Phytochrome-Induced Synthesis of Ribonuclease *de novo* in Lupin Hypocotyl Sections

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1. Density-labelling with 99 atoms % of $^2\text{H}_2\text{O}$ distinguished pre-existing from newly synthesized ribonuclease molecules in sections of developing hypocotyl tissue. 2. Activity profiles of enzyme extracted from the fraction pelletable at 100000g showed heterogeneity after isopycnic centrifugation in CsCl gradients. 3. Measurement of density shifts of the entire heterogeneous band shows that ribonuclease protein is synthesized *de novo* in both continuous far-red light and darkness. 4. A twofold increase in enzyme activity after irradiation was accompanied by band-broadening and a significantly faster rate of labelling than in darkness. 5. The conclusion is drawn from the experimental evidence and theoretical arguments presented that phytochrome regulates the synthesis of new enzyme molecules against a background of continuous (dark-rate) synthesis and degradation. 6. Further information has been deposited as Supplementary Publication SUP 50033 (3 pages) at the British Library Lending Division (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1973), 131, 5.

The object of the work presented below was to determine whether physiologically active phytochrome exerts control over synthesis when it increases enzyme activity from a low basal condition present in dark-grown plant tissue. Generally, three methods of regulating amounts of cellular enzyme activity are recognized; (1) control over synthesis, (2) control over degradation (Schimke *et al.*, 1964) and (3) activation of pre-existing enzyme molecules.

Filner & Varner (1967) used density-labelling to show that an externally applied stimulus, in this case gibberellic acid, induced the synthesis of α -amylase in barley aleurone cells. Their approach was to compare activity profiles of isopycnicly banded enzyme from two growth regimes, (a) with stimulus but without heavy isotope (H_2^{18}O) (unlabelled control) and (b) with both stimulus and density marker. Two of the prerequisites for demonstrating control over synthesis *de novo* are that label and stimulus be applied together and that the labelling period be short compared with the lifetime of the enzyme. Assuming that barley α -amylase has a half-life greater than 18h, these conditions were met. The third prerequisite is that turnover of the enzyme should not be faster than the rate of equilibration of the available amino acid pool with density marker (Quail & Scandalios, 1971). Otherwise labelling will be strictly a function of the state of the amino acid pool and will give no indication of the dynamics of the enzyme population. The degree

of labelling of the amino acid pool will increase with time until it is equal to the number of atoms % applied. The third condition can therefore be met in one of two ways: (1) by demonstrating the presence of a mixed population of differently labelled molecules [i.e. band-broadening in isopycnic density gradients (Quail *et al.*, 1973)] or (2) by demonstrating the maximum theoretical density shift (i.e. rapid amino acid pool equilibration in terms of the labelling period used). On the basis of these parameters, enzyme synthesis *de novo* in response to a stimulus is shown if incorporation of not less than the theoretical maximum amount of label is accompanied by a uniformly labelled enzyme population [see, e.g., Filner & Varner (1967) and also Jacobsen & Varner (1967)].

The key to Filner & Varner's (1967) approach lies in the method which they adopted to show that amino acid pool equilibration was rapid compared with the lifetime of the enzyme (assumed to be long). This is because band-broadening would not permit a distinction between any of the three regulatory mechanisms. However, maximum incorporation of an isotope which antagonizes cellular processes (i.e. $^2\text{H}_2\text{O}$) is unlikely. This limits the technique in its present form to ^{18}O and ^{15}N , thereby restricting its usefulness to tissues rich in reserve protein and/or the generation of relatively small density differences.

With respect to the current problem we require a label that can be incorporated into proteins synthesized in the absence of reserve protein, and that can generate a large density shift. We have therefore

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chosen $^2\text{H}_2\text{O}$ as our density marker. To test for any restriction imposed on incorporation of this isotope by the rate of deuteration of the available amino acid pool, we have designed our experiments to include further controls. An additional control of the test enzyme (c), in which tissue is subjected to label in the absence of the stimulus (no-stimulus control), taken together with the band-broadening test, has been used to show that the rate of deuteration of the amino acid pool is not limiting to the density shift because enzyme turnover is more rapid than turnover of the pool. Thus more labelling of the test enzyme in the presence of the stimulus than without, accompanied by band-broadening in both the labelling treatments (b) and (c), indicates control over the rate of synthesis *de novo*. This approach is possible because in plants amounts of enzyme activity are usually sufficiently high in the unstimulated condition to permit meaningful experimentation. Another way in which the state of the amino acid pool could influence the density shift is if the stimulus itself affected the rate of equilibration (or led to dilution of the label) to give a differently deuterated amino acid pool in the presence and absence of the stimulus. To test for this possibility we have run a parallel investigation of an enzyme whose activity is unaffected by the stimulus. Thus, taken in conjunction with the no-stimulus control of the test enzyme (c), this permits an estimation of the effect of the stimulus, if any, on the rate of equilibration of the amino acid pool. On the basis of these theoretical arguments it will be shown that light stimulates the rate of synthesis *de novo* of phytochrome-controlled (Acton, 1972) ribosome-bound acid RNAase* (Acton *et al.*, 1970) in lupin hypocotyl sections (Acton, 1974).

