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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

II. Indoleacetic Acid Oxidase Activity and Growth

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Abstract

1) Exposure to light of dark-grown gherkin seedlings causes an expansion of the cotyledons and inhibits the elongation of the hypocotyls. As was found earlier for the light-induced synthesis of phenolic compounds, at least two photoreactions seem to be involved: photoresponse I, the "high energy reaction", and photoresponse II, the phytochrome reaction.

2) The gherkin seedlings contain an enzyme which, purified, oxidizes indoleacetic acid (IAA) [1]) slowly. The IAA oxidase activity of a crude homogenate depends on phenolic cofactors; derivatives of p-coumaric acid. Ferulic acid derivatives, flavonols and ascorbic acid, the latter two only from the cotyledons, antagonize this stimulation. The IAA oxidase activity of a homogenate from cotyledons changes on standing due to enzymatic reactions involving the modifiers.

3) The photo-inhibition of hypocotyl elongation can be counteracted by supplying IAA externally, indicating that light lowers the level of IAA. Application of the naturally occurring p-coumaric acid cofactor of IAA oxidase to hypocotyl sections from dark-grown gherkin seedlings results in growth inhibition.

4) Qualitatively there is agreement between light-stimulated synthesis of the p-coumaric acid ester and inhibition of hypocotyl elongation, and between synthesis of IAA oxidase inhibitors and expansion of the cotyledons. The postulated causal relationship between light-dependent phenol synthesis and photoinhibition of hypocotyl elongation can only exist, however, if but a fraction of the phenolic compounds participates in the IAA regulating system, and the enzymatic oxidation of IAA is coupled to the destruction of phenols.

5) In at least one case the IAA oxidase hypothesis seems inadequate to explain the phenomena: the lag for inhibition of hypocotyl elongation in blue light is much shorter than for phenol synthesis.

Introduction

The IAA-destroying activity can be determined either in a homogenate or *in vivo* (Pilet, 1961). Both methods have serious disadvantages. In the first method reactive constituents of the living cell, which are normally separated from each other, are brought together. Measurements *in vivo*, on the other hand, are difficult to

[1]) Abbreviations: IAA, indoleacetic acid; HRP, horse-radish peroxidase; GP, gherkin peroxidase, the partly purified peroxidase-IAA oxidase from gherkin seedlings.

interpret because of wound effects and transportation problems, and no information is gained about the mechanism of the inactivation of IAA. Although IAA oxidase has been studied for many years, its physiological function is still a matter of controversy. BRIGGS *et al.* (1955) have cited data from the literature which are difficult to reconcile with the concept of auxin destruction as a morphogenetic factor. They showed that there is a lack of correlation between auxin destruction *in vitro* and auxin destruction *in vivo* in Osmunda leaves. On the other hand support for the view that the IAA oxidase system actually operates *in vivo* has been given by ZENK and MÜLLER (1963) who showed that the inactivation of carboxyl-labelled IAA by *Avena* coleoptiles is influenced by the concentration of applied p-coumaric acid. It may be possible that many of the discrepancies found in the literature are due to insufficient knowledge of the complications inherent in the IAA oxidase determination *in vitro*.

The following procedure was adopted in this study on the nature of the relationship between the IAA oxidase activity and the growth phenomena.

1) Model systems were investigated in order to develop a suitable method for the determination of IAA oxidase activities in homogenates. The modifier activity of three different hydroxycinnamic acids, p-coumaric, ferulic and caffeic acid, was tested under different conditions with two IAA oxidases, HRP, an enzyme studied by, among others, HINMAN and FROST (1961) and YAMAZAKI and PIETTE (1963), and with a partly purified IAA oxidizing enzyme from gherkin cotyledons.
2) The naturally occurring components which directly or indirectly participate in the inactivation of IAA were analysed.
3) The manner in which the concentrations of these components are affected by different light treatments was studied. For the phenolic modifiers the results were published in the previous paper (ENGELSMA and MEIJER, 1965a).
4) The results were compared with the effects of the same light treatments on the growth phenomena in order to check whether there is a direct correlation, or whether a correlation becomes plausible when certain assumptions are made with regard to differences between the *in vitro* and *in vivo* systems.

METHODS AND MATERIALS

Plants and irradiation

All the experiments were carried out with gherkin seedlings (Cucumus sativus, "Venlose niet plekkers", strain Tercken VI) grown from the same seed supply as used in the previous paper (ENGELSMA and MEIJER, 1965a). The irradiation set-ups were also the same as described there.

Measurement of the length of the hypocotyl

Measurements in darkness were carried out with a green electro-

luminescent shield (Philips, El. 20012/010 combined with "Plexiglas" (yellow No 3) and "Cinemoid" filters (Nos 39 and 16) as a background. A transparent ruler was used. The luminescent safe-light did not influence the elongation, as was verified experimentally. The photoinhibition of the hypocotyl growth as given in fig. 1 is expressed as the decrease in elongation as a percentage of the elongation in darkness.

Growth meter

To determine the length of the lag for the photoinhibition in different light qualities, a growth meter was used. It consisted of a lever with the seedling attached to the shorter arm and a pen to the longer arm. The growth rate was recorded on paper affixed to a clock-driven drum.

Enzyme preparation

The gherkin peroxidase was purified by a procedure partly based on the method of TANG and BONNER (1947). A crude extract was prepared from 600 gr of cotyledons of four-days old seedlings germinated in darkness. They were frozen with liquid nitrogen and ground in a mortar. The resulting powder was suspended in 1050 ml of 0.2 M phosphate buffer pH 6.0 and this crude homogenate was centrifuged at 25000 g at 2–5° C for 25 min. The precipitate was discarded. Further purification consisted of:

Heat precipitation: small portions of the supernatant were kept at 50° C for 15 min. After cooling, the precipitate was centrifuged off (25000 g, 25 min) and discarded.

pH precipitation: the pH of the supernatant was lowered to 4.0 by adding acetic acid. The inactive precipitate was removed as described above.

Ammonium sulphate precipitation: $(NH_4)_2SO_4$ was added to the supernatant up to 25 % of the saturation value. The precipitate obtained after centrifugation for 25 min at 20000 g showed very little IAA oxidase activity and was discarded. More $(NH_4)_2SO_4$ was added to obtain 75 % saturation. The resulting precipitate containing the IAA oxidase was redissolved in distilled water for a second fractionation with the same $(NH_4)_2SO_4$ concentrations.

The final precipitate was taken up in 50 ml distilled water and dialysed against distilled water for 5 days, the water being replaced every day. The product was a clear yellow-brown solution.

Horse-radish peroxidase, Sigma, Type V, RZ approximately 2.8, was also dialysed against distilled water for 5 days.

