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INDUCTION OF RAPID CHLOROPHYLL ACCUMULATION IN DARK GROWN SEEDLINGS. II. PHOTOREVERSIBILITY

C. W. RAVEN and C. J. P. SPRUIT

Laboratorium voor Plantenfysiologisch Onderzoek, Landbouwhogeschool, Wageningen*

SUMMARY

A short pre-irradiation with red light some hours before the start of continuous illumination with white light eliminates the lag phase in chlorophyll-*a* (Chl-*a*) accumulation. In seedlings of several cultivars of bean, pea, and maize, grown in complete darkness, this inductive action of red light is hardly reversible by far red. Most cases of low reversal can be explained by the considerable inductive capacity of far red light itself. However, far red reversibility of the effect induced by red increased considerably with increasing duration of the period of dark incubation between pre-irradiation and continuous white light. On the other hand, more or less complete red-far red reversal was observed in plants de-etiolated by a pre-irradiation given some hours prior to the normal inductive treatment. Even relatively short exposures to a green safelight caused a significant degree of de-etiolation with concomitant increase in subsequent red-far red antagonism. It is concluded that the biosynthetic pathway leading to protochlorophyll (Pchl) and Chl-*a* is not directly under phytochrome control. In excised leaf material the rate of Chl-*a* accumulation in continuous light proved strongly depressed and completely insensitive to irradiations that were inductive in intact plants.

1. INTRODUCTION

In photomorphogenesis of potentially green plants red-far red reversible light reactions are generally brought together under the heading "low energy reaction" or simply "phytochrome reaction". This implies that red light in low or very low doses (BRIGGS & CHON 1966; BLAAUW *et al.* 1968; RAVEN & SPRUIT 1972) may induce a physiological response that can be cancelled more or less completely by subsequent irradiation with far red light. Historically, these red-far red reversible reactions have led to the discovery of the pigment phytochrome (BORTHWICK *et al.* 1952). This reversible photochromism forms the basis for its spectrophotometric detection (BUTLER *et al.* 1959), whereas it also became the most important criterion for phytochrome involvement in light-mediated responses (e.g. SCHOPFER & MOHR 1972). Numerous physiological responses showing more or less complete red-far red reversals have been described during the last two decades. They include the induction of rapid Chl-*a* accumulation in seedlings of higher plants previously grown in darkness (e.g. WITHROW *et al.* 1956) and the control of Pchl regeneration in darkness (AUGUSTINUSSEN & MADSEN 1965; RUDOLPH 1965; AKOYUNOGLOU 1970). It is reported that in these reactions far red light sometimes is only weakly or not at all antagonistic

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to red. Far red reversal of red induction of rapid Chl-*a* accumulation in wheat is only weak (VIRGIN 1961) and the initial rates of Pchl regeneration in dark grown seedlings of bean, pea, and maize show no significant far red reversibility (SPRUIT & RAVEN 1970). We therefore have studied in more detail the induction of rapid Chl-*a* accumulation in dark grown seedlings of bean, pea, and maize. An action spectrum for induction of rapid Chl-*a* accumulation in pea pointed to the red-absorbing form of phytochrome (P_r) as the photoreceptor. However, the induction by red could be reversed only slightly by subsequent far red light (RAVEN & SPRUIT 1972). We will now discuss this low red-far red reversibility in more detail. Since we have invariably found low reversals of red induction of rapid Chl-*a* accumulation by subsequent far red light in all plant species studied earlier (SPRUIT & RAVEN 1970; RAVEN & SPRUIT 1972), we also include data about 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, since we have not been able to confirm these results.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of *Phaseolus vulgaris* L. cv. 'Widusa' and *Pisum sativum* L. cv. 'Krombek' were obtained from Nunhems Zaden N.V. (Haelen, Holland); seeds of *Phaseolus vulgaris* L. cv. 'Widuco' from Ruiter's Zaden (Andijk, Holland), seeds of *Zea mays* L. cv. 'Caldera 402' from Van der Have N.V. (Kapelle-Biezelinge, Holland), and 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' from W. Atlee Burpee Co. (Philadelphia, Pa, U.S.A.).

Except in a few cases indicated in the text, intact seedlings grown in flower pots at 20 °C in absolute darkness were used.

