*Proc. Natl. Acad. Sci. USA*
Vol. 73, No. 11, pp. 4017–4021, November 1976
Cell Biology

# Phytochrome-mediated *de novo* synthesis of phenylalanine ammonia-lyase: An approach using pre-induced mustard seedlings

**(enzyme synthesis versus activation/density labeling)**

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*Communicated by Winslow R. Briggs, July 12, 1976*

**ABSTRACT** **The molecular mechanism of enzyme (phenylalanine ammonia-lyase; EC 4.3.1.5) formation mediated by phytochrome in mustard seedlings was investigated by labeling the enzyme with deuterium followed by high resolution CsCl density gradient analysis. A favorable relationship between induced rise of activity and turnover of this short-lived enzyme (half-life 3–4 hr) was achieved by labeling pre-irradiated seedlings. The time course of deuterium incorporation during the light-mediated rise in enzyme activity that can be derived independently from density shifts and bandwidth changes demonstrates a stimulation of synthesis and degradation by phytochrome. When synthesis is faster than degradation, enzyme accumulates.**

Labeling *in vivo* with a heavy isotope ($^{15}N$, $^{18}O$, or $^{2}H$) followed by isopycnic density gradient centrifugation (1, 2) is a powerful method for direct investigation of the mechanism of enzyme formation. In the past a displacement towards higher density of the enzyme obtained from cells supplied with label during an experimentally induced rise of enzyme activity has been generally considered as evidence for the initiation of enzyme synthesis *de novo* (3–9). In higher organisms, however, the situation is usually complicated by the occurrence of enzyme turnover (10–12) and by the existence of a significant basal amount of enzyme in the unstimulated tissue that is not necessarily related to the inducible pool (13). Dilution of label through amino acid metabolism (12) is a further limitation of the method, especially if a tissue with extensive protein turnover is being labeled with $^{2}H_2O$, a heavy isotope precursor that is useful in other respects.

In the present paper we are studying a system that is typical for enzyme formation in higher organisms, including the experimental difficulties encountered in its investigation. Light, acting through the physiologically active form of phytochrome ($P_{fr}$), is a potent effector of enzyme formation in plants (14, 15). The molecular mechanism(s) of the operationally defined (11, 14, 15) increase in assayable enzyme activities ("enzyme levels") is currently a matter of intense investigation and debate in several laboratories (16–23). In contrast to other enzymes, contradictory conclusions have been reached by different laboratories for $P_{fr}$-mediated induction of phenylalanine ammonia-lyase (Phe $NH_3$-lyase, EC 4.3.1.5), an electrophoretically homogeneous (24) key enzyme of phenylpropanoid biogenesis in plants (25).

On the basis of density labeling experiments with $^{2}H_2O$, Attridge *et al.* (21) concluded that the $P_{fr}$-mediated increase in Phe $NH_3$-lyase activity involves the activation of pre-existing enzyme protein in the cotyledons of mustard seedlings. On the other hand, Wellmann and Schopfer (19) demonstrated $P_{fr}$ control of Phe $NH_3$-lyase synthesis *de novo* in a suspension culture of parsley cells using $^{15}N$ labeling. The experiments of Attridge *et al.* (21) can be criticized on the grounds that (*i*) no suitable density marker enzyme was used, (*ii*) the labeling periods (24–48 hr) were too long for a short-lived enzyme like Phe $NH_3$-lyase (half-life 3–4 hr) to prevent saturation of labeling in both light and darkness, (*iii*) the use of a high $^{2}H_2O$ concentration (100%) for labeling 48-hr-old seedlings produced an artificial 24-hr delay of the light-mediated rise in enzyme activity, making the $^{2}H_2O$-treated tissue unrepresentative with regard to the water control, and (*iv*) determination of density shifts was not supplemented by bandwidth measurements to show the state of heterogeneity in the enzyme populations centrifuged to equilibrium on a density gradient (10, 12). Under experimental conditions avoiding these limitations, Acton and Schopfer (18) were unable to reproduce the principal results of Attridge *et al.* (21). Instead, the reinvestigation (18) revealed that maximal incorporation of $^{2}H$-labeled amino acids into Phe $NH_3$-lyase was reached after 6–12 hr of labeling in both light- and dark-grown cotyledons. Prior to this time, the light-mediated increase in enzyme activity was too small to permit the demonstration of differential labeling. Thus, while rendering the activation of a pre-existing enzyme protein or inhibition of enzyme degradation by $P_{fr}$ unlikely, these experiments led to the conclusion that rapid turnover of Phe $NH_3$-lyase prevented the detection of any light-mediated increase in the rate of enzyme synthesis if it were present. In the current paper we will show how this unfortunate situation can be overcome and present data indicating that $P_{fr}$ indeed mediates an increased rate of Phe $NH_3$-lyase synthesis *de novo* also in mustard cotyledons.

