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TRITIUM LABELLING OF THE AMINO ACIDS OF *SINAPIS ALBA* IS IDENTICAL IN DARKNESS AND FAR-RED LIGHT

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Key Word Index—*Sinapis alba*; Cruciferae; mustard; phytochrome; amino acids; tritium labelling.

Abstract—Earlier reports that irradiation for 3 hr with continuous far-red light stimulated the incorporation of deuterium from deuterium oxide into the free amino acids of the cotyledons of *Sinapis alba* have not been confirmed by studies using tritium oxide. Possible explanations of the discrepancy are discussed.

INTRODUCTION

Studies on the mechanism of the UV or fungal elicitor-induced rise in phenyalanine ammonia lyase (PAL) (EC 4.3.1.5) in cultured parsley cells have produced results which are entirely consistent with synthesis *de novo* of the enzyme [1]. However, there is controversy as to whether the phytochrome-mediated increase in the activity of PAL in the cotyledons of *Sinapis alba* is due to activation [2, 3] or synthesis *de novo* [4, 5].

Attridge *et al.* [2] reported that PAL, extracted from the cotyledons of *Sinapis alba* treated with deuterium oxide and irradiated with continuous far red light, was density labelled to a lesser extent than PAL from cotyledons of seedlings treated with deuterium oxide in the dark. The authors argued that this result could not be explained by synthesis of the enzyme *de novo*, but was probably due to activation of an inactive form of PAL. Acton and Schopfer [4], estimated the half-life of PAL in mustard cotyledons as 3–4 hr and criticised Attridge *et al.* [2] on the grounds that labelling over $\geqslant 6$ enzyme half-lives can hardly provide meaningful results. Subsequently Tong and Schopfer [5] used seedlings of *Sinapis alba* in which PAL had been pre-induced by far red light, and observed more rapid density shifts in PAL extracted from the cotyledons of far-red light treated seedlings, than from seedlings grown in the dark. They interpreted their results as far-red light stimulated turnover of PAL in which the stimulation of synthesis was greater than the stimulation of degradation.

The apparent contradictions in these three publications are readily attributed to the theoretical and technical difficulties which are inherent in the density labelling method, as discussed for example by Johnson [6]. However, the report [3] that continuous far-red light, acting through phytochrome, stimulates the rate of incorporation of deuterium from deuterium oxide into the *free* amino acids of the cotyledons of *Sinapis alba* questions the validity of using density labelling for the study of far-red light stimulated enzyme synthesis.

In this paper we examine the effect of far-red light on the metabolism of amino acids, paying particular attention to the incorporation of ^3H from ^3H_2O into the total and individual amino acids of *Sinapis alba*. We used conditions essentially similar to those employed by Johnson and Smith [3] who reported that the greatest difference between deuterium labelling of free amino acids from dark and far-red light treated cotyledons occured after 3 hr of irradiation.

RESULTS

Before studying the effect of far-red light on the incorporation of tritium into amino acids, it was necessary to establish that the light conditions employed were adequate for the photoinduction of PAL. The spectral irradiance at seedling height is shown in Fig. 1a and the effect of the far-red light on the activity of PAL in the cotyledons is shown in Fig. 1b. These results agree closely with those of Acton and Schopfer [4] and show that the batch of seeds that we used responds in a similar manner to far-red light with regard to PAL activation.

Table 1 shows normalized results from 13 experiments using different levels of radioactivity in the incubation mixture and different volumes of water for germination, in which we measured tritium incorporation into non-volatile compounds in the ammonia eluate from the cation exchange column. Table 1 also shows results from two experiments where the ammonia eluate was subjected to amino acid analysis. We observed no significant differences in the specific activity of the free amino acid pool, or in the tritium incorporation into compounds in the ammonia eluate, which would correspond to free amino acids plus ethanolamine and the unknown radioactive peak observed on amino acid analysis.

It should be noted that the fraction identified as soluble amino acids by Johnson and Smith [3] was in fact an aqueous extract of the seedlings from which most apolar molecules had been removed by treatment with organic solvents. The amino acid fraction used in our experiments was purified by ion exchange chromatography from aqueous extracts prepared according to Johnson and Smith.

To facilitate the comparison between our results and those of Johnson and Smith [3] we have also measured the incorporation of tritium into the total aqueous extract and those compounds not positively charged at acid pH.