2.2. Irradiation

The standard light sources for the red-far red experiments were two Leitz 'Prado' 500 W slide projectors; spectral bands were isolated by means of interference filters (Balzers, Liechtenstein, type Filtraflex B 40). The light intensity at the level of the leaves was about 3000 erg/cm² sec at 651 nm and about 4150 erg/cm² sec at 739 nm. Far red, applied to abolish the effect of a red induction, was already switched on a few seconds before the end of the red irradiation in order to avoid a dark interval. In a few experiments a darkroom green safelight was used as inductive light source, giving an intensity at the level of the leaves of about 10 erg/cm²sec (RAVEN & SPRUIT 1972). Its spectral energy distribution is shown in *fig. 1*.

Usually these exposures were followed by a 16-hour dark incubation period at 20 °C before the seedlings were transferred to a standard white light field at 25 °C, when greening followed during continuous exposure for 5 hours at an intensity of about 1500 erg/cm² sec. Thereafter leaf samples were taken and extracted with acetone. Detailed descriptions of the extraction procedure and

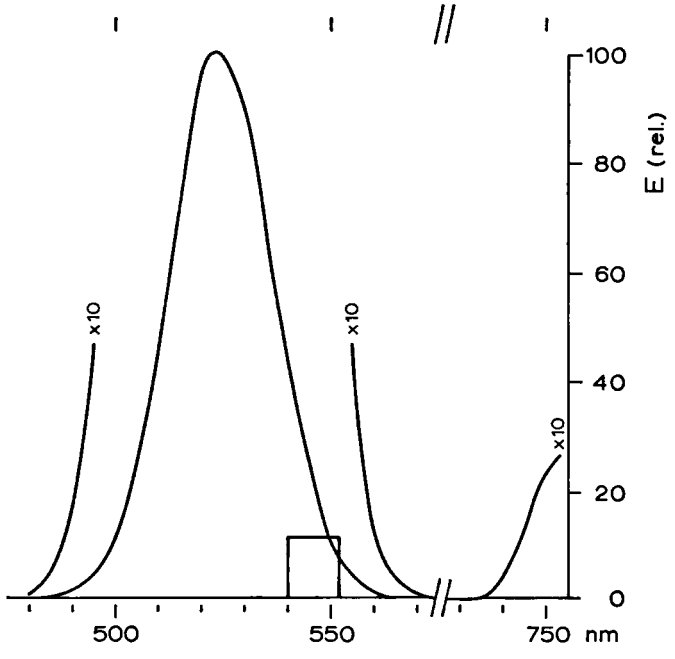

Fig. 1. Relative spectral energy distribution of the green safelight (For detailed description: RAVEN & SPRUIT 1972).

calculation of pigment concentration are given elsewhere (SPRUIT & RAVEN 1970; RAVEN & SPRUIT 1972).

3. RESULTS

3.1. Red-far red reversibility

In *fig. 2* results obtained with different types of red and far red treatments in 7 day old dark grown pea seedlings are summarized. The data are expressed as per cent of the induction obtained with a single one-minute red (651 nm) irradiation. As pointed out in the introduction, we observed hardly any reversal of the inductive effect of this standard red light treatment by subsequent irradiation with far red (739 nm) light (*fig. 2*, top). Remarkably, the remaining inductive action after red-far red is almost equal to the induction by one minute far red light alone. This indicates that this far red dose is 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 reverting 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 our pea material to far red light (RAVEN & SPRUIT 1972). Therefore we attempted to increase reversibility of the red-induced effect either by

