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THE INFLUENCE OF LIGHT OF DIFFERENT SPECTRAL REGIONS ON THE SYNTHESIS OF PHENOLIC COMPOUNDS IN GHERKIN SEEDLINGS IN RELATION TO PHOTOMORPHOGENESIS

III. HYDROXYLATION OF CINNAMIC ACID

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ABSTRACT

1. Light stimulates the conversion of L-phenylalanine and of trans-cinnamic to p-coumaric acid in hypocotyl segments from dark-grown gherkin seedlings.
2. Inhibition by cycloheximide of the increase in the rate of hydroxylation of cinnamic acid in response to light indicates that *de novo* synthesis of enzyme, probably cinnamic acid hydroxylase, is involved.
3. The responsivity to light is higher in the apical part than in the basal part but the spectral sensitivity does not alter significantly over the length of the hypocotyl. The photoreceptor directly responsible for the effect of blue light seems to be different from phytochrome.
4. Particularly in red and far-red light an effect of the cotyledons on the hydroxylase activity to be obtained in the hypocotyls becomes apparent.
5. There is a general correlation between the influences of light quality, light intensity and duration of irradiation on the hydroxylase activity and the accumulation of hydroxycinnamic acids in the gherkin hypocotyl.
6. The implications of this photo-inducible reaction for the regulation of lignification and cell elongation are discussed.

1. INTRODUCTION

The photocontrol of the accumulation of hydroxycinnamic acids in the hypocotyls of gherkin seedlings shows characteristics which are typical of several other light-dependent developmental processes in plants (ENGELSMA and MEIJER, 1965a). The biosynthesis of these phenols seems well suited for a study on the mechanism of light action in photomorphogenesis because of the relative simplicity of this phenomenon. Fairly detailed knowledge about the metabolic pathway is already available (NEISH, 1964).

Feeding experiments with trans-cinnamic acid and glucose enable us to study the influence of light on only a small part of this pathway, the synthesis of the glucose ester of p-coumaric acid from these compounds. In investigations carried out with segments from the gherkin hypocotyl we have tried to find an answer to the following questions.

1) In how far does hydroxylation of cinnamic acid play a key role in the light-dependent accumulation of hydroxycinnamic acids in the hypocotyl of intact seedlings? An enzyme responsible for this reaction, cinnamic acid hydroxylase, has been demonstrated by NAIR

and VINING (1965) in spinach leaves. The question is whether there is a correlation between the effects of light on the hydroxylase activity and the accumulation of phenols.

2) Besides phytochrome, are there other pigments involved in this process? If there are different photoreceptors with different distribution patterns, experiments with segments cut from various parts of the hypocotyl could supply an answer to this question.

3) Is the effect of light translocated intercellularly or does the site of ultimate response always coincide with the site of light perception? This can possibly be solved by varying on the one hand the part of the plant which is irradiated, and on the other hand the interval between light treatment and excision of the segment.

In view of the close connection between phenol synthesis, lignification and cell elongation (ENGELSMA and MEIJER, 1965b) changes in hydroxylase activity are expected to have an important bearing on the latter two phenomena too. A preliminary account of some of the results has been published elsewhere (ENGELSMA, 1965).

2. METHODS AND MATERIALS

The experiments were carried out with segments excised from the hypocotyls of gherkin seedlings (*Cucumus sativus*, "Venlose niet plekkers", strain Tercken VI) raised as described before (ENGELSMA and MEIJER, 1965a). Unless otherwise stated 5 mm segments were used which were cut with the aid of a mechanical device immediately below the plumular hook of three-days-old dark-grown seedlings. All manipulations were carried out under dim green safe-light obtained with a fluorescent lamp (Philips "TL" 17) filtered with "Plexiglas" yellow No. 3 and "Cinemoid" Nos. 39 and 16. Usually the experiments were performed in petri dishes with lots of 30 segments which were floated on 10 ml of distilled water or precursor solution. The latter consisted of an aqueous solution of 10^{-3} M trans-cinnamic acid (BDH) or another p-coumaric acid precursor and 1 % glucose at pH 5.0. The glucose was added as a routine in order to avoid that this compound might become rate-limiting. Irradiations were carried out at 25° C with the same light and filter combinations as used previously (ENGELSMA and MEIJER, 1965a). The p-coumaric acid glucose ester was determined spectrophotometrically as described in the same paper. All data refer to segments kept in the precursor solution for 24 hours at 25° C. Calculations are based on a molar absorbance of 3.10^4 cm²/mmole at 350 mμ for the ester at pH > 10.0. Each point in the graphs represents the mean of at least three experiments, each run in duplicate.