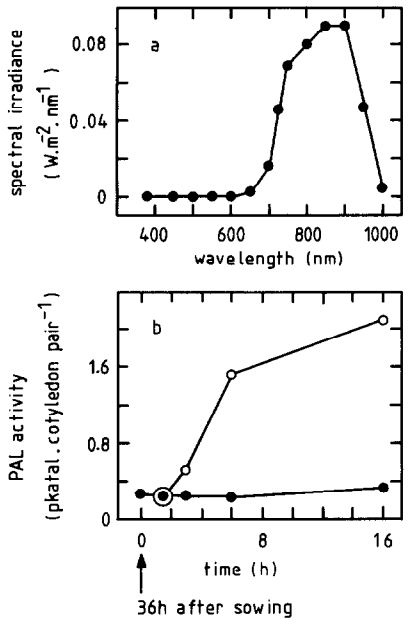

Fig. 1. (a) Spectral irradiance at seedling height. (b) Far-red light-induced rise in PAL activity in *Sinapis* cotyledons. Seeds were incubated for 36 hr in darkness as described in the Experimental, then 1.0 ml of water was added with simultaneous transfer to darkness or far-red light for the times shown. Far-red light ○; darkness ●.

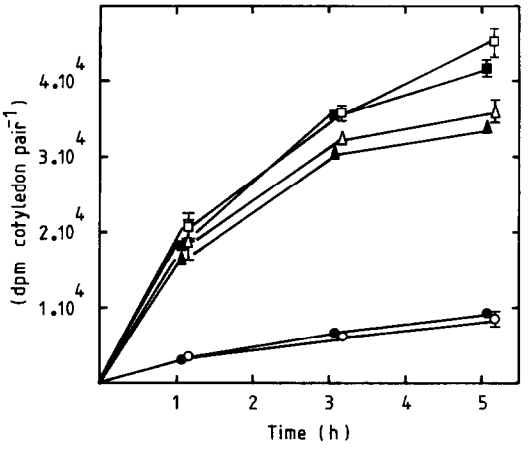

Fig. 2. Incorporation of tritium into the non-volatile compounds in aqueous extracts from cotyledons of *Sinapis alba*. The experiment was done essentially as described in the experimental section except that after 36 hr dark, seedlings were soaked in tritiated water (4 mCi.ml^{-1}) for 2 min, then transferred to petri dishes containing 1.5 ml of tritiated water of the same specific activity. Closed symbols, dark; open symbols, far red light. ■ □, Tritium incorporated into non-volatile compounds in total aqueous extract; ▲△, tritium incorporated into ammonia eluate from cation exchange column; ● ○, tritium incorporated into water wash from cation exchange column. Each point represents the results from two determinations. The error bars are s.d., and are shown when the s.d. was larger than the symbol.

As shown in Fig. 2 far-red light appears to have no effect on the incorporation of ^{3}H into either fraction, after 1.0, 3.0 or 5.0 hr incubation/irradiation times.

In a total of five experiments for the 3 hr time point similar to that shown in Fig. 2 but using various amounts of germination water and tritium the acid and neutral compound fraction was expressed as a percentage of total radioactive compounds, 16.2 ± 4.4 (3 hr dark) and 15.4 + 1.5 (3 hr far-red light). Note that the measured tritium incorporation into the total aqueous extract corresponded closely with the sum of acid and neutral compounds plus compounds in the ammonia eluate, indicating that these two fractions account for all compounds into which radioactivity was incorporated.

Table 1. Incorporation of tritium into the free amino acids of *Sinapis alba* cotyledons

	Experimental treatment 36 hr dark—plus	Tritium in ammonia eluate from cation exchange column (dpm. cotyledon pair^{-1})	Tritium in amino acids* (dpm. cotyledon pair^{-1})	Total free amino acid pool (nmol. cotyledon pair^{-1})	Specific activity of amino acid pool (dpm. nmol.$^{-1}$)
Experiment 1†	3 hr dark (control)	24 353	19 504	284	69
	3 hr far red light	25 529	19 555	338	58
Experiment 2†	3 hr dark (control)	53 814	34 367	525	66
	3 hr far red light	51 734	34 827	470	74
Normalized results from 13 experiments‡ ± s.d.	3 hr dark (control)	100 ± 5.7	—	—	—
	3 hr far red light	106 ± 12.0			

The size of the free amino acid pool was determined by amino acid analysis.

*Tritium in the amino acids was estimated by subtracting the tritium in ethanolamine and the unknown peak (determined by amino acid analysis, see Fig. 2) from the value for radioactivity in the ammonia eluate from the cation exchange column.

