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Turnover of Phytochrome in Pumpkin Cotyledons[1]

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ABSTRACT

By using density labeling, it was found that the protein moiety of phytochrome is synthesized *de novo* in the red-absorbing form in cotyledons of dark-grown pumpkin (*Cucurbita pepo* L.) seedlings, as well as those irradiated with red light and returned to the dark. The rate of synthesis appears to be unaffected by the light treatment. Turnover of the red-absorbing form was also detected in dark grown seedlings using density labeling, while turnover of the far red-absorbing form is already implied from the well known "destruction" observed in irradiated seedlings. In both cases, true degradation of the protein is involved, but the rate constant of degradation of the far red-absorbing form may be up to two orders of magnitude greater than that of the red-absorbing form. The data indicate that, in pumpkin cotyledons, phytochrome levels are regulated against a background of continuous synthesis through divergent rate constants of degradation of the red and far red-absorbing forms and the relative proportions of the two forms present.

Previous data indicate that newly appearing phytochrome molecules in the hook of pumpkin seedlings are synthesized *de novo* (16). Evidence has also been presented that "turnover" of Pr occurs in this organ, but the continuously changing cell population during growth makes it impossible to determine whether this represents intracellular turnover or turnover of the whole cell population.

The cotyledon, on the other hand, appears to have a more or less stable cell population (20) and is therefore a more suitable object for the study of cellular protein levels. Therefore, using the technique of density labeling (5, 8), an attempt has been made to understand the mechanism controlling phytochrome levels in pumpkin cotyledons. A report of *in vivo* measurements of changes in phytochrome levels in pumpkin seeds during germination has recently been published (22).

MATERIALS AND METHODS

Growth, Irradiation, and Extraction of Seedlings. Growth, irradiation, and extraction of seedlings were as previously described for hooks (16). Crude extracts were prepared from 1 g (fresh weight) lots of cotyledons. However, the specific activity of phytochrome per mg protein in these extracts was lower by a factor of five than in the hook extracts. This precluded the use of the crude cotyledon extracts directly for density gradient centrifugation, because aliquots containing sufficient phytochrome for subsequent measurement in gradient fractions caused protein overloading and distortion of the phytochrome profiles. Therefore, the pellet from a 33% ammonium sulfate cut was resuspended in 50 mM tris buffer containing 10 mM 2-mercaptoethanol (pH 7.5) and passed through Sephadex G-25 equilibrated with the same buffer using the technique previously described (16). This procedure resulted in a 10- to 12-fold increase in the specific activity of the phytochrome with greater than 95% recovery. This fraction was used for density gradient centrifugation. As previously, all manipulations were performed under dim green safelight. Protein was determined by the method of Lowry *et al.* (9).

Density Gradient Centrifugation. The procedure was as previously described (16). An aliquot of the G-25 eluate containing about 3 mg of protein was used in each gradient. This protein load resulted in more or less symmetrical phytochrome profiles. However, the recovery of phytochrome from the gradients was reduced to 55 to 60%, apparently as a result of the $(NH_4)_2SO_4$ step. This procedure also led to sharper density gradient profiles, as well as decreased apparent absolute densities of both phytochrome and LDH,[3] in comparison to the crude hook extracts previously used. This last effect is apparently because the $(NH_4)_2SO_4$ step eliminates substances which contribute to the refractive index readings. The relative densities of the LDH and unlabeled phytochrome however remained unchanged.

P_{tot} Measurements. P_{tot} measurements were as previously described using $CaCO_3$ as a scattering medium (16). In the time course experiments, P_{tot} was measured in at least four replicate extracts with at least two measurements from each extract. On the other hand, because of volume limitations, only one estimate from each density gradient fraction was possible.