3. RESULTS

Precursors of p-coumaric acid biosynthesis

In the hypocotyls of gherkin seedlings two hydroxycinnamic acids are synthesized, p-coumaric acid and ferulic acid (ENGELSMA and

MEIJER, 1965a). Light stimulates the synthesis of these compounds. It is generally accepted that in higher plants the aromatic compounds are formed via the shikimic acid pathway (NEISH, 1964). We tested a number of potential precursors for their ability to increase hydroxycinnamic acid synthesis in hypocotyl segments from gherkin

TABLE 1

Effect of different metabolites of the shikimic acid pathway on the synthesis of hydroxycinnamic acids, mainly glucose ester of p-coumaric acid, in gherkin hypocotyl segments during 24 hours in darkness and during 8 hours in blue light (700 μW/cm^2) followed by 16 hours of darkness. All solutions at pH 5.0 with NaOH. Numbers are mμmoles of hydroxycinnamic acid/segment

Solution:	24D	8B16D
Water	1.0	5.0
1 % Glucose	1.4	5.1
Quinic acid (10^{-3} M) + 1 % glucose	1.3	4.8
Shikimic acid (10^{-3} M) + 1 % glucose	1.7	5.0
Phenyllactic acid (10^{-3} M) + 1 % glucose	2.0	6.0
L-Phenylalanine (5×10^{-3} M) + 1 % glucose	2.7	12.5
Tyrosine (10^{-3} M) + 1 % glucose	1.7	5.8
trans-Cinnamic acid (10^{-3} M) + 1 % glucose	8.3	29.7
p-Coumaric acid (10^{-3} M) + 1 % glucose	3.5	11.4

seedlings. The results are presented in Table 1. Segments were floated on the precursor solution, and kept in darkness for 24 hours, or were irradiated with blue light (700 μW/cm^2) for 8 hours and subsequently kept in darkness for 16 hours. Only phenylalanine and trans-cinnamic acid gave a clear increase in phenol synthesis. The effect was stimulated by blue light. The same was found for red and far-red light but this part of the spectrum proved to be less effective.

Surprisingly, quinic acid and shikimic acid, which were found to be precursors in the synthesis of aromatic compounds in a number of plants (NEISH, 1964), did not cause any significant stimulation in our system. The compounds were tested over a range of different concentrations, acidities of the medium and light conditions, but the possibility can not be excluded that they fail to stimulate phenol synthesis in the gherkin hypocotyl because they do not reach the active site.

Optimal phenol synthesis was obtained at concentrations of 5×10^{-3} M L-phenylalanine or 10^{-3} M trans-cinnamic acid respectively. These compounds are almost exclusively converted to the glucose ester of p-coumaric acid, very little ferulic acid being formed. Cycloheximide (5 μg/ml) and chloramphenicol (2 mg/ml) block the light stimulation, indicating that *de novo* enzyme synthesis is involved (ENGELSMA and MEIJER, 1965a; ENGELSMA, 1965).

Distribution of the light sensitivity

An important problem in photomorphogenesis is to what extent other pigment systems besides phytochrome are involved. In the hope that if the effects of different wavelength regions are mediated through

different pigment systems these systems might not be equally distributed, we excised segments from different parts of the hypocotyl to determine the distribution of the spectral sensitivity for the induction of enzyme synthesis. From three-days-old dark-grown seedlings three segments were cut: the plumular hook, about 5 mm in length, a segment, 5 mm long, immediately below the plumular hook, and a segment, 5 mm long, about 5 mm above the implantation of the roots. The segments received the light treatment during 24 hours while floating on the cinnamic acid, glucose solution.