Experimental

Materials

Chemicals. Deuterium oxide (Uvasol, 99.75 atoms % of $^2\text{H}_2\text{O}$), CsCl and sucrose low in RNAase activity (for maceration medium) (all Suprapur grade) were supplied by E. Merck, 61 Darmstadt 2, Postfach 4119, West Germany. Bovine serum albumin (Pentex BV0162) was supplied by Fluka AG, Buchs SG, Switzerland, and pig muscle lactate dehydrogenase (L-lactate-NAD⁺ oxidoreductase, EC 1.1.1.27) was obtained from Boehringer Mannheim G.m.b.H., 6800 Mannheim 31, Postfach 51, West Germany. Other chemicals were of the highest purity available.

Plants. Hypocotyl sections (2.5 cm long) were cut under dim green light (Mohr & Appuhn, 1963) immediately below the cotyledonary node of 5-day-old etiolated *Lupinus albus* L. seedlings which had been

previously germinated at 25°C as described by Acton (1974). Growth was entirely by cell extension (Acton *et al.*, 1970), hence equal portions cut from standardized etiolated plants had an equal and constant cell population. The far-red light-source used (Mohr, 1966) maintained a low and constant amount of the physiologically active far-red absorbing form of phytochrome (Hartmann, 1966) and has not been found to induce the development of detectable photosynthetic activity (Masoner *et al.*, 1972).

Methods

Preparation of extracts. Ribosome-enriched 100000g (2 h) pellets were prepared from washed sections at 4°C as previously described (Acton, 1974), taken without further purification, and homogenized with streptomycin sulphate (final concentration of 12.4 mM) by using a hand-driven Potter-Elvehjem homogenizer. Solid CsCl (380 mg/ml) was added to the supernatant obtained after the streptomycin-precipitated material had been sedimented (100000g for 2 h), and taken as the RNAase extract. The soluble cell fraction [the supernatant obtained after spinning the hypocotyl homogenate at 100000g for 2 h (see Acton, 1974)] was used as the acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) extract after the addition of solid CsCl (380 mg/ml).

Equilibrium sedimentation. RNAase extract (0.4–1.2 ml, containing 200–800 µg of protein) and 70–140 units (µmol/min) (pyruvate as substrate) of lactate dehydrogenase were mixed with CsCl in approx. 0.1 M-sodium acetate adjusted at 23°C to pH 5.6 with acetic acid (buoyant density 1.285 kg/l) and saturated with sodium dodecyl sulphate, to give a final volume in the centrifuge tube of 5 ml. Acid phosphatase extract (90 µl, containing about 300 µg of protein) and 7 units (µmol/min) (pyruvate as substrate) of lactate dehydrogenase were dispersed in CsCl in approx. 0.1 M-sodium citrate adjusted at 23°C to pH 6.5 with 0.1 M-citric acid (buoyant density 1.285 kg/l), the final volume in the centrifuge tube being 5 ml. Tubes were filled with paraffin and proteins banded (conditions are given in the legend to Fig. 2) by isopycnic centrifugation in short-path-length CsCl gradients (5°C) (see Johnson *et al.*, 1973). After centrifugation, fractions of 35 µl or 27 µl were collected by piercing the bottom of the tube and forcing paraffin into the top by means of a peristaltic pump. Every tenth fraction was taken for refractive index determination and the values obtained converted into density units by using the equation of Ifft *et al.* (1961). The detergent was necessary to prevent proteins in RNAase extracts from sticking to the walls of centrifuge tubes (see Flamm *et al.*, 1969), but inhibited RNAase activity in preparations from both etiolated and irradiated material by approx. 60%.

* Abbreviation: RNAase, ribonuclease [resembles the EC class ribonuclease II: ribonuclease 3'-oligonucleotidohydrolase (EC 3.1.4.23)].

Assays

Fractions. RNAase was assayed by the Kunitz (1946) method by incubating for 48 h at 40°C, acid phosphatase by the method of Bessey *et al.* (1946) by incubating for 24 h at 25°C, and lactate dehydrogenase by the procedure of Kornberg (1955) by incubating for 3 min at 25°C. Reaction velocities were approximately linear with time at maximum enzyme concentration. Recoveries from gradients of both labelled and unlabelled enzymes were greater than 95% for RNAase and 60–70% for acid phosphatase.