†Expt 1, Seedlings germinated on 1.2 ml water, and 1 ml of tritiated water (8 mCi.ml^{-1}) added after 36 hr: effective specific activity 3.6 mCi.ml^{-1}. Expt 2, Seedlings germinated on 1.5 ml water and 1 ml of tritiated water (4 mCi.ml^{-1}) added after 36 hr: effective specific activity 1.6 mCi.ml^{-1}.

‡Results were normalized such that the mean value for the dark control was equal to 100.

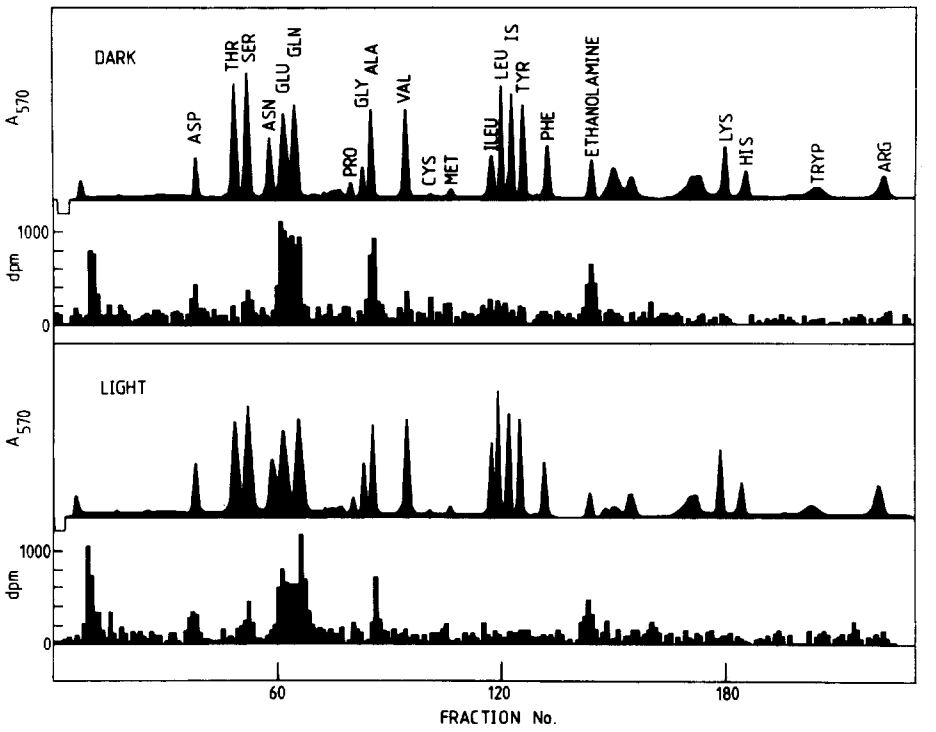

Fig. 3. Incorporation of tritium into the free amino acids from cotyledons of *Sinapis alba*. The fractions eluted from the cation exchange columns with ammonia (experiment 1, Table 1) were dried and resuspended in amino acid analyser start buffer. 100 μl, corresponding to material from 0.6 cotyledon pairs and containing about 15000 dpm, was injected. Dark, Dark control; light, 3 hr far-red light; IS, internal standard (norleucine).

To determine whether far-red light affected the incorporation of tritium into particular amino acids we separated the amino acids by ion exchange chromatography and measured the incorporation of tritium into each fraction. The results of a typical experiment are presented in Fig. 3, which shows that tritium is mainly incorporated into the amino acids, aspartate, serine, glutamate, glutamine and alanine, with significant incorporation into ethanolamine and an unknown compound which did not gave a ninhydrin positive reaction and ran near the start of the elution. There appears to be no effect of far-red light on the incorporation of tritium into any of the compounds.

DISCUSSION

Our results are clearly opposed to those of Johnson and Smith [3] who reported *ca* 100% stimulation of deuterium into the amino acid pool of *Sinapis alba* cotyledons which had been given 3 hr of continuous far-red light compared with the dark control. There are a number of possible explanations for the discrepancy, which relate to the somewhat unusual method used by Johnson and Smith [3] to measure the incorporation of deuterium into amino acids. They prepared crude extracts containing deuterium labelled amino acids from mustard cotyledons and fed them to *Escherichia coli*. They then measured the density shift of the β-galactosidase synthesized by *E. coli* to calculate the deuterium incorporation into the amino acids extracted from the mustard.

