOWINSKY and O'BRIEN, 1972), it should be expected that this pigment contributes in some way to the observed action spectrum for greening.

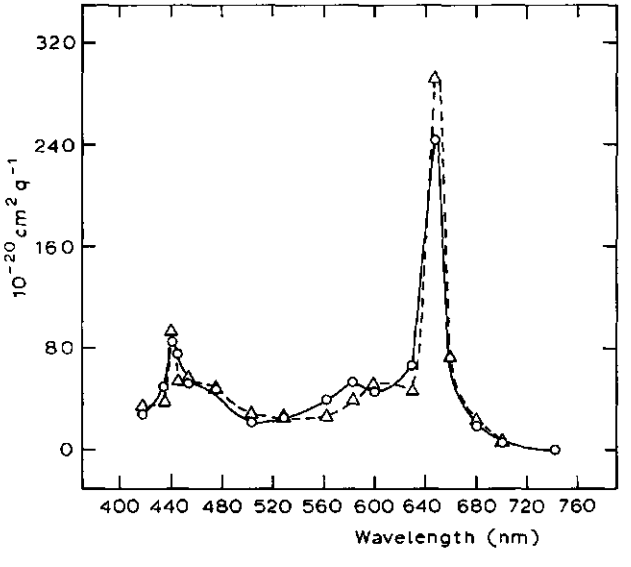

FIG. 31. Action spectra for Chl-*a* accumulation (○——○) and carotenoid formation (△ - - △) during continuous illumination in leaves of 7-day old pea seedlings of cv. Krombek. Ordinate: reciprocal of quantum dose.

We decided, therefore, to re-examine in more detail the exact peak position and shape of the red action band of the spectrum of fig. 30. In this context, the form of Pchl *in vivo* absorbing around 636 nm (SHIBATA, 1957) deserved attention, since this pigment, probably being non – or only slowly – phototransformable, seemed a potential candidate for a photoreceptor function under prolonged irradiation, more that Pchl-650 itself. For reasons of comparison (cf. section 7.3.), we also used this experiment for a simultaneous study of the fresh weight accumulation of leaves. Moreover, greening was studied in seedlings in which the lag phase in Chl formation (which proved to be under control of the phytochrome pigment system, see Chapters 8 and 9), was eliminated by pre-irradiation. To that end, the seedlings were briefly pre-illuminated with red light at 16 hours prior to the continuous monochromatic irradiations. In order to create optimal experimental conditions for greening and fresh weight accumulation, only pea seedlings, grown in flower pots were used.

Fig. 32 gives dose-response curves for Chl-*a* accumulation and increase in fresh weight of the leaves respectively, in 651 nm light of different intensities. In agreement with fig. 29, optimal Chl-*a* accumulation is observed at relatively low light intensities. This effect proved even more pronounced for the change in fresh weight. In the seedlings pre-irradiated with red (fig. 33), Chl-*a* accumulation reaches saturation at much higher light intensity and at an elevated level as compared with non-pre-irradiated plants, figs. 29 and 32. The dose-response

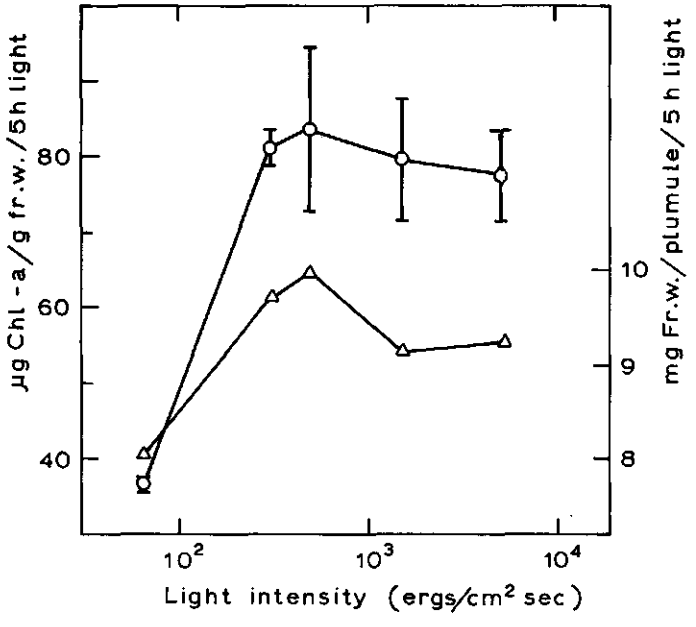

FIG. 32. Dose-response curves for Chl-*a* accumulation (○) and fresh weight accumulation (△) during 5 hours red (651 nm) light at 25 °C in leaves of 7-day old pea seedlings of cv. Krombek.

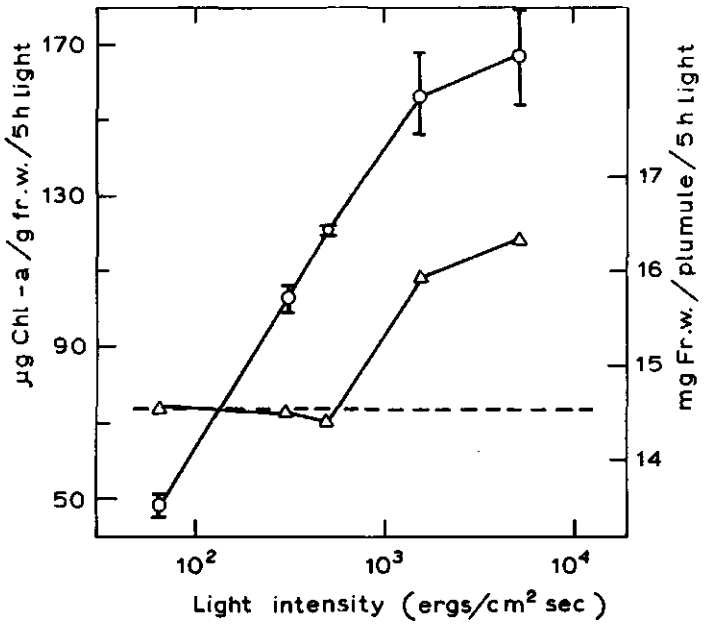

FIG. 33. Dose-response curves for red light induced Chl-*a* accumulation (○) and red light induced fresh weight accumulation (△) during 5 hours red (651 nm) light at 25°C in leaves of 7-day old pea seedlings of cv. Krombek; - - - - = 'dark' level of fr. w./plumule following 1 minute red (651 nm, 3000 ergs/cm² sec) and 16 hours darkness at 20°C.

curve for the fresh weight of the plumules indicates that low light intensities ($< 10^3$ ergs/cm² sec) are ineffective in influencing the growth of the plumule to a larger extent than that already induced by the red pre-exposure. This means that plumule growth is light-insensitive over this intensity range. At higher intensities, possibly due to photosynthetic cooperation, the fresh weight of the leaves rises above the red induced 'dark' control.

The action spectrum based upon the slope of the standard dose-response curve for Chl-*a* formation shown in fig. 32, is presented in fig. 34. Though more detailed, it is very similar to the red peak of fig. 30. The increased number of wavelengths used in this experiment, enables to estimate more accurately the peak position which is at about 646 nm. A very similar action peak was obtained with the red light pre-irradiated seedlings and is shown in fig. 35. These results imply that the action spectrum for greening is not influenced by conditions in which several reactions such as the elimination of the lag phase and the increase in capacity for Chl accumulation have been saturated by pre-exposure to red. This renders it unattractive, to explain the action maximum at 646–651 nm as a combination of two action peaks, e.g., that of phytochrome at 665 nm, and a second peak at shorter wavelengths, around 635–640 nm (RAVEN, 1972b). For, in that case, the action spectrum for the red pre-irradiated plants in which

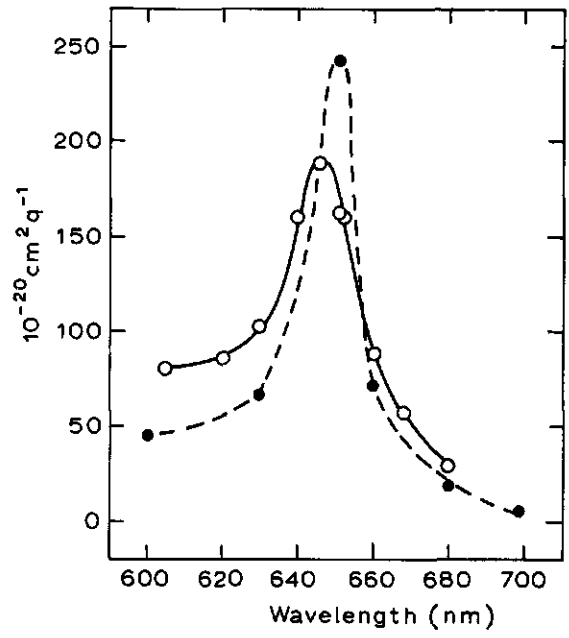

FIG. 34. Action spectrum for Chl-*a* accumulation (○——○) during continuous illumination in leaves of 7-day old pea seedlings of cv. Krombek. For comparison: the red action band of the spectrum of fig. 30 (●– – –●). Ordinate: reciprocal of quantum dose.

activation of the phytochrome reaction is already saturated, should have shown only an action peak corresponding to the short-wavelength component. In other words, a shift in the position of the spectrum to shorter wavelengths should have been expected.

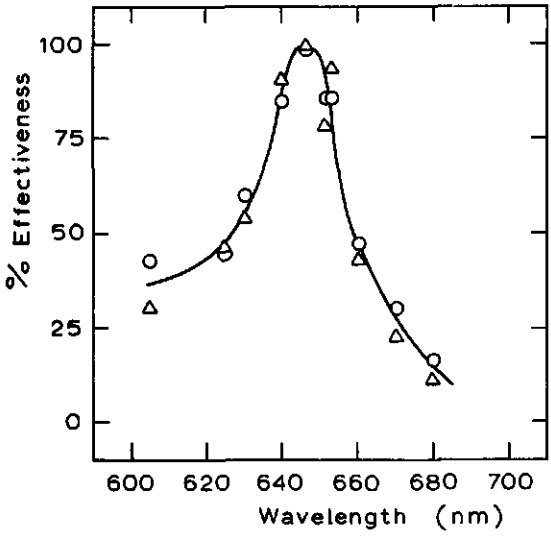

FIG. 35. Action spectrum for red light induced Chl-*a* accumulation (△) during continuous illumination in leaves of 7-day old pea seedlings of cv. Krombek. For comparison: the action spectrum of fig. 34 (○).

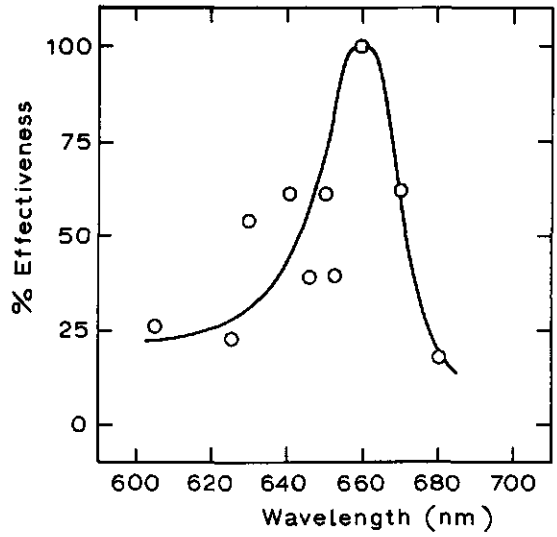

FIG. 36. Action spectrum for fresh weight accumulation in leaves of 7-day old pea seedlings of cv. Krombek during continuous illumination.

On the other hand, a case in which phytochrome indeed may act as the photoreceptor, is the action spectrum for fresh weight accumulation in pea plumules, shown in fig. 36, having its peak decidedly at higher wavelengths, probably around 660 nm. This indicates that a phytochrome type action spectrum can be found under our experimental conditions, thus emphasizing the probability that phytochrome indeed was not directly involved in the spectra discussed above.

7.2.3. *Ultrastructural development in etioplasts during greening*

During the first 5 hours of greening of 7-day old pea leaves in low intensity white fluorescent light, about 80% of the prolamellar bodies become fully dispersed and their membranes fuse to form primary lamellae (plate 2). Occasionally, some lamellae in their turn fuse to form grana. However, the number of grana per plastid is still very low, and the number of partitions per granum does not exceed three. In white light of high intensity (plate 3), the sequence of structural changes seems to be the same, although the time course is delayed. After 5 hours greening most prolamellar bodies are still more or less intact, although they have lost their crystalline character. Primary lamellae extrude in all directions, but doubling is hardly detectable. Vesicle dispersal and the building of grana in blue light (plate 4) follows a pattern and time course very similar to that in white light of low intensity (plate 2). A great number (5–8) of primary lamellae are arranged in roughly parallel layers and formation of grana is very clear. In green light (plate 5), only about 20% of the prolamellar bodies are fully dispersed and the number of grana per plastid and partitions per granum is

rather low. In red light (plate 6), the number of primary lamellae appears to be remarkably depressed. Also, hardly any doubling of the sheets is observable. On the other hand, 70% of the prolamellar bodies are completely dispersed. The action of the different wavelength regions is, therefore, different both quantitatively and qualitatively. It should be emphasized that light doses in the different spectral regions were such that ultimate Chl accumulation was the same in all spectral regions. Although these results cannot claim to represent an action spectrum of any completeness, it is clear that blue light enables the highest degree of structural differentiation in this respect.

7.3. Discussion

The results presented in this Chapter repeatedly point to damaging side effects of irradiation on completely dark-grown seedlings (e.g. figs. 23, 24, 25, 29, 32, and plate 3). As such, we mention that the duration of the lag phase in accumulation of Chl-*a* (fig. 23) and Chl-*b* (fig. 24) is prolonged by high intensity white light. A similar observation has been ascribed by Virgin (1955) to strong photodestruction of pigment. Our data suggest that Pchl regeneration continues with at least the dark rate throughout the lag phase (fig. 25, and p. 39). Chl accumulation also depends on the light intensity during subsequent stages of the greening process, although after very much prolonged greening, the differences become less apparent (fig. 23). This appears mainly due to the decrease in the Chl-*a* accumulation rate after about 10 hours greening in the low intensity white light. We would like to suggest that around this moment, photosynthesis starts contributing noticeably to the energy balance of the seedlings and, hence, to Chl formation. By that time, reserve substrates provided by the plant during the initial stages of greening may become depleted. The lower Chl-*a* accumulation rate during phase IV (fig. 20) may then be attributed to light-limitations of some photosynthetic activities; one of the things one might think about could be cyclic photophosphorylation (Dodge *et al.*, 1971).

High intensity white light was found to delay the structural development of plastids (plate 3), whereas even low intensity white light depressed the fresh weight of pea plumules during the first hours of irradiation (fig. 21). With respect to the latter observation, we may consider the possibility that there is a connection between this phenomenon and the occurrence of the lag phase in Chl accumulation.

The increase in fresh weight of the pea plumule in continuous monochromatic red light, shows a definite optimum at rather low intensity (fig. 32). Similar optima were observed for Chl accumulation in red light (figs. 29 and 32). This seems to confirm earlier conclusions (section 5.2.; Spruit and Raven, 1970) that this wavelength effectively bleaches a Chl-*a* type absorbing around 685 nm. This pigment, probably being Chlide-*a*, is known to be readily photooxidized (Anderson and Robertson, 1961; Goedheer, 1961; MacWilliam and Naylor, 1967; Ziegler and Schanderl, 1969).

The occurrence of a lag phase in Chl-*a* accumulation has often been interpreted as an indication that some component(s) essential for Chl synthesis and (or) chloroplast development lacks during this period. We have made some preliminary attempts to identify possible components limiting Chl synthesis during the lag phase and afterwards (fig. 26). In this experiment with 3-cm pea cuttings, we noticed that both the rate and the capacity for Chl formation were strongly depressed in detached leaves. Some improvement resulted from the application of sucrose, but complete restoration was by no means obtained. The duration of the lag phase, on the other hand, appeared completely insensitive to sucrose. Surprisingly, incubation with a 5 mM solution of the Pchl precursor ALA overnight and during the illumination with white light slightly decreased the quantity of Chl over a 24-hour irradiation period (fig. 26). No significant effect of ALA on Chl formation in weak red light (320 ergs/cm^2 sec) nor on Pchl synthesis in prolonged darkness was detected. An explanation of this fact might be either that ALA does not readily penetrate into our plant material or that the required level of precursors does not act as a limiting factor in the case studied.