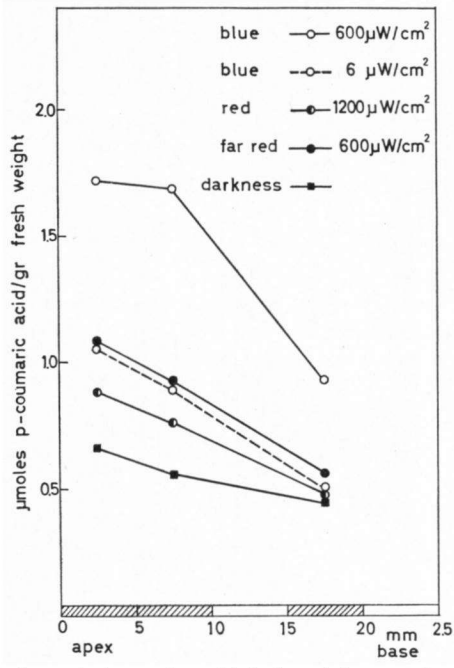

Fig. 1. Hydroxylation of cinnamic acid induced by continuous irradiations for 24 hours with blue, red and far-red light respectively in segments excised from different parts of the hypocotyls from dark-grown gherkin seedlings.

The results presented in Fig. 1 show that in segments kept in the dark the capacity to convert cinnamic acid into p-coumaric acid decreases from apex to base. This pattern remains the same in the light-treated segments. Blue light is much more effective than red and far-red light but there is no clear evidence that the distribution of the sensitivity for this light is different from that for the other spectral regions. If different pigments are involved, they must be rather equally distributed.

Dependence of light quality, light intensity and duration of irradiation

The influence of the duration of irradiation in blue, red and far-red light on the hydroxylation of cinnamic acid in hypocotyl segments

