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EVIDENCE FOR A POOL OF INACTIVE PHENYLALANINE AMMONIA-LYASE IN *CUCUMIS SATIVUS* SEEDLINGS

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; gherkin; phenylalanine; ammonia lyase; inactive enzymes.

Abstract—Evidence is presented which suggests that an inactive form of PAL exists in dark grown gherkin seedlings and may be activated by the application of protein synthesis inhibitors. The significance of this finding is discussed with reference to current views on the regulation of PAL activity.

INTRODUCTION

THERE is considerable evidence in the literature which suggests that the enzyme phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5) may be induced by light of various wavelengths.[1–7] In most plants investigated, the increase in lyase activity may be partially or entirely prevented by the application of cycloheximide.[8,9] Confirmatory evidence has been supplied from the density labelling experiments that this increase requires PAL synthesis.[10–12] However, it is too early to state categorically that light causes, or stimulates the *de novo* synthesis of PAL.

Engelsma demonstrated that if dark grown gherkin seedlings are exposed to blue light, a transient increase in PAL level results which reaches a maximum at about 4–6 hr and then declines. If cycloheximide is applied at suitable times, both the rise and subsequent decline in lyase level could be prevented.[9] These results have been taken to suggest that the decline also depends in some way on the *de novo* synthesis of protein. Subsequently, Engelsma demonstrated that if gherkin seedlings, which had been irradiated with blue light for 24 hr at 25°, were placed in the dark first at 4° for 24 hr and then transferred to 25°, a rise in enzyme activity occurred which was of the same magnitude as that originally induced by the blue light treatment. Unlike the first rise, however, this increase in response to temperature-transfer treatment was cycloheximide insensitive.[13]

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The hypothesis was proposed that there exists in gherkins a proteinaceous inactivator of PAL which is synthesized after PAL induction and which brings about the decline of the enzyme level by the formation of an inactive complex. The presumed PAL-inactivator complex may be dissociated *in vivo* by the temperature-transfer treatment, releasing active PAL, thus providing the cycloheximide-insensitive increase in enzyme level.

The evidence presented in this investigation is consistent with the concept of a specific PAL-inactivator, but requires some modifications of the details as originally proposed.

RESULTS

The data presented in Fig. 1 demonstrate that the temperature transfer experiment of Engelsma is readily reproducible.[13] When 4-day-old dark-grown gherkin seedlings are placed in blue light the level of extractable PAL increases, reaching a maximum between 4 and 6 hr after which the level declines. After 24 hr of blue light irradiation the plants were transferred to 4° in the dark for 24 hr. After this low temperature treatment the plants were transferred to 25° in the dark. An increase in the PAL level was observed which reached a maximum 4–6 hr after transfer.

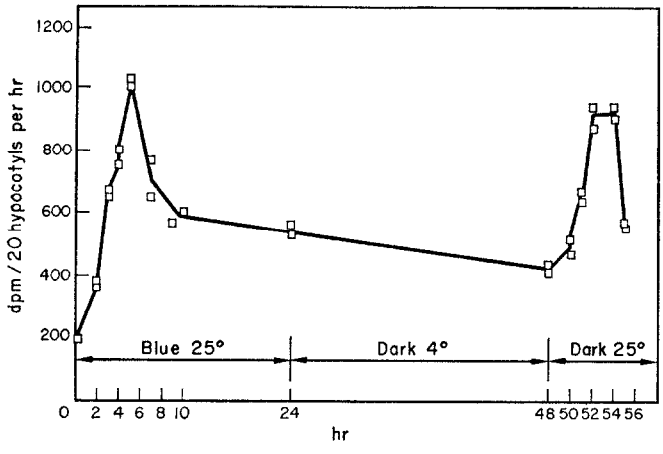

FIG. 1. EFFECT OF TEMPERATURE TRANSFER TREATMENT ON DARK GROWN GHERKIN SEEDLINGS AFTER BLUE LIGHT IRRADIATION.

4-Day-old dark grown gherkin seedlings were placed in blue light and samples of 20 hypocotyls 2 cm long were taken. After 24 hr the plants were transferred to the dark at 4° for 24 hr and then to the dark at 25°.

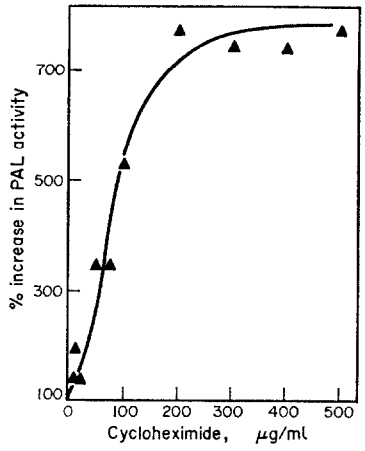

FIG. 2. EFFECT OF CYCLOHEXIMIDE CONCENTRATIONS ON PAL LEVEL IN DARK GROWN GHERKIN SEEDLINGS.