The low Chl accumulation rate in detached leaves is probably to a large extent due to lack of one or more specific substrates originating in other parts of the plants, such as the cotyledons. Also substances normally supplied by the roots may influence both growth rate of leaves and Chl synthesis. They may e.g. be an important source of cytokinins, that seem to affect Chl formation (FLETCHER and MACCULLAGH, 1971).

The rates of Chl-*a* formation in intact pea leaves were neither influenced by the total amount of phytochrome present during the irradiation, about by the $P_{fr}/P_{tot.}$ ratio (fig. 28). The same conclusion was reached by KENDRICK and HILLMAN (1972). However, this does not provide sufficient proof that phytochrome is not involved in the greening process, since its action might be saturated at very low P_{fr} concentrations (RAVEN and SPRUIT, 1972a). A slight delay in termination of the lag phase in Chl-*b* formation in mixed red-far red light might be attributable to phytochrome action.

It is obvious that one has to be careful in interpreting effects of wavelength upon Chl-*a* synthesis in the absence of any further knowledge about the course of dose-response curves involved. As shown in fig. 29, at a light intensity of about 1250 ergs/cm^2 sec, which is not very high, the Chl-*a* accumulation rates appear roughly the same in red and green monochromatic light, whereas at lower intensities, the rates are quite different. Obviously, it is not allowed to conclude that green light is as effective as red light in the greening process (MILLER and ZALIK, 1965). Probably, the same holds true with respect to situations in which blue light was found to be as effective as red in Chl formation (e.g. VIRGIN, 1958; FARINEAU, 1968; JACQUES, 1968).

The action spectra for greening of pea leaves (figs. 30, 34, and 35), point to a pigment spectrally resembling Pchl as the photoreceptor. The ratio of the maximum effectivity in the red to that in the blue is about the same as for Pchl photoconversion (section 5.3.). This result was rather unexpected, since we attempted to avoid Pchl phototransformation to act as the rate-limiting step in

pigment accumulation. This precaution, obviously, was not taken in the case of the spectra published by FRANK (1946), KOSKI and coworkers (1951), and SIRONVAL *et al.* (1968) which clearly are to be ascribed to Pchl limitation. Action spectra for prolonged greening in high quantum flux densities exist for *Euglena gracilis*. They point to either Pchl-636 (WOLKEN and MELLON, 1956) or chlorophylls (NISHIMURA and HUZISIGE, 1959) as photoreceptors. However, the rate of Pchl phototransformation in *Euglena* is very slow (50% conversion in about 24 minutes) and may limit rapid Chl formation. This may be ascribed to the presence in this organism of only the shorter-wavelength-absorbing Pchl type (BUTLER and BRIGGS, 1966). The observation of WOLKEN and MELLON (1956) draws our attention to Pchl-636. This pigment is non – or only slowly – phototransformable, according to data published by SHIBATA (1957) and SPRUIT (1965, 1966), whereas the stationary concentration of Pchl-650 should be extremely low during prolonged irradiations. However, under no conditions we have been able to detect a red action peak, solely attributable to Pchl-636 (fig. 35). On the other hand, we hesitate to exclude the possibility that the action maximum in fig. 34, situated around 646 nm, is the result of actions of both Pchl-636 and Pchl-650. A possible mode of action of Pchl pigments in regulating the Chl accumulation during prolonged greening is difficult to understand. We may, e.g. make the hypothesis that the initial rate of Pchl regeneration is regulated by the ultimate level of remaining Pchl molecules maintained under the continuous influx of quanta. A similar type of regulation has been proposed for the de novo synthesis of phytochrome (CLARKSON and HILLMAN, 1967). This means that the lower the stationary concentration of Pchl, the faster its initial regeneration rate which determines the Chl accumulation rate. Support for this idea might be found in the work of KALER *et al.* (1969). The problem then remains how Pchl can regulate carotenoid synthesis and Chl synthesis at the same time (fig. 31). It could be that enhanced Chl formation induces accelerated growth and development of plastids, which in turn promote carotenoid synthesis. However, regulation of plastid development by accumulated Chl is a hypothesis that has been examined, and emphatically rejected by several investigators (e.g. EILAM and KLEIN, 1962). Further evidence against this hypothesis can be found in plate 6 that shows that the internal structural development of plastids in red light is poor, despite the fact that this wavelength region is most effective in Chl formation. In this respect, the observation of HENNINGSEN (1967) that an action spectrum for extrusion of lamellae from the prolamellar body has a sharp band around 450 nm belonging to an unidentified photoreceptor which solely absorbs in the blue, may be of interest. We also found continuous blue light (section 7.2.3.) very effective with respect to the internal structural development of etioplasts. A positive interaction of successive treatments with red and blue light in promoting protein synthesis and enzyme activity was described by BRADBEER (1971). On the other hand, the action spectrum for fresh weight accumulation in pea plumules (fig. 36) points to phytochrome activity. THORNE (1971b) reported that, in bean, accumulation of carotenoids kept step with the expansion of the leaves. We therefore may still consider the possibility that accumulation of Chl

and carotenoids depends partly on the phytochrome system and partly on a second (Pchl type) photoreceptor system with a maximum at 640–650 nm.

Summarizing, it can be said that at least three photoreceptor systems may be involved in complete de-etiolation of dark-grown seedlings by continuous illumination (see also BRADBEER, 1971). Clearly this makes it the more difficult to detect the specific rôle of each of these photoreceptors separately as well as their absorption characteristics, since they may interact.

8. INDUCTION OF RAPID CHLOROPHYLL ACCUMULATION

8.1. INTRODUCTION

The lag phase in Chl accumulation can be eliminated if the dark-grown seedlings are pretreated with a low dosage of light some hours before the start of the continuous irradiation. This phenomenon is called induction of rapid Chl accumulation (section 3.1.). Many experiments to be described in this Chapter were made with pea as plant material for the following reasons: firstly, leaves of pea, grown in darkness are not covered by the seed coats 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 proved extremely sensitive towards inductive light. Thirdly, in contrast with monocotyledons, no action spectrum for induction of rapid Chl-*a* accumulation in dicotyledons had been published when we started our work.

In this Chapter, we will further describe experiments in which induction by red light is compared in leaves on intact seedlings and in detached leaves. The light sensitivity of pea will be compared with that of other plant species in relation to their phytochrome content, as determined spectrophotometrically.

8.2. RESULTS

8.2.1. *Duration of the dark incubation period*

The kinetics of red light induced Chl accumulation are presented in fig. 37. They illustrate that, following a relatively short dark interval, the induction by red affects only the duration of the lag phase and not the rate of Chl formation during the rapid phase of greening. Upon extension of the dark incubation period, also the rate of Chl accumulation during the rapid phase of greening becomes enhanced. In fig. 38, the Chl-*a* content of primary bean leaves, as measured after 5 hours of continuous white light, is plotted against the length of the dark incubation period following 5 minutes of red light. The leaves were harvested at the end of the period of continuous irradiation. 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 and decline afterwards. In bean leaves no evident maximum can be found. Dark intervals even as long as 48 hours result in a high Chl-*a* accumulation rate, upon exposure of the seedlings to continuous light. When, on the other hand, the calculation of Chl-*a* content is based upon a constant weight of leaves, a shallow maximum becomes demonstrable at 24 hours darkness. A completely different response is observed, when detached bean leaves, put in petri dishes on moist filter paper, are briefly irradiated with red

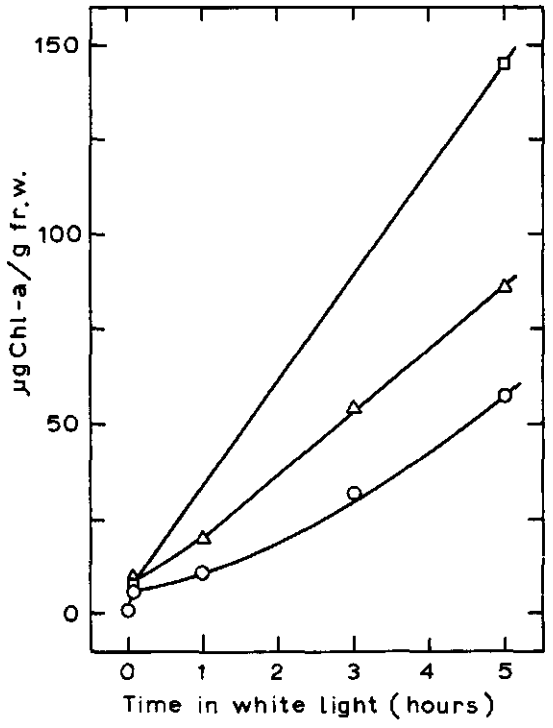

FIG. 37. Time course of Chl-*a* per g. fr. w. in red light induced leaves of 7-day old pea seedlings of cv. Krombek (grown in seed-pans) in continuous white fluorescent light of low intensity (1500 ergs/cm² sec) at 25 °C; ○ = dark control, △ = 5 minutes red (651 nm, 3000 ergs/cm² sec) followed by 4 hours darkness, □ = 5 minutes red followed by 24 hours darkness.

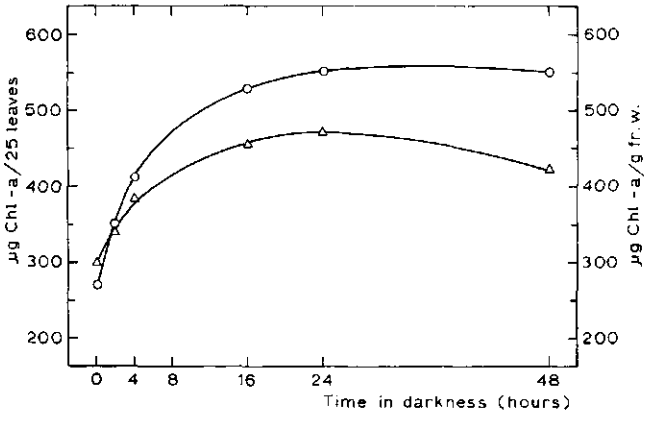

FIG. 38. Effect of duration of dark incubation period, following 1 minute red (651 nm, 3000 ergs/cm² sec) inductive light upon Chl-*a* accumulation during 5 hours continuous white light in 10-day old bean seedlings of cv. Widuco; ○ = µg Chl-*a*/25 leaves, △ = µg Chl-*a*/g fr. w.

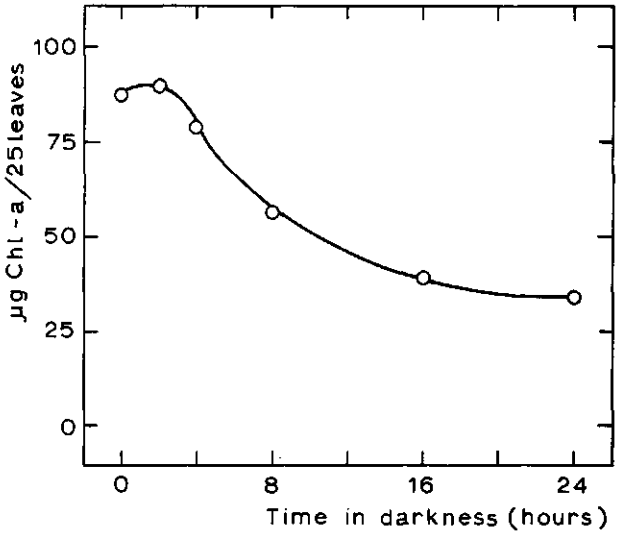

FIG. 39. Effect of duration of dark incubation period, following 1 minute red (651 nm, 3000 ergs/cm² sec) inductive light upon Chl-*a* accumulation during 5 hours continuous white light in detached leaves of 10-day old bean seedlings of cv. Widuco.

light and are exposed to continuous withe light after dark periods of varying length (fig. 39). With this material, prolonged dark incubation periods proved to be unfavourable for the biosynthetic system forming chlorophylls. This can be concluded from the low levels of Chl-*a* ultimately reached in continuous

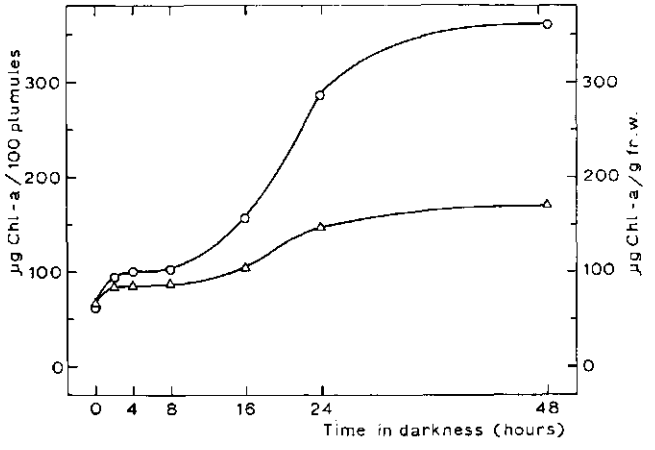

FIG. 40. Effect of duration of dark incubation period, following 5 minutes red (651 nm, 3000 ergs/cm² sec) inductive light upon Chl-*a* accumulation during 5 hours continuous white light in 7-day old pea seedlings of cv. Krombek; ○ = µg Chl-*a*/100 plumules, △ = µg Chl-*a*/g fr. w.

illumination as compared with intact seedlings. As already shown in table 2 (section 7.2.1.2.), the Chl content in the leaves, not pre-irradiated with red is also strongly depressed in detached leaves compared with leaves left on the seedlings.

Neither in the absence nor in the presence of sucrose in cuttings from 8-day old pea plants, pretreated with a saturating dose of red light, 16 hours prior to the continuous irradiation, did we observe any significant deviation from the greening kinetics shown in fig. 26. Contrarily, in leaves of intact pea plants, extension of the dark incubation period to 48 hours still gives rise to a marked increase in subsequent Chl-*a* formation (fig. 40), especially when the Chl-*a* content is considered in relation to the number of plumules.

8.2.2. *Duration of the dark incubation period for chlorophyll-a formation in relation to dry weight accumulation*

Additionally, we have studied the time course of dry weight of pea plumules following a brief pre-exposure to red light: fig. 41. At the moments 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 results in a considerable increase in dry weight

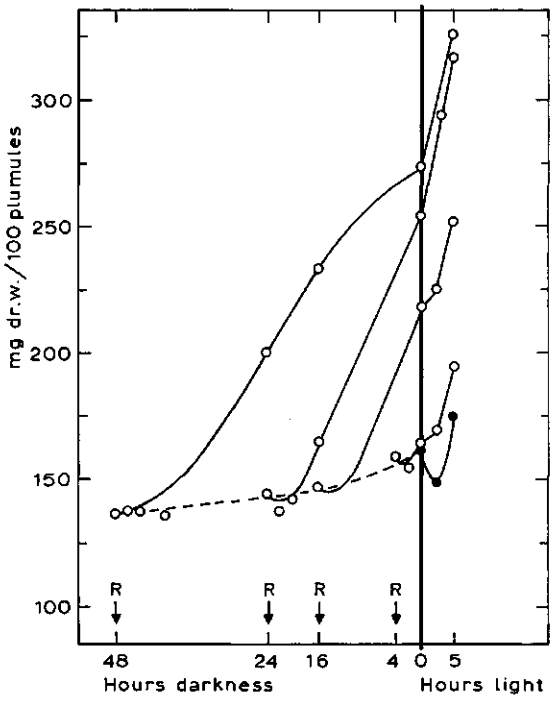

FIG. 41. Time course of dry weight of 100 plumules of pea cv. Krombek during darkness at 20 °C, following 5 minutes red (651 nm, 3000 ergs/cm² sec) inductive light, and during 5 hours continuous white light at 25 °C. The short red light impulse was administered to the whole pea seedlings at the moments indicated by arrows; - - - = non-induced seedlings in darkness; ● = non-induced seedlings in white light; ○ = red light pretreated seedlings.

of plumules in darkness, starting after a lag of about 4 hours. This rapid dry weight accumulation lasts for about 24 hours, thereafter the rate slows down. Upon transfer to continuous light the increase in dry weight accelerates again. After long (> 16 hours) dark incubation periods no lag phase in dry weight accumulation in continuous white light can be observed, i.e., there is an immediate increase in dry weight. Without pre-irradiation, a considerable initial decrease in dry weight occurs first in continuous light. Even after a short exposure to red light a temporary decrease in dry weight of the plumules in darkness is sometimes observed.