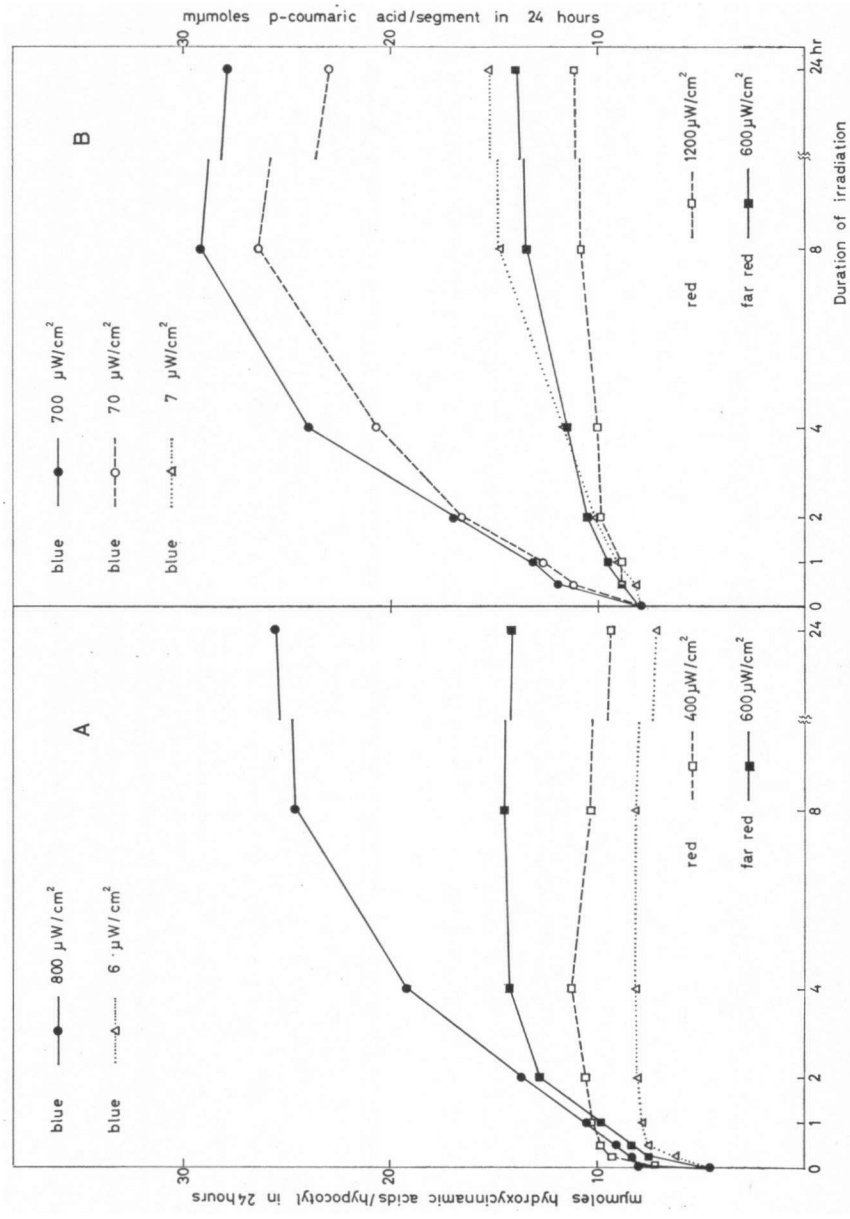

Fig. 2. Phenol synthesis as a function of the duration of irradiation with blue, red and far-red light in the hypocotyls of intact seedlings (A) and in hypocotyl segments floated on an aqueous solution of 10^{-3} M trans-cinnamic acid and 1 % glucose (B).

is given in Fig. 2B. Lots of 30 segments were floated on aqueous solutions of 10^{-3} M trans-cinnamic acid and 1 % glucose (pH 5.0), irradiated for the times indicated in the graphs, and subsequently kept in darkness until 24 hours from the beginning of the light treatment. In a number of aspects the results are remarkably parallel with those relating to the effects of light on the accumulation of hydroxycinnamic acids in hypocotyls of intact seedlings (Fig. 2A) (ENGELSMA and MEIJER, 1965a). Particularly in blue light we find the same dependence on light intensity and duration of irradiation. Saturation occurs after about 8 hours of irradiation irrespective of light intensity. On the other hand very short irradiations, especially with blue and red light, have a relatively marked effect on phenol accumulation in the hypocotyls of intact plants whereas the effect on the hydroxylase activity in segments is negligible. Moreover, also for irradiations of longer duration the relative effectiveness of red and far-red light is much lower in the latter case. Their influence on the induction of hydroxylase activity in segments reflects rather the induced phenol synthesis in the hypocotyls of decotyledonized plants than in those of intact plants (Fig. 3).

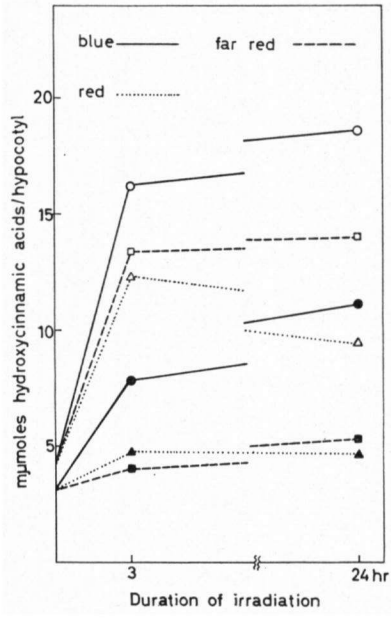

Fig. 3. Synthesis of hydroxycinnamic acids in the hypocotyls of intact seedlings (open symbols) and of decotyledonized seedlings (closed symbols) as a function of the duration of irradiation with blue, red and far-red light of 400 μW/cm^2 each.

The low responsivity to red light makes it difficult to study the occurrence of a red/far red antagonism. From the experiments with

segments no direct evidence could be obtained that phytochrome participates in this process. Maintaining the segments on ice during the irradiation did not increase the results. According to HOPKINS and HILLMAN (1965) this technique prevents a rapid loss of phytochrome.