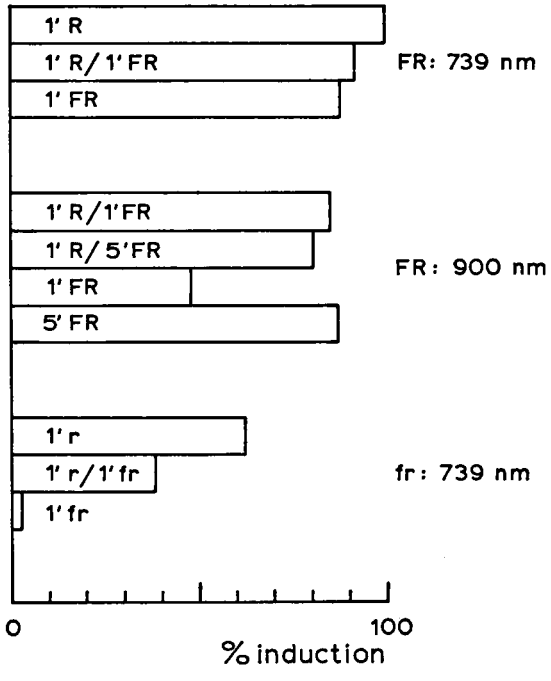

Fig. 2. Induction of rapid Chl-*a* accumulation by various light treatments in 7 day old, dark grown pea seedlings of cv. 'Krombek'.
R: 651 nm, 3000 erg/cm²sec.
r: 651 nm, 0.2 erg/cm²sec.
FR: 739 nm, 4150 erg/cm²sec.
FR: 900 nm, 4000 erg/cm²sec.
fr: 739 nm, 1.5 erg/cm²sec.

using far red light of longer wavelengths or by applying intensities sufficiently low to produce no appreciable induction themselves. Reversibility experiments with 900 nm light of high intensity (4000 erg/cm²sec) failed to give more reversion and had even marked inductive capacity (*fig. 2*, middle). On the other hand, irradiations with low intensities of far red (739 nm, 1.5 erg/cm²sec) gave somewhat better reversion, but only if the induction proper had also been performed with a suboptimal dose of red light (651 nm, 0.2 erg/cm²sec) (*fig. 2*, 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 the total amount of carotenoids showed a similar response as Chl-*a* synthesis.

To establish whether these findings were 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 1*. From the high levels of induction obtained with red irradiation immediately followed by far red light, it is clear that low reversibility is not peculiar to the pea cultivar 'Krombek'. Again it is remarkable that in most cases high levels of induction 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 showed a similar response pattern. Lack of red-far red reversibility seems, therefore, to be rather common in several aspects of seedling development

Table 1. 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

* Light intensities: see Materials and methods.

and this is related to the high inductive activity of far red. These observations make it all the more difficult to understand the numerous reports of more or less complete reversibility under experimental conditions that seem to be comparable to ours (e.g. WITHROW *et al.* 1956; PRICE & KLEIN 1961; FURUYA & THOMAS 1964; HENSHALL & 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 these experiments were carried out with 10 day old dark grown seedlings of the bean cv. 'Widuco'.

3.2. Effect of duration of dark incubation period in bean

In *fig. 3*, the Chl-*a* content of the leaves as measured after 5 hours continuous light is shown in relation to the length of the dark incubation period, following a pre-exposure to a saturating dose of red light. We confirmed our earlier observation (RAVEN & SPRUIT 1972) that there is no evident optimum at 4–6 hours (e.g. VIRGIN 1957) in the incubation period required for completion of the induction of rapid Chl-*a* accumulation. Dark intervals even as long as 48 hours resulted in a high Chl-*a* accumulation rate upon subsequent exposure of the seedlings to continuous light. An optimum at 24 hours darkness for Chl-*a* content per unit leaf weight can be detected. Optimal dark incubation periods are also observed after induction with red immediately followed by far red, or with far red alone *(fig. 4)*. Thus, the reverting action of far red given immediately after a red inductive irradiation upon Chl accumulation becomes increasingly manifest if dark periods of more than 16 hours are inserted between the inductive irradiation and continuous white light. During these extended dark periods the inductive action of far red only, correspondingly decreases.

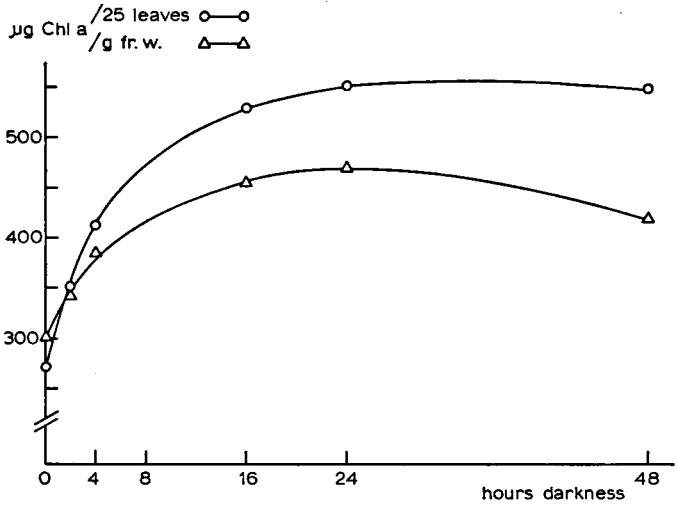

Fig. 3. Effect of duration of dark incubation period, following 1 minute red (651 nm, 3000 erg/cm²sec) inductive light upon Chl-*a* accumulation during 5 hours continuous white light in 10 day old bean seedlings cv. 'Widuco'.