## MATERIALS AND METHODS

The experimental materials, including the batch of mustard (*Sinapis alba L.*) seeds and germination procedures, are described elsewhere (18). Far-red light (3.5 $W \cdot m^{-2}$) was obtained from a standard source establishing a photostationary state of phytochrome with a few percent $P_{fr}$ in the mustard seedling (14, 26). Light of 756 nm (7.0 $W \cdot m^{-2}$) was obtained using a Schott AL interference filter (photostationary state of phytochrome approximately 0.1% $P_{fr}$; ref. 27). Enzyme extracts were prepared from 25 pairs of cotyledons using 6 ml of borate buffer. Aliquots (1.5 ml) containing equal amounts of protein (1.5 mg) and 1–8 pkat of enzyme activity were subjected to isopycnic density gradient centrifugation in a fixed-angle rotor at 110,000 × *g* for 36 hr using β-galactosidase from *Escherichia coli* as a density marker (1.3000 $kg \cdot liter^{-1}$) (18). (A katal is the amount of activity that converts 1 mole of substrate per sec.) Unlabeled controls were always processed in parallel. Phe $NH_3$-lyase activity was assayed as described (18, 19). Density values were calculated from refractive index measurements and corrected according to the position of the marker.

Abbreviations: $P_{fr}$, physiologically active form of phytochrome; Phe $NH_3$-lyase, phenylalanine ammonia-lyase.

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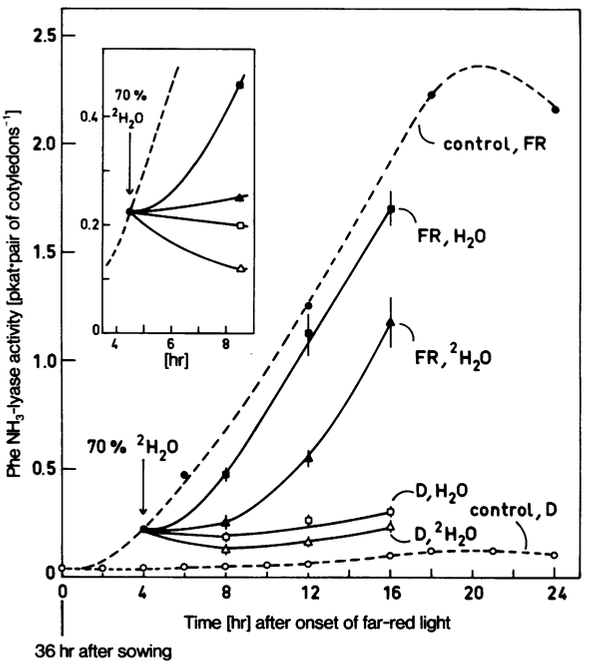


FIG. 1. Time course of the appearance of Phe $NH_3$-lyase activity in the cotyledons of mustard seedlings maintained on water or 70 atom % of $^2H_2O$ in either darkness or under far-red light. The seedlings were first irradiated with 4 hr of far-red light and then treated as follows: (a) $^2H_2O$ and far-red (FR) light (▲ — ▲), (b) water and far-red light (■ — ■), (c) 5 min of 756-nm light followed by $^2H_2O$ and darkness (D) (△ — △), (d) 5 min of 756-nm light followed by water and darkness (□ — □). Enzyme appearance in unincubated control seedlings in darkness (○ - - ○) and under far-red irradiation from 36 hr after sowing (● - - ●) are included for comparison (means of seven independent experiments). The inset shows the first 4 hr of labeling on an enlarged scale.