8.2.3. *Dose-response curves for induction*

Fig. 42 shows some dose-response curves for induction of rapid Chl-*a* accumulation with red (653 nm) light as observed in different plants. The degree of induction was calculated from the 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 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 standard saturating 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

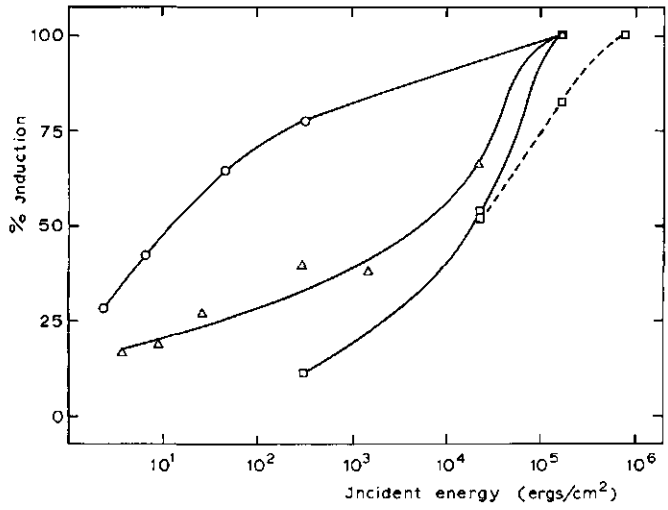

FIG. 42. Dose-response curves for induction of rapid Chl-*a* accumulation with red (653 nm) light in previously dark-grown seedlings; ○ = 7-day old pea cv. Krombek, △ = 9-day old bean cv. Widusa, □——□ = 9-day old maize, □- - -□ = 17-day old maize.

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.

It is clear that there is a widely divergent range in sensitivity to the wavelength applied, for different plant species. The seedlings of pea cv. Krombek are especially sensitive towards this inductive red light. Marked differences in the shape of the dose-response curves are 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). We have tried to correlate total 'spectrophotometric phytochrome' with sensitivity to induction as shown in fig. 42. Fig. 43 gives the total amounts of spectrophotometrically measurable phytochrome in leaves of pea, bean, and maize. It shows that pea leaves are quite rich in spectrophotometrically measurable phytochrome as compared with, e.g., leaves of maize.

Fig. 44 shows that Alaska-peas are 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 and CHON's (1966) experiments on the alteration of the photo-

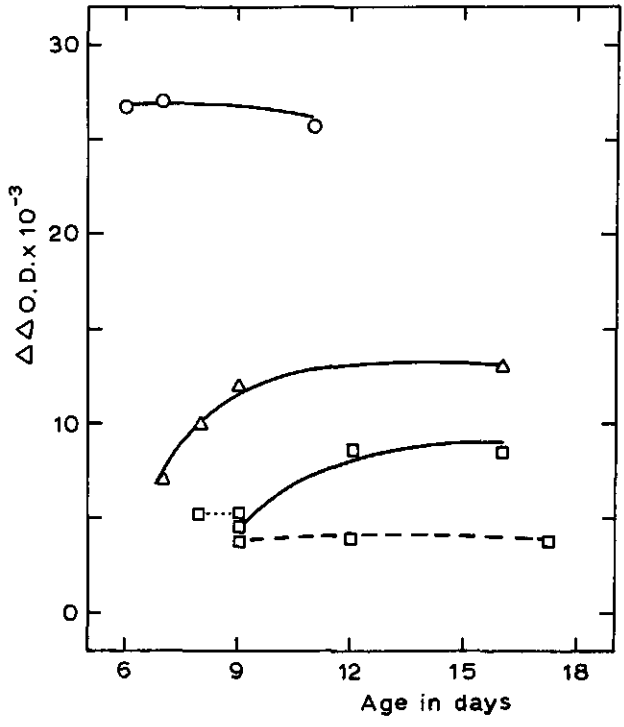

FIG. 43. Total amount of spectrophotometrically measurable phytochrome in leaves of dark-grown seedlings of varying age; ○ = pea cv. Krombek, △ = bean cv. Widusa, □ = maize. In case of maize also other parts of seedlings were tested: □·····□ = coleoptiles with leaves inside, □- - -□ = coleoptiles, □——□ = leaves. Depth of samples 4 mm. Ordinate optical density changes at 730 nm relative to 807 nm in response to actinic irradiations with red and far red light.

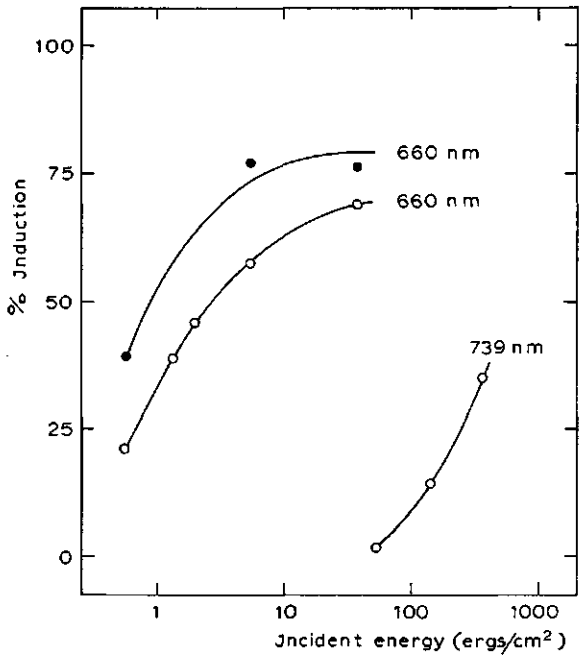

FIG. 44. Dose-response curves for induction of rapid Chl-*a* accumulation with red (660 nm) and far red (739 nm) light in 7-day old pea seedlings; ● = cv. Alaska, ○ = cv. Krombek.

tropic sensitivity in corn coleoptiles. However, in contrast with the findings of CHON and BRIGGS (1966), induction of Chl accumulation in pea is also relatively

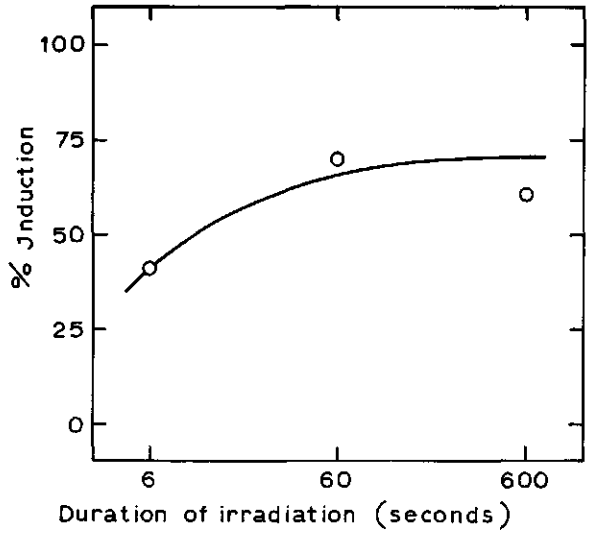

FIG. 45. Dose-response curve for induction of rapid Chl-*a* accumulation with green safelight (10 ergs/cm² sec) in 7-day old pea seedlings of cv. Krombek.

TABLE 3. Test for reciprocity of induction of rapid Chl-*a* accumulation in leaves of 7-day old pea seedlings of cv. Krombek.

Irradiance (I)	Time of irradiation	
	6 seconds	60 seconds
$10 \times I_{blue}$	38%	–
$1 \times I_{blue}$	–	36%
$10 \times I_{green}$	36%	–
$1 \times I_{green}$	–	38%
$10 \times I_{red}$	33%	–
$1 \times I_{red}$	–	37%

Blue = 442 nm, 0.8 ergs/cm² sec; green = 529 nm, 0.9 ergs/cm² sec; red = 653 nm, 0.1 ergs/cm² sec.

sensitive to far red. After these observations, it was hardly surprising that even an exposure as short as 6 seconds to the full intensity of our darkroom green safelight (section 4.3.3. and fig. 6) is sufficient to induce rapid Chl-*a* accumulation in pea up to about 50% of the maximum (fig. 45).

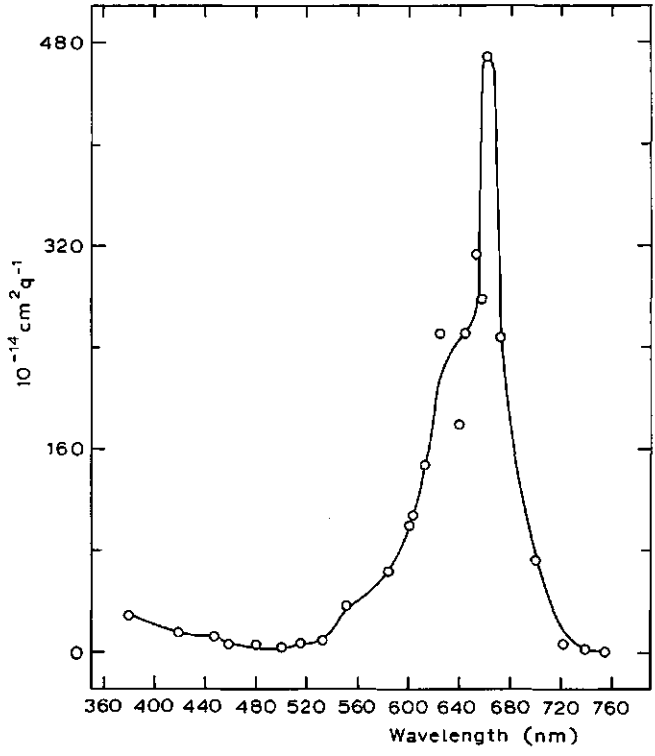

FIG. 46. Action spectrum for induction of rapid Chl-*a* accumulation to a level of 25% of the maximum in 7-day old pea seedlings of cv. Krombek. Ordinate: reciprocal of quantum dose.

Accumulation of Chl-*b* and carotenoids, as well as gain in fresh weight of the plumules, follow a similar pattern in their response to all inductive light treatments.

8.2.4. *Action spectrum for induction of rapid chlorophyll accumulation*

Although the reciprocity law appeared to hold for induction of rapid Chl accumulation up to an intermediate (about 35%) level (table 3), the duration of all inductive irradiations used for the construction of an action spectrum was kept constant at 60 seconds. Therefore, only the intensity of the light was changed in order to obtain for each wavelength dose-response curves or parts of dose-response curves, as shown in figs. 42 and 44. For the construction of the action spectrum shown in fig. 46, the reciprocal of the quantum dose required for induction to a level of 25% of the maximum was plotted against the wavelength. The action peak at about 660 nm suggests that the red absorbing form of phytochrome (P_r) acts as the photoreceptor.

8.3. Discussion

The induction of rapid Chl-*a* accumulation by a brief exposure to red light in pea (figs. 37 and 40) is accompanied by a pronounced rise in dry weight of the plumules, fig. 41. We may draw the following conclusions from this observation: firstly, the prolonged increase in dry weight of the leaves of whole pea seedlings parallels the persistence of the inductive capacity of a pre-exposure over long dark incubation periods (figs. 38 and 40). There may be a causal relation between these two reactions. Only in very young (4-day old) bean seedlings, Akoyunoglou (1970) could find a similar increase in stimulatory effect of a short pre-irradiation over a 24-hour dark incubation period. If translocation of metabolites is involved, this would be impossible in excised leaves, which would explain results as illustrated in fig. 39. Virgin (1957) and Akoyunoglou (1970) reported for their excised leaf material optimal dark intervals of rather short duration. This should then reflect exhaustion of small quantities of substrates, present in the leaves.

Secondly, the rise in dry weight of the leaves also appears to run parallel to an increase in the capacity of the biosynthetic system forming Pchl. A red light induction causes not only the complete elimination of the lag phase, but, upon prolonged incubations enhances also the rapid phase of subsequent greening (fig. 37). According to Mego and 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. In our opinion, the dry weight accumulation of the leaves in darkness upon a brief pre-irradiation as well as in the relatively short period of continuous low intensity white light (fig. 41) has to be ascribed to translocation processes from the cotyledons and other organs to the leaves since photosynthesis can be excluded (Bradbeer, 1969). According to the

effect of the red pre-illumination, these translocation processes are triggered by relatively small light doses. In agreement with the data for fresh weight, as shown in fig. 21, a considerable initial decrease in dry weight was observed, when no brief irradiation preceded the continuous light treatment. This decrease in dry weight apparently coincides with the lag phase in Chl-*a* accumulation, stressing the possibility already expounded in section 7.3., that there is a connection between them.

Striking differences between different plants were observed with respect to the sensitivity of dark-grown seedlings to red light (fig. 42). At first glance, a positive correlation seems to exist between the particularly high $\Delta\Delta$ O.D. values in pea leaves (fig. 43) and their extremely high red light sensitivity. Closer examination of these data, however, makes the existence of a real 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 leaves of dark-grown maize increases slightly over a long period of time, whereas light sensitivity decreases.

A direct comparison between phytochrome values is hampered by a technical difficulty. Since we are dealing with different plant materials and different parts of the same plant (maize), such comparisons are only permitted if the scattering properties of the different samples are identical (BUTLER, 1964; ROMBACH and SPRUIT, 1968). In fact, nothing is known about the effective optical pathlengths in these highly scattering samples. Moreover, differences in pigment distribution may influence the optical density readings for total phytochrome content (SPRUIT, 1972; SPRUIT and SPRUIT, 1972). For these reasons, data as shown in fig. 43, 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.

From the high sensitivity of pea to short irradiations with far red light (fig. 44) it can be concluded that 'a virtually stationary concentration of the active phytochrome (P_{fr}) maintained over a considerable period of time' (KASEMIR and MOHR, 1967) seems not to be required to obtain this photomorphogenic effect. Rather, it points to an extremely low P_{fr} requirement of the induction reaction. Similar high sensitivities to both red and far red were reported by BLAAUW and coworkers (1968) for inhibition of growth in the *Avena* mesocotyl. They constructed an action spectrum for the far red-irreversible inhibition of the initial 15% of the growth rate, which showed 'a general similarity with that obtained for phytochrome-mediated processes'. Their action spectrum is very similar to ours, as given in fig. 46. For instance, the effectivities of 660 nm and 739 nm in our spectrum have a ratio of about 300, in good agreement with the data published by BLAAUW *et al.* (1968) and the same as the ratio of the absorbancies of purified extracts of phytochrome in the red absorbing form for these wavelengths (calculated from data of KROES, 1970). On the other hand, many action spectra or dose-response curves, published in literature, point to a greatly variable ratio of the effectivities at 660 nm and at around 735 nm. For the 20%-

inhibition of stem growth in barley seedlings, BORTHWICK *et al.* (1951) reported a red-far red ratio of about 50. The irreversible red light induced inhibition of flowering in *Pharbitis* at the middle of 16-hour dark periods, showed a ratio of 40 (NAKAYAMA *et al.*, 1960). The action spectrum for the elimination of the lag phase in greening of wheat (VIRGIN, 1961) yielded the same ratio. The latter response also was hardly red-far red reversible. Several phytochrome-mediated reactions are known that are more or less completely red-far red reversible. In such cases, far red light appears either several orders of magnitude less effective than red in obtaining the same degree of response or it is completely inactive. Some examples: the hypocotyl hook opening in bean (WITHROW *et al.*, 1957), the red repromotion of far red inhibited flowering in *Pharbitis* at the beginning of 16-hour dark periods (NAKAYAMA *et al.*, 1960), the alteration of the phototropic sensitivity in corn coleoptiles (CHON and BRIGGS, 1966), and the 50%-inhibition of mesocotyl growth of *Avena* seedlings (BLAAUW *et al.*, 1968). We will discuss this matter further against the background of our own red-far red reversibility experiments in Chapter 9.

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 (fig. 45). In fact, this green light causes some de-etiolation, a phenomenon of great importance for the study of phytochrome physiology (section 9.3.). It demonstrates that such 'safelights' should be distrusted as long as rigorous tests have not shown them to be really safe. In this respect, the mere demonstration that safelight irradiation as such is not followed by directly visible physiological reactions, does not constitute sufficient proof of its inactivity. A similar opinion has also been proclaimed repeatedly by BLAAUW and coworkers (l.c.). It is possible that exposures to green safelight during the rearing of seedlings induce a response that masks the light sensitive part of a dose-response curve. This may be so e.g. for the dose-response curve of the red induced plumule growth in pea cv. Alaska, as reported by FURUYA and THOMAS (1964).