More or less the same holds true with respect to the light-induced increase of fresh weight of the leaves. Obviously, in this 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.

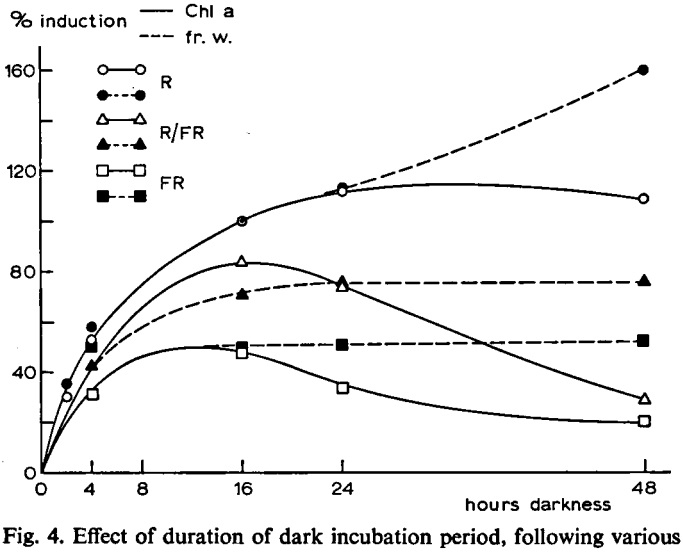

Fig. 4. Effect of duration of dark incubation period, following various inductive light treatments upon Chl-*a* accumulation and gain in fresh weight of 25 primary bean leaves. Responses measured after 5 hours of continuous white light.

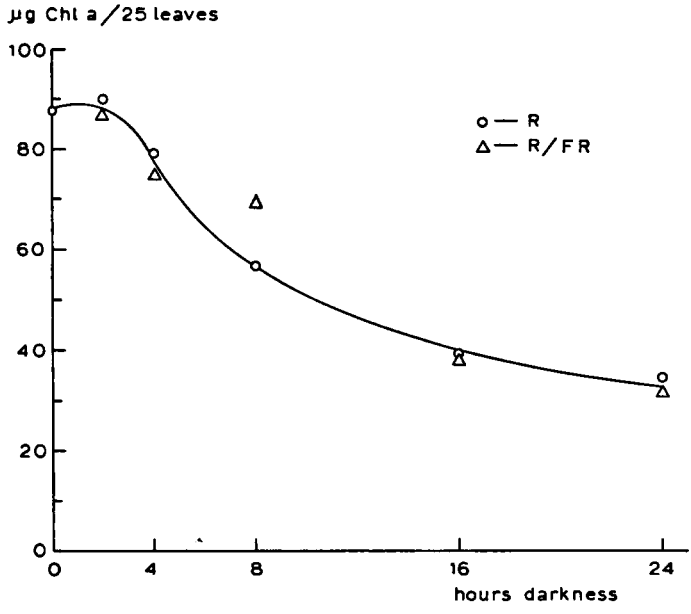

Fig. 5. Effect of duration of dark incubation period, following red and red-far red light upon Chl-*a* accumulation in continuous white light in detached leaves of 10 day old bean seedlings.

Several investigations have made use of excised parts of the dark grown seedlings. We have tried to check whether this could be responsible for the observed differences in optimal duration of the dark incubation period in the red-far red antagonism. Detached bean leaves *(fig. 5)* in petri dishes on 2 layers of filter paper (Whatman no. 4) moistened with distilled water were irradiated immediately with either red or red followed by far red. After dark periods of varying lengths the samples were exposed to continuous white light for 5 hours. However, prolonged dark incubation periods turned out to be very unfavourable to the biosynthetic system forming chlorophylls, as can be concluded from the lower levels of Chl-*a* ultimately reached in continuous illumination. Neither could significant differences in response to the pre-exposures with either red or red-far red be observed. Moreover, the Chl content in the non-pretreated leaves was already depressed by 70% as compared with leaves left intact on the plant (cf. *figs. 3* and *5*). Similar results were obtained with excised leaves of pea seedlings. Their capacity to synthesize Chl-*a* was partly restored by feeding sucrose, indicating that depletion of substrate might be responsible for these effects.