## RESULTS

### Timing of the light response in $^2H_2O$ and water

As in related investigations (8, 18, 21), continuous irradiation with far-red light was used to produce a sizable enzyme increase within a short period of time. This light quality is generally considered to act exclusively via phytochrome (26). To achieve a favorable relationship between the minimum length of the labeling period (determined by the rapidity and size of the response) and the extent of labeling by basal turnover (determined by the half-life of the enzyme), we adopted the following experimental program (Fig. 1): 36-hr-old dark-grown seedlings were irradiated for 4 hr with far-red light. This pre-irradiation permanently overcomes the lag phase of Phe $NH_3$-lyase induction (26) and raises the enzyme level to about six times the dark level. Pre-irradiated seedlings were transferred to 70 atom % of $^2H_2O$ (or water) and returned to far-red light for various lengths of time. For dark controls, pre-irradiated seedlings were first treated with a saturating (5 min) pulse of 756-nm light to convert nearly all of the remaining $P_{fr}$ back to $P_r$, the inactive form of phytochrome (19), and then transferred to $^2H_2O$ (or water) and placed in darkness. It has been shown (26) that the increase of enzyme activity rapidly ceases after $P_{fr}$ has been eliminated from the tissue. Fig. 1 shows that the increase of enzyme activity in $^2H_2O$-treated seedlings resumes the rate of the water controls after about 3 hr in far-red light. In seedlings transferred to darkness (after removal of $P_{fr}$) the enzyme level remains essentially constant. The lower level in the presence of $^2H_2O$ may be in part due to a reduction by $^2H_2O$ of the transient aftereffect of $P_{fr}$ on enzyme formation (28) since a similar inhibition cannot be observed in seedlings that are not pre-irradiated (18). Since $^2H_2O$ reduces Phe $NH_3$-lyase in irradiated and dark-grown seedlings to the same extent, it can be assumed that the relative isotopic effect exerted by $^2H_2O$ is similar in both cases.

The essential advantages of this irradiation program for triggering the response are the following: (*i*) although there is some unavoidable inhibition in the presence of $^2H_2O$ (affecting the system quantitatively similarly in light and darkness), an experimentally exploitable (about 100%) difference of enzyme activity between light and darkness is achieved after only 4 hr of incubation. This is not possible if the lag phase is included in the labeling period (18). Because of the increase of Phe $NH_3$-lyase activity by light in the water control and since $^2H_2O$ is generally known to slow down metabolic processes, this difference is most likely due to an increased appearance of enzyme activity in far-red light in the presence of $^2H_2O$. (*ii*) The enzyme of the dark control seedlings is largely of the light-induced type. Therefore, essentially the same pool of enzyme molecules is labeled in both light and darkness and the difficulties arising from the possibility of different pools of Phe $NH_3$-lyase in induced and noninduced seedlings (13) can be avoided.

### Kinetics of deuterium incorporation in light and darkness

Under steady-state conditions with respect to metabolic utilization of $^2H$, net labeling of a turning-over population of enzyme molecules with deuterium from externally supplied $^2H_2O$ can be expected to proceed until a saturation level is reached (physiological saturation). This level is generally considerably lower than the theoretical saturation calculated on the basis of the exchange of all carbon-bound hydrogen atoms in the protein. Since $^2H_2O$ rapidly exchanges with the water of the tissue, this discrepancy must be largely due to an extensive use in protein synthesis of unlabeled metabolites that are derived more or less directly from storage compounds. In mustard cotyledons large amounts of storage protein and fat are mobilized during the first few days of seedling development. Consequently the maximum density shift of Phe $NH_3$-lyase obtainable by labeling the enzyme with 80 atom % of $^2H_2O$ from inhibition of the seeds corresponds to not more than about 20% of the theoretical maximum amount of label in both darkness and far-red light (8, 18). Since the physiological saturation can be expected to be a function of seedling age, light treatment, isotopic stress, etc., density shifts measured after long labeling periods are not adequate for characterization of the dynamics of a population of enzyme molecules with rapid turnover and can lead to puzzling results (21).

In addition to the production of a mean density increase, the introduction of labeled molecules into an unlabeled enzyme population results in the appearance of heterogeneity with respect to density, which transiently produces an increased bandwidth of the enzyme profile on an isopycnic density gradient. Although the *size* of this bandwidth change again depends on the physiological saturation level of labeling, the *kinetics* of this effect show the balance of enzyme synthesis and degradation (provided the labeling of the amino acid pools is rapid compared to enzyme synthesis). If enzyme synthesis is increased by a stimulus, one should observe a faster progression through a state of increased profile bandwidth compared to the unstimulated control. On the other hand, if the stimulus preferentially retards enzyme degradation or activates a preformed (unlabeled) protein precursor, the outcome should be a slower progression through a state of increased profile bandwidth compared to the unstimulated control (18).

