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DENSITY-LABELLING STUDIES ON THE PHOTOCONTROL OF PHENYLALANINE AMMONIA LYASE LEVELS IN *CUCUMIS SATIVUS*

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; phenylalanine ammonia lyase; *de novo* enzyme synthesis density-labelling; photomorphogenesis.

Abstract—The *de novo* synthesis of PAL is demonstrated to occur sometime between imbibition and the end of a 4-hr white light treatment. H_2O–D_2O transfer experiments indicate that PAL synthesis may occur during the light period whilst D_2O–H_2O transfer experiments indicate that synthesis of inactive PAL may occur during dark growth followed by activation by light. Neither of these observations is conclusive. *De novo* synthesis of PAL occurs in excised hypocotyls of gherkin and tuber discs of potato either in darkness or in light. It is concluded that there is as yet no evidence which definitively shows that light controls PAL levels by regulating the rate of *de novo* synthesis.

INTRODUCTION

PHOTOMORPHOGENESIS in many seedlings is accompanied by dramatic changes in the extractable activities of certain enzymes.[1] Such changes have been used as model systems for investigations into the molecular mechanisms through which light mediates the ultimate control of developmental processes. One case which has been studied in great detail is the photoregulation of phenylalanine ammonia-lyase (PAL; E.C.4. 3.1.5) levels.[2–12] Increases in the extractable activities of PAL under the influence of various wavelengths of light have in many tissues been suppressed by inhibitors of nucleic acid and protein synthesis and these results have generally been taken to imply not only that PAL synthesis is taking place, but also that light exerts its influence by stimulating the rate of PAL synthesis.[3–5,8–13] In view of the demonstrated lack of specificity of these inhibitors in plant materials[14] such conclusions must be viewed with caution.

A specific and unequivocal demonstration of *de novo* PAL synthesis is clearly required. Three techniques offer themselves for this purpose: immunology, radioactive labelling, and

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density labelling. The first two methods require the purification of PAL to a single homogeneous protein, a procedure which is tedious, time-consuming and fraught with difficulties. Zucker has, in fact, used the radiolabelling method to obtain evidence for Pal synthesis in *Xanthium* leaf discs[15,16] but in this case it appears to be mediated by photosynthesis.

The third available method, density labelling, overcomes the major disadvantage of immunology and radiolabelling since prepurification of the enzyme is not necessary. Density labelling was first used in plants by Filner and Varner[17] to demonstrate the *de novo* synthesis of α-amylase in gibberellin treated barley aleurone cells. Since then it has been used for isocitrate lyase[18,19] and malate synthetase[19] in germinating peanut cotyledons, newly-appearing peroxidases in germinating barley embryos,[20] catalase in germinating maize seeds[21] and nitrate reductase in tobacco suspension cell cultures.[22] Density labelling has also already been used for PAL. Schopfer and Hock[23] have shown that PAL in mustard seedlings germinated and grown in D_2O in darkness for 85 hr and then treated with continuous far-red light, becomes density-labelled. This was claimed as evidence that *de novo* synthesis of PAL occurs during the light treatment, thus supporting the view that phytochrome acts to derepress the gene for PAL. It is quite possible, however, for PAL to have been synthesized during the period of dark growth in an inactive state and subsequently activated by the light treatment, in which case a density-labelled enzyme would also be observed. Attridge and Smith[24] have recently shown that dark-grown gherkin seedlings contain considerable pools of PAL in an inactive state. Recently, Sacher *et al.*[25] have shown that potato tuber discs incubated in D_2O either in darkness or in white light yield density-labelled PAL, indicating that *de novo* synthesis occurs in both the presence and absence of light. It is possible that wound responses are important here.

The experiments reported here have attempted to determine, by transferring seedlings from H_2O to D_2O (and vice versa) at various times, whether PAL synthesis occurs during early growth of the seedlings in darkness, or is stimulated to occur by light treatment.