3.3. De-etiolation pretreatments

Experiments on the light sensitivity of pea seedlings had already shown that even short exposures to a so-called darkroom safelight could induce a significant increase in the rate of Chl-*a* accumulation in continuous white light (RAVEN

& SPRUIT 1972). This observation led us to repeat the reversibility experiments, including de-etiolated material. This was obtained by giving a saturating dose of red light to dark grown bean seedlings at various points of time prior to the usual scheme of red and red-far red light treatments, followed by 16 hours darkness *(fig. 6)*. Also in this type of experiment the inductive capacity of a single saturating standard dose of red light followed by 16 hours of darkness as compared with a dark control has been taken as 100%. For the calculation of the degree of induction resulting from the different light treatments (see RAVEN & SPRUIT 1972). For comparison we have included data already shown in *fig. 4*. Obviously, induction of rapid Chl-*a* accumulation can be markedly increased if the saturating standard red light exposure 16 hours prior to the continuous illumination is preceded by another treatment with red light, causing "de-etiolation". 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.

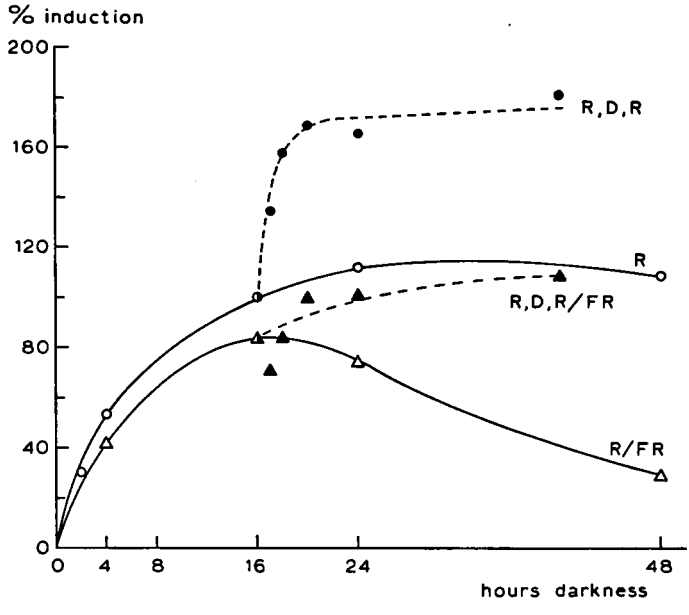

Fig. 6. Effect of duration of dark incubation period and de-etiolation with red light upon the reversibility of rapid Chl accumulation in bean.
○——○: 1'R followed by dark incubation of various duration.
△——△: 1'R/1'FR followed by dark incubation of various duration.
●- - - -●: 1'R- dark interval of various duration -1'R, 16^{h} darkness.
▲- - - -▲: 1'R- dark interval of various duration -1'R/1'FR, 16^{h} darkness.
Chl-*a* content was measured after 5 hours white light.

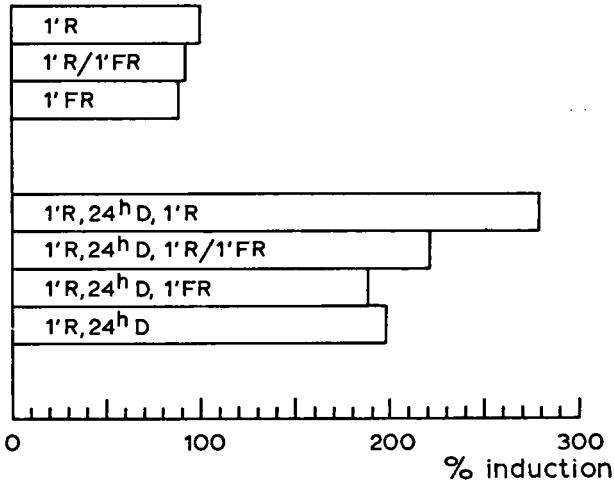

Fig. 7. Effect of de-etiolation with red light upon the induction and reversion of rapid Chl accumulation in 7 day old pea seedlings of cv. 'Krombek'. All irradiation schemes were followed by 16 hours of darkness and 5 hours of white light.