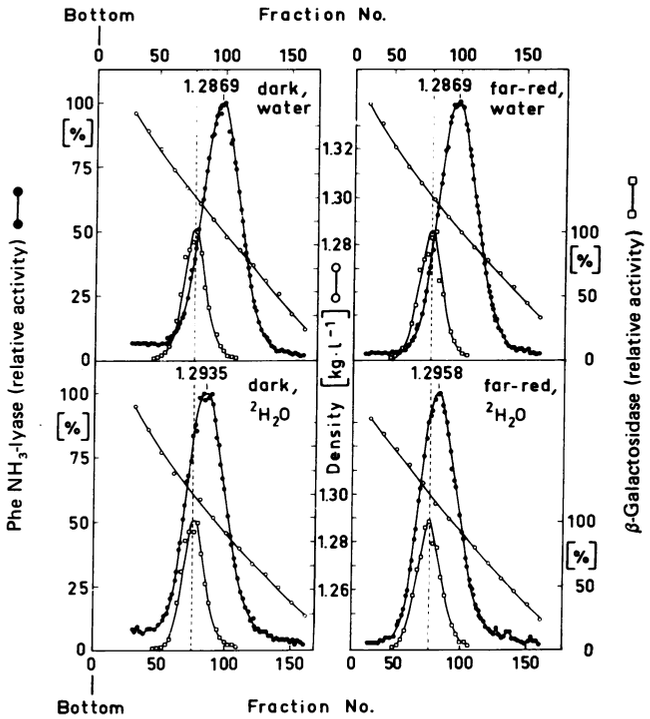


FIG. 2. Activity profiles of isopynically banded Phe $NH_3$-lyase (●) extracted from cotyledons of pre-irradiated mustard seedlings incubated for 8 hr on $^2H_2O$ or water in far-red light or darkness (see Fig. 1). (□) β-Galactosidase (density marker); (○) density of CsCl gradient (kg·liter$^{-1}$).

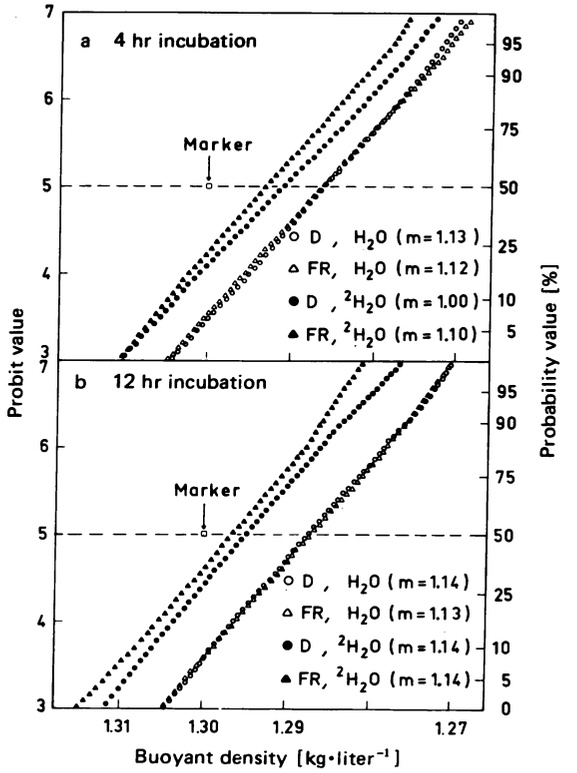


FIG. 3. Activity profiles of isopycnically banded Phe $NH_3$-lyase after 4 hr (a) and 12 hr (b) of labeling (see Fig. 1) converted into straight lines by probit transformation. D, darkness; FR, far-red irradiation. The steepness (probits·liter·kg$^{-1}$·10$^{-2}$) of the lines is indicated in parentheses.

With these considerations in mind we analyzed the kinetics of the density shift and the bandwidth change caused by 70 atom % of $^2H_2O$ in cotyledons of pre-irradiated seedlings over a period of 12 hr in far-red light and darkness. Enzyme profiles of a typical set of experiments are shown in Fig. 2. Due to the nonlinearity of the CsCl gradient established by centrifugation in a fixed-angle rotor, the profiles display some asymmetry which can be removed by plotting enzyme activity against CsCl density rather than against fraction number. Displacement and width of bands can be most accurately determined from a probit plot of the change in profile area (12). By this plot a gaussian distribution is converted into a straight line with an intersection at probit 5 (50 % area, equal to the peak position) and a steepness that is related to the bandwidth. Typical sets of data documented in this way are shown in Fig. 3. An increased bandwidth (see Fig. 3a, $^2H_2O$, darkness) of an enzyme profile compared to the water control is indicated by a reduction in the steepness of the linearized distribution. Density shifts and bandwidth changes during the course of light induction of Phe $NH_3$-lyase activity in pre-irradiated seedlings, together with the dark controls, are compiled in Table 1. In Fig. 4 the kinetics of these changes are plotted on a relative scale. In agreement with earlier results (18), labeling of the enzyme approaches the physiological saturation level available under the particular conditions of our experiments after about 12 hr of incubation in both light and darkness. At this point in time the period of increased bandwidth is surpassed under both regimes, indicating the exchange of virtually all unlabeled enzyme molecules by newly synthesized ones. In accordance with the time course of the average density increase (density shift, Fig. 4a) there is a significantly enhanced progression of the enzyme population through a state of maximal heterogeneity (maximal bandwidth) in far-red light (Fig. 4b).