After dialysis both enzyme systems had a small IAA-oxidizing activity which did not decrease on further dialysis. The pH optimum of 5.6-5.7 (citrate phosphate buffer) for the gherkin enzyme depends slightly on the conditions of preparation. Horse-radish peroxidase shows the highest activity at the lowest value of the pH range studied: pH 3.0.

Peroxidase activity

Horse-radish peroxidase and gherkin peroxidase were assayed by determining pyrogallol peroxidation according to the indications of the Sigma Chemical Company.

1.0 ml of a peroxidase solution in 0.1 M phosphate buffer, pH 6.0 was added to a reaction mixture at 20° C consisting of 2.0 ml of 5 % pyrogallol (freshly prepared in water), 1.0 ml of 0.147 M H_2O_2 (freshly prepared by diluting 1.67 ml of 30 % hydrogen peroxide to 100 ml with water), 2.0 ml of 0.1 M phosphate buffer pH 6.0 and 14.0 ml of water. After exactly 20 seconds at 20° C 1.0 ml of 2N H_2SO_4 was added to stop the reaction. The solution was extracted 5 times with 10 ml portions of ether. The extracts were made up to 100 ml with ether. With a Cary recording spectrophotometer the optical density at 420 mμ was determined, using ether as reference. Peroxidase activity was calculated by the use of the following formula

$$\frac{\text{O.D. } 420 \times 8.5}{\text{Light path in cm}}$$

= mg purpurogallin per 100 ml of ether
= units (PU) per ml of peroxidase solution.

IAA oxidizing activity

IAA was determined with the Salkowski reaction according to the method of GORDON and WEBER (1951) with a reagent made up of 1 vol. part of 0.5 M $FeCl_3$ and 50 vol. parts of 35 % $HClO_4$. A standard assay of IAA destruction contained IAA (10 μmole), peroxidase (1PU) and modifier (as indicated in the figures) in 20 ml of 0.05 M phosphate citrate buffer. The reaction mixture was shaken at 30° C in the water bath of a Gyratory Shaker. Immediately after addition of IAA to the reaction mixture, and again after 30 min, 1 ml samples were taken and added to 2 ml of the Salkowski reagent. Optical densities at 522 mμ were measured in a Vitatron Colorimeter after keeping the reaction mixture at 30° C for 20 min and for at least another 20 min at room temperature. All IAA oxidase tests were carried out in dim green safe-light.

RESULTS

Photomorphogenesis of the gherkin seedling

Exposure to light of the gherkin seedling results in an increase in fresh weight of the cotyledon and a decrease in elongation of the hypocotyl. This is shown in fig. 1 for seedlings for which the accumulation of phenolic compounds under different light conditions was described in the previous paper (ENGELSMA and MEIJER, 1965a, figs. 6 and 7). In that experiment the lengths of 15 randomly chosen seedlings were measured immediately before transfer to the light and after 8, 24 and 48 hours. The fresh weights of the cotyledons at these times, given in fig. 1, are from the plants used for the spectrophotometric measurements.

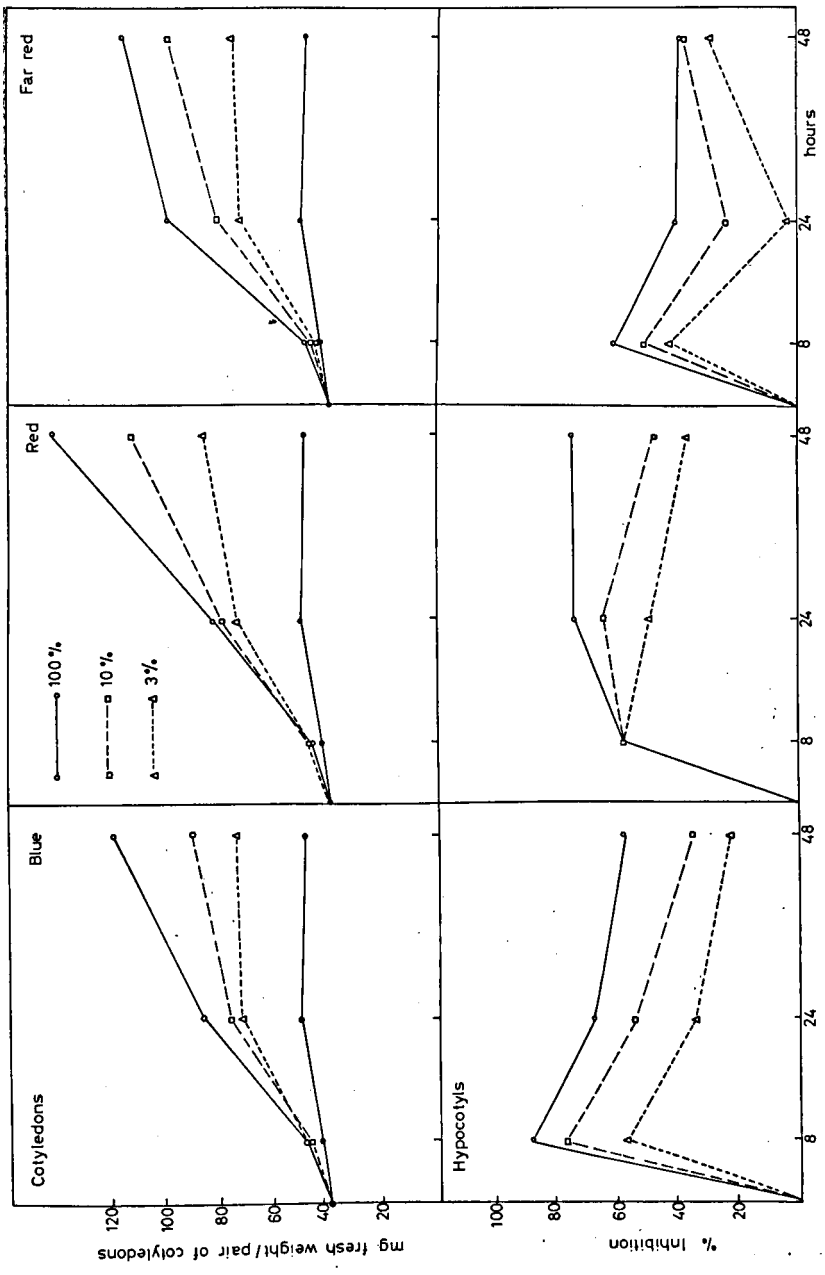

Fig. 1. Expansion of the cotyledons and inhibition of hypocotyl elongation of gherkin seedlings under different light conditions. Highest light intensity (100 %) for the three wavelength regions 400 μW/cm^2.