Fig. 7 shows the same response for pea. Whereas completely dark grown pea seedlings show hardly any reversal (*fig. 7*, top), de-etiolation with red light 24 hours prior to the normal scheme of red and red-far red exposures increased far red reversibility of the second red irradiation (*fig. 7*, bottom). Interestingly, after de-etiolation far red light given alone loses its inductive capacity, as compared with completely dark grown seedlings. Similar results were obtained with bean seedlings.

When green 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. 8*). This de-etiolation by green safelight did not induce any directly measurable physiological effect, which we ascribe to the rather low light sensitivity of bean (RAVEN & SPRUIT 1972). Thus dark controls and seedlings pretreated with "safelight" appear indistinguishable with respect to the Chl 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, the intercalation of only a 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. 9* shows that little further increase in induction is obtained by increasing the number of 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 the red irradiations following 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.

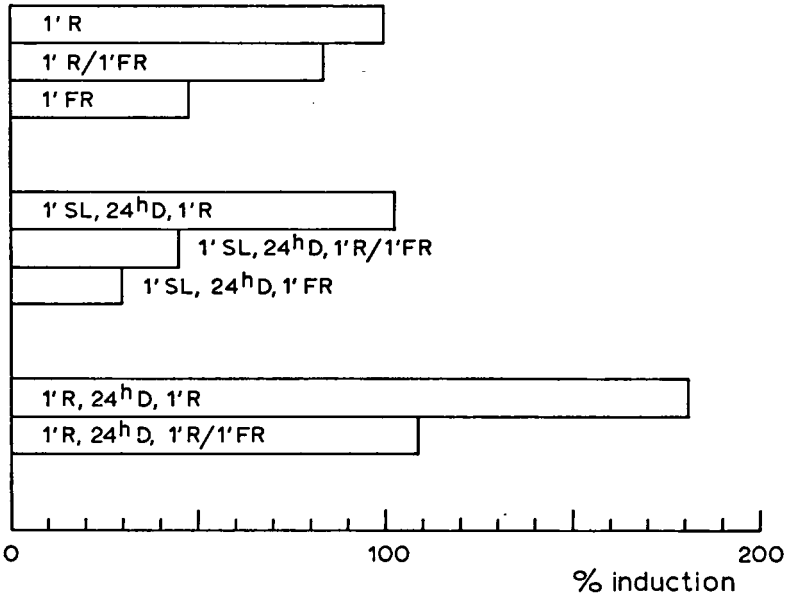

Fig. 8. Effect of de-etiolation with red light and green safelight (SL: 10 erg/cm²sec) upon the induction and reversion of rapid Chl accumulation in 10 day old bean seedlings of cv. 'Widuco'.

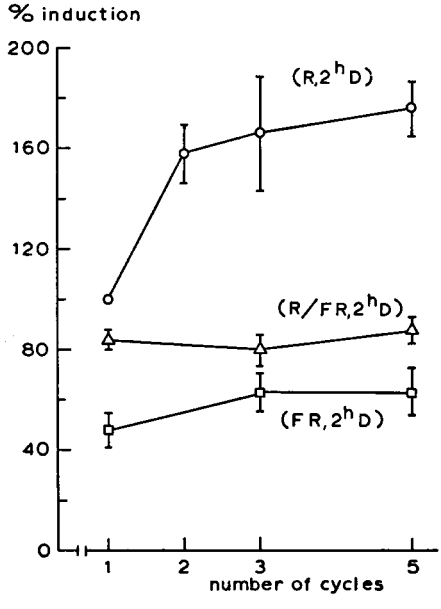

Fig. 9. Effect of different numbers of cycles, each consisting of an inductive irradiation followed by 2 hours darkness, upon Chl-*a* accumulation in continuous light. A 14-hour dark incubation period was inserted between the final inductive cycle and continuous illumination.

