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Phytochrome-mediated *de Novo* Synthesis of Phenylalanine Ammonia-Lyase in Cell Suspension Cultures of Parsley[1]

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ABSTRACT

After a preirradiation with ultraviolet light, phenylalanine ammonia-lyase activity in cell suspension cultures of parsley (*Petroselinum hortense* Hoff.) is controlled by phytochrome (red/far red photoreversibility). Isopycnic CsCl density gradient centrifugation, after labeling with ^{15}N (90 atom %) under inductive and noninductive conditions, was used to investigate the mode of action of phytochrome in this response. After a 5-hour labeling period, a buoyant density shift of 0.009 kg·l^{-1} (0.7%) without band-broadening (indicating close to maximal labeling of the enzyme), was observed in irradiated cells. In dark-grown controls, the density shift was 0.004 kg·l^{-1} (0.3%), accompanied by significant band-broadening, indicating turnover of about half of the enzyme pool during 5 hours. These results are taken as evidence that phytochrome controls *de novo* synthesis of this enzyme over a background of basal turnover.

Changes in the level of extractable enzyme activities (commonly referred to as enzyme induction or repression) are well established molecular events in phytochrome-mediated photomorphogenesis of higher plants (23, 27). The molecular mechanisms responsible for these regulatory processes are currently under intensive investigation in several laboratories. A basic question to be answered is whether control over synthesis of enzyme protein (*de novo* synthesis) is involved in the phytochrome-mediated induction of enzyme activity. A powerful and convenient procedure to demonstrate enzyme *de novo* synthesis is the *in vivo* density labeling technique (8, 15, 21). The application of this method has recently provided rigorous evidence for the control of enzyme *de novo* synthesis by phytochrome in the case of ribonuclease in lupin hypocotyl (1) and of ascorbate oxidase in mustard cotyledons (2, 4).

The present investigation was undertaken to demonstrate control by phytochrome of *de novo* synthesis in PAL[2], an enzyme that has been intensively investigated in molecular photomorphogenesis (6, 23, 27). In the cotyledons of mustard seedlings, *de novo* synthesis of PAL has been deduced from experiments with protein synthesis inhibitors (25) and by density labeling with deuterium, using an extended labeling period (28). Both experimental approaches independently show that protein synthesis takes place during the experimental period. As pointed out previously (28), this result does not provide unequivocal evidence for the control by phytochrome of PAL *de novo* synthesis. Attridge *et al.* (3) recently have dismissed this concept and, on the basis of their long time (days) labeling experiments with D_2O, reached the conclusion that phytochrome control is exerted on the activation of PAL from an existing pool of inactive proenzyme molecules in mustard cotyledons.

From work of this laboratory with the mustard seedling (Acton and Schopfer, in preparation) it appears that there are several serious disadvantages in using this system, which may invite misleading interpretation of D_2O density labeling data, especially when relatively long labeling periods are used (3). Some of these disadvantages follow. (*a*) Unlabeled amino acids derived from the mobilization of storage protein (which is enhanced by phytochrome [10]) compete with labeled amino acids in protein synthesis to an unknown extent. (*b*) Severe isotopic stress by D_2O is observed, which may influence dark- and light-grown seedlings differently with respect to development of PAL. (*c*) Mustard cotyledons are highly differentiated organs containing possibly more than one pool of PAL controlled by different mechanisms (24). (*d*) PAL displays a rapid turnover in dark-grown seedlings, leading to a rapid saturation of incorporation in the unstimulated control. Since these drawbacks are difficult to circumvent, we chose a cell suspension culture labeled with ^{15}N ammonia as an experimental system that is less liable to these errors. (*a*) The parsley culture, grown on a simple medium containing inorganic N as the sole source of nitrogen is devoid of reserve protein. (*b*) ^{15}N does not exert any detectable isotopic stress. (*c*) The light-induced cells respond with a rapid increase in PAL activity within a few hours, allowing short term labeling periods (shorter than the lifetime of the enzyme). (*d*) The culture provides cells in a homogeneous state of differentiation making different pools of PAL (24) unlikely.