The inhibition patterns for hypocotyl growth in blue and far-red light are very similar and differ from the patterns obtained in red light. In the former wavelength regions the inhibition is an increasing function of the light intensity and is highest during the first 8 hours of irradiation. It appeared in all experiments that in red light the inhibition during the first 8 hours was almost independent of the light intensity in the 10–600 μW/cm^2 range. The results are in agreement with the earlier finding that at relatively low light intensities red light is more active in growth inhibition, whereas at higher intensities blue light is more active (MEIJER, 1959). In the case of red light a partial reversal by a subsequent far-red irradiation can be obtained.

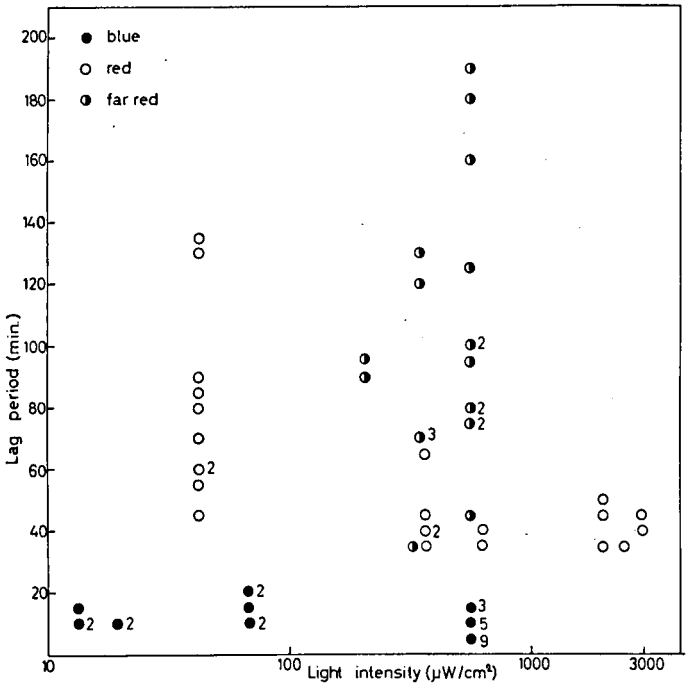

Fig. 2. Length of the lag period between beginning of irradiation and detectable growth inhibition for different light intensities in the blue, red and far-red wavelength regions. Figures behind the marks indicate number of coinciding points.

In investigations where the inhibition pattern was recorded with a growth meter, it was found that in blue light of 10–700 μW/cm^2 the lag between the beginning of the irradiation and the growth inhibition was shorter than 10 minutes (fig. 2). There was no indication that the length of the lag depended on light intensity. In red and far-red light the length of the lag period is variable, but always significantly higher than in blue light. This is also the case for light intensities that cause a subsequent growth inhibition which is large compared to the average inhibitions in blue light of low intensity. Leaving the

possible influence of light intensity out of consideration, the lag periods in blue, red and far-red light are less than 10 min, about 1 hour, and 1½ to 2 hours respectively. The investigations with the growth meter confirmed that, particularly in light of low intensity, the growth inhibition goes through a maximum.

Fig. 1 shows that the increase in expansion of the cotyledons is very slow during the first 8 hours of irradiation. The low initial rate at which this photoresponse develops makes a more exact determination of the lag period just as difficult as for the phenol synthesis in the cotyledons, discussed earlier.

IAA oxidase model systems

Before trying to relate the developmental responses to changes in IAA oxidase activity, it seems useful to determine what we actually measure in an IAA oxidase test. For this purpose the influence of pH and modifier concentration on the enzymatic destruction of IAA was studied in model systems with p-coumaric acid as an example of a cofactor and ferulic acid and caffeic acid functioning as inhibitors. As a basis for comparing the characteristics of the enzyme obtained from gherkin cotyledons with HRP, we used in these tests enzyme concentrations which have equal peroxidase activities in the purpurogallin test. The results of these investigations, presented in fig. 3, can be summarized as follows.

The cofactor activity of p-coumaric acid goes through a maximum. The optimum concentration for cofactor activity increases upon lowering the pH. The highest activity is obtained at pH 4.0 at a concentration of 10^{-4} M p-coumaric acid.

Ferulic acid and caffeic acid are inhibitory at pH $\geqslant 5$, but a lower pH and at low concentrations they stimulate IAA breakdown.

HRP and GP behave similarly with respect to the phenolic modifiers. Both enzymes are rapidly inactivated at pH < 3.

It should be mentioned here that the concentrations of the hydroxycinnamic acids as given in fig. 3 are initial concentrations since it has been shown that destruction of these compounds is coupled to oxidation of IAA by HRP and GP (ENGELSMA, 1964).

From these results it is obvious that the rate of destruction of IAA, as measured in crude homogenates of plants, is largely determined by the pH and concentration of the extract. The IAA oxidase test, therefore, has to include a variation of these factors. On the other hand, addition of artificial cofactors to a system containing naturally occurring phenolic modifiers will only complicate the picture and has therefore been omitted.

IAA oxidase system of the cotyledons

In freshly prepared homogenates from cotyledons of etiolated or light-pretreated gherkin seedlings IAA is not destroyed. After storage at 2° C for 24 hours, however, the homogenates show high IAA oxidase activity. This activity gradually decreases upon dialysis against