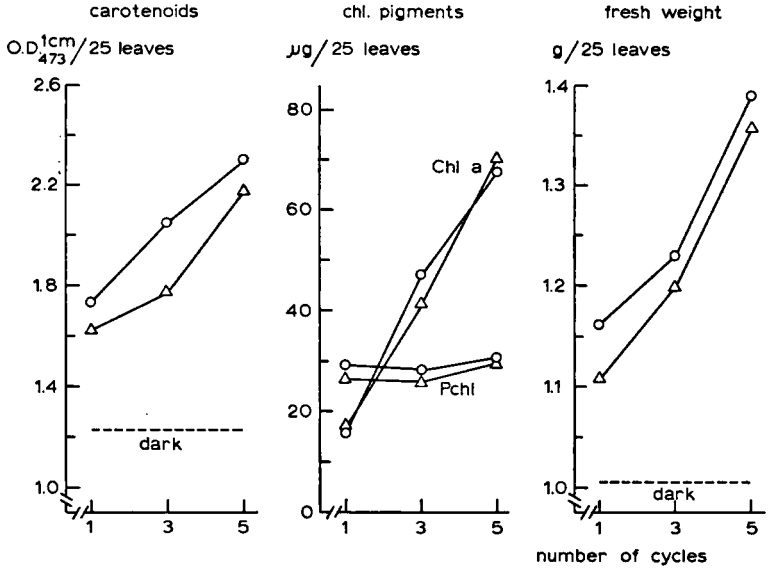

Fig. 10. Effect of different numbers of cycles of red (○——○) and red-far red (△——△) upon the level of carotenoids, Pchl, Chl-*a*, and fresh weight of bean leaves. Measured directly after 14-hour dark incubation period.

Repeated irradiations may also give useful information about the point of attack of phytochrome. *Fig. 10* 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, before the onset of continuous light, no important differences in response to red and red-far red could be observed. However, as shown in *fig. 11*, after exposing the seedlings to continuous white light for 5 hours marked differences between the results of repeated red and red-far red pre-irradiations become apparent, not only in Chl-*a* accumulation rate but also with respect to the rise both in fresh weight and in carotenoids.

4. DISCUSSION

The experiments reported above demonstrate that in a number of cultivars of pea, bean, and maize, phytochrome-induced rapid Chl accumulation shows no 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. E.g. NAKAYAMA *et al.* (1960), in order to explain the lack of reversibility of the inhibition of flowering in *Pharbitis*, suggested the possibility that irreversible physiological reactions occurred already during the short red irradiation. More or less similar conclusions were reached by VIRGIN (1961) regarding the induc-

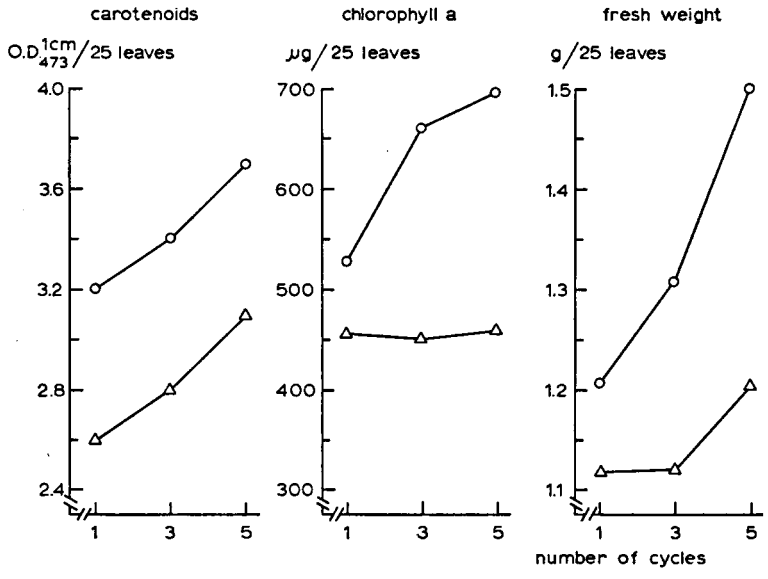

Fig. 11. Effect of different numbers of cycles of red (○——○) and red-far red (△——△) upon the level of carotenoids, Chl-*a*, and fresh of weight bean leaves. Measured after additional illumination of the seedlings with 5 hours of white light.

tion of Chl formation in wheat, and by HAUPT (1969) for inhibition of growth of pea internodes. The latter investigator also considered the possibility of long-lived intermediates of the phytochrome photoreactions being involved (HAUPT *et al.* 1970). Our data do not support 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. Similar 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 of even more than 16 hours gave rise to an increased red-far red antagonism (*fig. 4*). The observation that complete reversibility can be obtained after de-etiolation (e.g. *fig. 6*) also points to the possibility of other interpretations.