The regulation by white fluorescent light of flavonoid biosynthesis and related enzyme activities in parsley cell cultures is well established (12, 14). These cells incorporate ^{35}S methionine into PAL subunits after irradiation with white light (11). It can be shown that the UV part of the spectrum plays an essential role in this response to white light and that the operation of the phytochrome system (red/far red reversibility) in these cells requires a preirradiation with a small dose of UV light (30, 31).

MATERIALS AND METHODS

Chemicals. $(^{15}NH_4)_2SO_4$ (98 atom % ^{15}N) and $K^{15}NO_3$ (97 atom % ^{15}N) were supplied by the British Oxygen Company, London. CsCl (Suprapur) was obtained from Merck, Darmstadt, and pig muscle lactate dehydrogenase (EC 1.1.1.27) was obtained from Boehringer, Mannheim. All other chemicals were of highest purity available from Merck or Boehringer.

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[2] Abbreviations: PAL: phenylalanine ammonia-lyase; LDH: lactate dehydrogenase; P_{tot}: total phytochrome (Pr + Pfr).

Cell Cultures. Sterile suspension cultures of parsley (*Petroselinum hortense* Hoffm.; ref. 30) were propagated in Erlenmeyer flasks on a modified B5 medium (9), agitated by a rotary shaker at 25 C in darkness. Seven-day-old cultures (about 8 g fresh weight per 40 ml suspension) were used for all experiments.

Irradiation. Packed cells (1.5 g) (30) were transferred to irradiation vials and suspended in 15 ml of the original medium (13). The vials were continuously shaken until harvest. For red (660 nm, 0.67 $w \cdot m^{-2}$) and far red (equivalent to 718 nm monochromatic light, 3.5 $w \cdot m^{-2}$) light, standard sources were used (22). The long wavelength far red source (758 nm, 4.5 $w \cdot m^{-2}$) was a 500-w projector fitted with an AL type interference filter (Schott, Mainz). The UV source (350 nm, 1.2 $w \cdot m^{-2}$) has been described previously (31).

Density Labeling. Two hours after onset of irradiation, 1.5 ml of a sterile solution containing 38 mg of $K^{15}NO_3$ and 23 mg of $(^{15}NH_4)_2SO_4$ were added to each vial (ineffective green light). The supplement increased the original concentration of KNO_3 and $(NH_4)_2SO_4$ by a factor of 2 and 10, respectively. This application procedure ensured a maximum dilution of ^{14}N in the medium without any detectable effect on PAL activity, protein content, and growth during 5 hr (14, 30). Controls were treated in the same way with ^{14}N compounds. Because cell cultures are known to incorporate nitrate only after ammonia has been exhausted from the medium (5), the isotopic enrichment of ammonia will be essential for short term labeling experiments.

Preparation of Enzyme Extracts. Cells were harvested by filtration. Samples of 1-g packed cells were homogenized with 5 ml of Na borate buffer (0.1 M, pH 8.8) in a Sorvall Omni-Mixer for 1 min at full speed. Extracts were clarified by centrifugation at 35,000*g* for 10 min and purified from low molecular components by Sephadex G-25 chromatography (29). Samples of irradiated cells (labeled and unlabeled) and dark controls (labeled and unlabeled) were always obtained from the original culture and processed simultaneously. Enzyme extracts were stored at −20 C for several months without change of PAL properties.

Isopycnic Equilibrium Centrifugation. Crude extracts from irradiated (0.2–0.4 ml, 0.6–1.2 mg of protein) or dark-grown (0.8–2.0 ml, 2.5–6 mg of protein) cells were made 380 $g \cdot l^{-1}$ with respect to CsCl by adding solid salt. After adding 5 μg of LDH, the solution was adjusted to 5 ml with borate buffer containing CsCl (refractive index adjusted to 1.361). After mixing, the centrifuge tubes were filled with paraffin oil and centrifuged in a 30° fixed angle rotor (18) of a WKF-P50K ultracentrifuge at 40,000 rpm (maximum 150,000*g*) for 36 hr at 5 C. Gradients were fractionated into 1 drop (27 μl) fractions by inserting a hypodermic needle at the bottom of the tube and pumping paraffin on top of it. Every tenth fraction was used for refractive index (25 C) and every second to fifth fraction for LDH (density marker) determination. The position of the marker enzyme varied slightly, depending on the amount of total protein loaded on the gradient. Experiments with different protein loads showed that PAL and LDH positions on the gradient are influenced to the same extent by this experimental factor. Protein loads above about 2 mg measurably increased the bandwidth of PAL profiles (Table I), probably because of an increase in viscosity. Therefore, labeled PAL samples and the corresponding unlabeled controls were always made equal with respect to protein contents (and PAL activity), and centrifuged in the same run. This procedure greatly decreased experimental variation and granted strict comparability of samples to be compared with respect to bandwidth. Recovery of PAL activity from gradients was about 60% for all four treatments.