It should first be pointed out that the very reactions (transaminations) which lead to the incorporation of deuterium into the amino acids of mustard when grown in ^2H_2O would be expected to remove the deuterium when the *E. coli* is grown in H_2O. An alternative possibility is that the density shifts reported [3] were due to the effect of light on the incorporation of deuterium into the non-amino acid components of the extracts (e.g. carbohydrates, organic acids, etc.) isolated from the mustard. However, our results appear to exclude this possibility and we are forced to the conclusions (a) that the density shifts reported in *E. coli* β-galactosidase are not relevant to the incorporation of deuterium into the soluble amino acids of *Sinapis alba*; (b) that far-red light has little or no effect on the metabolism of amino acids in *Sinapis alba*; (c) within its limitations deuterium labelling coupled with density gradient centrifugation is a valid method for the study of red light stimulated enzyme synthesis in *Sinapis alba*; (d) that the case presented for far-red light stimulated synthesis *de novo* of PAL [4, 5] is supported by the data presented in this paper.

EXPERIMENTAL

Green safelight. All manipulations of seedlings, from sowing to grinding of cotyledons in liquid nitrogen inclusive, were done where necessary, under green safelight. This consisted of one 60 W bulb filtered through one layer of No. 5 orange, one layer No. 19a dark blue and one layer of No. 24a dark green cinemoid (Rank Strand, Brentford U.K.), and a 1 cm layer of 10% $CuSO_4 \cdot 5H_2O$ w/v plus 0.2% H_2SO_4 v/v [7] between two sheets of clear glass. Using a dimmer control the irradiance at seedling

height due to the green light was maintained at less than 0.001 Wm^{-2}, measured with a Li Cor Model Li-85 Quantum Radiometer.

Plant material and growth conditions. Seeds of *Sinapis alba* (Carter, Llangollen, U.K.), 40 seeds per batch, were surface sterilized with a solution of sodium hypochlorite (1%), rinsed with sterilized deionized water and germinated in the dark for 36 hr at 25° in covered 5.0 cm plastic petri dishes on two 5.5 cm discs of Whatman No. 1 filter paper moistened with a measured quantity of sterilized deionized water. The dishes were wrapped in two layers of Al foil as a further protection against stray light. After 36 hr a measured quantity of ^3H_2O (see Table 1 and Fig. 2 footnotes for details) was introduced into the dishes via a hypodermic needle which was fitted through the lid of the dish before seed germination. Seedlings for far red irradiation were placed under a light source consisting of 4 × 300 W single coil tungsten bulbs filtered through 10 cm of running water, one layer of No. 14 ruby and one layer of No. 19a dark blue cinemoid. Spectral irradiance at seedling height was measured with an ISCO Model SR spectroradiometer. The cabinet temperature was maintained at 25°. The dishes containing the dark control seedlings were wrapped in a further two layers of Al foil and placed in the same cabinet as the irradiated seedlings. Labelled water was added simultaneously with transfer to light and to the dark control seedlings. After irradiation the seedlings were rinsed with deionised water, frozen in liquid N_2 and stored at −70°.

Extraction and analysis of free amino acids. Each seedling was thawed individually by rinsing on a Buchner Funnel, the cotyledons dissected and immediately refrozen in liquid N_2 and quantitative extraction of H_2O soluble compounds effected as described in ref. [3]. The resulting aq. extract was passed through a 0.5 ml column of Bio-Rad AG50 W-X 8 (Bio-Rad Laboratories, Watford, U.K.) previously equilibrated with H_2O, washed with 25 ml H_2O, and amino acids and other compounds positively charged at acid pH eluted with 5 ml 1.8 M ammonia. The amino acid fractions and aliquots of the total aq. extract and H_2O wash from the column were dried under red. pres. at 40° and stored at −20° before analysis.

Amino acid analysis was done on an LKB model 4400 analyser. An unknown ninhydrin positive compound was found to coelute with the internal standard, so external standards were run and the results calculated accordingly. To determine tritium incorporation into individual amino acids, fractions (1 min, 0.67 ml) were collected from the amino acid analyser, placed under bright light for 2 days and 200 μl aliquots of each fraction mixed with 5 ml of Picofluor 30 (Packard, Illinois, USA) for scintillation counting.

Measurement of PAL activity. Extraction of PAL was carried out at 1–5°. Batches of frozen cotyledons (40 pairs), prepared simultaneously with those used for the amino acid analyses, were ground in liquid N_2 and the resulting powder extracted with 2 ml of 0.1 M sodium borate buffer (pH 8.7) containing 1 mM dithiothreitol. The sample was vortex mixed, then centrifuged for 5 min at 20000 *g*. The resulting supernatant was desalted on a small column of Sephadex G25. The protein-containing fraction was assayed for PAL spectrophotometrically as described by Acton and Schopfer [4] at 30° with 10 mM phenylalanine as substrate.

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