9. RED-FAR RED PHOTOREVERSIBILITY OF RAPID CHLOROPHYLL ACCUMULATION

9.1. INTRODUCTION

A well-known phenomenon in the photomorphogenesis of potentially green plants is the red-far red reversible light reaction, also indicated as low energy reaction or phytochrome reaction. These terms imply that red light, administered to the plants in low doses, induces a physiological response, whereas the inductive effect of red light can be cancelled more or less completely by subsequent irradiation with far red light. Historically, this phenomenon of red-far red reversibility has led to the discovery of phytochrome (BORTHWICK *et al.*, 1952). For further information see section 2.3.

Numerous physiological responses showing more or less complete red-far red reversal have been reported during the last two decades. Among them are the induction of rapid Chl-*a* accumulation in seedlings of higher plants as well as the control of Pchl regeneration rates in darkness (section 3.1.). However, as already expounded in section 6.2., we have been unable to detect any significant control by phytochrome of the initial rate of Pchl regeneration in seedlings of bean, pea, and maize. In this Chapter we will discuss the far red reversibility of the red induction of Chl-*a* accumulation.

Since we had invariably found weak reversals of red induction by subsequent far red in all those plant species studied earlier (section 6.2.), we also included in our study cultivars of pea and bean that have been reported in the literature as showing more or less complete reversals of various red induced morphogenic responses. We have not been able to confirm such reports, however. A discussion of the above-mentioned divergency in experimental results will form the main object of this Chapter.

9.2. RESULTS

9.2.1. *Red-far red reversibility*

In fig. 47, results are summarized that were obtained with different types of red and far red light treatments in 7-day old dark-grown seedlings of pea cv. Krombek. The data are expressed as per cent of the induction resulting from a saturating one minute irradiation with red light (section 8.2.3.). As pointed out in the introduction (section 9.1.) we observe hardly any reversal of the inductive effect of this standard red light treatment by subsequent irradiation with far red light (fig. 47, top). It is also remarkable that the induction remaining after this red-far red light treatment is almost equal to that of one minute far red (739 nm) light alone. This forms an indication that the far red dose is already active as such in reducing the length of the lag phase in Chl-*a* formation. It thus appeared possible that this inducing capacity of far red light masks a concomitant revert-

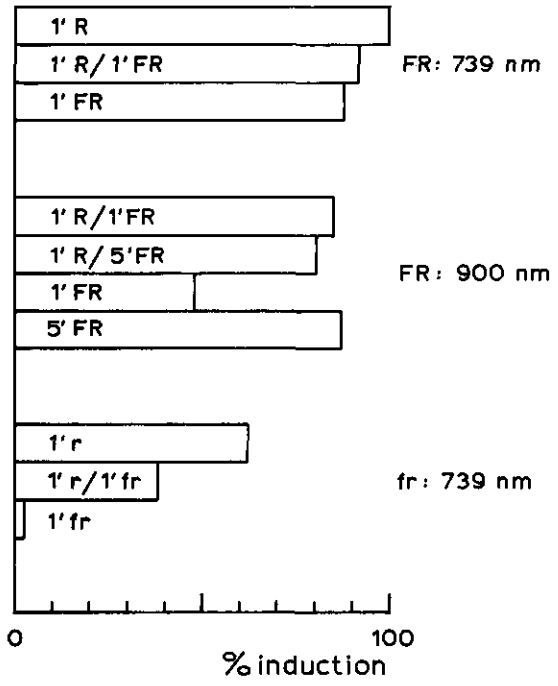

FIG. 47. Induction of rapid Chl-*a* accumulation by various light treatments in 7-day old, dark-grown pea seedlings of cv. Krombek.
R : 651 nm, 3000 ergs/cm² sec
r : 651 nm, 0.2 ergs/cm² sec
FR: 739 nm, 4150 ergs/cm² sec
FR: 900 nm, 4000 ergs/cm² sec
fr : 739 nm, 1.5 ergs/cm² sec
All irradiation schemes were followed by 16 hours darkness and, thereafter, 5 hours continuous white light.

ing effect of the same light quality, far red thus exerting a dual action (DE LINT, 1957). Dose-response curves for the induction of rapid Chl-*a* accumulation indicated a high sensitivity of this pea material to far red light (fig. 44). Therefore, we have attempted to increase reversibility of the red induced effect either by using far red light of longer wavelengths or by applying intensities sufficiently low to produce no appreciable induction by themselves.

Experiments with 900 nm light of high intensity (4000 ergs/cm² sec) failed to give more reversion and had even marked inductive capacity (fig. 47, middle). On the other hand, irradiation with low intensities of far red (739 nm, 1.5 ergs/cm² sec) gave somewhat better reversion, but only if the induction proper had also been performed with an extremely low dosage of red light (651 nm, 0.2 ergs/cm² sec) (fig. 47, bottom). Shortening the duration of both red and far red irradiations at constant intensity did not increase reversibility. The light induced increase in fresh weight of pea plumules as well as the increase in total amount of carotenoids showed a response similar to the one of Chl-*a* synthesis.

TABLE 4. Inductions resulting from red, immediately followed by far red and from far red only.

Plant material	Age in days	1'R/1'FR Induction in % of 1'R only	1'FR Induction in % of 1'R only
Pea			
cv. Krombek	7	92	88
cv. Alaska	7	100	100
Bean			
cv. Widusa	9	89	36
cv. Red Kidney	9	82	54
cv. Res. ASGROW Valentine	9	75	71
cv. BURPEE's Stringless Green-Pod	9	100	67
Maize			
cv. Caldera	9	78	60
cv. Caldera	17	39	12

R : 651 nm, 3000 ergs/cm^2 sec.
FR: 739 nm, 4150 ergs/cm^2 sec.

To establish whether these results are specific for the species or cultivar used, another cultivar of pea and several cultivars of bean as well as maize seedlings of different ages were tested. The results are summarized in table 4. The uniformly high levels of induction obtained with red irradiation immediately followed by far red light, demonstrate that low reversibility is not peculiar to the pea cv. Krombek. Again, it is remarkable that in most cases appreciable inductions are also obtained with far red only. The data for maize indicate that far red reversibility increases with increasing age of the seedlings. Moreover, in all cultivars studied, increase in fresh weight of leaves and accumulation of carotenoids follow a similar pattern. Lack of complete red-far red reversibility seems, therefore, to be rather common in several aspects of seedling development and this is related to the high inductive activity of far red. This makes it all the more difficult to understand the numerous literature reports of almost complete reversal under experimental conditions that seem to be comparable to ours (e.g. WITHROW *et al.*, 1956; PRICE and KLEIN, 1961; FURUYA and THOMAS, 1964; HENSHALL and GOODWIN, 1964; AKOYUNOGLOU, 1970). We, therefore, have paid attention to possible effects of those details of experimental conditions that traditionally have been regarded as of minor significance, e.g., differences in the duration of the dark incubation period, the use of excised parts instead of intact seedlings, and exposure to 'safelight'. Most of the experiments were carried out with 10-day old dark-grown seedlings of the bean cv. Widuco.

9.2.2. *Duration of the dark incubation period*

Fig. 48 illustrates that the slightly reverting action of far red given immediately after a red inductive irradiation, becomes increasingly pronounced if dark periods of more than 16 hours are inserted between the inductive irradiation

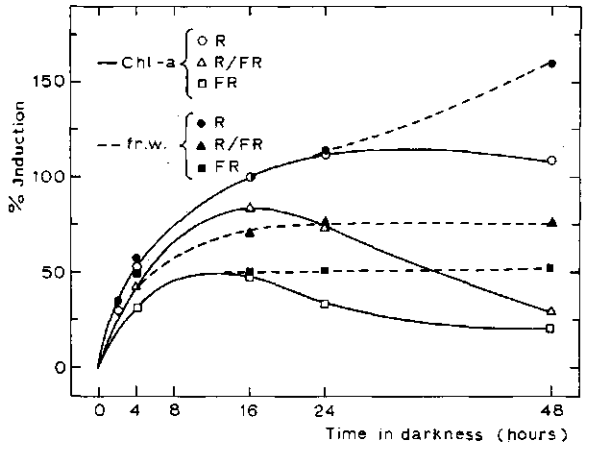

FIG. 48. Effect of duration of dark incubation period, following various inductive light treatments upon Chl-*a* accumulation and gain in fresh weight during 5 hours continuous white light in leaves of 10-day old bean seedlings of cv. Widuco.

and the application of continuous white light. During these extended dark periods, the inductive action of far red only correspondingly decreases. More or less the same holds true with respect to the light induced increase of fresh weight of the leaves. Obviously, in the latter case, the increase in reversibility is to be ascribed to the induction of a long-lasting growth response by the brief red irradiation. Maximum induction of the increase in fresh weight with red-far red or far red only is reached in relatively short dark periods.

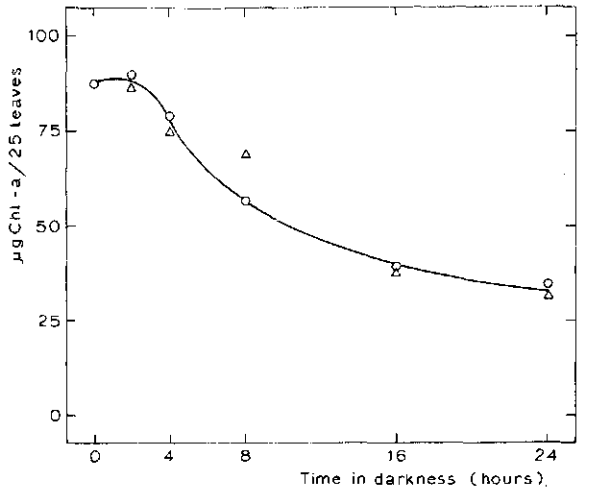

FIG. 49. Effect of duration of dark incubation period, following red (○) and red-far red (△) light upon Chl-*a* accumulation during 5 hours continuous white light in detached leaves of 10-day old bean seedlings of cv. Widuco.

In detached bean leaves, treated according to the method described in 8.2.1., no significant differences in response to pre-exposures with either red or red-far red could be observed even upon prolonged incubations (fig. 49).

9.2.3. *De-etiolation pretreatments*

Experiments on the light sensitivity of pea seedlings had already shown that even short exposures to a green safelight could induce a significant increase in the rate of Chl-*a* accumulation in continuous white light (fig. 45). This observation led us to repeat the reversibility experiments, including de-etiolated (see p. 1) material. The de-etiolated stage was reached in dark-grown bean seedlings by giving them a saturating dose of red light at various moments prior to the standard experimental schedule of red and red-far red light treatments, followed by 16 hours darkness (fig. 50). In these experiments, the inductive capacity of a single saturating standard dose of red light followed by 16 hours of darkness as manifest after 5 hours of white light and in comparison with a non-pre-irradiated but otherwise similarly treated dark control has again been taken as '100%'. For comparison we have included data already shown in fig. 48. Obviously, induction of rapid Chl-*a* accumulation can be markedly increased if the saturating standard red exposure 16 hours prior to the continuous illumination

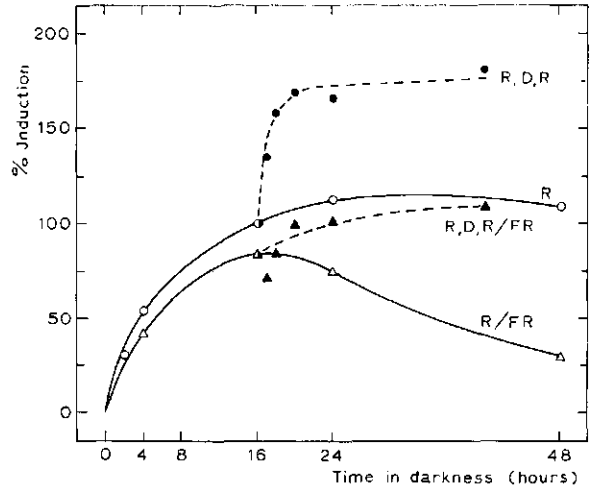

FIG. 50. Effect of duration of dark incubation period and de-etiolation with red light upon the induction and reversion of rapid Chl-*a* accumulation in 10-day old bean seedlings of cv. Widuco.

○——○: 1'R followed by dark incubation of various duration.

△——△: 1'R/1'FR followed by dark incubation of various duration.

●- - - -●: 1'R – dark interval of various duration – 1'R, 16h darkness.

▲- - - -▲: 1'R – dark interval of various duration – 1'R/1'FR, 16h darkness.

Chl-*a* content was measured after 5 hours continuous white light following the above treatments.

is preceded by another treatment with red light. Maximum response is already observed upon intercalation of relatively short dark periods between both red irradiations. However, this *additional* increase in induction is fully cancelled if the standard (i.e.: second) red exposure is followed by far red light. Thus a de-etiolating pre-irradiation can increase far red reversibility of a second red irradiation up to 100% in contrast to what is observed in completely dark-grown material.

Fig. 51 shows the same response for pea. Whereas completely dark-grown pea seedlings show hardly any reversal (fig. 51, top), de-etiolation with red light 24 hours prior to the normal scheme of red and red-far red irradiations increased far red reversibility of the second red exposure (fig. 51, bottom). Interestingly, after de-etiolation far red light given alone loses its inductive capacity, as opposed to its action on completely dark-grown seedlings. Similar results were again obtained with bean seedlings.

When green darkroom safelight was administered to bean seedlings during one minute, 24 hours prior to the red-far red irradiations, an increase in reversibility similar to the one caused by red could be demonstrated (fig. 52). The de-etiolation by green safelight did not induce any directly measurable physiological effect. This is in agreement with the rather low light sensitivity of bean (fig. 42). Thus, dark controls and seedlings pretreated with 'safelight' appear indistinguishable with respect to the Chl-*a* accumulation rate in subsequent continuous white light. The action of the irradiation with safelight is therefore latent and manifests itself only by rendering the inductive action of subsequent red irradiation more readily reversible by far red. Again, intercalation of only a

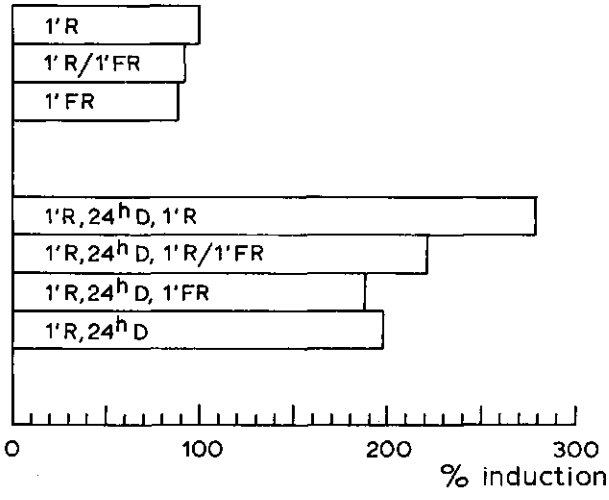

FIG. 51. Effect of de-etiolation with red light upon the induction and reversion of rapid Chl-*a* accumulation in 7-day old pea seedlings of cv. Krombek. All irradiation schemes were followed by 16 hours darkness and, thereafter, 5 hours continuous white light.

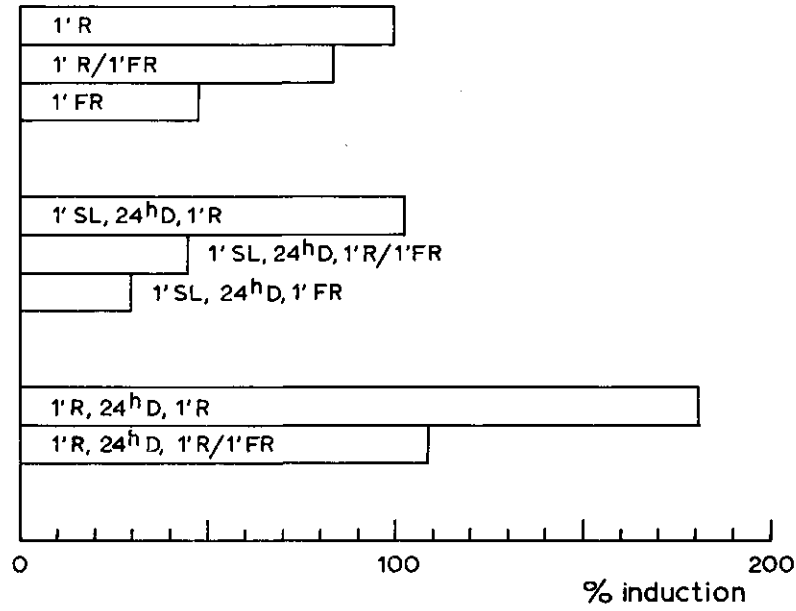

FIG. 52. Effect of de-etiolation with red light and green safelight (SL: 10 ergs/cm² sec) upon the induction and reversion of rapid Chl-*a* accumulation in 10-day old bean seedlings of cv. Widuco. All irradiation schemes were followed by 16 hours darkness and, thereafter, 5 hours continuous white light.

few hours darkness between safelight pretreatment and red-far red irradiations was sufficient for obtaining maximum response.