Analytical Methods. PAL activity in enzyme extracts was assayed spectrophotometrically at 30 C after Zucker (33). The absorbance increase ΔA_{290} was linear with time for more than 5 hr and proportional to the enzyme concentration. PAL activity in gradient fractions was assayed by adding 1 ml of substrate (20 mM phenylalanine in borate buffer) to each fraction and measuring ΔA_{290} after an incubation time of 96 hr at 25 C (28). The enzyme activity was constant for more than 5 days under these conditions and linearly related to the amount of enzyme up to $\Delta A_{290}/cm = 0.5$. LDH activity was assayed according to Kornberg (19). The refractive index was measured with an Abbé refractometer (Zeiss, Model A) at 25 C and converted to density units (17). Values are not corrected for the presence of borate. Protein was determined by the biuret method (20).

RESULTS AND DISCUSSION

Control of PAL Activity by Phytochrome. Red/far red photoreversibility of PAL induction (as well as of other enzymes of flavonoid biosynthesis [31]) in the parsley cell culture can be demonstrated only after the cells have been preirradiated with a small dose of UV light (30, 31). The photochemical role of UV light in making the system sensitive to visible light is currently under investigation. For the present investigation it is important to note that the UV irradiation is able to saturate the system with respect to Pfr in the same way as red light ($Pfr/P_{tot} = 0.8$). Subsequent irradiations with far red ($Pfr/P_{tot} \approx 0.03$) or long wavelength far red (758 nm, $Pfr/P_{tot} \approx 0.002$), which reduce the level of Pfr considerably, reverse the effect of UV light, while final red irradiation raises the PAL level to that obtained after UV light alone (Fig. 1). The 4-fold increase (after a lag phase of about 2.5 hr) over the dark level of PAL activity observed after UV (+ red) irradiation can be regarded as completely under the control of phytochrome. PAL formation in parsley cells, as is the case in mustard cotyledons (29), is very sensitive to low levels of Pfr. The data in Figure 1 prompted the following program for the labeling experiments: (*a*) 15 min

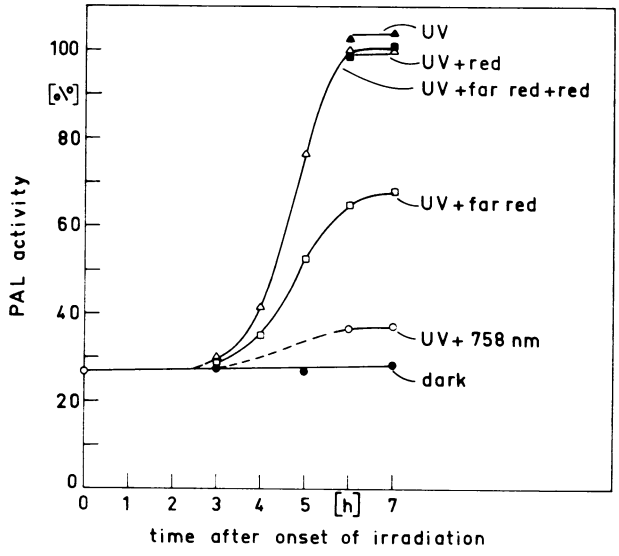

FIG. 1. Control of PAL induction in parsley cells by phytochrome after preirradiation with UV. Cells were irradiated for 15 min with UV immediately followed by 10 min of red, far red, or long wavelength far red (758 nm) light as indicated. Addition of N compounds 2 hr after the onset of irradiation had no measurable influence on the time courses of PAL activity.

