

Red and Far Red Effects on Phenylalanine Ammonia-Lyase in *Raphanus* and *Sinapis* Seedlings Do Not Correlate with Phytochrome Spectrophotometry¹

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ABSTRACT

In seedlings of *Raphanus sativus* (radish) and *Sinapis alba* (mustard), irradiation for 6 hours with far red light significantly increases the extractable activity of phenylalanine ammonia-lyase by the end of the light period. A schedule of 10 minutes red light-110 minutes darkness-10 minutes red-110 minutes darkness-10 minutes red-110 minutes darkness has no effect as compared to dark controls. However, the red light program maintains a level of far red-absorbing phytochrome always measurable by *in vivo* spectrophotometry during the 6-hour experimental period. We conclude that the far red effect on this enzyme and for this specific material cannot be explained solely by formation and maintenance of far red-absorbing phytochrome.

The idea that the photomorphogenetic effects of prolonged exposures to far red light result from the maintenance of a low but effective level of Pfr³ during the entire exposure has been formulated in several versions (*e.g.*, 8, 17). It also figures prominently in several recent papers either as an explicit postulate or an implicit assumption (2, 14). The work to be described represents a test of the basic form of this hypothesis.

Continuous irradiation with far red light increases the extractable activity of the enzyme phenylalanine ammonia-lyase (EC 4.3.1.5) in seedlings of mustard (13) and radish (1). In both cases this effect was ascribed to the formation and maintenance of a low level of Pfr according to the model worked out by Hartmann (8) for lettuce photoresponses. Both direct spectrophotometric determinations (5, 10) and results of simultaneous irradiations with two wavelengths (9) show that Pfr is formed in seedlings upon far red irradiation. It is very likely that the same thing occurs in the case of radish and mustard, but the presence of Pfr in tissues is not *per se* a proof of relation with the photoresponse.

If far red light acts on PAL activity by maintaining a low level of Pfr, it should be possible to bring about the same effect

with a different light quality programmed in such a way that a Pfr concentration similar to or higher than under far red light is established during the period in question. In this paper we compare the effect of continuous far red light and repeated brief red light exposures on PAL activity and on the formation of Pfr detectable by *in vivo* spectrophotometry.

MATERIALS AND METHODS

Plant Materials. Seeds of *Raphanus sativus* L. Cherry belle and of *Sinapis alba* L. Fine white were purchased, respectively, from W. Atlee Burpee Co., Philadelphia, Pa., and from Thompson and Morgan, Ipswich Ltd., England. Seeds for a complete series of experiments were stored at about 4 C. The seeds were washed with distilled water and sown in plastic boxes (30 × 20 × 10 cm) on one layer of cellulose and two layers of filter paper moistened with 100 ml of distilled water. The boxes were sealed with transparent plastic foil and placed in a constant temperature darkroom (25-26 C).

Light Sources. The dim green safelight was as described by Hillman (10). The red and far red sources were as described by Fox and Hillman (7). Whole seedlings in intact boxes were exposed to light. Temperature during all treatments was 25 to 26 C.

Phytochrome Assay. At selected times irradiated seedlings and dark controls were removed from boxes and dissected under the safelight. We used 20 hypocotyls (with hook) of radish and 50 whole seedlings of mustard for each determination. Phytochrome concentrations were measured in a two-wavelength difference spectrophotometer (Ratiospect R-2) as described by Hillman (10), using aluminum cells with 13- and 6-mm internal bore for mustard and radish, respectively. In both cases measuring beams of 735 and 800 nm were used to eliminate interference from the absorption of chlorophylls (12). All measurements of total phytochrome were divided by 0.8 to correct for incomplete conversion of Pr to Pfr by red light (4).