Since induction of rapid Chl accumulation could be markedly increased by repeating the red inductive irradiation after a relatively short dark period, we also studied the effect of repeated short exposures at 2-hour intervals. Fig. 53 shows that little further increase in induction is obtained by increasing the red exposures beyond two. The inductive effect of repeated red-far red exposures does not significantly surpass that of a single red-far red treatment, which confirms the conclusion that all red irradiations except the first are fully reversible by far red. The response to repeated far red irradiations did not surpass that of a single inductive far red cycle, either.

Repeated irradiations may also give useful information about the point of attack of phytochrome. Fig. 54 shows the effect of a varying number of red or red-far red cycles, followed by 14 hours darkness, upon accumulation of carotenoids, Pchl, Chl-*a*, and fresh weight in bean seedlings. Obviously, no important differences in response to these series of brief red and red-far red preirradiations become apparent. However, after exposing these seedlings to 5 hours continuous white light (fig. 55) marked differences between seedlings, repeatedly irradiated with red and those, irradiated with red followed by far red became apparent, not only in accumulation of Chl-*a* and carotenoids, but also with respect to the rise in fresh weight.

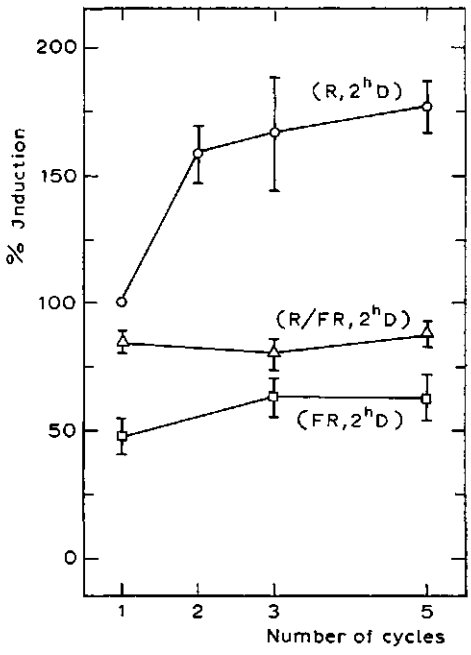

FIG. 53. Effect of different numbers of cycles, each consisting of a short light impulse followed by 2 hours darkness, upon Chl-*a* accumulation during 5 hours continuous white light in 10-day old bean seedlings of cv. Widuco. A 14-hour dark incubation period was inserted between the final inductive cycle and continuous illumination.

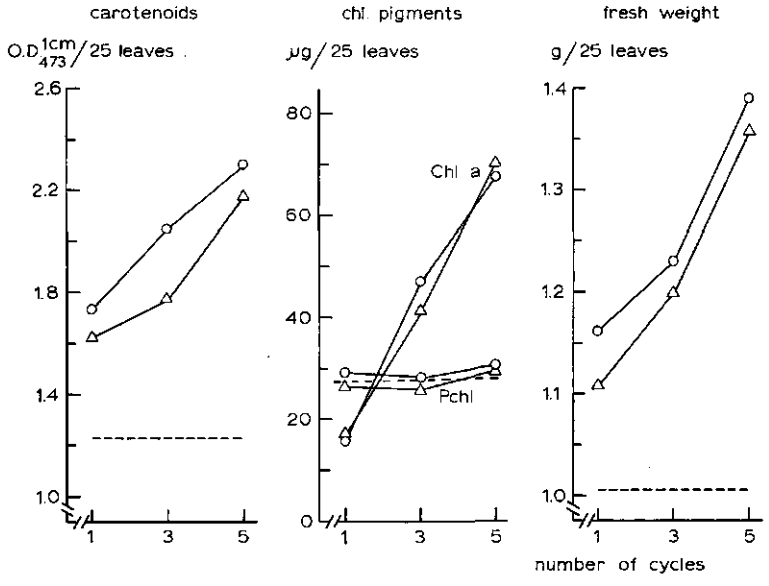

FIG. 54. Effect of different numbers of cycles of red (○——○) and red-far red (△——△) upon the level of carotenoids, Pchl, Chl-*a*, and fresh weight of leaves of 10-day old bean seedlings of cv. Widuco. Measured directly after 14-hour dark incubation period. Carotenoids were extracted in 25 ml acetone-80%. Dark control: - - - - - - - -

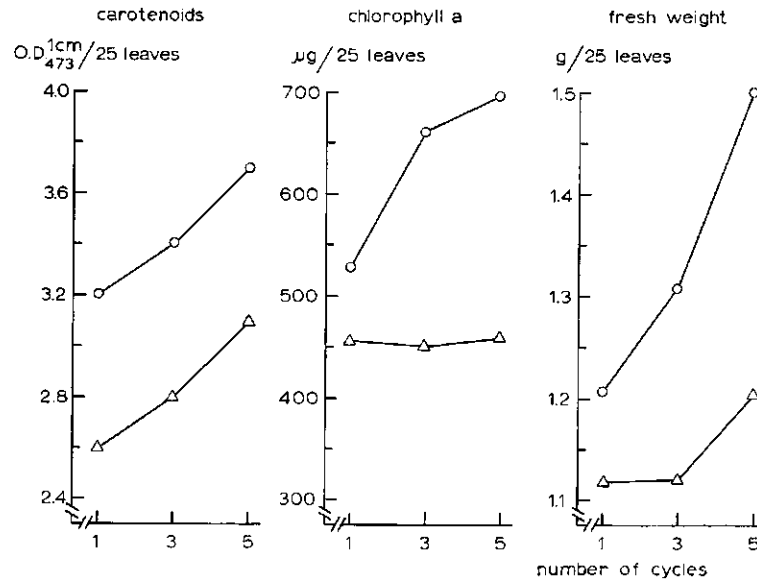

FIG. 55. Effect of different numbers of cycles of red (○——○) and red-far red (△——△) upon the level of carotenoids, Chl-*a*, and fresh weight of leaves of 10-day old bean seedlings of cv. Widuco. Measured after additional illumination of the seedlings with 5 hours continuous white light. Carotenoids were extracted in 25 ml acetone-80%.

9.3. DISCUSSION

In this Chapter we have shown (table 4) that in a number of cultivars of pea, bean, and maize rapid Chl accumulation induced by red light did not obey the operational criterion of complete red-far red antagonism. Increase in carotenoids and in fresh weight responded in a similar way. Reports in the literature on red-far red reversibility are rather contradictory and different explanations have been put forward. NAKAYAMA *et al.* (1960), in order to explain the lack of reversibility of the inhibition of flowering in *Pharbit is*,e.g. suggested the possibility that irreversible physiological reactions occurred already during the short period of red irradiation. In 1964, FREDERICQ offered experimental support for such a fast induction mechanism. More or less similar conclusions were reached by VIRGIN (1961) regarding the induction of Chl formation in wheat, and by HAUPT (1969) for inhibition of growth of pea internodes. The latter author also considered the additional possibility of phytochrome intermediates being involved (HAUPT *et al.*, 1970). Our data do not appear to fit these hypotheses: on the one hand, reducing the duration of both red and far red irradiations down to 6 seconds each at constant intensity did not increase reversibility. Equally negative results were reported by BOTTOMLEY (1970) for induction of RNA polymerase activity in etioplasts of dark-grown pea seedlings. On the other hand,

intercalation of dark incubation periods, considerably in excess of 16 hours, gave rise to an increased red-far red antagonism (fig. 48). The observation that complete red-far red reversals can be obtained after de-etiolation (e.g. figs. 50 and 51), also points to the possibility of other interpretations.

BLAAUW *et al.* (1968) and BOTTOMLEY (1970) have suggested that absence of photoreversibility may point to involvement of photoreceptors other than phytochrome. However, the close resemblance between our action spectrum for induction (fig. 46) and the absorption spectrum of purified phytochrome as discussed in 8.3., makes it unattractive, in our opinion, to accept this view. We want to emphasize that failure of far red to antagonize the effect of a red irradiation, does not disprove phytochrome as the receptor pigment. On the contrary, an extremely low P_{fr} requirement of the induction reaction and the presence of completely dark-grown tissue may explain the observed high inductive capacity of far red light when given alone as well as absence of far red reversal. The difference in photoreversibility between completely dark-grown and de-etiolated tissue, as shown in e.g. figs. 50, 51 and 53, may be more easily understandable if we ascribe these phenomena to migration of phytochrome during the process of de-etiolation.

We suppose that in seedlings grown in complete darkness, phytochrome is present in the red absorbing form P_r, in a comparatively large fraction of the cell volume, the simplest, and probably oversimplified assumption being that the distribution is homogeneous (RAVEN and SPRUIT, 1973, SPRUIT *et al.*, submitted). This is schematically represented in fig. 56, a. Possibly, in this stage, the localization of the phytochrome molecules is at or near cytoplasmic membranes (cf. HAUPT, 1972). Furthermore, we assume that there are, in the plant cells, certain predestinated sites of restricted capacity where phytochrome can become functional. These sites are assumed to have the possibility to initiate different physiological processes and will be called: 'reaction centres'. We also assume that upon irradiation of completely dark-grown tissue (fig. 56, b), the far red absorbing form of phytochrome (P_{fr}) is able to migrate to these sites, and, in this way, will become locally concentrated in the cell (fig. 56, c). An important aspect of this may be that phytochrome inside these restricted areas may escape spectrophotometric detection, which may as well offer an explanation for so-called phytochrome decay (section 2.3.) (SPRUIT, 1972; SPRUIT *et al.*, submitted). It is not unlikely, that there is a large number of reaction centres in the cell, so that the migration process may involve translocation of phytochrome molecules over only relatively small distances. In this respect, one could e.g. think of transport of P_{fr} from the cytoplasm to adjacent membranes of certain cell organelles, or of transport along and concentration at membranes on which phytochrome may already be present in a more dilute fashion in the dark-grown state. Support for the latter possibility has been given by e.g. SINGER and NICOLSON (1972). It is generally assumed that the photoconversion of phytochrome is accompanied by conformational changes in the apoprotein (ROUX and HILLMAN, 1969; HOPKINS and BUTLER, 1970; KROES, 1970). Possibly, there is a connection between this phenomenon and the ability of P_{fr} for migration.

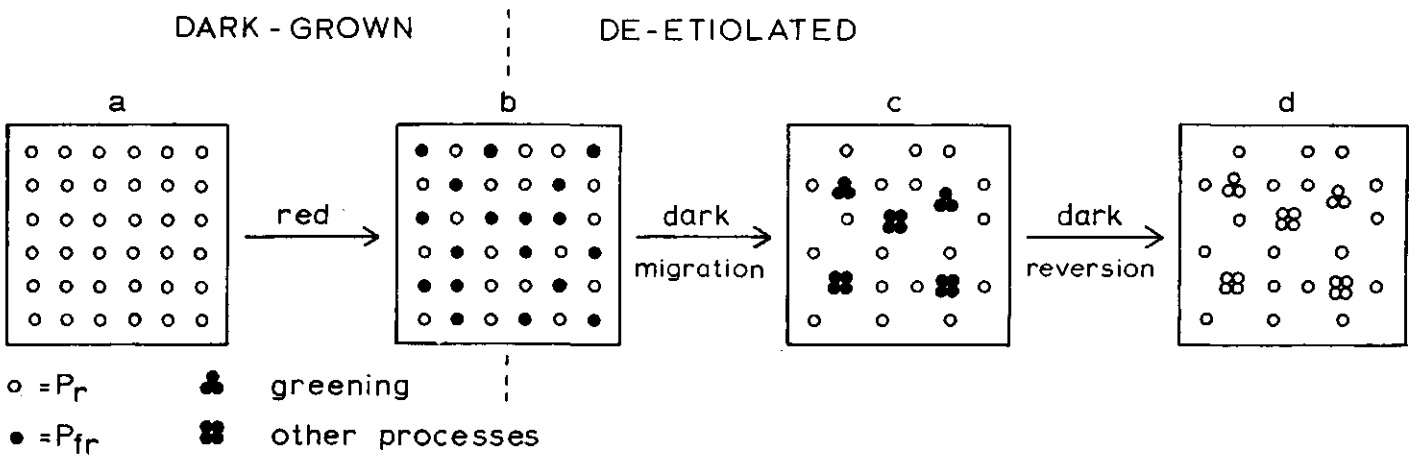

FIG. 56. Schematic representation of intracellular P_{fr} migration to and concentration at predestinated sites during the de-etiolation of dark-grown tissue. These sites are assumed to be associated with different physiological processes (indicated by different symbols). The squares schematically represent the parts of the cell accessible to phytochrome. For further explanation see text.

Upon arrival at the reaction centre, P_{fr} is assumed to become bound in some way so that return to its original site is no longer possible. Dark reversion to P_r, however, remains possible which results in only P_r at the reaction centre after prolonged darkness (fig. 56, d). With the special aim to consider in more detail the fate of P_r and P_{fr} during the de-etiolation phase and the connection with subsequent physiological response, i.e. in our case especially the greening process, the model described above is illustrated still more schematically, but in more detail in fig. 57. The seven squares in this figure again indicate the parts of the plant cell accessible to phytochrome. Initially, in the completely dark-grown state (fig. 57, a), phytochrome is assumed to be present only in the compartment, marked R, in the red absorbing form P_r (cf. fig. 56, a). The rectangular compartment at the right side of the cell represents the reaction centre associated with the induction of rapid Chl accumulation (groups of three small circles in fig. 56, c and d). The rectangle at the bottom of the cell represents other places were phytochrome may become bound (cf. groups of four small circles in fig. 56, c and d). The relatively small quantities of active phytochrome inside the Chl reaction centre (p_r and p_{fr}) are indicated by r and f respectively. The larger amounts of inactive phytochrome outside the centre (P_r and P_{fr}) have been denoted by capitals R and F. We have attempted to indicate the relative amounts of phytochrome involved by different sizes of these characters.

Starting with completely dark-grown tissue (fig. 57, a), a brief red exposure gives rise to a certain percentage P_{fr} (fig. 57, b). In darkness this is followed by migration of P_{fr} to the reaction centre for Chl induction as well as to other reaction centres, and by reversion of P_{fr} to P_r (fig. 57, c). As soon as the concentration of p_{fr} inside the reaction centre rises above a certain threshold level for a sufficient period (MOHR, 1970), the physiological induction process is assumed to start. Light regimes that experimentally (section 9.2.) resulted in a stimulation of the Chl-*a* accumulation rate above the dark control have been indicated by a plus sign (fig. 57, d).

Phytochrome in the reaction centre (p_{fr}) is assumed to undergo dark reversion to p_r. As soon as this reaction is completed, we have reached the de-etiolated state which in our model differs from the initial dark-grown state in that there is now p_r in the reaction centre (fig. 57, e).