General assays. Ribosomes, and RNAase activity ($\mu\text{g}/\text{min}$ at 37°C) determined by the acid-precipitation method, were assayed as previously described (Acton *et al.*, 1970). Protein was determined by the procedure of Lowry *et al.* (1951), with Fluka dry crystalline bovine serum albumin as standard.

Other procedures

Lactate dehydrogenase was used as an external marker of known buoyant density (1.253 kg/l at 25°C) to correct for high apparent buoyant densities of RNAase and acid phosphatase arising from substances in extracts which contribute to refractive index values (see Quail *et al.*, 1973), water activity (see Filner & Varner, 1967) and small differences in gradient composition (see Jacobsen & Varner, 1967). Reproducibility within each run with RNAase extract was tested by running duplicate gradients for each preparation, the second gradient of each pair containing 1.5 or 2 times the amount of sample protein. Only experiments in which density values of duplicates agreed within $\pm 0.002 \text{ kg/l}$ after adjustment for any shifts in the external marker were used to calculate the mean density values given in Table 1. Fraction numbers have been converted into distances across each gradient with the tube lying at 30° to the axis of rotation, i.e. as in the rotor before re-orientation. To estimate the molecular weight of banded RNAase a plot of molecular weight against bandwidth at half peak-height was constructed for five unlabelled enzymes (mol.wt. 12000–330000) which had been centrifuged to equilibrium under identical conditions. Bandwidths of external marker profiles were taken as an index of the consistency of centrifuging conditions between runs. Calculations on the deuteration of lupin RNAase are based on the amino acid composition of RNAase T₂ (Egami & Nakamura, 1969).

Results and Discussion

Effect of $^2\text{H}_2\text{O}$ on the light-mediated development of bound RNAase

Justification for using hypocotyl sections is derived from observations that wounding and aging have no

Fig. 1. Effect of $^2\text{H}_2\text{O}$ on the time-course of development of RNAase activity associated with the 100000g pellet

(a) Batches of 30 hypocotyl sections were cut from 5-day-old etiolated seedlings and preincubated in 15 ml of water in sterilized Petri dishes for 6 h in darkness. After being blotted dry, the sections were transferred to 12 ml of water containing streptomycin sulphate (final concentration 15 $\mu\text{g}/\text{ml}$) and exposed to continuous far-red light (3.5 J/m² per s) for various lengths of time or retained in continuous darkness. Details of the preparation of pellets are given in the Experimental section. (b) As for (a) except that 99 atoms % of $^2\text{H}_2\text{O}$ replaced the water. The preincubation in darkness gave an endogenous concentration of $^2\text{H}_2\text{O}$ of 50–70%, calculated from the ratio tissue water volume/applied $^2\text{H}_2\text{O}$ volume and confirmed by the increases observed in fresh weight. This leads to an endogenous concentration of $^2\text{H}_2\text{O}$ greater than 80% during the experimental growth period. (c) Changes in RNAase activity of pellets plotted as a function of duration of irradiation. RNAase was assayed by an acid-precipitation method (Acton *et al.*, 1970). Δ , Continuous far-red light; $\blacktriangle$, continuous darkness; $\blacksquare$, light-mediated increase in activity in water; $\bullet$, light-mediated increase in activity in $^2\text{H}_2\text{O}$.

profound effect on the development of bound acid RNAase activity in water-infiltrated tissue (Acton, 1974) and from the data presented in Fig. 1. Pre-incubation with isotope for 6h in the dark was used to ensure that a large proportion of amino acids available for protein synthesis would be deuterated at the time of transfer to light. This is permissible if the basal enzyme component, present in unilluminated tissue, does not have a short half-life.

The only isotopic effect was a lengthening of the lag phase from 9h (H_2O) to 36h ($^2\text{H}_2\text{O}$) before the rise in enzyme activity (cf. Hock, 1970), shifting the maximum of the response from 2 days (H_2O) to 3.5 days ($^2\text{H}_2\text{O}$) of continuous light (Fig. 1). On the other hand, rapid development of activity in darkness, which normally commences 7 days after sowing in hypocotyls of intact seedlings as well as in water-infiltrated sections (Fig. 1), was not observed within the lifetime of sections subjected to the isotope.