## DISCUSSION

Theoretically, the labeling of a protein by $^2H$ from $^2H_2O$ can be limited by its rate of turnover or the rate of $^2H$ incorporation into relevant amino acid pools. Only if amino acid labeling is significantly more rapid than protein turnover, heterogeneously labeled protein molecules will be detectable by the bandwidth test. Thus, while the time course of the density shift (Fig. 4a) alone permits no decision with respect to the rate-limiting (i.e., controlling) step in Phe $NH_3$-lyase labeling, the time course of the bandwidth increase (Fig. 4b) clearly shows that the active amino acid pools are equilibrated with label at a significantly faster rate than enzyme is synthesized and degraded in both irradiated and dark-grown cotyledons. Furthermore, the maximal bandwidth increase observed in dark-grown seedlings approaches 70% of the theoretical maximum (which can be calculated from a superposition of density profiles of fully labeled and unlabeled Phe $NH_3$-lyase). This, and the identical saturation level in Fig. 4a, is strong evidence against a significantly lower deuteration of the relevant amino acid pools in darkness compared to light. Results obtained with an internal marker enzyme (17, 21) are consistent with this view. On the basis of these arguments we reject the hypothesis that a light-dependent difference in amino acid labeling is responsible for the increased labeling of Phe $NH_3$-lyase in the light. The steady-state level of Phe $NH_3$-lyase in darkness (Fig. 1) can be described by $d[E]/dt = k_s - k_d[E] = 0$ ([E], enzyme level; $k_s$, zero order rate constant for synthesis; $k_d$, first order rate constant for degradation; ref. 29). It follows from this equation, the application of which in the present case is justified by the demonstration of first order kinetics of Phe $NH_3$-lyase degradation (unpublished results), that any increase of $k_s$ will necessarily lead

Table 1. Effect of far-red light on incorporation of $^2H$ into Phe $NH_3$-lyase (increase of mean density and bandwidth of isopycnically banded enzyme) in mustard cotyledons

| Experimental treatment 36 hr D + 4 hr FR *plus* | Density (kg·liter$^{-1}$, 25°C) | Density increase | Bandwidth increase | |
|---|---|---|---|---|
| | | | Phe $NH_3$-lyase | Galactosidase |
| 5 min 756 nm + 1.5 hr D | 1.2854[a] | 0.0011 ± 0.0003 | 0.0000 ± 0.0006 | 0.0002 ± 0.0005 |
| | 1.2865[b] | (0.09%) | (0%) | (1.4%) |
| 1.5 hr FR | 1.2849[a] | 0.0031 ± 0.0002 | 0.0023 ± 0.0007 | −0.0003 ± 0.0002 |
| | 1.2880[b] | (0.24%) | (10%) | (−2.1%) |
| 5 min 756 nm + 4 hr D | 1.2861[a] | 0.0050 ± 0.0003 | 0.0031 ± 0.0003 | 0.0001 ± 0.0003 |
| | 1.2911[b] | (0.39%) | (14%) | (0.8%) |
| 4 hr FR | 1.2860[a] | 0.0071 ± 0.0004 | 0.0013 ± 0.0004 | 0.0001 ± 0.0004 |
| | 1.2931[b] | (0.55%) | (6%) | (0.7%) |
| 5 min 756 nm + 8 hr D | 1.2873[a] | 0.0066 ± 0.0003 | 0.0015 ± 0.0004 | 0.0004 ± 0.0004 |
| | 1.2939[b] | (0.51%) | (7%) | (3.2%) |
| 8 hr FR | 1.2867[a] | 0.0090 ± 0.0004 | 0.0001 ± 0.0002 | −0.0001 ± 0.0007 |
| | 1.2957[b] | (0.70%) | (0.5%) | (−0.7%) |
| 5 min 756 nm + 12 hr D | 1.2868[a] | 0.0094 ± 0.0006 | −0.0003 ± 0.0002 | 0.0001 ± 0.0004 |
| | 1.2962[b] | (0.73%) | (−1.3%) | (0.7%) |
| 12 hr FR | 1.2864[a] | 0.0103 ± 0.0003 | −0.0001 ± 0.0002 | −0.0003 ± 0.0005 |
| | 1.2967[b] | (0.80%) | (−0.5%) | (−2.1%) |