Since it appears reasonable to assume that active phytochrome in the form p_{fr} is bound in some way, the kinetics of its dark reversion may well be quite different from those for the dark reversion of inactive phytochrome (KENDRICK and SPRUIT, in the press). We have found that in bean seedlings a renewed red irradiation, given after a dark interval of about 2 hours, enhances the inductive action of the first (fig. 50). A possible explanation for this observation may be that in the reaction centre dark reversion was already complete within this period. Alternatively, it may indicate that after that time the p_{fr} concentration is about to return to the threshold level or is already lower, while the system itself is still sensitive to further induction. We suppose, therefore, that the maintenance of the inductive capacity for Chl-*a* formation over a period of at least 48 hours (figs. 38, 40, and 48) does not reflect the continuous presence of p_{fr} at

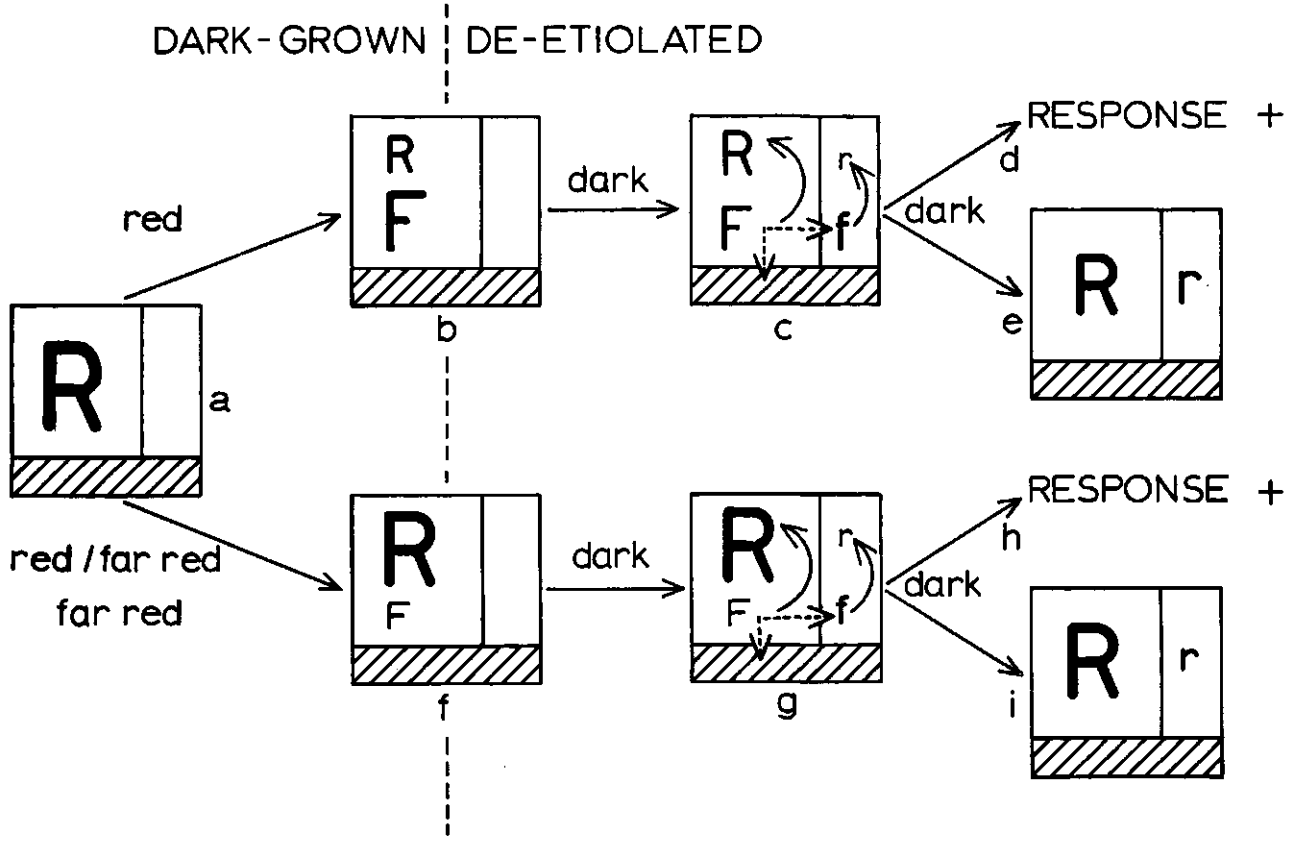

FIG. 57. Model illustrating processes during the establishment of the de-etiolated state by various light treatments. The squares represent the fraction of the cell volume accessible to phytochrome. Inactive phytochrome (P_r and P_{fr}) is indicated by R and F respectively; active phytochrome (p_r and p_{fr}) is indicated by r and f. Migration of P_{fr} is represented by dashed arrows, dark reversion by curved arrows. Other receptor sites than the one involved in induction of rapid Chl accumulation are indicated by the hatched rectangle forming the lower part of each square. For further explanation see text.

concentrations above the threshold level. On the contrary, we assume that the inductive capacity of a certain light dose as well as the duration of the period over which this induction will be maintained are determined by the extent to which the initial p_{fr} concentration exceeds the threshold level. The fact that the induction of Chl formation is maintained over such long dark periods may then be explained by the irreversible nature of the growth of the etioplasts, occurring simultaneously (MEGO and JAGENDORF, 1961). The latter process may be the basic feature of the Chl-*a* induction phenomenon (RAVEN and SPRUIT, 1972b; see also Chapter 10).

When red inductive irradiation of completely dark-grown tissue is immediately followed by far red, nevertheless a small amount of P_{fr} will remain as a result of the overlap of the absorption bands of the two forms of phytochrome (fig. 57, f). There is a competition between reversal of this P_{fr} to P_r and its transport to and concentration in the empty reaction centres during the following dark period (fig. 57, g). In these centres, it could still exert, in the form p_{fr}, a certain induction if its concentration rises above the threshold (fig. 57, h). It is understandable that in this situation no red-far red antagonism can be observed.

This model does not, of course, exclude the possibility of the existence of completely photoreversible reactions in dark-grown seedlings, since this may depend on both the relative capacity of the reaction centres involved and the height of their threshold requirement.

A similar explanation as for red followed by far red can be given for the inductive capacity of far red alone, when administered to completely dark-grown tissue (fig. 57, f-i).

In fact all standard red and far red light treatments, as described in the experimental sections 9.2.1. and 9.2.3., induced an increase in the Chl-*a* accumulation rate upon transfer to continuous light in completely dark-grown leaves of a number of cultivars of pea, bean, and maize. In our model, this indicates that in such cases, p_{fr} indeed surpassed the threshold level for induction as expressed by the plus signs in fig. 57.

An irradiation resulting in a de-etiolated state does not necessarily lead to a positive physiological response, as might be concluded from fig. 57: green safelight can act like red with respect to the increase in photoreversibility without inducing any directly measurable physiological response (fig. 52). We can ascribe this to the circumstance that P_{fr} after being produced in very small amounts by green light, is transported to the reaction centre so slowly that simultaneous dark reversion inside the centre prevents the p_{fr} concentration from rising above the threshold (SPRUIT *et al.*, submitted). Nevertheless, the slow accumulation at the centre of active phytochrome in the form p_r, establishes a more or less completely de-etiolated state, without, however, triggering the induction of rapid Chl formation. A similar experiment should be possible with light of very low intensities in wavelength regions other than green.

We will here recall that the term de-etiolated was defined as the state of a completely dark-grown seedling exposed to radiation for some time so that it has undergone a photobiological change of some sort. In the case described

above this change would be the slow accumulation at the reaction centre of phytochrome, first as p_{fr} and after dark reversion as p_r.

With tissues that are already de-etiolated by previous exposure to light, the responsiveness towards a second irradiation becomes quite different (fig. 58). In this case p_r is already present inside the reaction centre (fig. 58, a). A (second) red dose will therefore transform both active and inactive phytochrome (fig. 58, b). It is the immediate reappearance of p_{fr} above the threshold level inside the reaction centre (fig. 58, b-c) that gives a new impulse to a further increase in physiological response (fig. 58, d). It is of some importance to note that our model implies that a reaction centre, if already completely occupied by active phytochrome (as is the case upon saturating de-etiolation) cannot accommodate more P_{fr}. Molecules newly formed outside the reaction centre may, however, be transported to reaction centres associated with other physiological responses (fig. 58, c and g). One can also consider the possibility that certain phytochrome 'sinks' exist, where P_{fr} remains inactive or, eventually, its components might be used as substrate in some other biosynthetic chain (KENDRICK and HILLMAN, 1972).

These assumptions explain the difference in far red sensitivity and photoreversibility between dark-grown and de-etiolated tissues. Far red following red as well as far red alone give rise to a small amount of P_{fr} outside the reaction centre, as illustrated in fig. 58, f. Also at the centre some p_{fr} is formed. The ratios P_{fr}/P_r (and p_{fr}/p_r) established by this irradiation will, of course, be the same as in completely dark-grown material. Since, however, the completely filled reaction centre should not allow the penetration of any new P_{fr}-molecules, the absolute concentration of its p_{fr} will remain low enough not to surpass the physiological threshold (fig. 58, g-h). Obviously, this condition should also lead to complete photoreversals in de-etiolated tissue. To make this as clear as possible, let us assume e.g. that we start with completely dark-grown tissue. Irradiation with a particular far red source establishes a photostationary state with, say, 3 % P_{fr}. If this amount is adequate to saturate the centre after P_{fr} migration, the latter is fully occupied by phytochrome, all in the p_{fr} form. After dark reversion, the centre is still fully occupied, but now by p_r. A repeated irradiation with the same light source then establishes a photostationary state in the centre with 3 % p_{fr} and the p_{fr} concentration now levels off at 0.03 times the first or 9×10^{-4} of total phytochrome originally present, which should be far below the activation threshold. The original far red source now is no longer able to activate the centre and a shorter wavelength light source giving a much higher photostationary state would be required.

Predictions from this model can also be used to give possible explanations for some other paradoxical observations from the phytochrome literature (RAVEN and SPRUIT, 1973; SPRUIT *et al.*, submitted).

As shown in fig. 48, reasonably good reversals in completely dark-grown seedlings can be observed after prolonged periods (>16 hours) of dark incubation. This has to be expected, since the pool size of p_{fr} reached with red followed by far red falls sooner below the threshold level required for the maintenance of

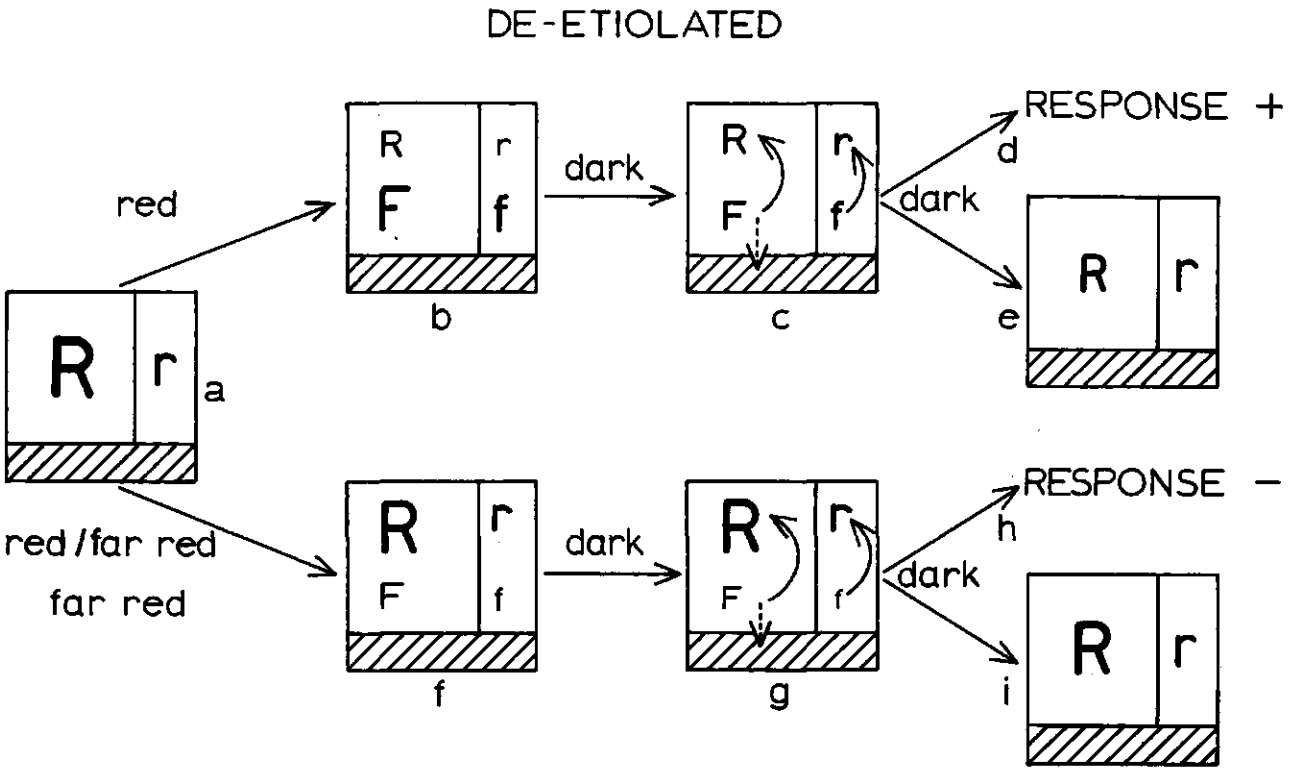

FIG. 58. Model illustrating processes that occur upon various light treatments in tissue, already de-etiolated by previous exposure to red (i.e. e in fig. 57). For details see fig. 57 and text.

the inductive capacity than would be the case after red irradiation only, because of the much lower initial concentration of P_{fr} outside the reaction centre while still empty.

10. GENERAL DISCUSSION

The starting point for the present investigation was the question, controversial at that time, whether or not the phytochrome pigment system was involved in the regulation of Chl-*a* formation in previously dark-grown seedlings. The existing literature contained a number of contradictory reports concerning the problem of complete far red reversibility of effects induced by red. In Chapters 8 and 9, we have presented experimental data that may reduce the core of this controversy to the question whether or not de-etiolated plant material was used in these studies. We observed that red-far red antagonism of the induction of rapid Chl-*a* formation in a number of plants was more pronounced in pre-irradiated seedlings than in those, raised in complete darkness (section 9.2.3.). We have attempted to explain this with a model assuming that upon the very first exposure of dark-grown seedlings to light, the phytochrome present migrates to and is concentrated in relatively small receptor sites in the plant cells which sites then become physiologically active (section 9.3.). Our data support the view that, quite generally, absence of far red reversibility of physiological reactions induced by red should not per definition be ascribed to the involvement of photoreceptors other than phytochrome (e.g. CLAES, 1967; BOTTOMLEY, 1970). This is equivalent to saying that one of the generally accepted criteria for phytochrome involvement, viz. red-far red reversibility, is a sufficient, but not a necessary one. Even, handling of dark-grown plants in the presence of green safelight may cause a marked degree of de-etiolation (sections 8.2.3. and 9.2.3.) and should, for this reason, be avoided as far as possible. If this precaution is neglected, the responsiveness of dark-grown plants towards light may alter even though no direct physiological effect of the exposure to safelight can be observed.

In Chapter 6, we have presented evidence that the initial rates of Pchl regeneration in darkness are not controlled by phytochrome. At first sight, these findings seem to disagree with those demonstrating red-far red control of Chl-*a* accumulation during continuous illumination. In order to expose more clearly the nature of this problem, we must discuss in some detail the possible modes of action of phytochrome in regulating the greening process.

From reports in the literature and from certain data presented in this paper, we conclude that the following are the most probable points of attack for the phytochrome system:

1. The biosynthetic chain leading to Chl-*a*
2. The biosynthesis of structural proteins
3. Protection of chlorophyllous pigments from photodestruction.

With respect to the first possibility, SISLER and KLEIN (1963), GASSMAN and BOGORAD (1967a), NADLER and GRANICK (1970), BEALE (1971), MURRAY and KLEIN (1971), and ZUCKER (1972) have assumed that the photoregulation of the duration of the lag phase in Chl formation occurs at the level of δ-ALA biosyn-

thesis. In many cases, this was concluded from observations that Chl formation in leaves treated with inhibitors of protein synthesis such as CAM and cycloheximide was restored by application of δ-ALA. At any rate, the duration of the lag phase would reflect the degree of depletion of the seedlings for this porphyrin-precursor. Generally, succinyl CoA synthetase (STEER and GIBBS, 1969) and δ-ALA synthetase (GRANICK, 1967; GASSMAN and BOGORAD, 1967a, 1967b; NADLER and GRANICK, 1970) are considered as the most plausible candidates for phytochrome control in the δ-ALA biosynthesis chain. As a matter of fact, STEER and GIBBS (1969) demonstrated a limited red-far red reversibility with respect to the light induced initial decrease in activity of succinyl CoA synthetase. A possibly more attractive candidate for phytochrome regulation would be δ-ALA synthetase. However, the main source of information about a possible occurrence of such an enzyme in higher plants so far have been indirect studies, such as of Pchl regeneration behaviour and mode of action of Pchl- and Chl-inhibiting antibiotics. The presence of this enzyme in higher plants has not yet been demonstrated directly (KIRK, 1970; BOGORAD *et al.*, 1971). It is possible that in plants δ-ALA synthetase may have a short lifetime (10–90 minutes) (NADLER and GRANICK, 1970; MURRAY and KLEIN, 1971; SÜZER and SAUER, 1971). The relatively short duration of the period of Pchl regeneration could be explained in this way. It could also explain observations that the period over which the inductive capacity of red light for the elimination of the lag phase was optimally maintained was rather short (4–6 hours) (VIRGIN, 1957; MITRAKOS, 1961; AKOYUNOGLOU, 1970). That this enzyme, if present, is under phytochrome control, was concluded from observations such as those of AUGUSTINUSSEN and MADSEN (1965), RUDOLPH (1965), SHLYK *et al.* (1969), AKOYUNOGLOU (1970), and MASONER *et al.* (1972) on red-far red antagonism of the Pchl regeneration rates.

