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In Vitro Phytochrome Dark Reversion Process

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Abstract. **Thermal reversion of the far-red absorbing form of phytochrome to the red absorbing form in darkness has been investigated in crude and partially purified isolates from a number of etiolated and light grown higher plants. The influence of temperature, aging and urea on the rate of reversion was also determined.**

Phytochrome isolated from all higher plants underwent reversion. The reversion proceeded in at least 2 distinct stages; a short rapid initial phase being followed by a slow phase which continued for many hours. Reversion rate was highest in phytochrome isolated from green leaves of parsnip (*Pastinacea sativa*) and lowest in that isolated from etiolated oats (*Avena sativa*). Although the rate of reversion could be changed by modifying the tertiary structure of the protein component, the large differences in rate appeared to be characteristic of the plant source. Observed *in vitro* rates of reversion are slower than those occurring *in vivo*. Removal of other buffer solubilized material during purification had little effect on the rate of reversion of phytochrome isolated from etiolated material.

Physiological evidence originally suggested that a thermal reversion of the far-red absorbing form of phytochrome (P_{FR}) to the red absorbing form (P_R) may occur in darkness (3). Other results thought to support this possibility have since been found to be caused by phytochrome (P_{FR}) decay (5). Because the reversion process may be an important part of the timing mechanism in photoperiodism, it is essential to obtain more direct evidence of the reactions occurring in green leaves. Interpretation of direct *in vivo* spectrophotometric data would be difficult because of the interference caused by large amounts of chlorophyll (20). Recent attempts to study the process in non-chlorophyllaceous tissues have produced a variety of conclusions. In etiolated seedlings (4) and in some plant tissues not previously exposed to light; *i.e.* brussel sprout receptacles (13) and gladiolus corms (15), P_{FR} shows a rapid and often complete destruction rather than reversion following its generation with red light. Even after inhibition of P_{FR} destruction with respiratory inhibitors such as CO, cyanide and azide, Butler and Lane (5) found little or no reversion of P_{FR} to P_R in etiolated maize seedlings. In etiolated peas however, Furuya *et al.* (10) suggest that inhibition of decay can be accompanied by an increase in what they interpret as reversion.

In some light grown or light stored tissues such as cauliflower curds (5) and parsnip roots (15), phytochrome reversion certainly occurs, and in these instances reversion is accompanied by little or no P_{FR} destruction. In these tissues it is difficult to envisage a function for phytochrome, and the question exists as to whether this is a normal phytochrome response.

Investigations using partially purified solutions of phytochrome have not clarified the situation. Bonner (1) reported that phytochrome isolated from etiolated peas could undergo a reversion of P_{FR} to P_R but the absorbancy change at 725 nm was less than that at 665 nm. A similar phenomenon has been reported in barley isolates (12), but Butler *et al.* (6) put these observations down to partial denaturation. They found for example, that phytochrome freshly purified from etiolated oats underwent no thermal transformations after several hours at room temperature, yet exhibited some dark reversion with unequal optical density changes after denaturation with urea. After excluding oxygen from his buffers Mumford (18) reports that a dark reversion of P_{FR} to P_R occurs in freshly prepared solutions of phytochrome from oats. The reaction is reported to be first order with respect to P_{FR}, and highly temperature dependent with a calculated P_{FR} half-life of about 9 hours at 25°.

Factors which may be responsible for these contradictions have been investigated. An attempt has also been made using *in vitro* data to extrapolate from measurable *in vivo* dark reversion rates (*i.e.* parsnip root) to the situation which may exist in green leaves.

Materials

Seeds were obtained from the following sources: *Avena sativa* cultivar "Achilles" (oat). Hodder and Tolley Ltd., Palmerston North, New Zealand. *Pisum sativum* cultivar "Greenfeast" (pea). Arthur Yates and Company, Auckland, New Zealand. *Pastinaca sativa* cultivar "All American" (parsnip). Ferry Morse Seed Company, U.S.A.

Oats were grown in vermiculite at 24° in complete darkness. After 5 days the coleoptiles (8 cm in length) and enclosed primary leaves were harvested under dim green light. They were usually frozen with the aid of dry ice before use. Frozen material was not stored for longer than 4 weeks. Peas were also grown in complete darkness at 24° in vermiculite. After 7 days the plumular hooks were harvested under dim green light and stored frozen.