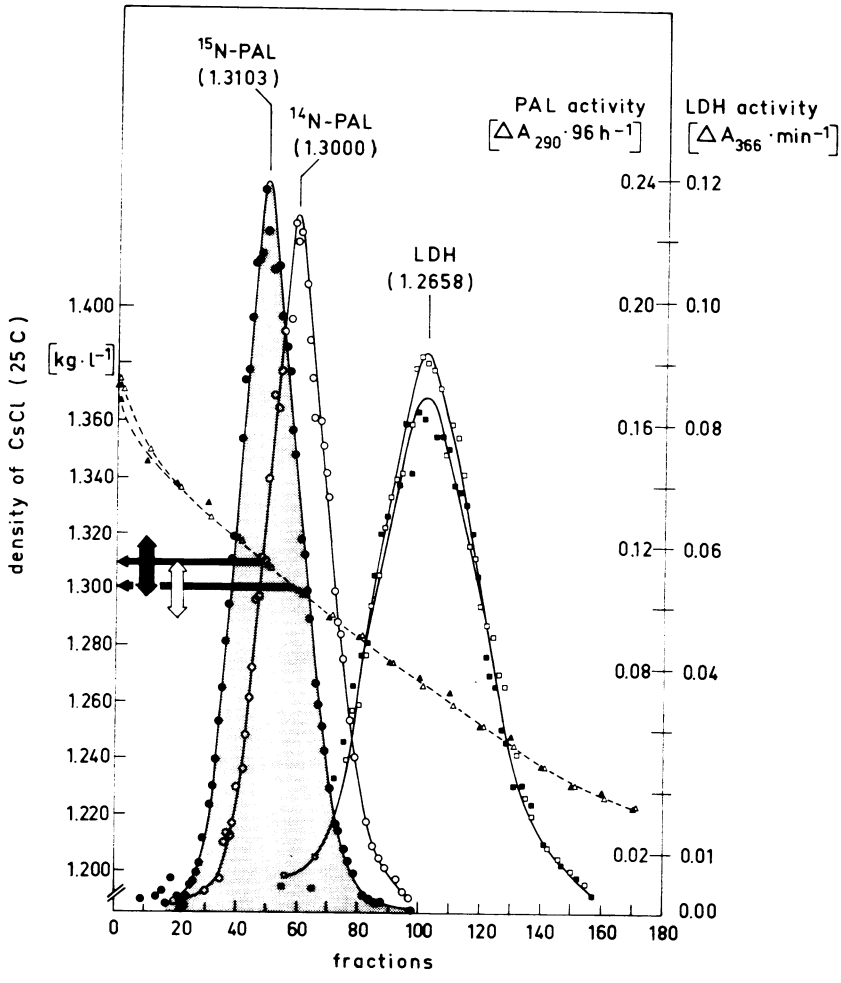

FIG. 2. Density gradient profiles of PAL extracted from irradiated parsley cells grown in the presence of ^{15}N or ^{14}N. Experimental program: 15 min UV + 10 min red + 1.5 hr darkness − addition of ^{15}N label − 5 hr darkness (^{15}N-PAL, closed symbols). Simultaneously a control culture (^{14}N-PAL, open symbols) was treated with the corresponding ^{14}N compounds. The profiles of the two tubes are superimposed on the basis of the CsCl gradients (triangles). Co-centrifuged LDH served as a density marker. Actual density values (*i.e.* unnormalized) of band centers are given in parenthesis and indicated on the CsCl density scale (left) together with the bandwidths at half peak height expressed in density units (vertical double arrows).

Table I. *Density Shift and Bandwidth Increase in PAL from Irradiated and Dark-grown Parsley Cells*

Cells were labeled with ^{15}N (means of seven independent experiments; Figs. 2 and 3). All density values (ρ^{25c} ± standard error) are normalized with respect to the density of the marker enzyme LDH (assumed value 1.2528 kg·l^{-1}; P. Quail, personal communication). Due to considerably higher protein loads of the gradients, experiments with dark-grown cells yielded significantly higher bandwidths compared to irradiated cells. However, bandwidth increases are not influenced by this systematical error.

Enzyme Preparation	Isopycnic Density	Density Increase	Bandwidth	Bandwidth Increase
	ρ [kg·l^{-1}]		$\Delta\rho$ [kg·l^{-1}]	$\Delta(\Delta\rho_i)$ [kg·l^{-1}]
Irradiated cells				
Unlabeled	1.2843 ± 0.0009	0.0093	0.0200 ± 0.0003	0.0006
Labeled	1.2936 ± 0.0009	(0.72%)	0.0206 ± 0.0003	(3 ± 3%)[1]
Dark-grown cells				
Unlabeled	1.2841 ± 0.0006	0.0041	0.0226 ± 0.0014	0.0020
Labeled	1.2882 ± 0.0005	(0.32%)	0.0246 ± 0.0011	(9 ± 2%)[1]

[1] Standard error calculated from $\Delta(\Delta\rho)$ values obtained in independent experiments.