PAL level was estimated 3 hr after the 4-day-old seedlings were sprayed with cycloheximide.

If 3-, 4- and 5-day-old dark grown gherkin seedlings are sprayed with antibiotics and harvested 4 hr later increases in the level of PAL result with those plants treated with cycloheximide and puromycin, whereas those treated with chloramphenicol give no significant reponse (Table 1). The largest increase is found with cycloheximide treatment and this response has been investigated further. The dose response curve presented in Fig. 2 shows that saturation is reached between 100 and 200 μg/ml cycloheximide.

A comparison of the kinetics of the response to cycloheximide and to blue light (Fig. 3) reveals that the response of dark grown gherkins to cycloheximide is much faster than that

to blue light. The response to cycloheximide has a lag phase of 40–60 min whereas that of the blue light response is 90–120 min. It is interesting to note that if cycloheximide is applied at the same time as exposure to blue light then the response has a lag phase of 90–100 min and the increase is slightly smaller than that of the blue light treatment, except in the early stages. There is clearly therefore some interaction between blue light and cycloheximide in their effects on PAL levels.

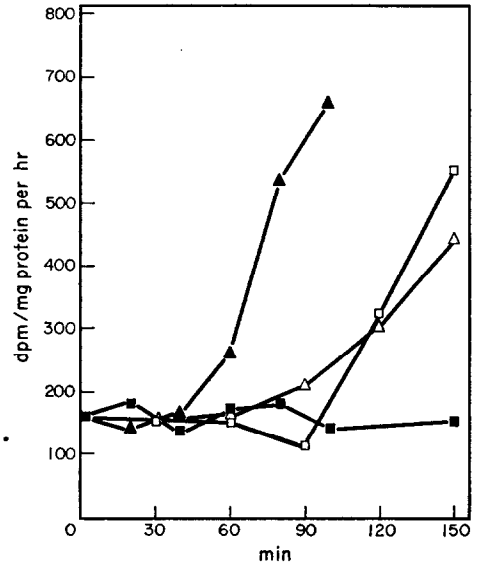

FIG. 3. COMPARISON OF KINETCIS OF THE RESPONSE OF PAL LEVEL TO CYCLOHEXIMIDE AND BLUE LIGHT IRRADIATION.

4-Day-old dark grown gherkin seedlings were treated either with blue light (□) or with blue light and cycloheximide 100 μg/ml (△) or with cycloheximide 100 μg/ml alone (▲) or left in the dark (■). Changes in PAL level were estimated at intervals for 150 min.

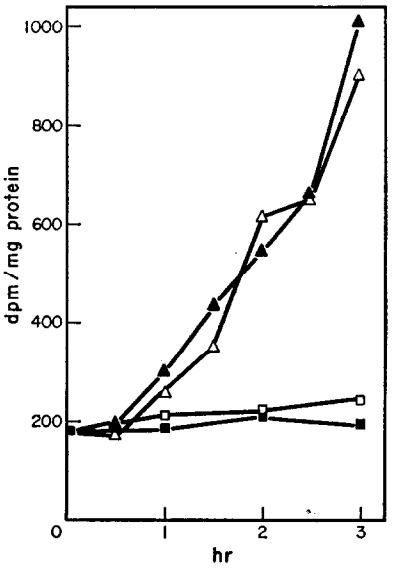

FIG. 4. EFFECT OF CYCLOHEXIMIDE ON SEEDLINGS PRETREATED WITH 12 hr BLUE LIGHT.

4-Day-old dark grown gherkin seedlings were treated with 12 hr blue light then, left in the blue light (□) or left in the blue light and sprayed with cycloheximide 100 μg/ml (△) or transferred to the dark (■) or transferred to the dark and sprayed with cycloheximide 100 μg/ml (▲).

In order to investigate this interaction, plants were first exposed to blue light for 12 hr. Such plants experienced a rise in PAL followed by a decline to levels similar to those in dark grown controls (see Fig. 1). The plants were divided into four sets. Two sets of plants were kept in the blue light at 25° one being sprayed with cycloheximide. The other two sets were transferred to the dark at 25° and again one set was sprayed with cycloheximide. Under these conditions the PAL levels remained constant in plants without cycloheximide, whereas in both sets of cycloheximide-treated plants, enzyme activity increased at equal rates after a lag of 60–90 min (Fig. 4).