Parsnip cultivar "All American" was used since it was known to be high in phytochrome (16). Parsnips were grown in a soil-pumice-peat mixture at 18° to 25° in a glasshouse with supplementary lighting to give continuous illumination. Plants were fed an Fe-EDTA modified Hoaglands nutrient solution every second day. Roots were harvested after 3 months when they were 7 inches long. Young leaves 6 to 10 inches long were taken at the same time.

Methods and Results

Phytochrome Estimations. The dark reversion of phytochrome in crude or partially purified solution was determined in a Perkin Elmer 450 recording spectrophotometer in 10 × 100 mm cylindrical cells. The sample holder was maintained at the desired temperature with a "Forma-Temp" constant temperature bath. Sample and reference compartments were purged with dry nitrogen to reduce condensation on the optical surfaces. Solutions containing phytochrome were irradiated with light obtained by passing the output of a projector (500W tungsten lamp) through heat absorbing glass (KG3- Jena Glaswerk Schott and Gen., Mainz, Germany) then through Baird-Atomic type B3 interference filters peaked at 660 and 725 nm. An irradiation time of 3 minutes was adequate to saturate the phytochrome conversion.

When solutions contained little light scattering material and no chlorophyll, reversion was determined from absorption spectra taken at intervals over a 24 hour period after saturating the phytochrome with red light. With more crude solutions, both reference and sample cells were filled with the same solution and phytochrome in the reference cell saturated with far-red light. Reversion was then determined from difference spectra obtained after irradiating the cell in the sample compartment with a saturating dose of red light. Light scattering characteristics of these crude solutions often changed slowly with time, so the sample was saturated with red light after each determination of reversion to allow a more accurate measure of the ratio of optical density changes at the peak wavelengths.

Isolation and Reversion of Phytochrome from Etiolated Oats. Frozen oat coleoptiles were ground in a Waring Blendor. Short bursts were used with the blendor, and a plastic bottle was held down in the brei to reduce the vortex and consequent dispersion of air into the sample. Grinding was always performed in 0.1 M sodium pyrophosphate buffer (pH 7.8) containing 29 mM 2-mercaptoethanol, 1 mM K^+ EDTA and 10 mM cysteine. Subsequent purification steps were carried out in potassium phosphate buffer pH 7.8 containing 29 mM 2-mercaptoethanol and 1 mM K^+ EDTA. All water was distilled, deionized and boiled before use. All operations were carried out in a cold room at 1°. Samples were kept on ice in the dark whenever possible, but no attempt was made to completely exclude light from the sample during the purification procedures. After grinding the homogenate was strained through several layers of nylon mesh and centrifuged for 15 minutes at 12,000 × *g*. Phytochrome in the supernatant was partially purified by the method of Taylor and Bonner (23). In some cases gradient elution from brushite (21) was substituted for the final elution from DEAE-cellulose but this did not appear to have any effect on the reversibility of the phytochrome. The blue green pellet obtained by centrifugation of the final $(NH_4)_2SO_4$ precipitate was resuspended in 0.1 M potassium phosphate buffer (pH 7.8) containing 29 mM 2-mercaptomethanol and 1 mM K^+ EDTA.

Phytochrome prepared from oats in this manner was capable of undergoing a significant degree of reversion (see fig 1) at 1°. The reaction was not first order with respect to P_{FR} although rate "constants" calculated for longer periods of reversion (*i.e.* 13–66 hr) span those given by Mumford (18) for partially purified oat phytochrome at 10°. The phytochrome was relatively stable showing a 14 % loss in optical reversibility at 1° over a period of 6 days, during which time it was subjected to repeated photoreversals. The ratio of the optical

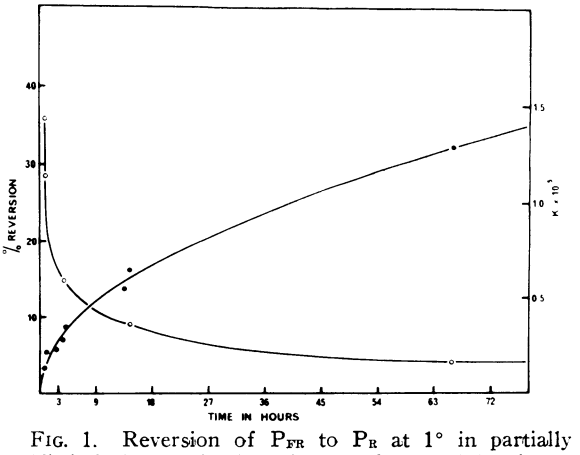

FIG. 1. Reversion of P_{FR} to P_R at 1° in partially purified isolates of phytochrome from etiolated oat coleoptiles. Reversion is expressed as a percentage (Δ $OD_{665\ rev.}$ + Δ $OD_{725\ rev.}$) of the total phytochrome (Δ $OD_{665\ tot.}$ + Δ $OD_{725\ tot.}$) determined at the start and end of each run. Also plotted are first order rate "constants" (K) derived from these data. ● — ● % Reversion. ○ — ○ K.