PAL Extraction and Assay. Forty radish hypocotyls (250-350 mg fresh weight) or 20 whole seedlings of mustard (460-510 mg fresh weight) were homogenized at 4 C in a mortar with 0.1 M borate buffer, pH 8.8, and quartz sand. After centrifugation at 20,000g for 30 min, PAL activity was measured spectrophotometrically (18) in the supernatant fluid. An appropriate amount of the enzyme solution, 200 mmoles of borate buffer, pH 8.8, 60 mmoles of L-phenylalanine, and distilled water for a total volume of 3 ml were mixed in a 1-cm path cuvette. Increase in absorbance at 290 nm against controls without phenylalanine was recorded with a Gilford spectrophotometer at 30 C for radish and 25 C for mustard. After an initial period this increase was linear for at least 2 hr

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³ Abbreviations: Pr and Pfr: the red- and far red-absorbing forms of phytochrome; PAL: phenylalanine ammonia-lyase.

and proportional to the volume of extract. An enzyme unit is that amount of enzyme producing an increase in absorbance at 290 nm of 0.001 in 100 min under the above conditions.

RESULTS

Experiments with Radish. Irradiation of seedlings started 48 hr after sowing, when the separation of hypocotyls from roots and cotyledons was very easy. PAL activity increased slightly but not significantly in the dark during the experimental period (Fig. 1); 6 hr of far red irradiation brought about a significant increase as compared to dark controls. Irradiation with 10 min of red light was without effect on enzyme activity after either 2 or 6 hr of darkness. Repeated 10-min red light exposures separated by about 2 hr of darkness were also ineffective, even if activity is expressed on a fresh weight basis.

Figure 2 shows the kinetics of phytochrome transformations for the radish hypocotyls. Total phytochrome level underwent no change in seedlings kept in darkness or under continuous far red light. Repeated 10-min red light exposures alternated with darkness caused a decrease in total phytochrome, and at the end of the treatment only 14% of the total initially photo-reversible phytochrome remained; Pfr was always measurable. Since the amount of Pfr in samples was determined from the change in absorbance following an initial exposure to actinic far red light, it is clear that the Pfr in hypocotyls so treated was never below the concentration of Pfr under far red light. The precise amounts are less easy to estimate. Hillman (10) measured the photostationary state of phytochrome in *Pisum* under a similar source of far red light. About 4% of the corrected total phytochrome was present as Pfr. Because of the

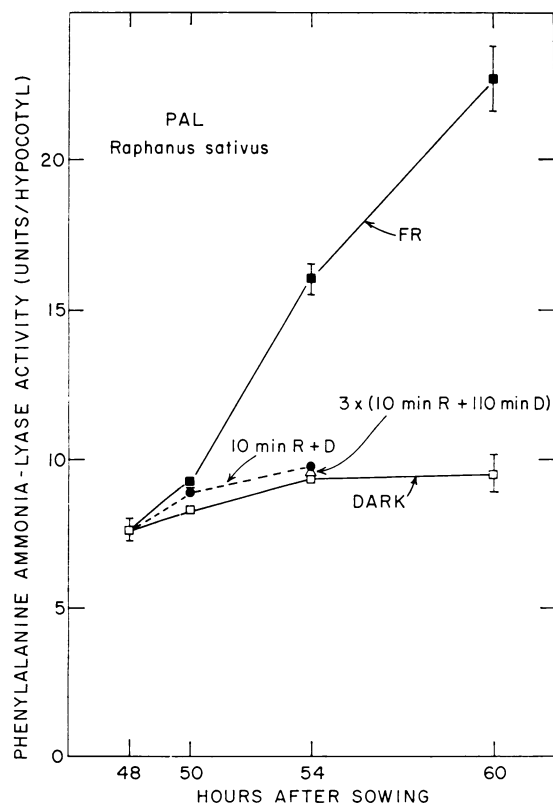


FIG. 1. Effects of continuous far red light or brief exposures to red light on the phenylalanine ammonia-lyase activity of radish hypocotyls. Each point represents the mean of five or six independent experiments; each determination was made on extracts from 40 hypocotyls. Brackets are equal to twice the standard error.