RESULTS

The changes in the level of extractable phytochrome per cotyledon up to 216 hr from sowing are shown in Figure 1. In dark-grown seedlings, an increase prior to about 150 hr is followed by a plateau up to 216 hr. This is essentially in agreement with published *in vivo* measurements of P_{tot} (22). In 96-hr-old cotyledons exposed to 2 hr of red light, followed by 15 min far red, and returned to the dark, P_{tot} declines with an apparent half-life of 58 min during red irradiation but then increases again in the dark to within 90% of the plateau level for dark-grown cotyledons. From 98 to 168 hr, the rate of increase of P_{tot} in irradiated seedlings is the same as in dark-grown ones. From 168 to 216 hr, the rate decreases by a factor

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[3] Abbreviations: LDH: lactate dehydrogenase (EC1.1.1.27); P_{tot}: total phytochrome.

of three. Seedlings given the same light program and returned to the dark at the beginning of the plateau (168 hr) exhibit the same pattern of P_{tot} decline and recovery as at 96 hr. However, the apparent half-time for P_{tot} disappearance is about 48 min, and the recovery rate is half that of the 96-hr-old seedlings. All newly appearing phytochrome is in the Pr form.

To test whether the accumulation of P_{tot} by dark-grown cotyledons results from *de novo* synthesis, seedlings were grown on either H_2O or 70% D_2O from sowing until 168 hr. The density gradient profiles are presented in Figure 2. The density shift of 0.015 g/cm³ observed indicates that the protein moiety of the phytochrome is synthesized *de novo*. This shift is smaller than that obtained in the hook (16) but may be due to a difference in the amino acid pools of the two organs.

Various explanations are possible for the disappearance ("destruction") of phytochrome in red light and its subsequent reaccumulation ("recovery") in the dark (Fig. 1). (*a*) Disappearance and regeneration of phytochrome could involve detachment and reattachment of the chromophore without net protein turnover (3) in a manner similar to that believed to be true for rhodopsin (4). (*b*) *In vivo* the Pfr formed by red irradiation might move to a "trap," and therefore occupy a smaller volume of the cell than the Pr previously did, thus leading to a decreased P_{tot} signal (19). Recovery in the dark would then presumably be the reverse process, and the whole cycle would require neither degradation nor synthesis of new molecules. (*c*) Destruction may indeed represent true degradation of the protein moiety and recovery new synthesis.

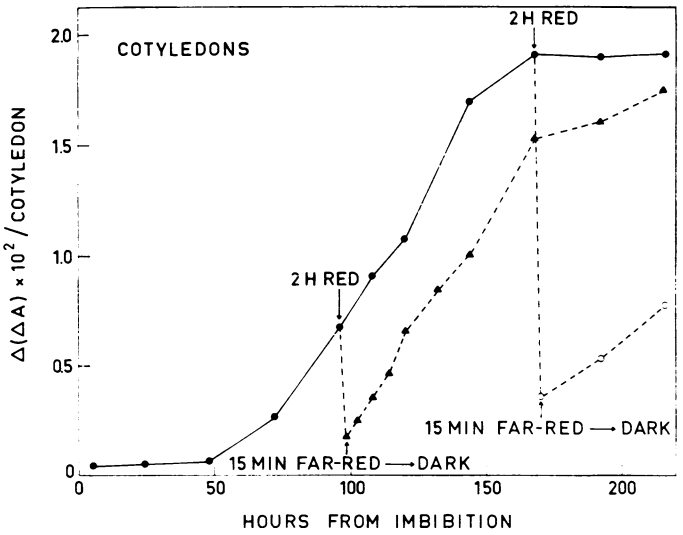

FIG. 1. Time course of the level of extractable phytochrome/cotyledon of dark-grown seedlings (●) and of seedlings irradiated at 96 hr (▲) or 168 hr (○) with 2 hr red light + 15 min far red and returned to the dark.