Activity distribution and buoyant density of unlabelled RNAase

A bimodal distribution of banded material, characteristic of molecular weight and density heterogeneity together (Meselson *et al.*, 1957), was obtained from unlabelled attached ribosomal proteins (Fig. 2a). Justification for using a heterogeneous distribution in the present context is based on the fact that the origin of the bulk of the enzyme activity present in irradiated sections (about 70% of the activity is new and mediated by phytochrome, see Fig. 1) is being investigated under conditions in which the bimodal distribution is always obtained [compare profiles (a), (b) and (c) for the three treatments given in Fig. 2]. The mean density position at equilibrium of the entire bimodal band therefore gives a measure of any gross changes in effective mass. Since this is within ± 1 fraction (± 0.01 cm) of the centre of the broad peak (Fig. 2), this has been taken as the index of effective enzyme mass. This would not be justified if the second peak,

nearest to the rotor centre, was the result of protein overloading or 'wall effects'. However, these two possibilities can be largely discounted because the protein loading of the gradients was low and detergent was included to prevent sample material from sticking to the walls of centrifuge tubes (see the Experimental section). It seems more likely that banding to form two peaks represents the separation of two reasonably homogeneous components, most probably a monomer or dimer of a large RNAase (the estimated mol.wt. from the profile bandwidth is 60000) and a ribosomal-protein-RNAase complex [calculated mol.wt. from the conditions required to give the minimum obtained (see Hu *et al.*, 1962) is greater than 650000].

Table 1 shows a buoyant density of 1.292 kg/l at the centre of the broad peak. The minor component, further from the rotor centre with greater effective density, equilibrates 0.012 kg/l higher. Neither the buoyant density nor the bandwidth differed appreciably from the values found in two separate runs for an unilluminated unlabelled control. Differences in density and bandwidth are not to be expected unless the action of light is to create an enzyme population which is more heterogeneous in molecular weight, since the step of isolating the attached ribosomal protein fraction should remove most substances present in cell homogenates, which could be influenced by irradiation, and which might therefore contribute differently to the observed density value.

The buoyant density obtained is lower than those previously reported for soluble RNAases extracted from plant tissue (Barker *et al.*, 1971; Bennett & Chrispeels, 1972).

Origin of the RNAase present in darkness

A density increase averaging 0.021 kg/l shows that RNAase protein is synthesized largely or entirely *de novo* in darkness (Fig. 2a, b; Table 1). This conclusion is permissible since (1) any gross differences in

Fig. 2. Activity profiles of RNAase extracted from 100000g pellets and spun to equilibrium in CsCl gradients

Batches of 30 hypocotyl sections were cut from 5-day-old etiolated seedlings and treated as follows: (a) preincubated with 15 ml of water for 6h in darkness, blotted dry, and transferred to 12 ml of water containing streptomycin sulphate (final concentration 15 $\mu\text{g}/\text{ml}$) and exposed to continuous far-red light (3.5 J/m² per s) for 2.5 days preceding the preparation of extracts; (b) as for (a) except that 15 ml and 12 ml of 99 atoms % of $^2\text{H}_2\text{O}$ replaced the water and each batch was retained in continuous darkness for 3.5 days after the 6h preincubation; (c) as for (b) except transferred to continuous far-red light for 3.5 days after the 6h preincubation in darkness. Details of the preparation of extracts and composition of gradients are given in the Experimental section. Equilibration was in CsCl gradients formed in a WKF 12 $\times$ 13 fixed-angle rotor and run for 48h at 5°C and 140000g (corrected for de-rating) (46000 rev./min, r_{av} 7.3 cm) in a WKF P-50K ultracentrifuge. The data presented in the panels (a), (b) and (c) relate to three separate experiments. A standard gradient, loaded only with the lactate dehydrogenase external marker, has been superimposed on each gradient and the diagrams are so aligned that the external marker profiles coincide. Relative activity means that all points on the marker profiles are expressed as a percentage of the highest point of the curve on the standard gradient. Buoyant-density values are given above the broad peak of each RNAase profile. Δ , CsCl distribution of the standard gradient; $\blacktriangle$, CsCl distribution of gradients run with extract; $\square$, lactate dehydrogenase of standard gradient; $\blacksquare$, lactate dehydrogenase of gradients run with extract; $\bullet$, RNAase.