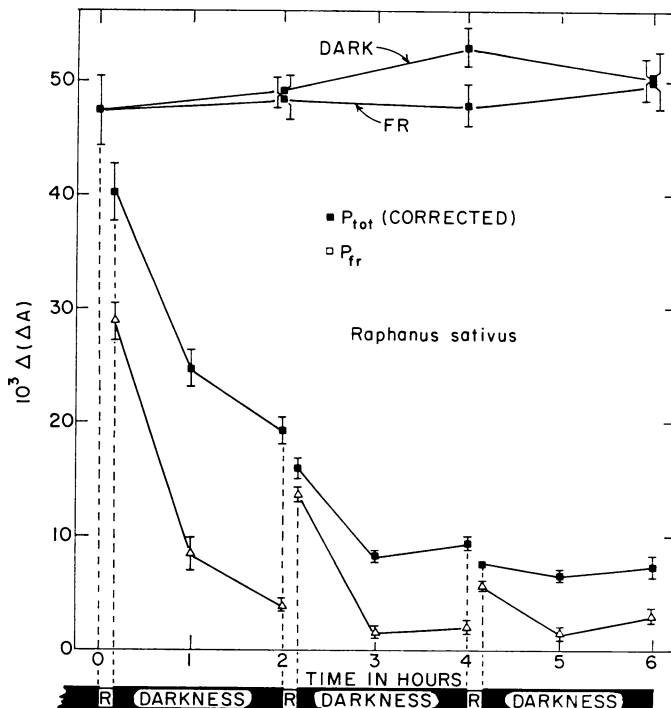


FIG. 2. Phytochrome transformations in radish hypocotyls after repeated exposures to 10 min of red light. Upper curves represent total phytochrome in hypocotyls grown in darkness or continuously irradiated with far red light; temperature was 25 to 26 C; first irradiation was started 48 hr after sowing. Pfr was measured after an initial exposure to far red light; P_{tot} was calculated by dividing the $\Delta(\Delta A)$ readings by 0.8 to account for incomplete conversion of Pr to Pfr by red light. Each point is the mean of six independent experiments; each determination was made on samples of 20 hypocotyls. Brackets are equal to twice the standard error.

Table I. Effects of Continuous Far Red Light or Repeated Brief Exposures of Red Light on Phenylalanine Ammonia-Lyase Activity of Whole Mustard Seedlings

Means and standard errors of five independent experiments; each determination was made on extract from 20 seedlings. D means darkness.

Treatment of Seedlings after Sowing	Enzyme Activity
36 hr D	units/seedling \pm SE
36 hr D + 6 hr D	29.3 \pm 1.2
36 hr D + 6 hr far red	51.3 \pm 1.6
36 hr D + 3 \times (10 min red + 110 min D)	76.2 \pm 2.1
	58.1 \pm 2.4

lower (about one-third) content of phytochrome in our hypocotyls, we cannot measure any significant value of Pfr under far red light. If we assume that this source also causes the formation of roughly 4% Pfr, the minimal Pfr concentration (as percentage of the total phytochrome in the corresponding dark control) under repeated light exposures is about 6.7%.

Experiments with Mustard. Similar studies were undertaken with mustard seedlings, in which PAL has been extensively studied with regard to phytochrome action (13). The effects of two kinds of irradiation programs on seedlings 36 hr old are reported in Table I. Enzyme activity extracted from the whole seedling after 6 hr of continuous far red light is significantly higher than in the corresponding dark controls. The

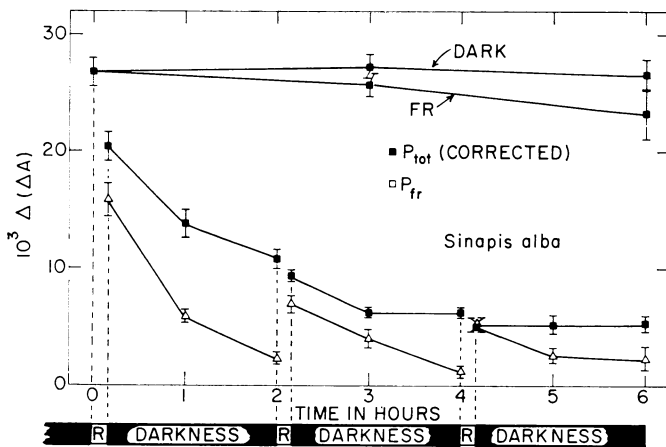


FIG. 3. Phytochrome transformations in whole seedlings of mustard after repeated exposures to 10 min of red light. Upper curves represent total phytochrome in seedlings grown in darkness or continuously irradiated with far red light; the first irradiation was started 36 hr after sowing and each determination was made on samples of 50 whole seedlings. Otherwise as in Figure 2.

effect of repeated red light is not significant, even on a fresh weight basis.