Effects of excision

In order to test in how far the differences in stimulation of phenol synthesis by small light doses between intact plants and segments are due to effects of excision the following experiment was performed. A box with at least 300 seedlings, having been picked for a uniform length, was irradiated for 10 min with blue (600 μW/cm^2) or red light (1200 μW/cm^2). Immediately after return to darkness, and after 1, 2, 3 and 6 hours respectively, segments were cut from the hypocotyls and transferred in duplicate lots of 30 segments each to petri dishes with 10 ml of the cinnamic acid, glucose solution. All samples remained in the precursor solution in darkness for 24 hours. For both light qualities it appeared that segments excised immediately after the light treatment showed a much lower capacity to convert cinnamic acid to p-coumaric acid than segments which remained part of the plant for at least one hour. No significant differences were observed between segments excised one hour after the light treatment and segments excised after six hours. The result of this experiment can be explained in different ways. For instance

1) A factor involved either in the enzyme induction or in the activation of the hydroxylase reaction moves from other parts of the plant to the part to be excised.
2) Excision introduces a factor which interferes with the enzyme induction.

An attempt to discriminate between these two possibilities and to evaluate the relative contribution of the effect of excision for the different spectral regions, was undertaken in the following experiment. From a box with three-days-old seedlings the cotyledons of 20 seedlings were covered with aluminium foil. From 20 plants the cotyledons and

TABLE 2

Influence of different covering and cutting treatments on the stimulation of the conversion of cinnamic acid into p-coumaric acid by blue, red and far-red light respectively. Numbers are mμmoles p-coumaric acid/hypocotyl segment accumulated during 24 hours in darkness

State of the seedlings during irradiation	Time between beginning of light treatment and excision (min)	Light treatment			
		10 min Blue 600 μW/cm^2	10 min Red 1200 μW/cm^2	30 min Far-red 600 μW/cm^2	Darkness
Hypocotyl segments		10.7	8.6	9.3	8.5
Intact seedlings	10	11.2	9.8		
Intact seedlings	120	13.2	11.4	13.0	
Cotyledons covered	120	11.0	10.1	10.8	
Cotyledons excised	120	10.6	9.7	10.3	
Cot. + pl. hook excised	120	10.0	8.4	10.3	

from another 20 plants cotyledons plus plumular hooks were excised. From other seedlings two lots of 20 hypocotyl segments were prepared and put in petri dishes with the cinnamic acid, glucose solution. One lot received together with the seedlings in the box the light treatment which consisted of 10 min of blue (600 μW/cm^2), 10 min of red (1200 μW/cm^2), or 30 min of far-red light (600 μW/cm^2) respectively. The other lot served as dark control. Immediately after the light treatment the aluminium foil was removed, and from 20 plants, of which both cotyledons and hypocotyls had been irradiated, segments were excised (except in the experiments with far red). From all other plants segments were cut two hours from the beginning of the irradiation. The accumulation of p-coumaric acid ester in the segments that were kept in the precursor solution in darkness for 24 hours is presented in Table 2. The data are averages of 9 to 12 experiments.

The results show:

1) Photo-stimulation of the hydroxylating capacity is much higher when the entire plant is irradiated instead of a segment, and the part to be excised remains in contact with the surrounding tissue for some time (about one hour) after the end of the (short) irradiation.
2) Covering or excision of the cotyledons lowers the capacity of the hypocotyl segments to convert cinnamic acid into p-coumaric acid.
3) The relative contribution of the excision effect on the photo-induction is higher in the case of irradiation with red or far-red light than with blue light.

4. Discussion

A generally accepted mechanism for hydroxycinnamic acid biosynthesis in higher plants is the shikimic acid pathway (Neish, 1964). However, in the gherkin seedlings the majority of the "proto-aromatic compounds" failed to stimulate the synthesis of p-coumaric acid. Stimulation was only obtained with L-phenylalanine and trans-cinnamic acid. The importance of L-phenylalanine as an intermediate in the formation of hydroxylated aromatic compounds has been established for a number of plants (Neish, 1964; Zenk, 1964; Zenk and Müller, 1964). It may be possible that at this stage of development of the gherkin seedling the entire shikimic acid pathway does not yet function and that the hydroxycinnamic acids and lignin are synthesized exclusively from phenylalanine originally stored in the seed.