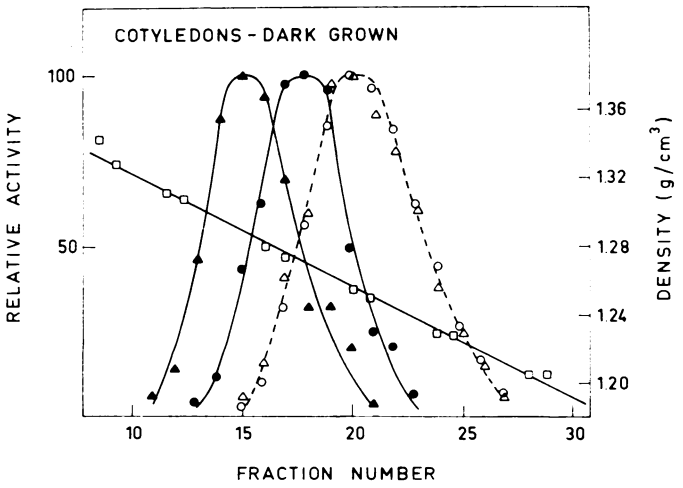

FIG. 2. Equilibrium distribution in CsCl gradients of phytochrome from cotyledons of pumpkin seedlings grown in the dark for 168 hr on either H_2O (●) or 70% D_2O (▲). LDH marker profiles from the labeled (△) and unlabeled (○) gradients have been superimposed and drawn as one. Density of CsCl gradients (□).

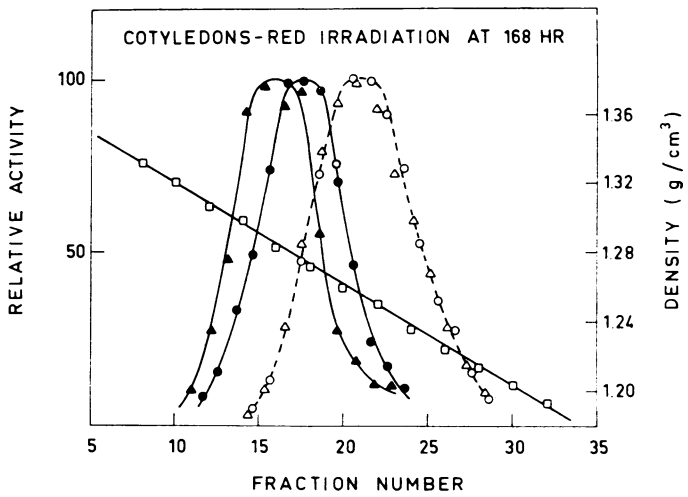

FIG. 3. Equilibrium distribution in CsCl gradients of phytochrome from cotyledons of pumpkin seedlings grown on H_2O in the dark for 168 hr, then either retained on H_2O (●) or transferred to 70% D_2O (▲), irradiated with 2 hr red light + 15 min far red and returned to the dark for a further 46 hr. LDH marker profiles from the labeled (△) and unlabeled (○) gradients have been superimposed and drawn as one. Density of CsCl gradients (□).

This question was investigated for both 96- and 168-hr-old seedlings. The seedlings were transferred to 70% D_2O at the above times, exposed to 2 hr of red light, followed by 15 min far red, and returned to the dark. (Previous experiments had shown that transfer of seedlings to 70% D_2O at 96 hr had no effect on the subsequent rate of P_{tot} accumulation up to 144 hr.) The 96-hr-old seedlings were extracted at 120 hr and the 168 hr seedlings at 216 hr. In both cases, the phytochrome became density labeled, indicating that the newly appearing molecules are synthesized *de novo*. Figure 3 shows the profiles for the 168 hr seedlings.

The cycle of phytochrome disappearance and recovery would appear therefore to involve, respectively, the degradation and synthesis of phytochrome protein. This is evidence against the first two alternatives outlined above, since both imply the absence of synthesis and degradation. Furthermore, since extractable phytochrome follows the same pattern of disappearance and recovery (Fig. 1) as that observed in *in vivo* measurements, phytochrome traps would not appear to be responsible for this process.

The synthesis of phytochrome in red-irradiated cotyledons while the P_{tot} level in dark-grown seedlings remains constant (168–216 hr) (Fig. 1) suggests that this plateau might represent a steady state turnover of Pr rather than a simple cessation of synthesis. To test this, 168-hr-old dark-grown seedlings were transferred to 70% D_2O and extracted at 216 hr. A small (0.005 g/cm³), but reproducible density shift indicates that there is indeed a turnover of Pr molecules during this time (Fig. 4).