RESULTS

PAL Changes in Response to White Light Treatment

When etiolated gherkin seedlings are treated with continuous white light, a marked increase in the extractable activity of PAL is seen (Fig. 1). This is a similar response to that reported by Engelsma[5] with continuous blue in that the rise in PAL is preceded by a lag of *ca.* 60–90 min, and reaches a peak at about 4 hr, after which a decline in activity occurs. During the period of the experiment (6 hr) only a very low level of PAL activity was extractable from dark-grown hypocotyls. White light has been used in these experiments in preference to blue light because the increases in PAL levels are considerably greater. High activities are important in density-labelling since the technique inevitably leads to significant losses in activity.

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Effects of D_2O on the Light-mediated Changes in PAL Activity

Density-labelling involves growing the plants in D_2O and thus it is essential to determine whether or not D_2O has any effect on the light-mediated changes in PAL activity. Concentrations above 70% (v/v) D_2O in H_2O completely prevented seedling growth. However, seeds imbibed and grown in 60% D_2O for 8 days had reached a comparable stage of development to seedlings grown in 100% H_2O for 3 days, although certain morphological abnormalities were apparent, e.g. thickened stems. Irradiating seedlings grown for 8 days in 60% D_2O produced a similar but lower rise in extractable PAL activity as with 3-day-old H_2O grown seedlings (Fig. 2). In certain density labelling experiments, seedlings were grown in 60% D_2O and transferred to H_2O at or before the onset of irradiation. This treatment had no significant effect on the time course of the response to light (Fig. 2). In all cases, however, the presence of D_2O at or before the time of irradiation significantly depressed the effect of light on enzyme activity.

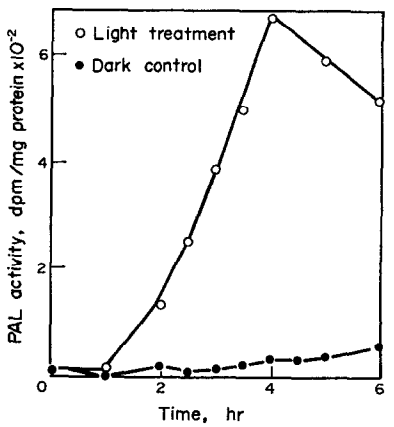

FIG. 1. EFFECT OF WHITE LIGHT ON PAL IN GHERKIN HYPOCOTYLS.

Time course of the increase in extractable PAL activity in the hypocotyls of 3-day-old gherkin seedlings, during continuous irradiation with white light (39·6 W . m^{-2}).

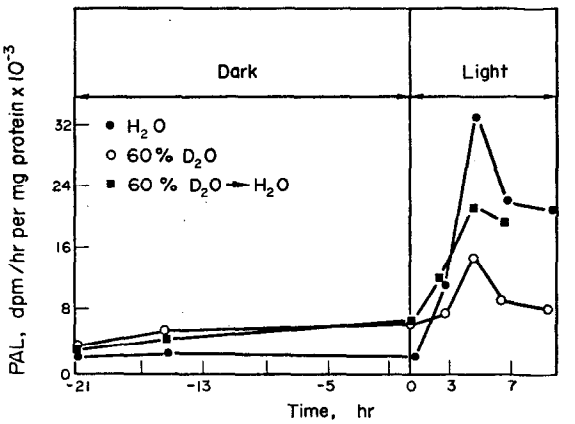

FIG. 2. EFFECTS OF D_2O ON WHITE LIGHT MEDIATED INCREASES IN PAL ACTIVITY.

Seedlings were either grown in H_2O (●), in 60% D_2O (○), or in 60% D_2O and transferred to H_2O at the time of light treatment (■). Time 0 was 72 hr from the start of imbibition for the H_2O grown seedlings and 192 hr for D_2O grown seedlings. Seedlings were given white light (39·6 W . m^{-2}) from time 0.