density shifts was 0.95 at the peak wavelengths during phototransformations (*viz.* $\triangle OD_{725}/\triangle OD_{665}$). During the dark reversion process the ratio of optical density shifts at the peak wavelengths ($\triangle OD_{725}/\triangle OD_{665}$) varied from 0.65 to 0.8.

Isolation and Reversion of Phytochrome from Parsnip Roots. An unequivocal reversion of P_{FR} to P_R occurs in parsnip roots (13). Since denaturation may play a part in the reversion process observed in oat isolates, an attempt was made to isolate phytochrome from parsnip roots and compare its *in vitro* rate of reversion to that reported *in vivo*.

Roots were washed free of dirt and cut into slices after cooling to 1°. Slices were immediately frozen in acetone at −72° and ground to a fine powder while frozen. The powder was resuspended several times in dry acetone at −20° and filtered on a Buchner funnel after each resuspension. Acetone and any residual traces of water were removed under high vacuum at −17° over P_2O_5. Dry powder (240 g) was stirred into pyrophosphate buffer (1600 ml) containing twice the usual amount of cysteine to ensure that potential oxidants were controlled. After centrifugation the clear straw colored supernatant appeared to contain large amounts of pectin, which blocked the porous polyethylene discs used in the gel filtration columns. The phytochrome and much of the protein was therefore precipitated with 30 % sat. $(NH_4)_2SO_4$. After resuspension of the pellets in 0.1 M pyrophosphate buffer the phytochrome was partially purified in an identical manner to the oat preparation. Gradient elution from brushite was used as the final chromatographic step. The phytochrome was finally precipitated with 30 % sat. $(NH_4)_2SO_4$ and the small grey protein precipitate resuspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing mercaptoethanol and K^+ EDTA at the concentrations used previously.

Absorption spectra of the red and far-red absorbing forms of phytochrome prepared from parsnip roots in this manner were reminiscent of those reported for *Mesotaenium* (23). The absorption maxima (λ max P_{FR} 715; λ max P_R 665 nm) were 5 to 10 nM towards shorter wavelengths than the absorption maxima of phytochrome isolated from etiolated material. Phytochrome isolated from *Mesotaenium* did not appear to undergo reversion however, while the phytochrome did revert when isolated from parsnip roots by very similar techniques. In figure 2 are shown plots of reversion against time at 4 different temperatures. The process is obviously not first order. At 13° 50 % reversion takes approximately 24 hours *in vitro,* which is in contrast to the 2 to 3 hours taken at 23° calculated from Hillman's (13) data.

Isolation and Reversion of Phytochrome from Parsnip Leaves. It seemed interesting to look at the reversion undergone by phytochrome isolated from a leaf grown in continuous light. If there are 2 forms of phytochrome (14) and the inactive one

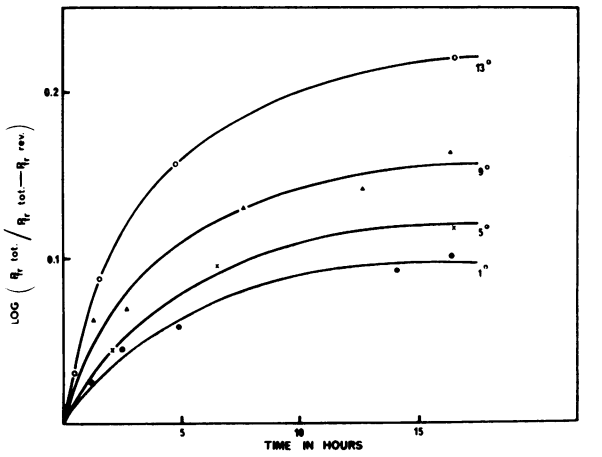

FIG. 2. Kinetics of the *in vitro* P_{FR} to P_R reversion process at different temperatures. Phytochrome was prepared from parsnip roots.

is characterized by P_{FR} decay, there may be little or none of this form present in tissues grown in continuous light.