UV + 10 min red + 6.5 hr darkness, and (*b*) addition of ^{15}N label 2 hr after onset of irradiation (end of the lag phase). There is no detectable change in fresh weight, protein content, and enzymes not involved in flavonoid biosynthesis over that period of time (14, 30).

Density Labeling of PAL in Phytochrome-induced Cells. A typical experiment conducted in this way is shown in Figure 2. From the difference in the peak position, a density shift of about 0.8% is evident. There is no significant difference in the bandwidth of the two PAL profiles, indicating that the population of PAL molecules from labeled cells (^{15}N-PAL) is essentially homogeneous with respect to the incorporation of label. Data of seven similar, independent experiments are given in Table I. The theoretical maximum of protein mass increase by ^{15}N incorporation is about 1%. Recycling of ^{14}N compounds in the cell tends to reduce this value. In tobacco cell cultures grown over 10 generations on ^{15}N (99 atom %) medium, the maximal density increase of nitrate reductase was 0.92% (32). An average density shift of 0.73% between ^{14}N-PAL and ^{15}N-PAL (Table I) obtained in the presence of 90 atom % ^{15}N can be taken as evidence for *de novo* synthesis of at least 80 to 90% of the PAL molecules present in irradiated cells at the time of harvest. The lack of a significant

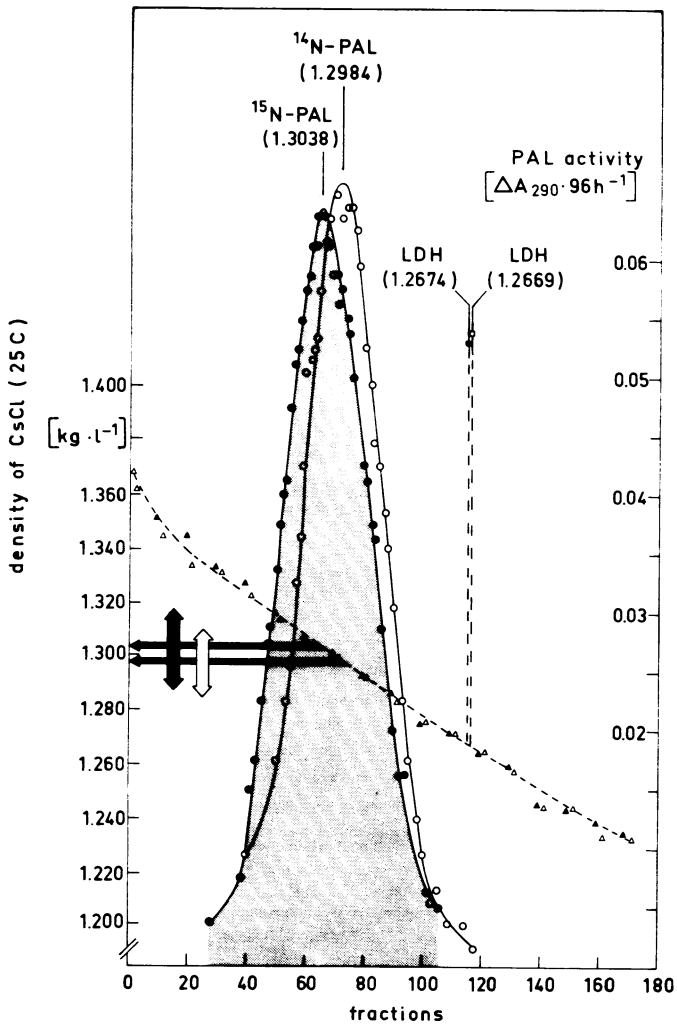

FIG. 3. Density gradient profiles of PAL extracted from non-irradiated parsley cells grown in the presence of ^{15}N or ^{14}N for 5 hr. Refer to Fig. 2 for further explanation. The LDH marker profiles are omitted for clarity.

bandwidth increase in these profiles supports this conclusion. This result clearly excludes the existence in unstimulated cells of significant amounts of preformed PAL proenzyme molecules that become activated after the light treatment (3).