Density-labelling of PAL in Intact Seedlings

In common with all previously published density-labelling experiments, density gradient centrifugation was here performed using a swinging-bucket rotor. This produces a very steep gradient, and because of the relatively long tubes, the time taken to reach equilibrium is very long. Since PAL is a relatively unstable enzyme, the above conditions are not ideal for demonstrating a density shift. However, it was found that 20 mM dithiothreitol maintained sufficient activity (e.g. *ca.* 63% activity after 67 hr) to enable the experiments to be carried out.

Initially, seedlings were imbibed and grown in either H_2O or D_2O solutions until comparable stages of development were reached, and then 4 hr of white light were given followed by enzyme extraction and density gradient analysis. It was found that considerable varia-

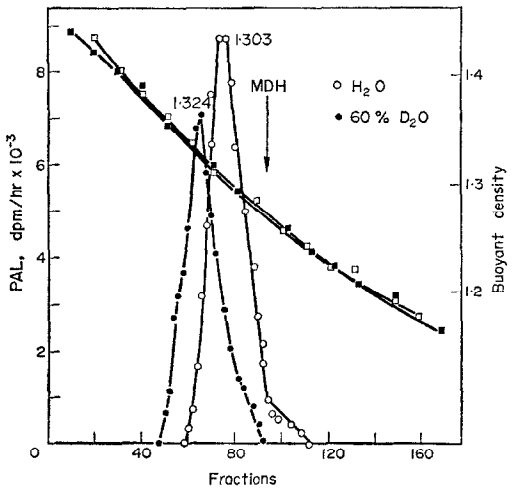

FIG. 3. DENSITY-LABELLING OF PAL IN SEEDLINGS GROWN IN D_2O FROM THE START OF IMBIBITION.

Seeds were imbibed and grown for 3 days in H_2O (○), or for 8 days in 60% D_2O (●) and irradiated for 4 h with 39·6 W . m^{-2} white light. PAL was extracted, layered together with marker MDH on CsCl of a buoyant density of 1·303 and centrifuged in the SW 50·1 rotor for 67 hr at 40 000 rev./min. After centrifugation, one-drop fractions were collected; even numbered fractions were assayed for PAL (● or ○ as above), odd numbered for MDH, and every 10th for refractive index (□ or ■). The peak positions of MDH are given by the vertical arrow.

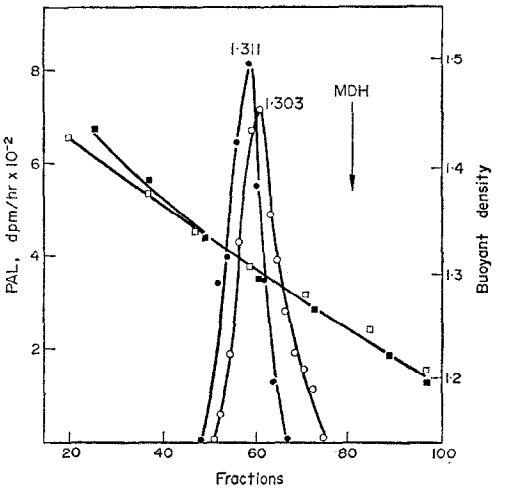

FIG. 4. APPARENT DENSITY-LABELLING OF PAL IN SEEDLINGS GROWN IN H_2O AND TRANSFERRED TO D_2O.

Seedlings were grown for 3 days in H_2O and one batch was transferred to 60% D_2O with vacuum infiltration (●); the rest were left in H_2O (○). Seedlings were then irradiated for 4 hr with 39·6 W . m^{-2} white light and PAL extracted. The enzyme extract, together with marker MDH was layered on CsCl of buoyant density 1·303 and centrifuged in the SW 50·1 rotor for 48 hr at 46 000 rev./min. Two-drop fractions were collected; even-numbered fractions were assayed for PAL (○ or ● as above), odd-numbered for MDH, and every 10th for refractive index (□ or ■). The peak positions of MDH are given by the vertical arrow.