However, our data on the initial rates of Pchl regeneration (Chapter 6) definitely disprove the view that phytochrome acts directly on the Chl-*a* biosynthesis chain since no short term red-far red effect on Pchl regeneration was observed, compatible with phytochrome activity on an enzyme, even a short living one. The same may be concluded from the observed ineffectivity of δ-ALA on Chl-*a* biosynthesis under various experimental conditions (sections 7.2.1.2., 7.3., and 8.2.1.). In this respect, it is also of interest to note that in young seedlings the level of Pchl, reached in prolonged darkness after a brief illumination, appeared to approach closely the level before this illumination (cf. figs. 16, 18, and 19). Similar findings were reported by VIRGIN (1955), AKOYUNOGLOU and SIEGELMAN (1968), and SÜZER and SAUER (1971); this was also observed when considerable growth of leaves occurred simultaneously (THORNE, 1971b; see also fig. 54) as well as in leaves after 24 hours of greening (SHLYK *et al.*, 1972). To us, these data suggest that Pchl regeneration takes place at a distinct number of regeneration sites, that can be used several times. The apoprotein molecule of the Pchl holochrome may well be considered to function as the regeneration site; similar hypotheses were proposed by e.g. BOARDMAN (1967), BOGORAD *et al.* (1968), and SUNDQVIST (1969, 1970). Restricted availability of regeneration sites,

rather than the short lifetime of δ-ALA synthetase should then limit Pchl regeneration capacity, causing cessation of pigment production whenever the initial pigment level is reached.

Our demonstration that the inductive capacity of a red pre-exposure on Chl formation is maintained over at least 48 hours (figs. 38 and 40) as well contradicts the hypothesis that phytochrome would act on some short living enzyme.

With respect to the second possible point of attack for action of phytochrome, it should be mentioned that a number of investigators (e.g. KIRK and ALLEN, 1965; KASEMIR and MOHR, 1967; BOGORAD *et al.*, 1968) have pointed to the importance of biosynthesis of structural proteins in Chl-*a* accumulation. For instance, these proteins could be used as materials for development of the internal structure of the etioplasts or as apoproteins for pigment holochromes. In support of this view, MEGO and JAGENDORF (1961) reported that growth of etioplasts of bean was controlled by phytochrome. Red-far red antagonism was also observed with respect to e.g. the lipid content and the amount of proteins of the leaves. Similarly, the internal structural development of the etioplasts in continuous light was accelerated as a result of pre-exposure to red. Subsequent irradiation with far red light more or less completely abolished the effect (KLEIN *et al.*, 1964; BERRY and SMITH, 1971; HOLOWINSKY and O'BRIEN, 1972; WELLBURN and WELLBURN, 1973). Similarly, the quantity of etioplast membranes is reported to be controlled by phytochrome (UNSER and MOHR, 1970). BOARDMAN and coworkers (1971), using sensitive fluorescence spectroscopic methods, concluded that, following a pre-exposure newly-formed Chlide-*a* was more rapidly removed from pigment units containing Pchl. They suggested that this could have been the result of an accelerated rate of formation of photosynthetic membranes. In our opinion, the following picture of how phytochrome may control the rate of Chl-*a* accumulation during the greening process may be derived from presently available information. We suppose that one of the first biosynthetic steps, resulting from a pre-exposure to red light, is the stimulation of the formation of structural proteins (cf. MEGO and JAGENDORF, 1961), leading to acceleration of the formation of plastid lamellae (cf. UNSER and MOHR, 1970). This may also be concluded from the data, presented above, that a pretreatment with red light enhances the internal structural development of etioplasts in continuous light. Furthermore, it appears likely that, following photoconversion, the Chlide-*a* formed is detached from the Pchlide-apoprotein molecule and moves to another site (e.g. CAPON and BOGORAD, 1962; BOARDMAN *et al.*, 1971). Somewhere during this process it is esterified with phytol, yielding Chlide-*a* ester. We would like to suggest, that the rate of release of Chlide-*a* from the Pchlide-apoprotein is determined by availability of new deposition sites for Chlide-*a* ester (cf. BOARDMAN *et al.*, 1971), while in its turn the availability of empty Pchlide-apoprotein molecules regulates the rate of Pchlide regeneration. The observation of GOEDHEER (1961) that the time course of the SHIBATA shift (section 2.2.) seems related to that of Pchl regeneration, may form additional evidence for this suggestion. Moreover, we observed striking similarities between the kinetics of esterification of Chlide-*a* and the rates of

Pchl regeneration in leaves of the same age (unpublished observations).

For these reasons, we assume that phytochrome control of Chl-*a* formation should primarily be at the level of the biosynthesis of structural proteins and etioplast lamellae. In its turn, the availability of sufficient deposition sites for Chl-*a* enables the Chl-biosynthesis chain to function in an optimal way.

SCHNARRENBERGER and MOHR (1969) similarly suggested a possible correlation between phytochrome-mediated carotenoid accumulation and the rate of plastid growth and development. SMITH and FRENCH (1963) and THORNE (1971b) ascribed the increased capacity for Chl formation following a pre-irradiation, to the expansion of the leaf.

In Chapters 5 and 7, we described the unfavourable effect of light of high intensity upon Chl-*a* formation. We suggested that this is due to photobleaching of a transient form of Chl-*a*, probably Chlide-*a*. We can, therefore, visualize acceleration of the phytolization process of Chlide-*a* as another possible aspect of phytochrome regulation of Chl-*a* accumulation. Stimulation of the phytolization reaction would decrease the stationary concentration of the photosensitive intermediate, thereby increasing the yield of Chl-*a*. LILJENBERG (1966) published an action spectrum for the light induction of rapid phytolization that shows a peak around 660 nm. This seems to point to the involvement of phytochrome despite the lack of red-far red photoreversibility. If this interpretation is correct, we may suppose that phytochrome regulates the availability of lamellar structures inside the etioplast in such a way as to enable rapid incorporation of Chlide-*a* ester. Alternatively, the effect of phytochrome on the phytolization rate may be due to an activation of the enzyme catalyzing the reaction, probably chlorophyllase (BOGORAD, 1966).

It has been reported that carotenoid pigments protect chlorophylls from photodestruction both *in vitro* (CLAES and NAKAYAMA, 1959) and *in vivo* (SMITH and KOSKI, 1948). Therefore, stimulation of accumulation of carotenoids via the phytochrome pigment system, as described by COHEN and GOODWIN (1962) and SCHNARRENBERGER and MOHR (1969) may favour the rate of Chl-*a* accumulation, especially in continuous white light of high intensity.

11. SUMMARY

The rôle of phytochrome in the regeneration of protochlorophyll (Pchl) in darkness following short exposures to light, as well as in the accumulation of chlorophyll-*a* (Chl-*a*) in continuous light in previously dark-grown seedlings of pea, bean, and maize has been the subject of the present investigation.

The *in vitro* red absorption peak of Chl-*a* was situated at shorter wavelengths, if a dark period was inserted between the moment of Pchl phototransformation and that of pigment extraction (Chapter 5, fig. 8). There was a considerable pigment photobleaching during Pchl photoconversion in high quantum flux densities (2.2×10^4 ergs/cm^2 sec) of red light (fig. 10). The effectivity of red (651 nm) light in Pchl phototransformation surpassed that of blue (442 nm) light about two times (fig. 11.).

The initial rates and ultimate level of Pchl regeneration were strongly depressed in older leaves (Chapter 6). In young seedlings Pchl regeneration in darkness after an illumination, generally, stopped after having reached its initial level (e.g. figs. 16, 18, and 19), which level (either expressed as pigment content per g fr. w. or per constant number of leaves) was not affected by simultaneous leaf growth over a prolonged period (fig. 54). Thus, we concluded that the number of Pchl regeneration sites per cell is constant during the first hours of greening. Red-far red control of the initial rates of Pchl regeneration could not be demonstrated (figs. 16, 17, 18, and table 1). In older maize leaves, an effect attributable to phytochrome was observed upon the final level of Pchl, reached in prolonged darkness (fig. 18). In intact bean seedlings, however, even five repetitive exposures to red and red followed by far red, at 2-hour intervals, did not significantly alter the final level of Pchl (fig. 19).

The duration of the lag phase in Chl-*a* formation was increased, and the ultimate rate of Chl-*a* accumulation depressed in pea seedlings continuously exposed to white fluorescent light of high intensity (60,000 ergs/cm^2 sec) (figs. 23 and 25) which we ascribe to photodestruction of freshly formed chlorophyllous pigments (Chapter 7). The duration of the lag phase in Chl-*b* formation was even more sensitive than that of Chl-*a* formation to the intensity of the light (fig. 24).

In detached leaves and leaves of seedlings with the cotyledons removed, Chl-*a* formation was very poor as compared with leaves on intact plants (section 7.2.1.2.). Chl-*a* synthesis of detached leaves was only partly restored by sucrose supply. Application of δ-aminolevulinic acid had no stimulating effect on the rate of Chl-*a* formation in the light (fig. 26 and section 8.2.1.) nor on the accumulation of Pchl in darkness (section 7.3.). This renders unlikely that synthesis of this compound is a bottleneck in Chl-*a* formation.

Continuous red light (646–651 nm) was the most effective wavelength range for Chl-*a* formation and accumulation of carotenoids; they were much weaker in the blue (442 nm) (section 7.2.2.). The nature of the photoreceptor pigment(s)

involved could not be established with certainty (sections 7.2.2. and 7.3.). The increase in fresh weight of pea plumules during continuous illumination is most likely mediated by phytochrome (fig. 36). Electron micrographs demonstrated that internal structural development of etioplasts was especially rapid in continuous blue light (section 7.2.3.). During the first hours of greening in white light, three photoreceptor systems may be simultaneously active.

In intact seedlings the stimulatory effect of brief pre-exposures to red light on rapid Chl-*a* accumulation in continuous light was retained during a dark period of at least 48 hours (Chapter 8, figs. 38 and 40). This may be related to the irreversible concomitant light induced rise in fresh and dry weight of leaves (figs. 48 and 41, respectively), which may be paralleled by equally irreversible growth and development of etioplasts (section 9.3.). Excised leaves were completely insensitive to irradiations of the type, inductive in intact plants (fig. 39).

Considerable differences in sensitivity to red light (fig. 42) were not accompanied by similar differences in spectrophotometrically demonstrable phytochrome (fig. 43).

Pea leaves were found extremely light sensitive (fig. 44): even relatively short exposures to weak green 'safelight' induced rapid Chl-*a* accumulation in subsequent continuous white light (fig. 45). The action spectrum pointed to phytochrome as the photoreceptor pigment (fig. 46).

Induction by red light was hardly reversible by subsequent far red in various cultivars of pea and bean, and in young maize seedlings (table 4), owing to considerable inductive capacity of far red (Chapter 9). Far red reversibility of the effect induced by red increased considerably with increasing duration of dark incubation between pre-irradiation and continuous white light (fig. 48). Fairly complete red-far red reversal occurred in plants de-etiolated by pre-irradiation some hours prior to the inductive treatment (fig. 50). Even relatively short exposures to green safelight caused de-etiolation with concomitant increase in subsequent red-far red antagonism (fig. 52). We define as de-etiolated the state of a completely dark-grown seedling treated with a photobiologically inductive amount of light (see p. 1, and p. 77).

In order to explain the difference between completely dark-grown and de-etiolated seedlings in their sensitivity to far red induction and red-far red photoreversibility, a model is presented, involving transport of phytochrome during de-etiolation to receptor sites of restricted capacity which then become activated to initiate the physiological response (figs. 57 and 58, section 9.3.).

As for the rôle of phytochrome in the greening process, it is concluded that the biosynthetic pathway leading to Pchl and Chl-*a* is not directly under phytochrome control. However, P_{fr} is postulated to increase the capacity of the biosynthetic system forming Pchl by stimulating synthesis of structural proteins, enabling rapid build-up of the photosynthetic apparatus as soon as Chl molecules are being continuously supplied by phototransformation of Pchl. During this process, Chl is supposed to be detached from the Pchl regeneration sites, and the availability of empty regeneration sites is supposed to activate Pchl biosynthesis.

Phytochrome-mediated enhancement of the rate of phytolization might well be another factor favouring Chl-*a* accumulation in continuous illumination by facilitating the protection of the freshly formed chlorophylls from photodestruction. A similar function may be ascribed to certain red light induced carotenoid pigments, at least for greening in light with an appreciable content of shorter wavelengths.

12. ACKNOWLEDGEMENTS

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13. SAMENVATTING

Met behulp van in het donker opgekweekte kiemplanten van erwt, boon en maïs werd een onderzoek verricht naar de rol van het phytochroom bij de regeneratie van protochlorophyl (Pchl) in het donker na korte belichtingen, alsook bij de vorming van chlorophyl-*a* (Chl-*a*) in continu licht (= groeningsproces).

Wanneer de photochemische omzetting van Pchl in Chl-*a* werd gevolgd door een periode van donker, voordat de pigmenten werden geëxtraheerd, had dit tot gevolg dat het rode absorptiemaximum van dit Chl-*a* *in vitro* zich bij een kortere golflengte bevond (Hoofdstuk 5, fig. 8). Tijdens de omzetting van Pchl met rood licht van hoge quantenstroomdichtheid ($2{,}2 \times 10^4$ ergs/cm^2 sec) trad aanzienlijke afbraak van pigment op (fig. 10); overigens was rood licht (651 nm) ongeveer tweemaal zo effectief als blauw licht (442 nm) bij de omzetting van Pchl (fig. 11).

Zowel de aanvangssnelheid als het eindniveau van de Pchl regeneratie werden geringer naarmate de kiemplanten ouder werden (Hoofdstuk 6). Na belichting van bladen van jonge kiemplanten stopte de regeneratie van Pchl in het donker meestal nadat het oorspronkelijke niveau bereikt was (b.v. fig. 16, 18 en 19); dit niveau (hetzij uitgedrukt in pigmentgehalte per gram vers gewicht van het blad of per constant aantal bladen) werd gedurende lange tijd niet beïnvloed door gelijktijdig plaatsvindende groei van het blad (fig. 54). Hieruit werd de conclusie getrokken, dat gedurende de eerste uren van de groening, het aantal plaatsen in de cel waar Pchl regeneratie mogelijk is, constant blijft. De aanvangssnelheid van de Pchl regeneratie bleek niet te worden beheerst door het rood-donkerrood reversibel pigmentsysteem (= phytochroom-systeem) (fig. 16, 17, 18 en tabel 1); uitsluitend in oudere maïsbladen werd het eindniveau van Pchl, zoals dat werd bereikt na lange tijd donker, beïnvloed door het phytochroom (fig. 18). In intacte kiemplanten van de boon waren zelfs 5 belichtingen met rood of rood direct gevolgd door donkerrood, waarbij telkens twee uur donker tussen iedere belichting werd ingelast, niet in staat om het eindniveau van Pchl significant te veranderen (fig. 19).

Wanneer erwtekiemplanten langdurig werden belicht met wit licht van hoge intensiteit (60.000 ergs/cm^2 sec) afkomstig van fluorescentiebuizen, werd de duur van de aanloopperiode in de Chl-*a* vorming verlengd, terwijl ook de uiteindelijke snelheid van Chl-*a* vorming lager bleef (fig. 23 en 25); dit werd toegeschreven aan de afbraak onder invloed van het licht van pas gevormde chlorophylpigmenten (Hoofdstuk 7). De duur van de aanloopperiode in de vorming van Chl-*b* was in nog sterkere mate dan bij Chl-*a* afhankelijk van de lichtintensiteit (fig. 24). De Chl-*a* vorming in afgeplukte bladen en in bladen van kiemplanten waarvan de zaadlobben waren verwijderd, was bijzonder gering in vergelijking tot bladen aan intacte planten (sectie 7.2.1.2.); door toediening van sucrose werd de Chl-*a* synthese slechts gedeeltelijk op het oude peil teruggebracht. Er werd geen stimulerende werking waargenomen van toegediend δ-

aminolevulinezuur op de snelheid van Chl-*a* vorming in het licht (fig. 26 en sectie 8.2.1.) noch op de synthese van Pchl in het donker (sectie 7.3.). Het is dus niet waarschijnlijk dat de biosynthese van deze verbinding de limiterende factor is bij de Chl-*a* vorming.