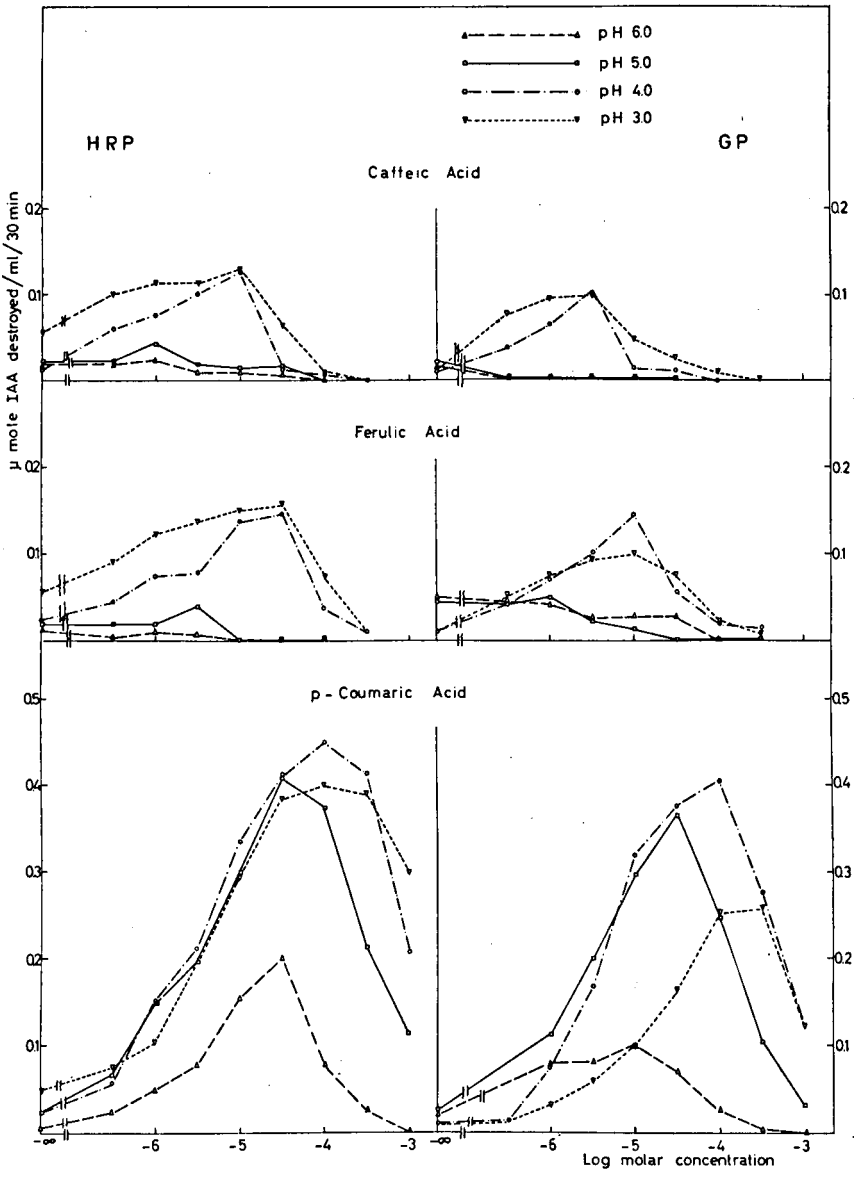

Fig. 3. Effects of hydroxycinnamic acids on the destruction of IAA by HRP and GP. Systems consisted of: enzyme (1 PU): IAA (10 μmole) and hydroxycinnamic acid in 20 ml of 0.05 M phosphate citrate buffer of pH, as indicated in the graphs.

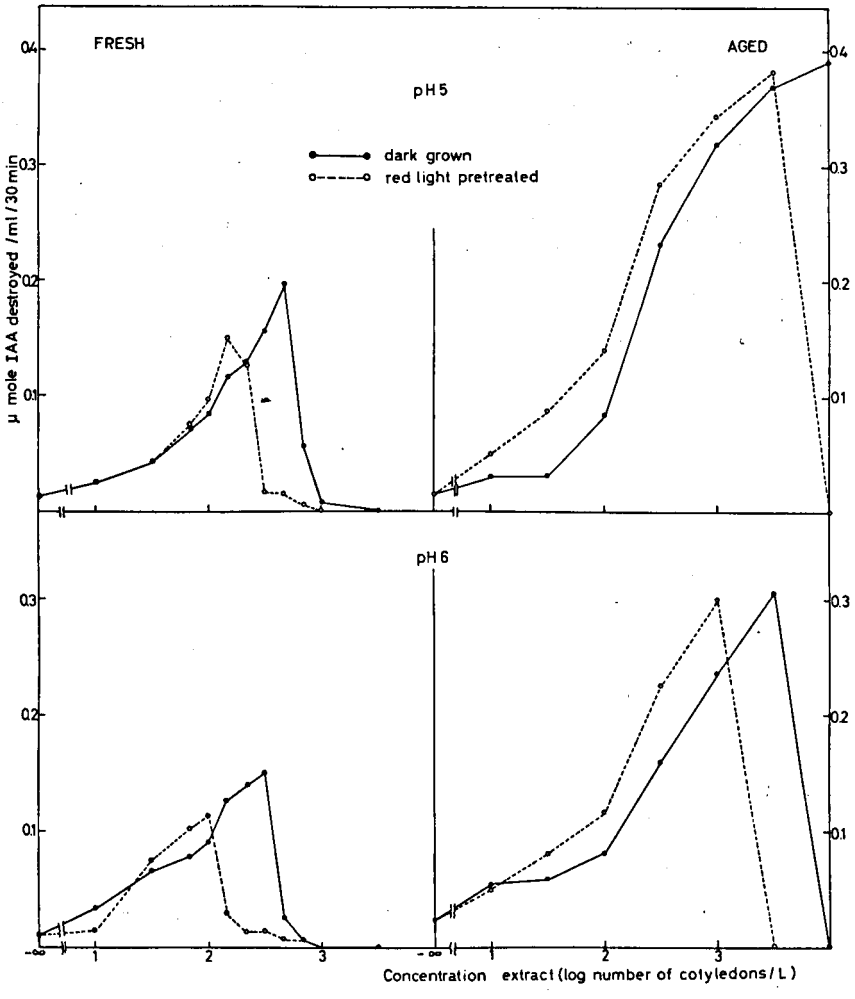

Fig. 6. Destruction of IAA for 30 min at 30° C in the system: HRP (1 PU), IAA (2.10^{-4} M) and extract from cotyledons in 20 ml of phosphate citrate buffer 0.05 M. The aged extract was obtained by storing the homogenate for 24 hours at 2° C before boiling. The pretreatment with red light was the same as given under fig. 2.

changes in the modifier mixture have occurred during storage. Before studying these changes we should pay attention to the fact that a number of non-phenolic reducing compounds like ascorbic acid, NADH, cysteine and glutathione inhibit IAA oxidase as well (WAYGOOD and MACLACHLAN, 1961; BETZ, 1963). We have found that fresh homogenates of cotyledons from three-days-old dark-grown seedlings reduce an amount of dichlorophenolindophenol corresponding to 35 mµmole ascorbic acid/pair of cotyledons. It was confirmed in model tests that ascorbic acid inhibits the IAA oxidase

activity of an aged extract can be reduced again by adding fresh extract which has been boiled for 5 min. In the previous paper it was shown that such an extract contains phenols, some of which activate IAA oxidase in a model test whereas others are inhibitory. Apparently the inhibitor activity predominates in fresh extracts. The IAA-destroying activity of purified GP cannot be activated at any concentration of added boiled extract, whereas the activity of p-coumaric acid as a cofactor in the standard test is counteracted by increasing amounts of the extract (fig. 5). Here it is also demonstrated