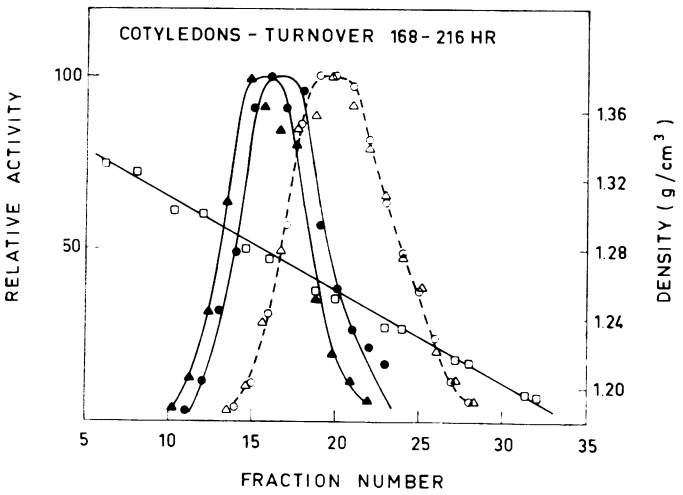

FIG. 4. Equilibrium distribution in CsCl gradients of phytochrome from cotyledons of pumpkin seedlings grown on H_2O in the dark for 168 hr then either retained on H_2O (●) or transferred to 70% D_2O (▲) for a further 48 hr in the dark. LDH marker profiles from the labeled (△) and unlabeled (○) gradients have been superimposed and drawn as one. Density of CsCl gradients (□).

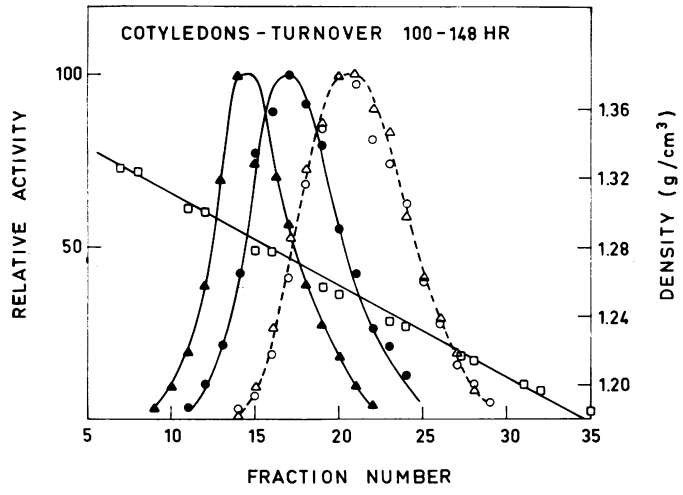

FIG. 5. Equilibrium distribution in CsCl gradients of phytochrome from cotyledons of pumpkin seedlings grown on H_2O in the dark for 100 hr then either retained on H_2O (●) or transferred to 70% D_2O (▲) for a further 48 hr in the dark. LDH marker profiles from the labeled (△) and unlabeled (○) gradients have been superimposed and drawn as one. Density of CsCl gradients (□).

Turnover during the plateau phase does not, however, necessarily mean that a degradative process is also operative while the level of P_{tot} is increasing. Therefore, a semiquantitative approach was devised to test for turnover prior to 168 hr from sowing. The 100-hr-old H_2O-grown seedlings were transferred to 70% D_2O and retained in the dark until 148 hr. A density shift of 0.012 g/cm³ with no peak broadening was obtained (Fig. 5). Since the net increase in P_{tot} level between 100 and 148 hr accounts for only 57% of the total present at 148 hr (Fig. 1), a density shift of this magnitude with no peak broadening (Fig. 5) indicates that substantial turnover of Pr molecules has occurred during the labeling period.