BLAAUW *et al.* (1968) and BOTTOMLEY (1970) have suggested that absence of reversibility may point to involvement of other photoreceptors. However, an action spectrum for the irreversible inhibition of the initial 15% of the growth rate in *Avena* mesocotyls showed "a general similarity with that obtained for phytochrome-mediated processes" (BLAAUW *et al.* 1968). A more or less similar action spectrum was found for the almost completely irreversible induction of Chl accumulation in pea leaves (RAVEN & SPRUIT 1972). For instance, the ratio of the effectiveness at 660 nm and 739 nm was about 300, in good agreement with the data of Blaauw *et al.* We are of the opinion that there is no need to

assume that photoreceptors other than the red-absorbing form of phytochrome are involved. A very low P_{fr} requirement of the induction and the presence of completely dark grown tissue seem to explain the observed high inductive capacity of far red light when given alone as well as the low reversals.

In a subsequent paper (RAVEN & SPRUIT, in preparation) we shall present arguments to explain the good reversibility observed in de-etiolated tissues.

Red-far red reversibility was completely absent in isolated bean leaves *(fig. 5)* and the Chl-*a* accumulation rate proved insensitive to otherwise inductive pre-exposures. This supports our earlier hypothesis that there is a parallel between increase in dry weight and induction of rapid Chl accumulation. Relatively little attention has been paid so far to the possible existence of quantitative or qualitative differences in response to light between isolated parts of seedlings and intact ones. BERTSCH & HILLMAN (1961) reported that the growth of excised stem segments of pea was insensitive to light, unless a sugar suitable as a nutrient for growth was included in the medium, whereas "elongation of excised tissue never approached the elongation of the same tissues left intact on the plant". SISLER & KLEIN (1963) demonstrated that Chl formation strongly depends upon the presence of cotyledons on apical tissue of bean seedlings.

We conclude that results obtained with excised material are to be approached with great caution since various factors influence the light sensitive processes both in a quantitative and qualitative way, as might have been predicted from general plant physiological evidence. Moreover, since preparation and handling of isolated tissues is often carried out under green safelight, it appears possible that de-etiolation is brought about (e.g. *fig. 8*).

The duration of dark incubation (*fig. 3*) affected red-induction in bean leaves left intact on the plants in a way very similar to that observed earlier for pea plumules. The inductive effect of short red pre-exposures upon subsequent Chl accumulation proved effective over very long dark periods. As shown by *fig. 4*, the induction of gain in fresh weight of the leaves was maintained even longer. However, upon red-far red as well as upon far red irradiation only, an appreciable decline in the inductive capacity was observed after long periods of dark incubation. A tentative explanation might be that the pool size of P_{fr} reached in this way falls sooner below the threshold level required for maintenance of the inductive capacity than would be the case after a short red irradiation (WAGNER & MOHR 1966).

The outcome of the experiments with repeated irradiation (*figs. 10* and *11*) raises a problem. Whereas phytochrome unquestionably is involved in Chl-*a* accumulation in continuous light *(fig. 11)*, the biosynthetic pathway leading to Pchl and, ultimately, to Chl-*a* shows no sign of a similar type of control. Chl-*a* accumulation resulting from repeated inductive irradiations, accompanied by as many phototransformations of Pchl and subsequent Pchl regenerations in darkness, did not show any red-far red antagonism *(fig. 10)*. This confirms our earlier conclusion (SPRUIT & RAVEN 1970) that Pchl biosynthesis is not directly phytochrome-controlled. In our opinion these data are, however, consistent with a model in which a phytochrome-controlled reaction is prepared in

darkness following the pre-irradiation(s). Its action upon Chl-*a* accumulation becomes manifest only during the subsequent prolonged irradiation period. A possible point of attack for such a reaction might be ultrastructural growth and development processes of the etioplasts. MEGO & JAGENDORF (1961) demonstrated red-far red controlled growth of etioplasts, if dark grown leaves of bean were repeatedly irradiated at 24-hour intervals during a period of 4 days. Since, however, this time scale appears inconsistent with our repetitive irradiation scheme, we have started experiments aimed at elucidating this point.

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