bility occurred in the apparent buoyant densities of PAL extracted from seedlings grown in either H_2O or 60% (v/v) D_2O followed by light treatment. This is partially due to the inaccuracy of reading off the buoyant density of the enzyme from the very steep gradients obtained, but also to the fact that it normally was not possible to allow all of the enzyme to attain equilibrium and thus Gaussian curves were not obtained. These criticisms also apply to all previously published reports and simply illustrate a shortcoming of the method. Figure 3 shows a representative density gradient analysis and the buoyant densities recorded in several different experiments are given in Table 1. The means of 1·301 for the H-enzyme and 1·321 for the D-enzyme represent a density increase of *ca.* 1·5%. In comparison with other recorded density increases in the literature this is a respectable shift and demonstrates that PAL is being synthesized sometime between imbibition and the end of the light period. These results therefore confirm the findings of Schopfer and Hock.[23]

D_2O–H_2O *Transfer Experiments*

The only way to demonstrate at which stage in seedling development PAL is being synthesized is to introduce the label during discrete time periods. This calls for transfer experiments in which seedlings grown in either H_2O or D_2O are transferred at specific times

to D_2O or H_2O respectively. Even though vacuum infiltration was used to facilitate penetration, it must be expected under these conditions that a much lower proportion of the newly-introduced hydrogen will find its way into protein and thus density differences would be expected to be much smaller. In this respect, the high degree of variability associated with buoyant density measurements using swinging-bucket rotors becomes critical.

TABLE 1

Experiment	Buoyant densities (g/cm³)		Experiment	Buoyant densities (g/cm³)	
	H_2O	D_2O		H_2O	D_2O
1	1·305	1·323	6	1·307	1·321
2	1·303	1·324	7	1·302	1·320
3	1·303	1·324	8	1·307	1·322
4	1·291	1·309	9	1·291	1·322
5	1·303	1·322			
Means ± standard deviations	1·301 ± 0·0061	1·321 ± 0·0036	Standard deviation as per cent of mean buoyant density shift	30·5%	23·0%

Buoyant densities of PAL from seedlings grown either in H_2O for 3 days (H_2O) or in 60% (v/v) D_2O for 8 days (D_2O) and treated with white light for 4 hr immediately prior to extraction. Data are from completely separate experiments.

When seedlings are grown in H_2O for 3 days and transferred to 60% (v/v) D_2O by vacuum infiltration just prior to 4 hr white light, a small increase in buoyant density is sometimes observed. Figure 4 shows an example of this experiment in which the observed density increase is 0·61%. Although this is the result to be expected if PAL synthesis is occurring during the light period, it is a very small shift indeed and could not be repeated reliably. This, in fact, is the largest shift we have observed in a H_2O–D_2O transfer experiment, the average shift in six experiments being 0·51%, and thus we cannot conclude unequivocally that *de novo* synthesis has occurred under the influence of light.

TABLE 2

Treatment	Buoyant density	Treatment	Byouant density
H_2O, 3 days	1·303	D_2O, 7·5 days; H_2O 12 hr.	1·320
D_2O, 8 days	1·324	D_2O, 6 days; H_2O 2 days	1·303
D_2O, 7·75 days; H_2O 6 hr	1·315		

Buoyant densities of PAL extracted from seedlings grown in 60% (v/v) D_2O and transferred to H_2O for various time intervals before being treated with 4 hr white light. All preparations were centrifuged in the same rotor and the marker enzyme (MDH) had a buoyant density of 1·278 in each case.

When the experiment is repeated in the reverse direction, i.e. with seedlings grown for 8 days in 60% (v/v) D_2O and transferred to H_2O just prior to irradiation, the observed buoyant density was always high (Fig. 5). A possible reason for this is that the vacuum infiltration failed to effect the entry of sufficient H_2O to significantly dilute the D_2O in the tissues. In subsequent experiments, seedlings grown in 60% (v/v) D_2O were transferred to H_2O at

various time intervals before light treatment. The results of these experiments (Table 2) show that it is necessary to transfer to H_2O for 48 hr before light treatment in order for the enzyme to reach the low buoyant density found in the H_2O controls.