Figure 3 shows the kinetics of phytochrome transformations in whole seedlings. In dark-grown seedlings the total phytochrome is nearly constant; a little decay is observed in far red-irradiated plants. In mustard irradiated three times with 10-min red light during 6 hr of darkness, destruction appears to be slower than in radish and about 20% of total phytochrome remains at the end of the experiment. The Pfr concentration is always measurable and the minimum observed (corrected as for radish) is about 7.7% of total.

DISCUSSION

As a test of the basic hypothesis outlined, our results seem clear: the promotion of PAL activity by prolonged exposure to far red light cannot be mediated by Pfr alone, since a red light program maintaining an equivalent or higher Pfr concentration during the same period of exposure is ineffective.

The results of an induction-reversion experiment for mustard PAL (13), indicating phytochrome control, are in apparent contrast with our findings. However, because of the sensitivity of the enzyme in question to many factors, even hormones (references in Ref. 1), this reported phytochrome effect may be an indirect one, and distinct from the far red effect here. This hypothesis is supported by the fact that four 5-min red light exposures evenly distributed over 24 hr were required for enzyme induction (13).

It is likely that the far red effect on PAL in *Cucumis sativus* seedlings is similar to that described for mustard and radish. Engelsma (6) could obtain no indication of an effect on PAL activity as measured after 10 min of red light plus 3 hr of darkness, although after 3 hr of continuous far red light activity increases about six times over the dark controls. Moreover, Hillman and Purves (11), working with another source of *Cucumis* and at a slightly higher temperature, showed that Pfr is formed in hypocotyls after 10 min of red light and decreases to about 10% of the initial content after 4 hr of darkness.

The conclusion that Pfr maintenance cannot account for our data involves the assumption that the phytochrome pools under far red light and in darkness after red light are qualitatively the same, differing only with respect to the percentage of Pfr. This assumption is absent from some formulations of the Pfr

maintenance hypothesis (e.g., 17) but is integral to the version most commonly encountered (cf. 2, 14, 16). A major variant, suggesting that the far red effects might "result from oscillation excitation of Pfr under conditions of competitive self-inhibition"—that is, from an excited state of Pfr which is more active than Pfr itself, though in the same manner—has been proposed specifically for materials in which Pfr destruction is absent or saturates at a low ratio of Pfr to total phytochrome (8). Thus it does not seem relevant here and at any rate does not figure in the other works cited. A related but distinguishable possibility, that the intermediates arising from phytochrome phototransformation (3) are involved, has been specifically rejected (9) although on grounds indicating merely that Pfr is required for the effects to occur, and not necessarily that it is the photoreceptor.

Recently a photosynthetic involvement in the photomorphogenic effect of far red light has been supported by inhibitor data (15); there are also data on the photosynthetic requirement for PAL induction in *Xanthium* leaves (18). Thus, the far red effect on radish and mustard PAL might well involve photoreceptors related either to the phytochrome intermediates or to the photosynthetic system. Our data provide no evidence for or against either view, nor is it our intention to propose an additional hypothesis. The point is simply that the effect of far red light described in this paper cannot be explained by the formation and maintenance of Pfr alone, at least, if by Pfr one means the far red-absorbing form of phytochrome detectable by *in vivo* spectrophotometry. Alternatively, to maintain the view that Pfr alone does indeed account for these results, one is forced to conclude again (10) that the spectrophotometric method does not provide information on the active fraction of the total phytochrome population.

Remarks on Phytochrome Transformations. A comparison between the phytochrome kinetics in mustard and radish is not possible because we used whole seedlings in the first case and hypocotyls in the second one. However, in both materials the phytochrome seems particularly stable after the third irradiation with red light (Figs. 2 and 3). No destruction is observable in darkness during the last 2 hr, while reversion persists. The same result was evident in samples (cotyledons plus hooks) from 60-hr-old mustard seedlings irradiated with the same program. These findings, although perhaps quantitatively questionable because of the very low ΔA and the unknown role of phytochrome synthesis, are comparable to those reported for phytochrome stability and dark reversion in cauliflower heads (4).

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