Enzymes which catalyze the steps from phenylalanine to p-coumaric acid have been isolated. Koukol and Conn (1961) have purified phenylalanine deaminase from barley, while Nair and Vining (1965) have demonstrated the existence of a cinnamic acid hydroxylase in spinach leaves. They found that the unstable enzyme requires tetrahydrofolic acid and a reduced pyridine nucleotide to obtain maximum activity.

As already stated, the major product of the feeding experiments in gherkin hypocotyl segments is the monoglucose ester of p-coumaric acid. Working with potato tuber tissue LEVY and ZUCKER (1960) have obtained evidence that hydroxylation of cinnamic acid is preceded by esterification. With the gherkin hypocotyl segments no direct influence of externally supplied glucose on the conversion of cinnamic acid into p-coumaric acid glucose ester could be found. Although no strict evidence is available, we assume that the effect of light in our system is on the hydroxylase step.

Experiments with cycloheximide and chloramphenicol have demonstrated the involvement of *de novo* enzyme synthesis (ENGELSMA and MEIJER, 1965a; ENGELSMA, 1965). Whether this concerns hydroxylase itself or, for instance, one or more enzymes involved in the synthesis of a co-factor has not yet been established. Evidence of induction of enzyme synthesis in higher plants in response to light has also been obtained for the formation of chlorophyll in beans and maize (BOGORAD and JACOBSON, 1964), anthocyanin in mustard seedlings (MOHR and LANGE, 1965) and chlorogenic acid in potato tuber slices (ZUCKER, 1963).

Up to a limit of about 8 hours the increase in hydroxylase activity in hypocotyl segments is roughly linear with the duration of the irradiation in blue light. The rate increases with increasing light intensity until a saturation level is reached. The induction apparently depends on a continuous excitation of a pigment by the light. The mechanism leading from light perception to enzyme induction is still obscure but two recent findings may be relevant. WILLIAMS and NOVELLI (1964) have discovered that ribosomes from maize shoots acquire an enhanced capability of incorporating C^{14}-leucine into protein two hours after the plants have been transferred from darkness to the light. And CLARK *et al.* (1964) have found that on exposing Brassica pekinenses leaves to light, a rise in polyribosomes can be detected within 4 minutes, which, at least in part, appears to be associated with the synthesis of new RNA.

Our experiments provide some evidence that a mobile factor is involved which enhances the hydroxylase activity in the hypocotyls. In particular the effect of covering the cotyledons does not lend support to the idea that the low responsivity of the segments to red and far-red light is due to wound artefacts interfering with the process of enzyme induction. In 8 out of 10 experiments with intact plants we found that the accumulation of hydroxycinnamic acids in the hypocotyls in response to a small dose of red light (3.5 kerg/cm^2) was lower when only the hypocotyls were irradiated (unpublished results). It should be noted, however, that covering the cotyledons may have a certain traumatic effect.

Whether the transported factor from the cotyledons acts on the level of enzyme induction or enzyme action is still unknown. Its relative contribution to the increase in hydroxylase activity in the hypocotyls seems higher in the case of irradiation with red and far-red light than with blue light. If this is taken into account we obtain a good

correlation between hydroxylase activity and accumulation of phenols under different light conditions. We do not yet know whether the enzyme activity in the preceding step, deamination of phenylalanine, is already so high that it never becomes rate-limiting, or that the phenylalanine deaminase activity is stimulated to an extent comparable with the stimulation of cinnamic acid hydroxylase activity.

The induction of hydroxylase activity seems to be an important link in the regulatory mechanism for lignification and hypocotyl elongation (ENGELSMA and MEIJER, 1965b). Influences of oxygen tension and of oxidizing and reducing agents on these phenomena (SIEGEL and PORTO, 1961) may be at least partly due to their effects on the hydroxylating enzyme. We found that supplying ascorbic acid to the segments has no effect on the induction of cinnamic acid hydroxylase but enhances its activity.