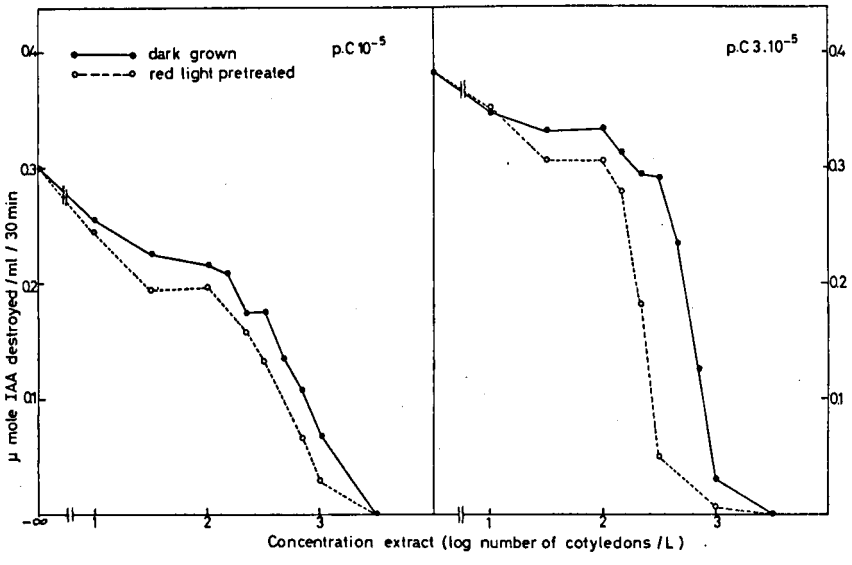

Fig. 5. Destruction of IAA for 30 min at 30° C in the system: gherkin enzyme (1 PU), IAA (2.10^{-4} M), p-coumaric acid (10^{-5} and 3.10^{-5} M) and boiled extract from cotyledons in 20 ml of phosphate citrate buffer, 0.05 M, pH 5.0. The seedlings pretreated with red light were irradiated for 2 hours (2300 μW/cm^2) and then kept in darkness for 22 hours before harvesting.

that an extract from cotyledons of red pretreated seedlings has a stronger inhibitory action than the extract from an equal amount of cotyledons of dark-grown controls. This procedure offers a method of determining changes in inhibitor content.

When HRP is used instead of the gherkin enzyme, low concentrations of the fresh modifier mixture activate destruction of IAA whereas higher concentrations are inhibitory (Fig. 6). The position and height of the peak for optimum cofactor activity shift to lower values when the seedlings have been irradiated, demonstrating again the increase in inhibitors.

When a boiled extract of a homogenate which has been stored at 2° C for 24 hours is used instead of the fresh modifier mixture an activation of the gherkin IAA oxidase is obtained. The activity of HRP is also greatly enhanced (fig. 6). This clearly demonstrates that

phosphate buffer (pH 6.0) (fig. 4). These phenomena can be explained in two ways:

1) The IAA-destroying enzyme is slowly released from particles in the homogenate.
2) Inhibitors blocking the enzyme activity are either destroyed or changed into cofactors during storage. From the loss of activity upon prolonged dialysis it may be presumed that the enzyme requires one or more cofactors.

All experimental results favour the second possibility. Immediate destruction of IAA takes place in a fresh homogenate when hydrogen peroxide instead of oxygen is used as a substrate. This indicates that the oxidizing enzyme is already present in an accessible form. The

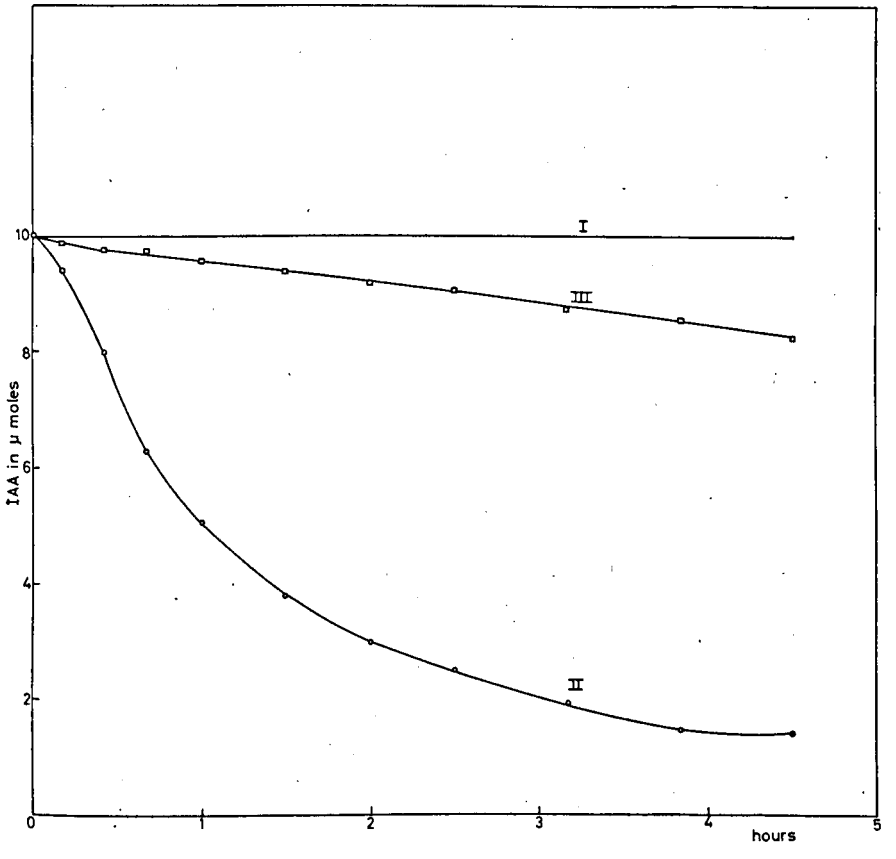

Fig. 4. Kinetics of IAA destruction in a crude homogenate from dark-grown cotyledons. Reaction mixture consists of the homogenate of 25 cotyledons and 10 μmole of IAA in 20 ml of 0.05 M phosphate citrate buffer pH 6.0. I. Assay started immediately after preparing the homogenate. II. Homogenate which has been kept at 2° C for 24 hours. III. Homogenate which has been kept at 2° C for 24 hours followed by dialysis for three days.

activity of GP and HRP. Light causes an increase in the ascorbic acid content of the cotyledons since 24 hours of blue, red and far-red irradiation enhanced the amount to 110, 105 and 130 mμmole/pair of cotyledons respectively. The concentration in the dark-grown controls had only risen to 45 mμmoles/pair of cotyledons during the same time. We found that the ascorbic acid gradually disappears on storage. But this cannot entirely explain the aging phenomenon, because a homogenate from which the ascorbic acid has disappeared does not yet activate destruction of IAA by the gherkin enzyme. Therefore changes in the phenolic compounds as well are expected to occur in the homogenate on storage.