Young parsnip leaves were rinsed thoroughly in distilled water, and dried as much as possible with filter paper before being frozen in acetone at −72°. A dry powder was prepared from this frozen tissue in the same manner as powders were prepared from parsnip roots. Dry powdered leaf material (100 g) was resuspended in 1800 ml of pyrophosphate buffer and after centrifugation, phytochrome in the green brown solution was partially purified in an identical manner to that used in the isolation of phytochrome from *Mesotaenium* (23). At the final stage of purification the sample was still faintly green and reversion studies had to be done by difference spectroscopy.

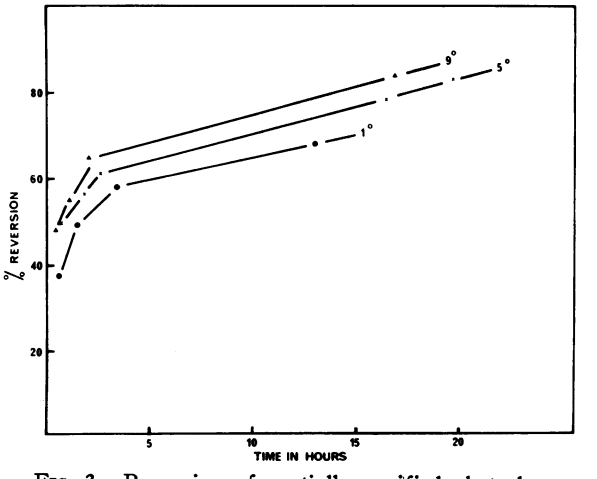

FIG. 3. Reversion of partially purified phytochrome isolated from young parsnip leaves grown in continuous light. Reversion has been calculated as in figure 1. Data was obtained by difference spectroscopy.

The yield of phytochrome from light grown parsnip leaves was calculated to be 2.5 × 10^{-3} △ (△OD)cc/g dry weight by assuming that the overall recovery was similar to the eventual 10% yield of phytochrome from etiolated peas put through a similar purification procedure. The absorption maxima of the red and far-red forms as seen by difference spectroscopy were very close to the respective λ max of phytochrome isolated from parsnip roots. The far-red absorbing form of the pigment had a faster rate of reversion than that seen in any previous phytochrome preparations (see fig 3); half the P_{FR} having reverted after one and one-half hours at 1°. The rate of the reversion process is obviously not proportional to the P_{FR} concentration. It appears to proceed in at least 2 distinct stages, a very rapid phase being followed by a very much slower phase.