Table 1. *Effect of continuous far-red light on the incorporation of label into RNAase and acid phosphatase spun to equilibrium in CsCl gradients*

Batches of 30 hypocotyl sections were cut from 5-day-old etiolated seedlings and preincubated in either 15 ml of water or 99 atoms % of $^2\text{H}_2\text{O}$ for 6 h in darkness, blotted dry, and transferred to 12 ml of water or 99 atoms % of $^2\text{H}_2\text{O}$ containing streptomycin sulphate (final concentration 15 $\mu\text{g}/\text{ml}$). Enzyme extracts were prepared after incubating sections for either 2.5 days in far-red light (water controls) or 3.5 days in continuous darkness (dark controls) or continuous far-red light. Details of the preparation of extracts and composition of gradients are given in the Experimental section. Proteins were spun to equilibrium in a 12×13 fixed-angle rotor of a WKF P-50K ultracentrifuge run for 46–48 h for RNAase extracts, or 24–26 h for acid phosphatase extracts, at 5°C and 140000g (corrected for de-rating) (46000 rev./min, r_{av} , 7.3 cm). In the labelling treatments RNAase and acid phosphatase were assayed from the same batch of sections. Data for the different treatments are from completely independent experiments. The buoyant-density values given are means of at least four separate experiments and are corrected for any shifts in the added lactate dehydrogenase standard. Percentage values in parentheses are the density shifts. RNAase was assayed by the Kunitz (1946) method.

Enzyme	Treatment of sections	Isopycnic density (kg/l)	$\pm$ S.D.	Increase in buoyant density (kg/l)	Far-red light-mediated increase in density (kg/l)
RNAase	Far-red light+water	1.292	0.0026 (8)	—	0.011 (0.85%)
	Unilluminated+ $^2\text{H}_2\text{O}$	1.313	0.0019 (10)	0.021 (1.63%)	
	Far-red light+ $^2\text{H}_2\text{O}$	1.324	0.0041 (10)	0.032 (2.48%)	
Acid phosphatase	Far-red light+water	1.323	0.0015 (4)	—	0.001 (0.07%)
	Unilluminated+ $^2\text{H}_2\text{O}$	1.331	0.0014 (5)	0.008 (0.61%)	
	Far-red light+ $^2\text{H}_2\text{O}$	1.332	0.0020 (5)	0.009 (0.68%)	

the rate of incorporation of label into newly synthesized proteins between batches of sections would have been detected in the acid phosphatase internal marker (Table 1); such a system is thus internally controlled; and (2) the absolute density of unlabelled RNAase (1.292 kg/l) is within the range expected for a non-glycoprotein (Anstine *et al.*, 1970). Further, (3) to obtain a density shift of this magnitude by attachment of a carbohydrate fraction, without synthesis of a protein moiety, would require that the molecule be at least 25% carbohydrate. (4) A similar argument indicates that deuteration of a lipid moiety could not explain the observed density shift.

Incorporation of one-third of the theoretical maximum of stable isotope [a 5.5% shift is possible, calculated assuming an endogenous concentration of 90 atoms % of $^2\text{H}_2\text{O}$ and taking into account that 28–35 non-labile hydrogen atoms of the pancreatic RNAase molecule are slowly exchangeable (Leach & Hill, 1963)] was accompanied by a band-broadening of 15% [means of bandwidths at half peak-height, $\pm$ S.D., were 0.049 ± 0.005 density unit (8) and 0.057 ± 0.002 density unit (10) without label and with label in darkness respectively]. This indicates that the constant amount of activity observed in darkness is regulated through a continuous process of synthesis and degradation of enzyme protein which has a half-life greater than 1.75 days.

Origin of the phytochrome-mediated enzyme activity

The density increase averaging 0.032 kg/l (Table 1), in internally controlled experiments, shows that

RNAase protein is synthesized *de novo* in the light. More variation in both the extent of the labelling (Table 1) and bandwidths of activity profiles was observed between runs than was found for the unilluminated labelling treatments. Band-broadening by 40% [the mean bandwidth at half peak-height, $\pm$ S.D., was 0.070 ± 0.010 density unit (10)] and more skewed distributions (see Fig. 2c) both indicate the presence of a mixed population of differently deuterated molecules.

The essential features of the results are that a two-fold increase in enzyme activity (Fig. 1) was accompanied by a 0.011 kg/l difference in density between the unilluminated and irradiated labelling treatments, and that a difference in bandwidth increase of 25% was observed. These observations can most reasonably be interpreted to mean that a larger population of newly synthesized molecules of a relatively stable enzyme exists in the light.

Taken in isolation, these data permit no firm conclusion as to the origin of the proposed larger enzyme population. However, given the constraint that acid phosphatase, the activity of which is unaffected by light (results not shown), was not significantly heavier after irradiation (Table 1), the possibility of photo-regulation through degradation, accompanied by heavier labelling of RNAase via a more heavily deuterated amino acid pool (i.e. a non-specific light-effect), can be largely discounted. This leads to the conclusion that light stimulates the rate of synthesis of RNAase molecules *de novo*. This is against a background of synthetic and degradative processes, the velocity balance of which is independent of light.

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