Paper chromatography of aged modifier extracts shows that the bands of the p-coumaric- and ferulic acid esters are no longer present. Instead, free p-coumaric acid and ferulic acid are found. It can be seen in fig. 7 that the activity-concentration curves of free p-coumaric acid and of the sugar ester isolated from the hypocotyls do not differ very much. Apparently, hydrolysis of the hydroxycinnamic acid esters has no important consequences for the activity of the modifier mixture.

Alterations in the flavonol mixture have been detected spectrophotometrically. A more detailed investigation of these changes must

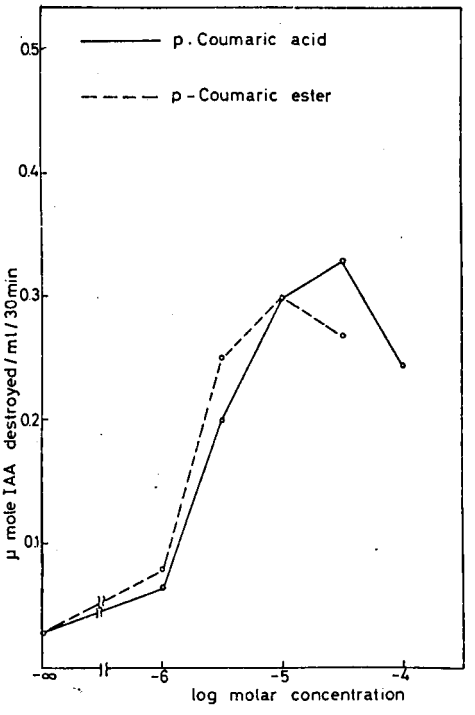

Fig. 7. Modifier activity of free p-coumaric acid and of its sugar ester from the hypocotyls. System consisted of GP (0.25 PU), IAA (2.5 μmole), and p-coumaric acid or its ester in 5 ml of 0.05 M citrate phosphate buffer pH 5.0.

await the elucidation of the structure of the flavonols. They may be caused by:

1) oxidation by polyphenoloxidase, particularly of o-dihydroxy compounds,
2) hydrolysis of the phenol glycosides followed by precipitation of the water-insoluble aglycone.

We have to consider also the possibility that a polyphenolic inhibitor is converted into a cofactor by virtue of preferred oxidation of its o-hydroxy groups.

In figs. 5 and 6 it can be seen that a red light pretreatment increases the inhibitor activity of a fresh homogenate of the cotyledons two to three times. The homogenate has to be diluted by this factor in order to have the activity-concentration curve coincide with the dark control. This is in agreement with the light-dependent increase in ascorbic acid and flavonols (ENGELSMA and MEIJER, 1965a, fig. 8). Similarly, a correlation may be seen between the enhanced cofactor

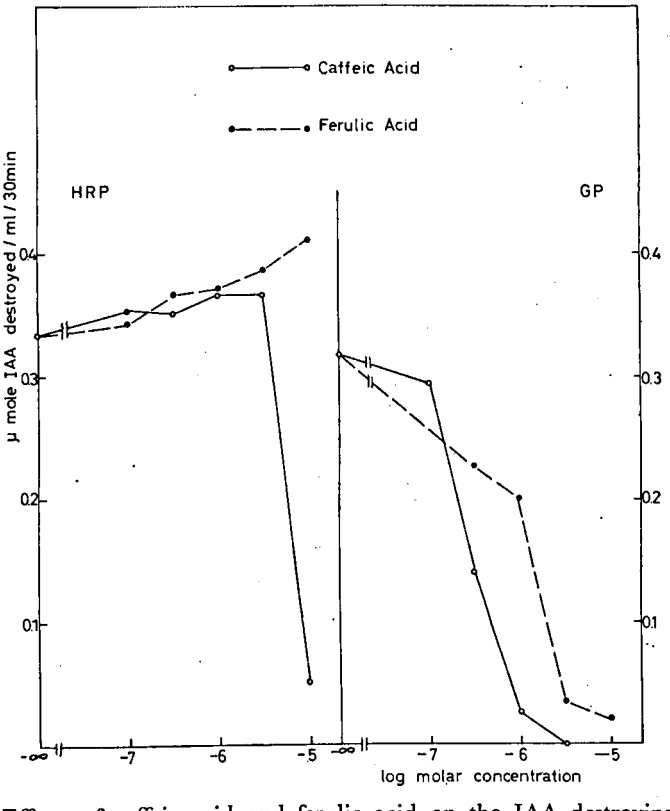

Fig. 8. Effects of caffeic acid and ferulic acid on the IAA destroying activity of HRP and GP activated by p-coumaric acid. Systems consisted of: enzyme (1 PU), IAA (2.10^{-4} M), p-coumaric acid (10^{-5} M) and caffeic or ferulic acid in 20 ml of 0.05 M phosphate citrate buffer pH 5.0.

activity of an aged homogenate from cotyledons irradiated in red light (fig. 6) and the increase in p-coumaric acid derivatives in these organs.

It is not clear as yet why HRP is activated by the mixture of naturally occurring modifiers and why GP is not. The observation that a p-coumaric acid activated IAA oxidase model system with GP is inhibited by much smaller amounts of caffeic acid and ferulic acid than a similar system with HRP (fig. 8) may have a significant bearing on this problem.

IAA oxidase system of the hypocotyls

A homogenate of the hypocotyls does not show the aging phenomenon. Immediately after preparation it has an IAA destroying activity which does not alter much on standing. This is apparently due to the absence of flavonoid inhibitors and to the very low concentration of ascorbic acid.

The activity of a homogenate from the apical section of the hypocotyl is much higher than that of a homogenate from the basal part. This is in agreement with the higher concentration of p-coumaric acid and the lower ratio of ferulic acid to p-coumaric acid in the apical part of the hypocotyl. Transferred from darkness to light the

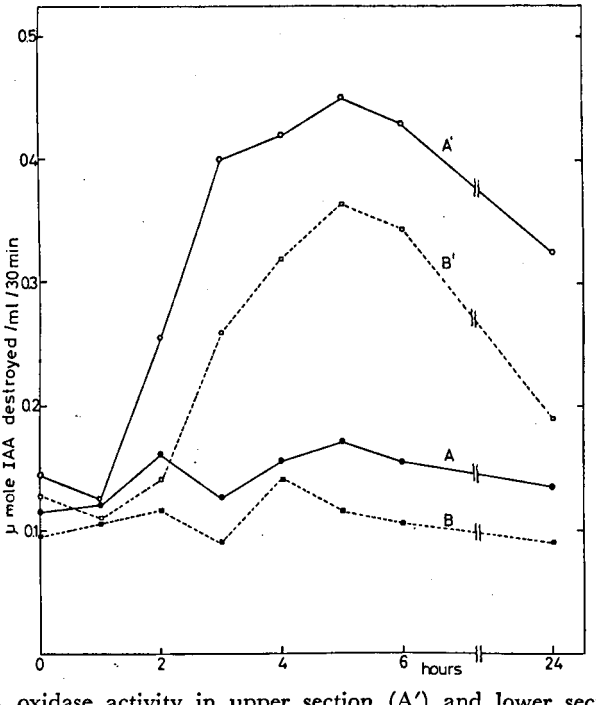

Fig. 9. IAA oxidase activity in upper section (A') and lower section (B') of gherkin seedlings transferred from darkness to blue light (700 μW/cm^2). A and B are dark controls. A and A': 30 mg of fresh weight/ml of IAA oxidase test solution. B and B': 120 mg of fresh weight/ml of IAA oxidase test solution.