Further experiments show that an increase in PAL can be obtained when completely dark grown seedlings are given a temperature-transfer treatment (Fig. 5). Furthermore, this rise in lyase activity is not merely insensitive to cycloheximide inhibition, it is in fact dramatically increased in the presence of the antibiotic. Figure 6 shows the result of an experiment designed to compare the changes in PAL in response to temperature-transfer treatment in fully dark grown seedlings and in seedlings pre-treated with 24 hr blue light. The dark grown

seedlings showed a slightly larger increase in PAL level than those pre-treated with blue light. When cycloheximide was applied at the time of transfer from 4 to 25° very large increases in PAL activity were observed both with blue light pre-treated and dark-grown plants. The increases occurred at approximately the same rate although the dark-grown seedlings reached a higher maximum than those pre-treated with blue light.

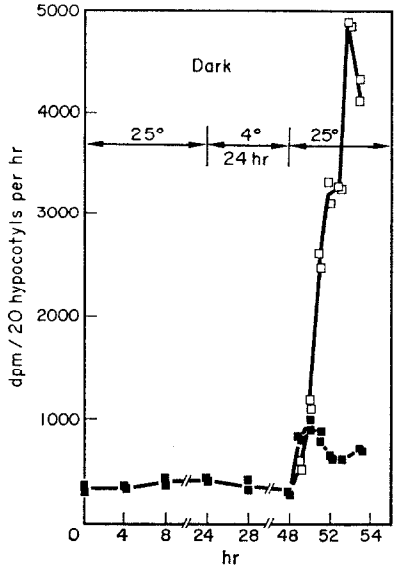

FIG. 5. EFFECT OF TEMPERATURE TRANSFER TREATMENT ON DARK GROWN GHERKIN SEEDLINGS.

PAL level in 4-day-old gherkin seedlings was estimated over 24 hr in the dark at 25°, then transferred to the dark at 4° for 24 hr and then transferred back to 25° in the dark. At the time of the last transfer one set of plants were treated with cycloheximide (100 μg/ml).

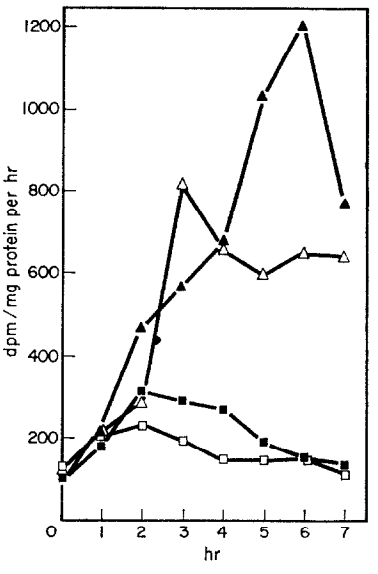

FIG. 6. COMPARISON OF KINETICS OF THE RESPONSE OF PAL LEVEL TO TEMPERATURE TRANSFER TREATMENT.

4-Day-old gherkin seedlings were given 24 hr of blue light irradiation transferred to the dark at 4° for 24 hr and then transferred to 25° in the dark (□). At the time of transfer from 4 to 25° one set of seedlings were sprayed with cycloheximide 100 μg/ml (△). These responses are compared to those of 4-day-old gherkin seedlings which have been given no blue light irradiation but give identical temperature transfer treatment (■). At the time of transfer from 4 to 25° one set of seedlings was sprayed with cycloheximide 100 μg/ml (▲).

DISCUSSION

It has been demonstrated by Engelsma that the decay of PAL activity in gherkin seedlings which occurs after blue light irradiation can be blocked by the application of protein synthesis inhibitors.[4,14] Similar observations have been made for PAL in potato tuber slices,[15] excised bean axes[16] and *Xanthium* leaf disks.[17] Engelsma[18] suggests that the mechanism of inactivation of PAL may be similar to that of potato invertase,[19] in which a proteinaceous

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inhibitor reacts specifically with the enzyme to give an inactive enzyme inhibitor complex. Although this is clearly the simplest explanation of the data, it is possible that the level of PAL activity is regulated by the presence of a metabolite, the production of which depends on the synthesis of an enzyme with a rapid turnover. Since there is no evidence to support the latter hypothesis, the data presented here has been interpreted in terms of the simpler hypothesis with slight modification of details.

TABLE 1. PAL IN GHERKIN HYPOCOTYLS EFFECTS OF PROTEIN SYNTHESIS INHIBITORS

Inhibitor (100 μg/ml)	Enzyme activity (% of control) Days 3	4	5
Cycloheximide	484	550	620
Puromycin	131	176	138
Chloramphenicol	102	105	94

It is shown in Table 1 that as early as 72 hr after imbibition PAL level may be increased by the application of cycloheximide to gherkin seedlings germinating in the dark. This may be taken to indicate that a pool of inactive PAL complex exists in dark grown gherkins, and that the maintenance of this pool in an inactive state depends on continuous protein synthesis. A number of questions remain unanswered. It is not known whether the pool of inactive PAL is inherited in the seed or rapidly formed during the early stages of germination either as inactive complex or as active PAL which is subsequently inactivated. The investigation of PAL levels in recently imbided seeds is difficult due to the presence of large quantities of polysaccharide material. As yet we have not been able to demonstrate increases in PAL level due to cycloheximide treatment much before 72 hr.