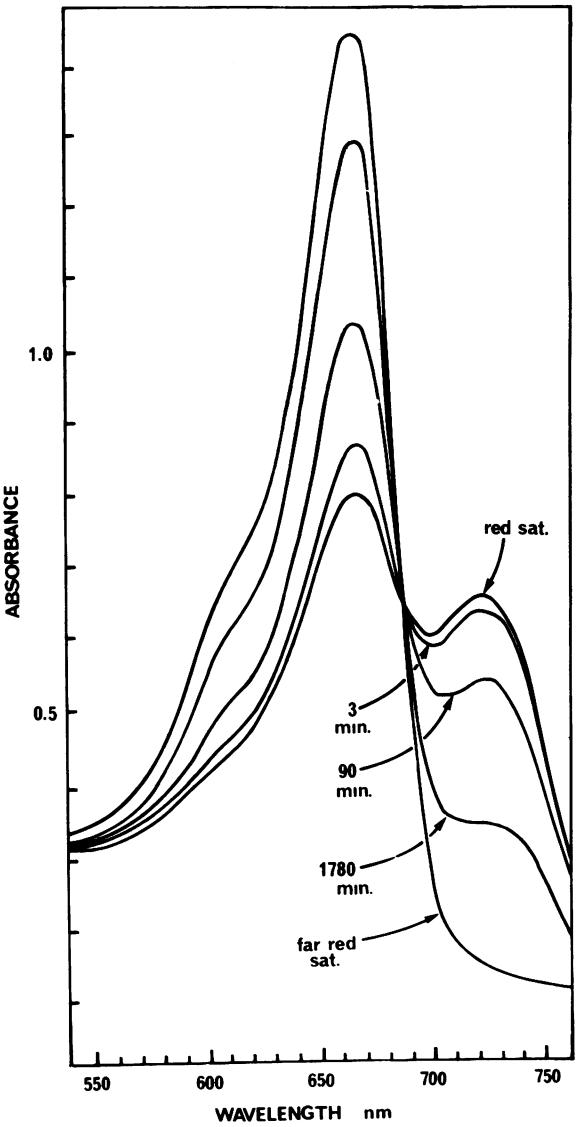

FIG. 4. Absorption spectra of the red and far-red absorbing forms of partially purified phytochrome isolated from etiolated pea plumules. Traces demonstrating dark reversion at 1° are shown 3, 90 and 1780 minutes after saturation of the sample with red light. A slight shift in the isosbestic point to shorter wavelengths can be seen during the reversion process.

Isolation and Reversion of Phytochrome from Etiolated Peas. Frozen dark grown pea plumules and plumular hooks were ground in pyrophosphate buffer (1°) by short bursts in a Waring Blendor. Similar precautions to those used in the isolation of phytochrome from etiolated oats were used to prevent the dispersion of air into the homogenate. The crude solution was centrifuged after filtering through nylon mesh. Phytochrome in the clear supernatant was partially purified in the same manner as isolates prepared from etiolated oats.

Phytochrome partially purified from peas in this manner underwent reversion of a very similar nature to that reported by Bonner (1). Figure 4 shows the absorption spectra of the red and far-red absorbing forms of the pigment and a number of successive traces of reversion of the sample. It is obvious that the optical density increase in the red is far greater than the decrease in the far-red over the first hour, and that over longer times this difference becomes less pronounced. This, along with a slight shift in the position of the isosbestic point to shorter wavelengths, suggest the presence of semi-stable intermediate(s) in the reversion process with absorption spectra differing from P_R and P_{FR}. Kinetic analysis of optical density changes at 665 and 725 nm are shown in figures 5a and b. They are complicates by overlapping rate constants but do show definite breaks presumably corresponding to steps in the process. No attempt has been made to characterize these intermediates further.

Reversion in Nonpurified Solutions. Results so far obtained in this work do not discount the possibility that isolation or purification induced denaturation of the phytochrome distorts the observed reversion process. Phytochrome was therefore isolated from etiolated peas and oats by 2 completely different techniques and the reversion rate of P_{FR} in nonpurified solutions compared with rates observed in partially purified isolates. Etiolated oats or peas were either acetone powdered (see isolation from parsnip roots) or ground fresh with sand in a mortar and pestle. Standard pyrophosphate buffer was used in the grinding or for resuspending the acetone powder. After clearing by centrifugation the crude solutions were passed through Sephadex G50 (equilibrated with 0.1 M phosphate buffer) to remove any low molecular weight oxidants which could distort the reversion process observed (11).

Rates of reversion of P_{FR} observed in crude isolates of oats obtained by these 2 very different procedures were very similar. Exposure of the phytochrome to the unfavorable pH of the cell sap for a few seconds during fresh grinding of the coleoptiles, does not appear to alter the rate or