activity begins to increase after a lag of one to two hours (fig. 9), to reach a maximum after about 5 hours. Qualitatively this can be explained if one considers:

1) the time course of the synthesis of hydroxycinnamic acids as given in fig. 4 of the preceding paper (ENGELSMA and MEIJER, 1965a);
2) the increase in the ratio of ferulic acid to p-coumaric acid as given in table 2 of the same paper.

The concentration-activity curve for the p-coumaric acid sugar ester has already been given (fig. 7). Fig. 10 shows how the activity decreases with increasing amounts of the ferulic acid derivative from the hypocotyls.

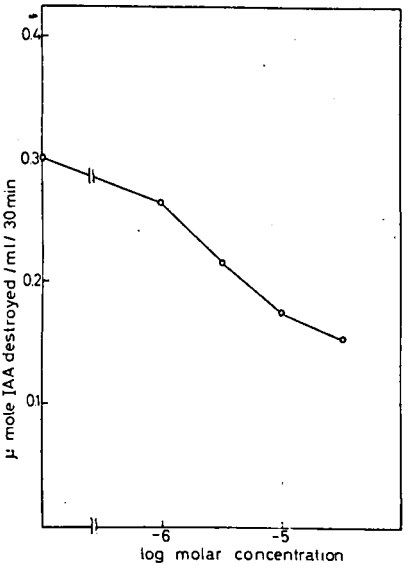

Fig. 10. Inhibitory effect of the ferulic acid sugar ester from the hypocotyls. System consists of GP (0.25 PU), IAA (2.5 µmole), p-coumaric acid sugar ester (0.125 µmole) and ferulic acid sugar ester in 5 ml of 0.05 M phosphate citrate buffer pH 5.0.

IAA level in light-treated hypocotyl sections

Evidence that light lowers the level of IAA in the hypocotyl was obtained as follows. From three-days-old seedlings grown in the dark, one 5-mm section per hypocotyl was cut immediately below the plumular hook. Samples of 20 sections, floating in petri dishes in solutions of 1 % sucrose and different concentrations of IAA, were kept in the dark or irradiated with 400 µW/cm² of blue, red and far-red light. The length of the sections was measured after 24 hours (table 1). Light inhibits the elongation of the sections. But growth can be made independent of the light conditions when sufficient IAA is supplied.

TABLE 1

Increase in length in millimetres of 5-mm hypocotyl sections in 1 % sucrose solutions with increasing IAA concentrations during 24 hours in darkness and in light of different wavelength regions of 400 μW/cm^2 (25° C).

	IAA					
	0	10^{-7}	3.10^{-7}	10^{-6}	10^{-5}	10^{-4}
Dark	2.45	2.5	2.5	2.8	3.1	2.9
Blue	1.4	1.6	1.9	2.4	3.25	3.15
Red	1.75	2.0	2.25	2.7	3.0	2.8
Far-red	2.05	2.2	2.55	2.6	3.0	2.8

Growth inhibition caused by IAA oxidase cofactors

Growth during 24 hours in the dark was studied on 5-mm hypocotyl sections of dark-grown gherkin seedlings, floating in solutions of 10^{-3} M p-coumaric acid, and solutions of 10^{-3} M p-coumaric acid sugar ester in phosphate citrate buffer, pH 5.0, at 25° C. At the end of this period the sections contained 275 and 140 mμmoles hydroxycinnamic acids/gr fresh weight respectively, compared with 75 mμmoles/gr fresh weight in the controls. The average inhibition of elongation was 28 and 13 % respectively. It was found that the elongation of the sections in the dark is also inhibited by trans-cinnamic acid (3.10^{-4} M), a precursor in p-coumaric acid synthesis in gherkin hypocotyls (ENGELSMA and MEIJER, 1965a).

DISCUSSION

When gherkin seedlings are transferred from darkness to light the growth rate of the hypocotyl decreases after a lag of less than 10 minutes to nearly 2 hours, depending on the light quality. This inhibition is strongest during the first 8 hours of irradiation, except for high intensities of red light. Phenolic compounds accumulate in the hypocotyls in this period. The question is whether these compounds play an active role in the transition from etiolated state to maturity, or whether they are merely waste products.

It was found that the growth of hypocotyl sections from dark-grown seedlings is inhibited by external application of p-coumaric acid and of the sugar ester of p-coumaric acid naturally occurring in gherkin hypocotyls. More extensive studies of the effect of hydroxycinnamic acids on the IAA-induced elongation of Avena first internode sections have been carried out by HENDERSON and NITSCH (1962) and NITSCH and NITSCH (1962). They found that monophenols inhibit IAA-induced growth, whereas ortho-diphenols show synergism. Qualitatively there is agreement with the effects of these compounds on IAA oxidase, but this does not exclude the possibility of other mechanisms (SIEGEL and PORTO, 1961).

We have found that in a homogenate the following components are somehow involved in the destruction of IAA.

a) An enzyme, resembling HRP, which causes oxidation of IAA in the presence of phenolic cofactors.
b) p-Coumaric acid sugar esters which activate this enzyme.
c) Derivatives of ferulic acid and flavonoid compounds (the latter only in the cotyledons) which antagonize the stimulation of the former components.
d) Ascorbic acid, also mainly in the cotyledons, which inhibits the IAA oxidase activity as well.
e) Hydrolyzing and oxidizing enzymes, probably ascorbic acid oxidase and polyphenoloxidase, which destroy the IAA oxidase modifiers or alter their activity.

In how far the latter reactions play a role in the regulation of the IAA concentration in the living cell cannot be decided.

In addition to the disturbing factors mentioned above, account has also to be taken of the following reactions in the *in vitro* determination of IAA-destruction.

1) Oxidation of IAA coupled to the oxidation of polyphenols by polyphenoloxidase (BRIGGS and RAY, 1956; TOMASZEWSKI, 1959).
2) Oxidation of phenolic modifiers coupled to oxidation of IAA by peroxidase (ENGELSMA, 1964).