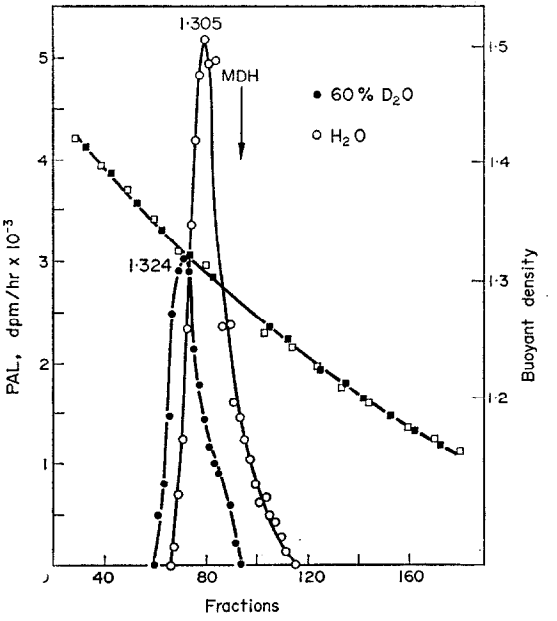

FIG. 5. DENSITY-LABELLING OF PAL IN SEEDLINGS GROWN IN D_2O AND TRANSFERRED TO H_2O AT TIME OF IRRADIATION.

Seedlings were grown in 60% D_2O for 8 days and then transferred with vacuum infiltration to H_2O (●); other seedlings were imbibed and grown in H_2O for 3 days (○). Seedlings were then irradiated for 4 hr with 39·6 $W.m^{-2}$ white light and PAL extracted. The enzyme extract, together with marker MDH was layered on CsCl of buoyant density 1·303 and centrifuged in the SW 50·1 rotor for 67 hr at 40 000 rev./min. One-drop fractions were collected; even numbered fractions were assayed for PAL (● or ○ as above), odd numbered for MDH, and every tenth for refractive index (■ or □). The peak positions of the MDH are given by the vertical arrow.

Density Labelling using Excised Hypocotyls

One of the possible interpretations of the above results is that PAL is synthesized in an inactive form in darkness and activated by light treatment. If this were the case, then the high buoyant densities observed in seedlings transferred from D_2O to H_2O 12 hr before light treatment would be accounted for by synthesis of PAL before that 12 hr dark period. It was therefore of interest to determine whether PAL could be density-labelled in dark grown tissues. Several attempts were made to carry out this experiment using intact seedlings, but the level of PAL activity in dark-grown intact seedlings is so low that reliable results could not be obtained. However, Engelsma,[26] has shown that excision of gherkin hypocotyls leads to a slow but steady increase in extractable PAL levels in either light or darkness. In addition, the increase in PAL under these conditions can be prevented by protein synthesis

[26] ENGELSMA, G. (1968) *Planta* **82**, 355.

inhibitors.[26] A further advantage of excised tissues is that entry of D_2O should be facilitated. Thus, although wound responses may be involved it was throught useful to determine whether or not *de novo* synthesis was occurring.

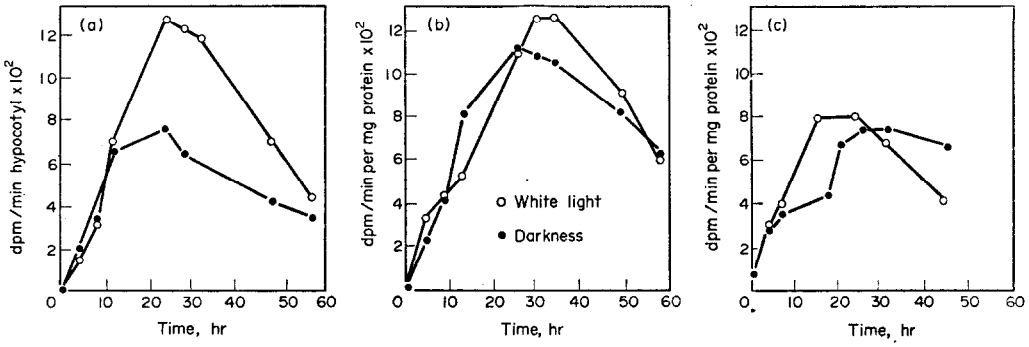

FIG. 6. PAL CHANGES IN EXCISED GHERKIN HYPOCOTYLS.