From the comparison of the kinetics of the response to cycloheximide and to blue light shown in Fig. 3 it is clear that the response to cycloheximide has a shorter lag-phase than that to light. This is consistent with the view that the blue light increase in PAL level involves *de novo* protein synthesis whereas the increase brought about by cycloheximide treatment occurs by activation of pre-existing enzyme. Those plants treated with both blue light and cycloheximide respond with the same lag-phase as those treated with blue light, but reach a lower maximum value.

In no experiment of this kind have we been able to inhibit entirely the rise in PAL in plants treated with both blue light and cycloheximide. There may be a case for suggesting that this behaviour is not merely PAL synthesis which has escaped inhibition by cycloheximide but rather a positive response to cycloheximide treatment which is less efficient in the presence of blue light. In experiments not presented here it has been possible to produce a 'dark cycloheximide' response from plants irradiated with blue light merely by applying cycloheximide a few minutes before the onset of light. If cycloheximide is sprayed at the same time as, or a few minutes after the onset of blue light, on the other hand the lag-phase is the same as that with blue light alone.

The interpretation of these experiments depends largely on the fate of the PAL inhibitor complex found in dark grown tissues after the onset of blue light irradiation. It is entirely possible that this pool would remain unaffected during blue light irradiation. Cycloheximide

and temperature transfer stimulated increases in PAL level may originate from this pool rather than from a pool of PAL which has been synthesized *de novo* due to the influence of blue light and subsequently complexed with inhibitor.

The increases in PAL level stimulated by cycloheximide after the increase due to blue light irradiation has declined may arise from either of the proposed pools of inactive enzyme. However, in Fig. 5 where dark grown tissue is subjected to temperature-transfer treatment together with cycloheximide treatment at the last transfer, only the dark inactivated pool is present and it must be from this pool that the increases in PAL level occur. When these responses are compared with the responses given by plants pre-treated with blue light (Fig. 6) the increases in PAL level are generally slightly larger in the dark pre-treated plants. Once again these results are consistent with the view that the responses which seem to be brought about by activation of pre-existing enzyme may originate from the pool of inactive enzyme present in dark grown tissue.

The response of PAL to the various treatments described in this paper have been examined using the technique of deuterium oxide density labelling, and the results will be described in a subsequent publication.

EXPERIMENTAL

Plant materials and growth conditions. Seeds of *Cucumis sativus* L. var. 'Venlo Pickling' were obtained from Suttons Seeds, Reading, England. Seedlings were grown on filter paper for 72 hr in covered boxes in a dark-room at 25°.

Light source and treatment. The source of blue light consisted of 9×30 W Phillips, Warm White Fluorescent tubes filtered through 2 layers of No. 20 Deep Blue (Primary) Cinemoid (Strand Electrics, London). The irradiance at plant height was 1·08 W m^{-2}, radiation jeasured being less than 722 nm. During the irradiation treatments the temp. of the cabinet was kept at 25° ± 1.

Enzyme extraction and assay. PAL was extracted from the upper 2 cm of the hypocotyl with the cotyledons removed but containing the terminal hook. 20 hypocotyls were frozen in liquid N_2, crushed to a powder which was then extracted with 1 ml of borate buffer pH 8·8 and the extract filtered through glass fibre paper (GF/A Whatman) under suction. For assay,[20,21] the extract (0·2 ml) was added to a mixture of 0·1 μg L-phenylalanine-[ring 4-T] (Radiochemical Centre, Amersham, England) in 1 mM L-phenylalanine, buffered with 0·1 M borate buffer pH 8·8 in a final vol. of 1·2 ml. The reaction mixture was incubated at 35° for 1 hr after and the reaction then stopped with 0·5 ml 0·1% (w/v) cinnamic acid in 0·05 M KOH and 1 ml 20% (w/v) trichloroacetic acid. The mixture was centrifuged and the supernatant decanted. Cinnamic acid was extracted with 3 ml toluene, mixing and centrifuging. 2 ml of the upper phase was added to 10 ml of scintillation fluid and counted in a Packard Tri-Carb Scintillation Counter with automatic quench correction. The scintillation fluid consisted of 1 l. toluene, 0·5 l. Triton X100 and 5 g, 2,5-diphenyloxazole (PPO). Protein was assayed using the Lowry[22] method.

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