The bandwidth was calculated from the density increment at half peak height. Apparent bandwidth changes of the marker galactosidase are included to demonstrate the accuracy of the method. Due to small systematical errors the absolute density values are less accurate than the differences calculated from gradients of the same run (internally controlled experiments). Means of four to five experiments ± standard error. D, darkness; FR, far-red irradiation.

[a] $H_2O$.

[b] $^2H_2O$.

to an increase of the enzyme level and consequently the rate of degradation, $k_d$[E]. (The rate of synthesis would not be affected in this way by a change of $k_d$.) In accordance with this prediction, the time for half labeling of the enzyme can be estimated to be about 2 and 4 hr in light and darkness, respectively (Fig. 4). Therefore, we conclude that phytochrome increases the rate of synthesis *de novo*, which is consequently accompanied by an increased rate of degradation. The induced rise of Phe $NH_3$-lyase activity has to be attributed to the fact that synthesis greatly exceeds degradation. For theoretical reasons, one cannot exclude the existence of modification ("activation") steps in the sequence of events leading from the completed polypeptide chains of Phe $NH_3$-lyase subunits to the active tetrameric enzyme which contains dehydroalanine in its prosthetic group (30). It can be concluded, however, that post-translational steps of enzyme processing are obviously not rate-limiting for the appearance of enzyme activity in light-stimulated mustard seedlings or parsley cells (19). While there appears to be no experimental approach to eliminate the assumption that phytochrome simultaneously mediates both an increase of enzyme polypeptide synthesis and the capacity of a subsequently operating activation mechanism (or similar complex mechanisms; ref. 31), the conclusion can be drawn unequivocally that phytochrome-mediated Phe $NH_3$-lyase accumulation involves a stimulation of synthesis *de novo* of enzyme protein.

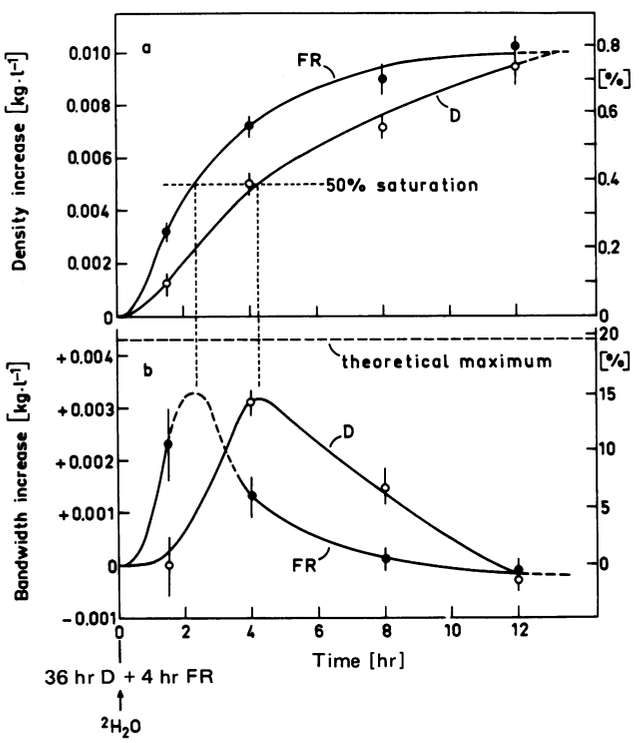


FIG. 4. Kinetics of density labeling of Phe $NH_3$-lyase in far-red light (FR) and darkness (D). (a) Band shift; (b) bandwidth change (data of Table 1, the bars represent standard errors). The dashed lines indicate half saturation of labeling in light (about 2 hr) and darkness (about 4 hr). It is assumed that maximal bandbroadening in FR is reached after half saturation of labeling indicated in the upper graph.

This work was supported by Deutsche Forschungsgemeinschaft (SFB 46) and Deutscher Akademischer Austauschdienst.

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