Excised hypocotyls from 3 day H_2O-grown seedlings were vacuum infiltrated in either H_2O (A and B) or 80% D_2O (C) and incubated in darkness ((●) or 39·6 $W.m^{-2}$ white light (○)). A, data expressed as PAL activity per hypocotyl; B and C, data expressed as PAL activity/unit protein.

Figure 6 shows the time course of the changes in PAL in light- and dark-treated excised hypocotyls from seedlings grown on H_2O when incubated in either H_2O or 80% (v/v) D_2O. (Excised hypocotyls appear to be able to tolerate a higher concentration of D_2O than intact seedlings.) Clearly, the increases in PAL activity are much slower than those elicited by light in intact tissues, but a very much greater overall effect is obtained. On a total activity basis (Fig. 6a), light appears to enhance the increase in PAL, but on a specific activity basis

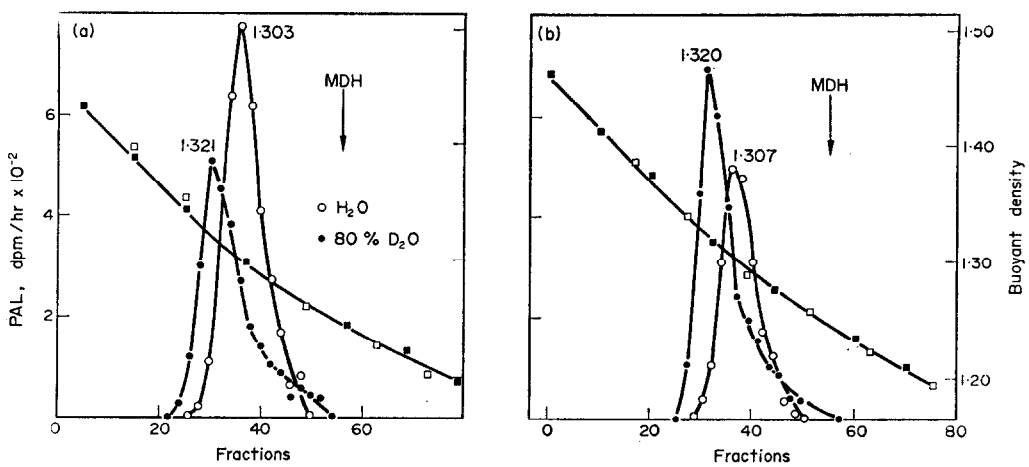

FIG. 7. DENSITY LABELLING OF PAL IN EXCISED GHERKIN HYPOCOTYLS.

Excised hypocotyls from 3 day H_2O-grown seedlings were vacuum infiltrated in either H_2O ((○) or 80% D_2O (●)) and incubated in either darkness (b) or 39·6 $W.m^{-2}$ white light (a) for 24 hr. Enzyme was extracted, layered with MDH on CsCl of buoyant density 1·303 and centrifuged in the SW 50·1 rotor for 48 hr at 46 000 rev./min. Two-drop fractions were collected; even numbered fractions were assayed for PAL (○ or ● as above), odd numbered for MDH, and every 10th for refractive index (□ or ■). The peak positions of MDH are given by the vertical arrows.

(Fig. 6b), no real difference can be detected. The effect of light in this case therefore appears to be relatively unspecific and may be related to a maintenance of general protein levels. The changes in activity in the presence of 80% (v/v) D_2O are smaller than in H_2O but nevertheless the same general response is observed (Fig. 6c).