Notwithstanding these complications, there is general agreement between the results of the *in vitro* determinations of IAA oxidase and those of the independent determination of the phenolic cofactors and inhibitors, which allows sufficient confidence in the interpretation of the data.

The effect of light on the expansion of the cotyledon and the inhibition of the hypocotyl appears to be brought about by the same types of photoreactions as observed for the phenol synthesis: photoresponse I, the "high-energy reaction", and photoresponse II, the phytochrome reaction. In all cases the responsivity to light intensity is much higher in blue and far-red light than in red light.

We have obtained an indication that light causes a decrease in the IAA content of the hypocotyl. Recently FLETCHER and ZALIK (1964) came to similar conclusions with Phaseolus vulgaris. This lowering of IAA concentration is in agreement with the increase in IAA oxidase activity due to synthesis of a p-coumaric acid derivative as a consequence of a light treatment. Fig. 9 shows that this activity goes through a maximum. A correlation may be seen with the concomitant photoinhibition of hypocotyl elongation, which also goes through a maximum (fig. 1), indicating that elongation might be governed by the ratio of ferulic acid to p-coumaric acid.

On the other hand, when we look at these phenomena in greater detail we come to a number of observations seemingly in disagreement with the above hypothesis.

1. The optimum p-coumaric acid concentration in a standard IAA oxidase test is 10^{-4} M or lower, depending on the pH. It does not change when the enzyme concentration is increased. We have

seen that concentrations in excess of 10^{-4} M are generally encountered in the apical section of the hypocotyl. This would mean that an increase in phenolic compounds would lead to a decreased IAA oxidase activity, which is in contradiction with the lowering of the IAA level. A similar inconsistancy can be brought forward for the cotyledons. It is not understood how an increase in inhibitors in the light can have a regulatory effect on the expansion of the cotyledons when the action of IAA oxidase in the cotyledons of dark grown seedlings is already completely blocked.

2. When seedlings are irradiated for a limited time and subsequently returned to darkness, they assume within 24 hours the same growth rate as the dark controls (MEIJER and ENGELSMA, 1965) although the IAA oxidase activity is much higher. The total amount of phenolic compounds accumulated by the former plants can be of the same order as in continuously irradiated plants (ENGELSMA and MEIJER, 1965a, fig. 5) which are strongly inhibited in their elongation.

3. In the case of photoinhibition of hypocotyl elongation the length of the lag period between the beginning of irradiation and the response is shorter or at the most equally long as the lag for the photoinduced phenolsynthesis. FURUYA and THOMAS (1964) came to a similar conclusion with regard to the correlation between light-stimulated flavonol synthesis and photoinduced development of etiolated pea plumules. For both responses they found a lag of about 4 hours.

These facts can either mean that phenol synthesis does not play the expected role in morphogenesis, or that the conception of the IAA oxidase mechanism is too simple. We therefore will try to determine whether the conflicting facts can be resolved by modifying the hypothesis. For this purpose we will assume that only a fraction of the total amount of phenolic compounds which can be isolated, participates in the IAA regulating system, and that destruction of the phenolic modifiers is coupled to IAA inactivation.

1) It has been shown for a number of plants that phenolic derivatives are stored in the vacuoles (BANCHOR and HÖLZ, 1960; BOYER, 1964). It is likely that these phenols do not participate in the IAA regulating system. If we assume that only the modifiers present in the protoplasm are effective and that this is the site of synthesis as well, we might expect a correlation between rate of accumulation of phenolics and concentration at the site of action. Comparing fig. 1 with fig. 6 of the preceding paper we see that, particularly for the initial period when the situation is not yet complicated by lignification, photosynthesis etc., there is a qualitative correlation between rate of phenol synthesis and growth inhibition.

The same can be said of the flavonol synthesis in the cotyledons and the increase in fresh weight of these organs.

2) Previously (ENGELSMA and MEIJER, 1965a) it has been argued that although monomeric phenols are no longer accumulated, phenol synthesis may continue to meet the demands of phenolconsuming

processes like lignification. Once we have replaced the static IAA oxidase hypothesis, correlation between IAA oxidase activity and length or fresh weight, by a dynamic relationship in which the IAA level becomes a function of the rate of synthesis of phenolic modifiers, an apparent discrepancy between the growth inhibition of continuously irradiated seedlings and of plants returned to darkness can be readily explained.

3) It has been demonstrated that *in vitro* destruction of hydroxycinnamic acids is coupled to enzymatic oxidation of IAA (ENGELSMA, 1964). The concentration of p-coumaric acid in an IAA oxidase model system can be kept at a very low level as long as IAA is present by adding the p-coumaric acid slowly to the system. If such a mechanism operates *in vivo* the initial synthesis of phenolic compounds might be obscured by increased destruction coupled to oxidation of IAA. A somewhat shorter lag for growth inhibition than for phenol synthesis is therefore not necessarily in contradiction with the IAA oxidase hypothesis. A prerequisite is that the material should respond very rapidly to changes in IAA concentration.

In blue light, however, the lag period for photoinhibition is very short compared to the lag for synthesis of the phenolics. The conclusion seems inevitable that the action of blue light on plant growth is at least partly due to another mechanism. A hypothesis which fulfils the requirement of the rapid response is the riboflavin-sensitized photodestruction of IAA (GALSTON, 1949; GALSTON and BAKER, 1949). It was found that hydroxycinnamic acids inhibit this reaction *in vitro* (ENGELSMA and MEIJER, to be published). This would offer an alternative explanation for the fact that, particularly in blue light, photoinhibition is very strong during the first hours of irradiation. The concentration of hydroxycinnamic acids is then still low. Support for this hypothesis may be the fact that hypocotyl sections release a heat-stable compound which sensitizes destruction of IAA in blue light but not in red and far red. We do not yet know whether this is a riboflavin or another blue-absorbing pigment.

To suggest that the developmental changes of the gherkin seedling might be regulated by only one growth hormone, IAA, is probably an unjustified simplification. These plants respond very well to gibberellic acids too. Application to the light-treated seedling results in strong elongation together with an increase in the amount of hydroxycinnamic acids per hypocotyl. The alternative approach, to explain the effects of light on plant growth by influence on the gibberellin metabolism (LOCKHART, 1961; 1964), was beyond the scope of these investigations.

Summarizing it can be said that qualitative agreement between IAA oxidase action and growth can only be obtained if certain assumptions are made about the distribution of the phenolic compounds within the cell and about the coupling of inactivation of IAA and IAA oxidase modifiers. Evidence for both assumptions is available but this does not necessarily have to be construed as proof of the IAA oxidase mechanism. In at least one case, i.e. the inhibition of

hypocotyl elongation in blue light, the phenomena seem incompatible with this hypothesis.

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