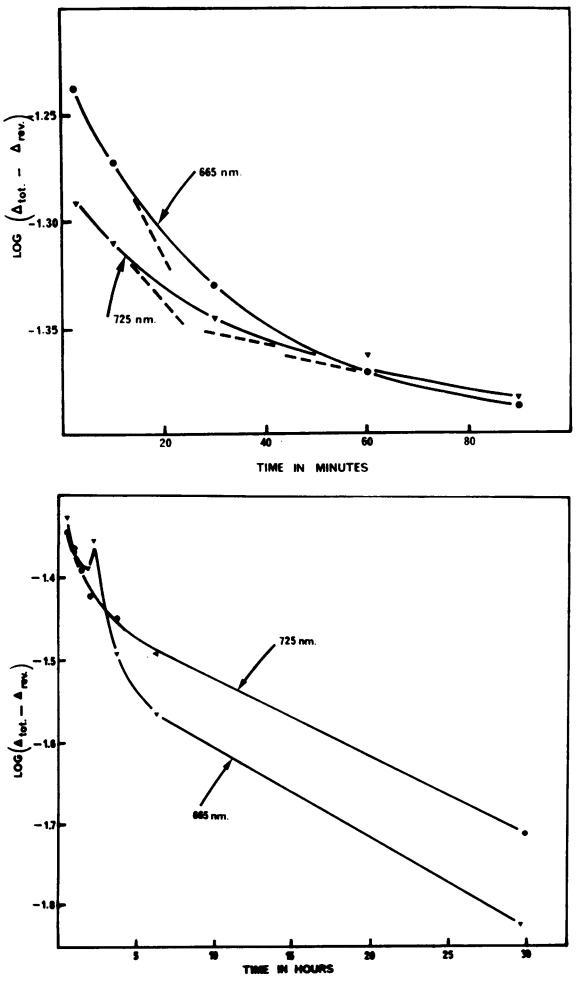

FIG. 5a and b. Kinetics of the reversion process undergone by the phytochrome sample shown in figure 4. Rates of change in absorbance occurring at the λ max of the red and far-red absorbing forms have been plotted separately. Breaks in the shape of the curves over short (fig 5a) and long (fig 5b) time intervals suggest the existence of metastable intermediates, in the process.

extent of the reversion process. The solution obtained from the acetone powder showed typical reversion figures of 4.2 % after 2 hours and 13.5 % after 16 hours at 1°. Although reversion in these crude isolates was not studied in detail, it was obvious that the extent of the initial rapid part of the reversion process was reduced compared to that seen previously in partially purified solutions. This can be seen from the values given if they are compared to those in figure 1.

The extent of the initial rapid phase of the reversion process is more pronounced in partially purified pea isolates. Crude solutions of phytochrome from etiolated peas were therefore prepared as above by fresh grinding and acetone powdering. The crude isolates were cleared of low molecular weight contaminants by passage through Sephadex G50. Reversion studies were performed by difference spectroscopy. Phytochrome isolated from etiolated peas by these 2 very different methods underwent reversion, though the rate of reversion appeared to change with time. Eighteen hours after preparation of the sample by fresh grinding, its hourly reversion rate had more than doubled from 5.5 % to 12.5 %.

This phenomenon was looked at in more detail and over longer periods in isolates produced from acetone powders, since they allowed the preparation of more concentrated protein solutions. In table I, the extent of the reversion in this preparation can be seen to decrease initially, then slowly increase over a period of several days.

Table I. *Influence of Time on the Reversion of P_{FR} to P_R in Nonpurified Solutions of Phytochrome Obtained from an Acetone Powder of Etiolated Pea Plumules*

Reversion has been measured during hourly periods over a number of days. Actual preparation of the sample took one and one-half hour though for simplicity the first reading has been denoted as time zero.

Time[1]	Hourly rates of reversion[2]	Total phytochrome
Hr	%	ΔΔ *OD* cc^{-1}
0	13.6	0.304
2	11.2	0.295
4	9.6	0.295
19	8.9	0.284
23	9.2	0.275
72	14.1	0.212
97	14.3	0.194
145	17.4	0.162

[1] Time after preparation of the sample.
[2] Reversion expressed as a percentage of the total photoreversible phytochrome produced at red saturation at these times.

Influence of Urea Induced Partial Denaturation on Reversion. Phytochrome was isolated from frozen etiolated pea plumules as previously. The changing rates of reversion of this sample over a period of days are shown in figure 6. After determination of the third sequence of points (*i.e.* 96 hr after preparation of the sample) an aliquot of the sample was converted to the red absorbing form and 10 M urea pH 7.8 added to a final concentration of 2 M. Urea was added slowly in the dark with thorough stirring. The reversion of the sample was then reinvestigated. It can be seen in figure 6 that the reversion rate of the P_{FR} has dropped dramatically in 2 M urea. If urea had not been added, the rate of reversion would have been between that exhibited by the sample 96 and 192 hours after its preparation. An obvious feature of the shape of the curves is that although urea has reduced the rate of the slow phase of the reversion slightly, its major influence has been to virtually

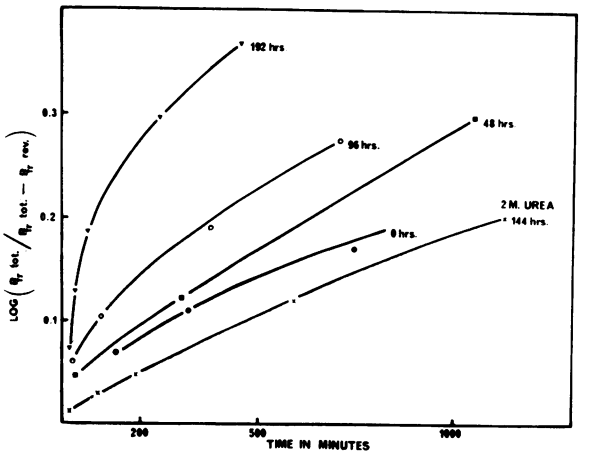

FIG. 6. Kinetics of the reversion process in a non-purified solution of phytochrome obtained from an acetone powder of etiolated pea plumules. The rate of the reaction at 1° has been determined at increasing times after preparation of the sample (0 hr). Urea (to a final conc of 2 M) was added to an aliquot of the sample 144 hours after its preparation. (See text for details).