Density Labeling of PAL in Dark-grown Cells. While unequivocally demonstrating synthesis of the bulk of the PAL protein from amino acids in irradiated cells, the data of Table I permit more than one hypothesis with regard to the mechanism by which phytochrome controls synthesis of this enzyme. Phytochrome may increase synthesis of PAL, inhibit the decay of PAL that is rapidly turning over in darkness, or activate a proenzyme with rapid turnover. To distinguish among these possibilities, information about the turnover of PAL in the unstimulated system is required. This can be obtained by density labeling of dark-grown cells. Figure 3 and Table I show the pertinent results. Under the assumption that the equilibration of amino acid pools with ^{15}N is not rate-limiting, the observed density shift of ^{15}N-PAL (0.32%, about half way compared to irradiated cells) indicates that only about half of the PAL pool becomes labeled during a period of 5 hr in darkness. The substantial increase in bandwidth (9%) documents heterogeneity of this enzyme population with respect to ^{15}N incorporation, indicating that the equilibration of amino acid pools with ^{15}N is indeed considerably more rapid than the enzyme turnover (1).

A model experiment with an artificial mixture (1:1 ratio) of unlabeled and homogeneously labeled PAL preparations leads to results similar to those from experiments with dark-grown cells (Fig. 4, Table II). Besides demonstrating the accuracy and reliability of the method, it shows that a half-way density shift of the PAL band in the gradient is accompanied with maximal increase of 40% in bandwidth. Incomplete labeling of molecules during the transient phase of amino acid pool equilibration with ^{15}N will lead to a reduction of this value *in vivo*. Although the data of Table I do not permit an accurate calculation of the half-life of PAL in dark-grown cells, they are certainly incompatible with half-lives shorter than about 2 hr.

Taken together, these data show that turnover of PAL in dark-grown cells is occurring. However, the turnover rate is considerably smaller than would be required by the hypotheses involving control over the rate of enzyme breakdown, or proenzyme activation as the mechanism of phytochrome control. To account for the maximal rate of PAL accumulation in phytochrome-stimulated cells (about 35% per hr), synthesis of PAL would have to be compensated by an equally high rate of destruction to yield the steady state observed in darkness (Fig. 1). This compensation through destruction would result in a half-life on the order of 20 min for PAL in dark-grown cells, a value excluded by the data of Figure 3 and Table I. The proenzyme activation hypothesis could only be reconciled with the data by making sophisticated additional assumptions (*e.g.* half-life of about 20 min only for the proenzyme but not for the enzyme which has to be "stabilized" after becoming active in darkness). We conclude, therefore, that the phytochrome-dependent increase of PAL activity shown in Figure 1 is produced by an increase of the rate of PAL *de novo* synthesis.

Our data do not permit the exclusion of the possibility that the rate of PAL destruction is increased after light stimulation. In fact, this has to be expected if the destruction reaction follows first order kinetics, as is generally assumed for enzyme inactivation (26) and has been shown for PAL in gherkin (7) and radish (16) seedlings. Ignoring this complication, and assuming 2 to 5 hr as the half life of PAL in dark-grown cells, we can calculate from Figure 1 a 5- to 10-fold increase in the rate of PAL synthesis that is induced by the light treatment. A concomitant increase in the rate of PAL destruction would necessarily make this factor even higher.

Table II. *Density Shift and Bandwidth Increase in Artificially Mixed PAL Populations*

The unlabeled and homogeneously labeled PAL were from irradiated cells; means of two experiments; density values normalized as in Table I. The expected values for the mixture were calculated from the superposition of the individual profiles (Fig. 4).