Lack of peak broadening in the turnover experiments (Figs. 4 and 5) indicates that the turnover rate of Pr is more rapid than the rate of equilibration of the amino acid pool with deuterium (15, 21). In fact, it can be estimated that, for no peak broadening to occur, the half-time of phytochrome turnover has to be at least three times faster than that of amino acid pool equilibratoin. Under these conditions, 87.5% of the phytochrome population would turn over during one half-life of amino acid pool equilibration leaving only 12.5% of the previous (unlabeled) population. This would not be detectable as peak-broadening within the error of measurement.

Accurate estimates of the true rate constant of Pr degradation from the density shifts in Figures 4 and 5 are therefore not possible. Minimum values are however obtainable in this way, and it was found that the rate constant of degradation of Pfr may be up to two orders of magnitude greater than for Pr. That the rate constant of Pfr degradation must be substantially greater than that of Pr is already evident from the considerably higher levels of phytochrome in dark-grown than in red-irradiated seedlings (Fig. 1). On the other hand, since true rate constants for Pr cannot be calculated, it is not known if the decreased accumulation rate of P_{tot} in 168-hr-old red-irradiated seedlings (Fig. 1) is due to a change in the rate of synthesis or degradation of Pr or both.

DISCUSSION

In higher organisms, the levels of many cellular proteins are regulated through a combination of the processes of synthesis and degradation (6, 18, 21). The present data indicate that phytochrome levels in pumpkin cotyledons are also regulated in this way. Phytochrome, however, has the additional property of existing in two forms, Pr and Pfr, interconvertible by light. The pigment is synthesized in the Pr form, but the rate of photoconversion of Pr to Pfr is infinitely more rapid at the irradiance levels normally employed than the rate of synthesis of Pr. The general formula describing the relationship between synthesis and degradation in establishing protein levels (18) can therefore be modified in the following way to accommodate the two forms of phytochrome:

$$\frac{dP}{dt} = k_s - k_d^{Pr} \cdot (1 - \phi) \cdot P - k_d^{Pfr} \cdot \phi \cdot P$$

where P = total phytochrome concentration; k_s = zero order rate constant of Pr synthesis; k_d^{Pr} = first order rate constant of Pr degradation; k_d^{Pfr} = first order rate constant of Pfr degradation; ϕ = proportion of P in the Pfr form. Pfr is absent from dark-grown tissue, and the relative proportions of the two forms in irradiated tissue depends on the photostationary state established by the wavelength(s) used (2, 7). This accounts for the higher steady state levels of phytochrome in dark-grown tissue than in tissue subjected to continuous irradiation and predicts that the steady state level of phytochrome under continuous irradiation will be strongly wavelength-dependent (17).

Phytochrome destruction (2) may then be viewed simply as a transition between steady state levels with degradation predominating. The dependence of the rate of P_{tot} destruction in dicotyledons under continuous irradiation on the photostationary state has been clearly documented (10–13) and is understandable in terms of the different rate constants of Pr and Pfr degradation. The recovery of P_{tot} levels in the dark following destruction may similarly be viewed as a transition between steady state levels with synthesis predominating.

The fact that the rate of phytochrome accumulation following 2 hr of red light at 96 hr parallels that of dark-grown seedlings (Fig. 1) suggests that k_s is unaffected by the light treatment. This was also found to be true in *Sinapis* cotyledons where seedlings transferred to the dark after 48 hr in continu-

ous far red light, accumulated phytochrome at the same rate as untreated dark controls (17). The k_d of Pfr is unaffected by previous light treatments or irradiance (14) and that of Pr would also appear to be unaffected as indicated by the almost total recovery of the P_{tot} level following red light at 96 hr (Fig. 1).

These data lend support to the concept that the phytochrome system of pumpkin cotyledons is a dynamic one in which the photoreceptor itself continually regulates its own level in response to the presence, absence, and quality of light. This appears to be accomplished within a framework of ongoing synthetic and degradative processes which are themselves unaffected by light. Support for the generality of this scheme, at least in its present simple form, is lacking, however, since instances of only weak (3) or limited (12) recovery of P_{tot} levels in dicotyledons have been recorded; and in monoctyledons the mechanism of Pfr destruction is apparently saturated at very low Pfr levels, resulting in zero order destruction kinetics (10).

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