Bij langdurige belichting bleek rood licht (646–651 nm) het meest werkzame golflengtegebied voor de vorming van Chl-*a* en carotenoïden te zijn; blauw licht (442 nm) was veel minder werkzaam (sectie 7.2.2.). Het was niet mogelijk om met zekerheid de aard van de hierbij betrokken photoreceptorpigment(en) vast te stellen (sectie 7.2.2. en 7.3.). Het is het meest waarschijnlijk dat tijdens de langdurige belichting de toename van het vers gewicht van de erwtepluimpjes wordt gereguleerd door phytochroom (fig. 36). De ontwikkeling van de interne structuur van de etioplasten, zoals dit met behulp van electronenmicroscopie werd bestudeerd, vond vooral snel plaats onder invloed van de langdurige belichting met blauw licht (sectie 7.2.3.). Het is dan ook mogelijk dat tijdens de eerste uren van de groening in wit licht drie photoreceptorsystemen gelijktijdig actief zijn.

Korte voorbelichtingen met rood behielden in intacte kiemplanten gedurende tenminste 48 uur donker hun stimulerend effect op de versnelling van de Chl-*a* vorming in continu licht (Hoofdstuk 8, fig. 38 en 40). Dit staat mogelijk in verband met de gelijktijdig door dit rode licht geïnduceerde, irreversibele toename van het vers gewicht en het droog gewicht van de bladen (resp. fig. 48 en 41); het is dan ook denkbaar dat dit op zijn beurt gepaard gaat met, eveneens irreversibele, groei en ontwikkeling van de etioplasten (sectie 9.3.). Afgesneden bladen waren volledig ongevoelig voor belichtingen welke inductief waren bij intacte planten (fig. 39).

De gevoeligheid van intacte kiemplanten voor inductie met rood licht liep sterk uiteen (fig. 42), hetgeen echter niet gekoppeld was aan identieke verschillen in hun gehalte aan spectrophotometrisch aantoonbaar phytochroom (fig. 43).

Bladen van intacte erwtekiemplanten bleken bijzonder gevoelig voor licht te zijn (fig. 44). Zelfs relatief korte belichtingen met een groen veiligheidslicht van een geringe intensiteit, induceerden een snelle vorming van Chl-*a* wanneer de planten vervolgens werden belicht met wit licht (fig. 45). Het actiespectrum voor de inductie van snelle Chl-*a* vorming duidde op phytochroom als het photoreceptorpigment (fig. 46).

In verschillende cultivars van de erwt en de boon, en in jonge kiemplanten van maïs was echter de inductie verkregen met rood licht nauwelijks reverteerbaar met direct daarop volgend donkerrood licht (tabel 4), hetgeen samenhing met de op zich reeds sterk inductieve werking van dit donkerrode licht (Hoofdstuk 9). De rood-donkerrood reversibiliteit nam aanzienlijk toe, wanneer de donker-incubatietijd tussen voorbelichting en continu wit licht werd verlengd (fig. 48). Nagenoeg volledige rood-donkerrood omkeerbaarheid trad op bij kiemplanten welke, enige uren voorafgaande aan de eigenlijke inductieve belichting, aan licht werden blootgesteld (= deëtiolering) (fig. 50). Deëtiolering werd zelfs teweeggebracht door een relatief korte belichting met groen veiligheidslicht, aangezien dit namelijk vergezeld ging van een versterking van het rood-donker-

rood antagonisme (fig. 52). Er is derhalve sprake van een gedeëtioleerde staat, wanneer een in het volstrekt donker opgekweekte kiemplant wordt blootgesteld aan een photobiologisch werkzame hoeveelheid licht (zie p. 1 en p. 77).

Met het doel om het verschil in gevoeligheid voor donkerrood licht en in rood-donkerrood photoreversibiliteit tussen kiemplanten die volstrekt in het donker zijn opgekweekt en kiemplanten, die vervolgens zijn gedeëtioleerd, te verklaren, werd een model ontwikkeld (sectie 9.3., fig. 57 en 58). Dit model is gebaseerd op de aanname dat, gedurende de deëtiolering, phytochroom naar bindingsplaatsen met slechts een beperkte capaciteit wordt getransporteerd, welke hierdoor worden geactiveerd om een physiologische reactie te doen aanvangen.

Met betrekking tot de rol van het phytochroom in het groeningsproces wordt geconcludeerd, dat de biosyntheseketen naar Pchl en Chl-*a* niet direct door het phytochroom wordt beheerst. Er wordt daarentegen gepostuleerd dat de physiologisch actieve vorm van het phytochroom (P_{fr}) de capaciteit van het Pchl-biosynthesesysteem vergroot en wel door stimulering van de synthese van structuureiwit. Hierdoor kan, in continu licht, op snelle wijze het photosyntheseapparaat worden opgebouwd zodra voortdurend Chl moleculen beschikbaar komen door de photochemische omzetting van Pchl. Hierbij wordt verondersteld dat tijdens dit proces het Chl-*a* wordt verwijderd van de plaats waar het Pchl regenereert, en dat het voortdurend snel beschikbaar komen van lege regeneratieplaatsen op zijn beurt de biosyntheseketen van Pchl activeert.

Het is bovendien mogelijk dat de Chl-*a* vorming tijdens langdurige belichting wordt bevorderd als resultaat van de onder controle van het phytochroom staande versnelling van de phytylering, waardoor pas gevormde chlorophyllen beter worden beschermd tegen afbraak door licht. Voor zover het groening betreft in licht dat een aanzienlijke hoeveelheid kortgolvige straling bevat, kan een dergelijke beschermende functie wellicht ook worden toegeschreven aan bepaalde carotenoïden, waarvan de vorming door rood licht geïnduceerd kan worden.

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PLATE 2. Internal structure of an etioplast in leaves of 7-day old pea seedlings of cv. Krombek after 5 hours greening in low intensity white fluorescent light (1500 ergs/cm² sec) at 25 °C. × 46,000.

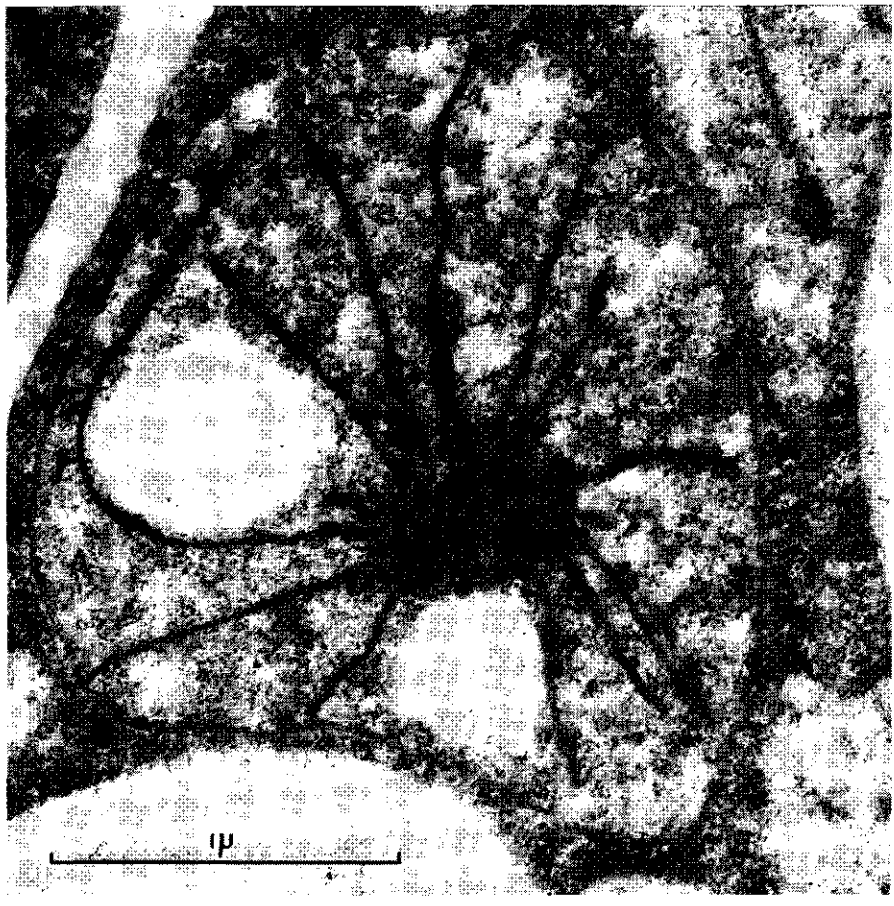

PLATE 3. Internal structure of an etioplast in leaves of 7-day old pea seedlings of cv. Krombek after 5 hours greening in high intensity white fluorescent light (60,000 ergs/cm² sec) at 25 °C. × 46,000.

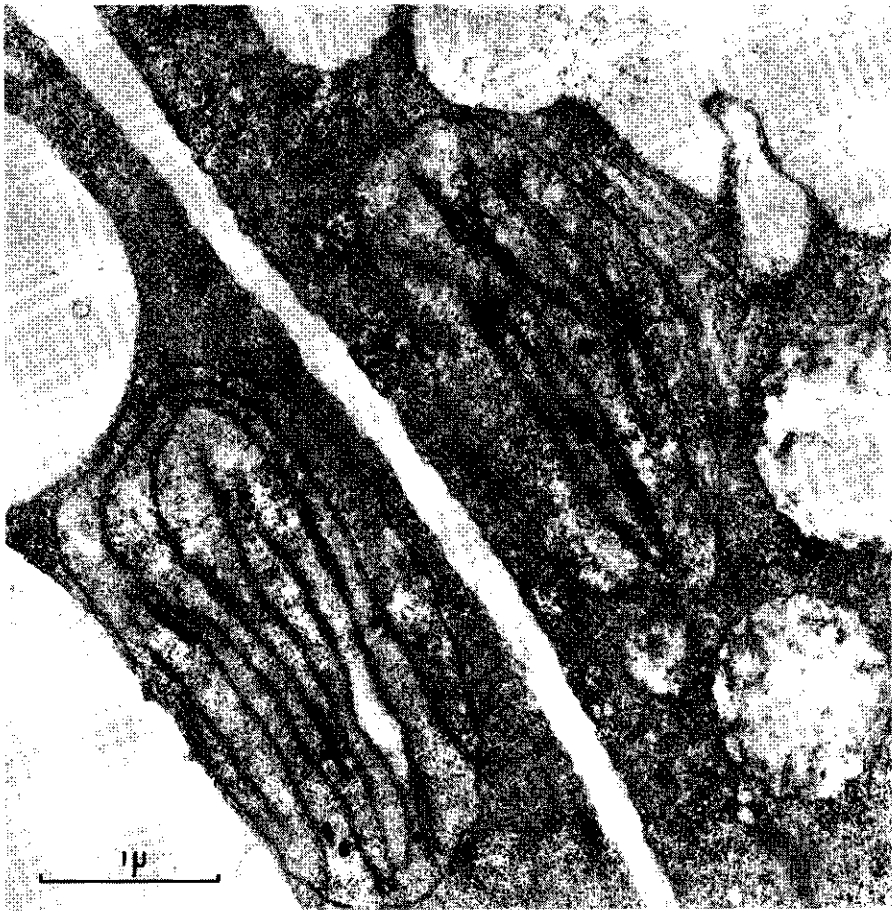

PLATE 4. Internal structure of etioplasts in leaves of 7-day old pea seedlings of cv. Krombek after 5 hours greening in blue light (442 nm, 335 ergs/cm^2 sec) at 25 °C. × 23,500.

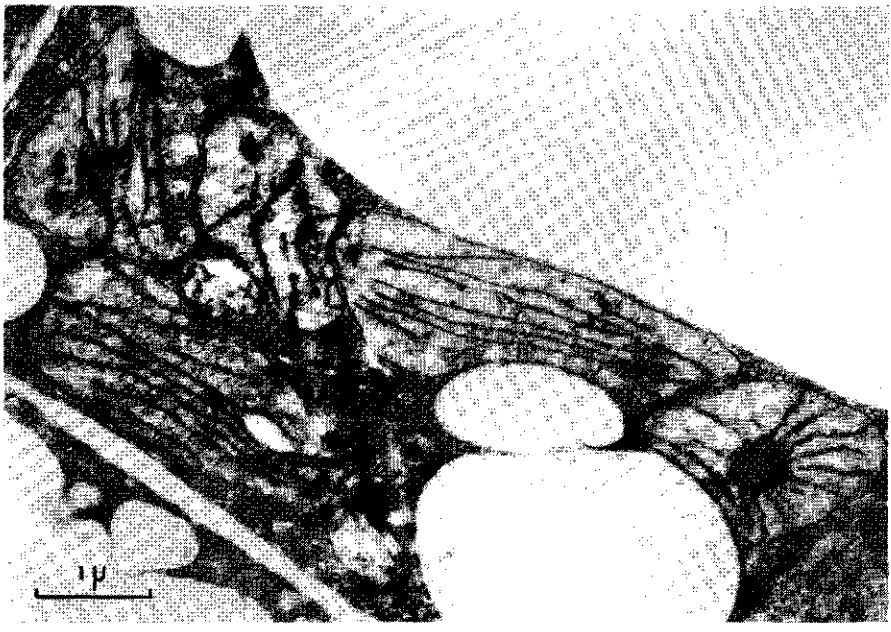

PLATE 5. Internal structure of etioplasts in leaves of 7-day old pea seedlings of cv. Krombek after 5 hours greening in green light (529 nm, 1200 ergs/cm² sec) at 25 °C. × 15,500.

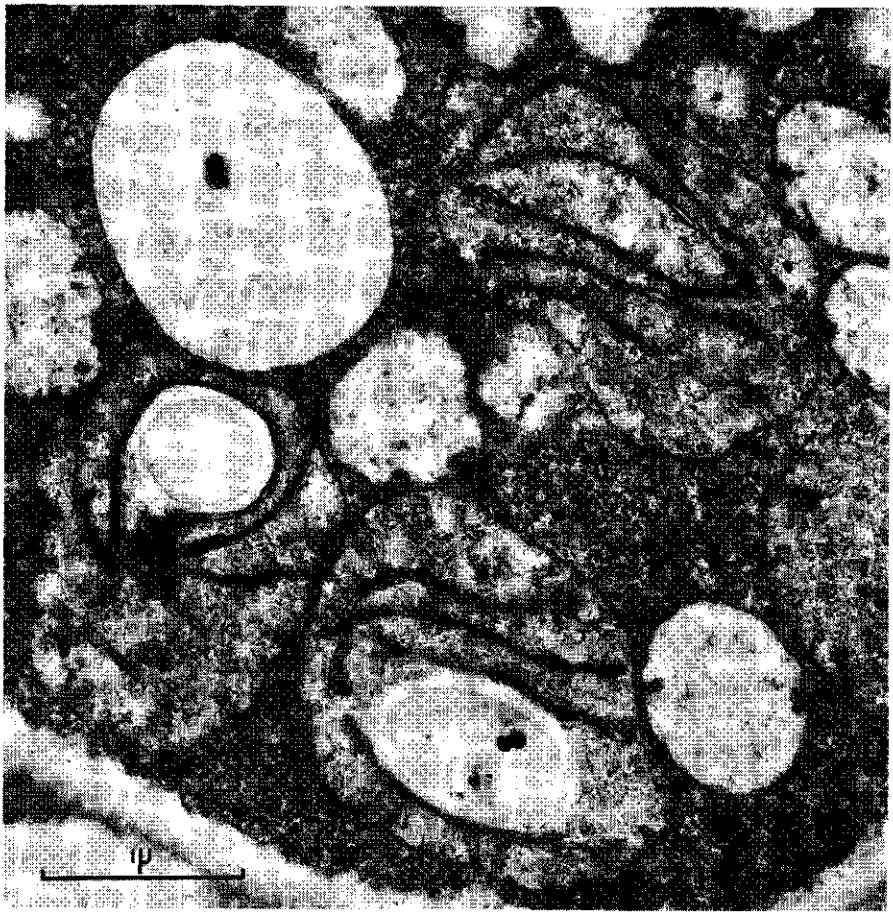

PLATE 6. Internal structure of etioplasts in leaves of 7-day old pea seedlings of cv. Krombek after 5 hours greening in red light (651 nm, 1550 ergs/cm² sec) at 25 °C. × 26,500.