Figure 7 shows the density gradient analysis of the PALs extracted from excised hypocotyls treated as above. It is quite clear that in both dark- and light-incubated hypocotyls, a significant density increase has occurred during the 24 hr of incubation. This demonstrates that PAL can be synthesized *de novo* in the complete absence of actinic light and furthermore, that density-labelling of H_2O imbibed tissues can be achieved by transferring to D_2O. This has only been reported once before by Sacher *et al.*,[25] who incubated discs of potato tubers in D_2O and obtained a density shift in PAL. We have confirmed their data with potato discs, using 80% (v/v) D_2O for 14 hr in the dark. The apparent shift in buoyant density in this case was 1·37% demonstrating again that *de novo* PAL synthesis can occur in darkness.

DISCUSSION

The aim of the experiments reported here was to determine whether or not PAL synthesis occurs during a period of irradiation. To do this, it is necessary to introduce a density label for restricted periods during the development of the seedling. This has clearly been only partially successful and unfortunately it is not possible as yet to arrive at a confident conclusion regarding the above question. However, it is confirmed that PAL is synthesized sometime between imbibition and the end of the light period, and is not inherited in an inactive form within the seed and later activated by light. The H_2O–D_2O transfer experiments could indicate that *de novo* synthesis occurs during the light treatment, but the low reliability of the buoyant density determinations does not allow that conclusion to be definite. The transfers from D_2O–H_2O, on the other hand, may indicate that PAL is synthesized in an inactive form during dark growth, and activated by light, as suggested recently on other grounds by Attridge and Smith.[24] Another possibility, however, is that D_2O, which has obvious inhibitory effects on plant development, may somehow affect metabolism so that large pools of deuterated amino acids build up. Upon transfer to H_2O, it would then be necessary to dilute the deuterated amino acids with H-amino acids before the buoyant density of newly synthesized enzymes would be reduced; such a dilution process could conceivably take many hours. Thus, it is not yet possible to state categorically that PAL synthesis occurs during light treatment.

It is, however, quite clear that under certain conditions PAL synthesis can occur in the total absence of actinic radiation. In excised gherkin hypocotyls, and in discs of potato tubers, significant density shifts were obtained both in dark- and light-incubated tissues. These results, of course, may have no relevance for the problem of the photomorphogenic control of enzyme synthesis, since in excised tissues the initiation of enzyme synthesis may be solely a wound response occurring only in damaged cells. Until it is possible to get direct evidence on the situation in intact seedlings, however, we must resign ourselves to extrapolation from excised tissues.

On the basis of this and other work[23,25] it seems quite clear that PAL synthesis occurs in intact seedlings sometime between imbibition and the end of the light period, and in excised tissues it occurs either in darkness or under irradiation. There are indications that synthesis may also occur both in dark- and light-treated intact hypocotyls, but neither of these observations is conclusive. Early results of this investigation were reported, somewhat

prematurely, in two recent review articles.[27,28] At that point in time the results appeared consistent with the view that a significant shift in buoyant density occurred when seedlings were grown in H_2O and transferred to D_2O upon irradiation. It was thus concluded[27] that *de novo* synthesis of PAL occurred during the light period. The results reported here show that this conclusion is erroneous and we withdraw it.

The unsatisfactory nature of density-gradient centrifugation in swinging bucket rotors exemplified in these results has stimulated us to experiment with fixed angle rotors as used by Flamm *et al.*[29] for DNA. The results of these experiments are reported in a separate paper[30] and indicate that owing principally to the shallow gradients set up, much greater resolution and accuracy can be attained. It may be possible, using these new techniques, to measure reliably the small density increases expected when limited amounts of density label are introduced into H_2O-imbibed tissues as in the transfer experiments reported in this paper. In this way it is hoped to provide definitive evidence on the photomorphogenic control of PAL synthesis.