Enzyme Preparation	Isopycnic Density	Density Increase	Bandwidth	Bandwidth Increase
	ρ [kg·l^{-1}]	$\Delta\rho$ [kg·l^{-1}]		$\Delta(\Delta\rho)$ [kg·l^{-1}]
Unlabeled	1.2856	0.0000	0.021	0.000
Labeled	1.2946	0.0090	0.021	0.000
50% unlabeled + 50% labeled	1.2903	0.0047	0.024	0.003
Expected	1.2901	0.0045	0.024	0.003

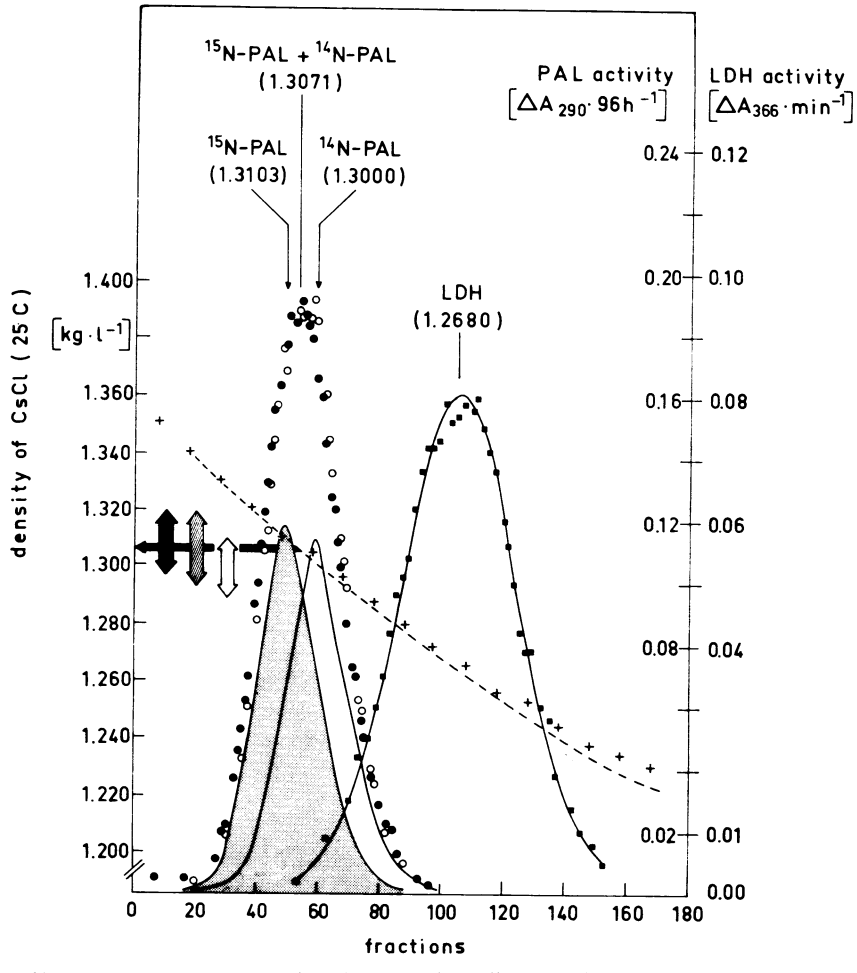

FIG. 4. Density gradient profiles of PAL extracted from irradiated parsley cells grown in the presence of ^{15}N (^{15}N-PAL, hatched peak) or ^{14}N (^{14}N-PAL), and of a 1:1 mixture prepared from the two enzyme extracts (closed symbols) and centrifuged in the same run. The open symbols matching the same profile were obtained from the sum of the two curves for the labeled and unlabeled enzyme. Refer to Fig. 2 for further explanation. The profiles of ^{15}N-PAL and ^{14}N-PAL are those of Fig. 2. (Gradient of tube with ^{15}N-PAL + ^{14}N-PAL [+++]; gradients of tubes with ^{15}N-PAL or ^{14}N-PAL [– – –]; *cf.* Fig. 2, the LDH profile refers to the gradient of mixed PAL extracts.)

CONCLUSION

Our experiments provide evidence for the control by phytochrome of PAL *de novo* synthesis in parsley cell cultures. Unambiguous results were made feasible by selection of a density label rapidly incorporated into amino acids without side effects, and a labeling period (5 hr) long enough to allow a reasonable increase (4-fold) in the induced enzyme level, but short enough to avoid complete exchange of enzyme molecules through turnover in the unstimulated control. This is a prerequisite for the conclusion that enzyme synthesis during basal turnover cannot account for the observed density increase in the stimulated cells (1). Labeling periods significantly longer than 5 hr would have made a decision with regard to the mechanism controlling this phytochome response impossible.

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