The accumulation of hydroxycinnamic acids in the hypocotyls of continuously irradiated seedlings becomes more or less saturated within 24 hours, as has been shown previously (ENGELSMA and MEIJER, 1965a, Fig. 6). Experiments with hypocotyl segments from such plants, irradiated for 24 hours, showed that a further accumulation occurs when they are supplied with phenylalanine or cinnamic acid. When experiments as presented in Fig. 2B of this paper are carried out over a period of 48 hours the amount of p-coumaric acid that accumulates is about 160 % of the amount obtained in the 24-hour experiments. This indicates that under natural conditions the hydroxylase activity does not become blocked but that phenol-producing and consuming reactions attain an equilibrium.

It is still far from understood which pigment systems are involved in photomorphogenesis and through which mechanism the only photoreceptor so far established, phytochrome, causes the observed biochemical and morphological changes. The responsivity to blue light of the process studied here is much higher than that to the other spectral regions, the difference being greater than for phenol synthesis and inhibition of hypocotyl elongation of intact seedlings. This would be difficult to understand on the basis of phytochrome, which has its main absorbance in the red, far-red region. Preliminary experiments on the determination of an action spectrum show that the highest sensitivity lies in the 430–470 mμ region where neither of the two forms of phytochrome has an absorption maximum (BUTLER, HENDRICKS and SIEGELMAN, 1965).

Experiments with a very sensitive growth meter have revealed that the lag for inhibition of hypocotyl elongation in blue light is even shorter than reported previously (ENGELSMA and MEIJER, 1965b), being less than one minute (Meijer, to be published). The recording of the growth rate as a function of time of a dark-grown seedling transferred to blue light differs characteristically from those obtained on exposing a dark-grown seedling to red or far-red light. This indicates that growth inhibition in blue light is also at least partly due to a process not mediated by phytochrome. The above does not imply that phenol synthesis and rapid growth inhibition in response

to blue light are necessarily mediated by the same pigment system.

Returning to the phenol synthesis in the hypocotyls of intact plants, we find that the effects of red as well as far-red light, in contrast to blue light, are mediated to a predominant extent by the cotyledons. This situation is not in disagreement with the hypothesis that both the effects of red and far-red light are mediated by the same photoreceptor, phytochrome (BUTLER *et al.*, 1963; HARTMANN, personal communication, see also BERTSCH and MOHR, 1965). To account for the energy dependence observed for far-red irradiations (ENGELSMA and MEIJER, 1965a, Fig. 6) it seems necessary to assume, however, that the process not only depends on the ratio of the two forms of phytochrome but also on a continuous perception of the light by this or another pigment as argued before for blue light.

The sensitivity of the different excised parts of the hypocotyl to red and far-red light for the stimulation of phenol synthesis shows a correlation with the distribution of chlorophyll accumulating in response to light. Similar conclusions have been reached by BJÖRN and VIRGIN (1958) for the influence of red light on the growth of pea seedlings. They state that the distribution of the areas giving the strongest response to red irradiation seems to agree with the sites for the formation of the green and yellow pigments of the plant. Whether, as these authors ask, the structural relationship between the light-absorbing groups of phytochrome and chlorophyll, both being pyrrole pigments, has any bearing on this, is still an open question.

A correlation between the intensity of the yellow colour of the etiolated hypocotyl and the sensitivity to blue light for the induction of hydroxylase activity can also be observed. Yellow pigments in the etiolated hypocotyl and chlorophyll in the light-treated hypocotyl show a similar distribution pattern. The assumptions that the effect of blue light is mediated by the yellow pigment(s), probably carotenoid(s), and those of red and far-red light by phytochrome, and possibly other pyrrole pigments, would explain the results presented in Fig. 1.

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Note added in proof. Since the preparation of this manuscript it has been found that light stimulates *de novo* synthesis of the enzyme fenytalanine deaminase in the gherkin hypocotyl to a similar extent as cinnamic acid hydroxylase.