EXPERIMENTAL

Preparation of plant material. Seeds of *Cucumis sativus* L. var. 'Venlo Pickling' were obtained from Suttons Reading. Seedlings were grown in Petri dishes on filter paper moistened with either H_2O or 60% D_2O and left in the dark at 25° for 3 or 8 days respectively. After this time they were treated with fluorescent white light (39·6 Wm^{-2}) for 4 hr. In the transfer experiments, intact seedlings were removed from the filter papers, blotted dry and placed in a 50 ml beaker containing 10 ml of either H_2O or 60% D_2O. They were then placed under a vacuum for 2 min during which time all the air in the solution and the seedlings was seen to bubble out. Seedlings were then replaced in petri dishes in contact with filter papers soaked in the transfer liquid. It was not found possible to determine the quantities of D_2O or H_2O taken up in this procedure, but 60% D_2O vacuum infiltrated in this way was observed to significantly inhibit subsequent growth, suggesting that some uptake had occurred. In the excised tissue experiments 10 mm segments were cut from immediately below the plumular hook. 30 segments were vacuum infiltrated with either H_2O or 80% (v/v) D_2O floated on 10 ml of the same medium, and exposed to the above white light source for 24 hr.

Extraction of PAL. 5·0 g of hypocotyl sections, 10 mm long, from just below the plumular hook were subjected to a steel hammer press previously cooled with liq. N_2. The resulting solidified material was taken up in 0·2 ml 20 mM dithiothreitol in 0·1 M borate buffer pH 8·8 and the homogenate filtered through Whatman GF/A glass fibre paper. The filtrate was then passed through a G25 fine Sephadex column 20 × 1 cm pre-equilibrated with the extraction buffer.

PAL assays. The assay employed was similar to that of Koukol and Conn.[31] Aliquots of the protein fraction (0·4 ml), obtained from desalting through Sephadex, were added to reaction mixtures containing 0·1 μCi L-3-phenylalanine-ring 4-H3 in L-phenylalanine, normally at a concentration of 1 mM and 0·1 M borate buffer pH 8·8 in a final vol. of 4 ml. Both the molarity and the amount of radioisotope were altered in various experiments. The reaction mixture was incubated at 35° for 1 hr after which time the reaction was stopped by the addition of 0·5 ml 0·1% (w/v) cinnamic acid in 0·05 M KOH and 1 ml 20% (w/v) trichloroacetic acid. The protein precipitate was centrifuged and cinnamic acid extracted from the supernatant by adding 3 ml toluene, mixing and centrifuging, 2 ml of the upper phase being added to 10 ml of scintillation fluid (5 g 2,5-diphenyloxazole, 500 ml Triton X100, 1 l. toluene). Samples were counted in a Packard Tri-Carb Scintillation Counter with automatic quench correction. Since L-phenylalanine has a slight but detectable solubility in toluene, blanks for this method contained ^{3}H-L-phenylalanine but no enzyme.

Protein estimation. Protein was determined by the method of Lowry *et al.*[32]

Equilibrium sedimentation of protein from gherkin hypocotyls in a caesium chloride density gradient. A CsCl solution of refractive index 1·3628 (ρ^{25} = 1·307) was pipetted into a 5 ml cellulose nitrate tube. To this

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was added 0·1 ml of a crude enzyme preparation of PAL containing 1–2 mg protein and 0·1 ml of commercially obtained malate dehydrogenase. The tube was then filled to within 4 mm of the top with liquid paraffin. Centrifugation was performed using a Beckman L2-65 Preparative Ultracentrifuge with a SW 50·1 rotor. The rotor velocities and times of run are specified in the figure legends. One or two drop fractions were collected from each tube. The refractive index of every 12th fraction was measured with a Zeiss Abbe refractometer at 25° and converted to buoyant density by means of a standard equation.[33] All even numbered fractions were assayed for PAL activity whilst all odd numbered fractions were assayed for malate dehydrogenase activity.

Enzyme assay for malate dehydrogenase. Malate dehydrogenase activity was determined by the method of Ochoa.[34]

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