abolish the initial rapid phase. It would be tempting to assume that 2 M urea has merely caused denaturation of the rapidly reverting fraction of P_{FR}. Yet after correction for dilution, the overall loss of photoreversible phytochrome during addition of the urea is only 4 %, while the rapidly reverting fraction appears to make up approximately 10 % of the P_{FR} in freshly prepared pea isolates.

It was difficult to be certain that the ratio of optical density changes during photoconversion and dark reversion were similar to that exhibited by the sample before addition of urea. A slow increase in absorbance at shorter wavelengths causes the whole baseline to tilt after urea addition. Nevertheless the extent of the optical density changes in the red and in the far-red did appear to be normal when an attempt was made to correct for this.

Discussion

It is clear that phytochrome prepared by several techniques from a number of etiolated and light grown plant sources and purified to different degrees, is capable of reverting in darkness from the far-red absorbing form to the red absorbing form. The reaction appears similar to that reported in isolates of etiolated peas by Bonner (1) and *in vivo* in cauliflower curds by Butler *et al.* (5). It proceeds in at least 2 stages, a short relatively rapid phase being followed by a very slow phase. A preliminary analysis of the reversion process occurring in isolates of etiolated rye seedlings has also led Edwards and Correll (9) to suggest that the mechanism is more complex than either a first or second order reaction. Mumford (18) maintains that the reversion process in oat isolates is first order but his rate constants appear to fall with time and he gives no "constants" calculated from short time intervals which would show up a possible 2 phase process most clearly. Buffers used throughout this work were freshly boiled, but there was no clear indication that removal of dissolved oxygen influenced the reversion process. When phytochrome isolated from etiolated oats reverts, optical density changes at the absorption maxima of the 2 forms are reasonably similar when measured over long time intervals. During the initial rapid phase of the process however, the ratio of $\triangle$OD 725 to $\triangle$OD 665 is significantly lower than unity. This variation in the ratio of optical density changes during reversion is more obvious in phytochrome prepared from etiolated peas where the initial rapid phase is more extensive and the reversion process as a whole is more rapid. This data is interpreted as meaning that intermediates exist in the *in vitro* dark reversion process and that these intermediates have absorption spectra differing from either P_{FR} or P_R. Data in this work are not sufficient to determine their number or absolute absorption spectra, but they can be seen to have half-lives several orders of magnitude longer than intermediates known to occur in $P_R \leftrightarrows P_{FR}$ phototransformations (17). Large differences in the ratio of optical density changes ($\triangle OD_{725}/\triangle OD_{665}$) during reversion of oat phytochrome reported by Butler *et al.* (6) are presumably due to massive denaturation of the sample.

It is difficult to be certain what part denaturation plays in the *in vitro* results obtained in this work. The author has been unable to prepare samples of oat phytochrome which did not revert whatever precautions were taken to prevent denaturation, and direct spectroscopy has demonstrated reversion *in vivo* in some non-chlorophyllaceous plant tissues (15). Molecular changes occurring in the reversible phototransformations of phytochrome may involve an isomerization (17) of the bilitriene (19) chromophore followed by a conformational change of the protein component (6). Since the reversion process is purely thermal, factors which influence protein configuration could influence the rate at which dark reversion occurs. There is some data which support this. Low molarities of urea (2 M) used in this work, actually reduce the rapid phase of the reversion process without appearing to irreversibly denature phytochrome isolated from peas. The rate at which phytochrome will revert in any preparation will however change and usually increase with time. This presumably means that some slow change in protein tertiary structure does occur in solution. The initial rapid drop in the rate of reversion of unpurified solutions of phytochrome prepared from dry powders of etiolated peas is difficult to understand. The total amount of phytochrome has stayed virtually the same during this time so the drop cannot represent denaturation of the rapidly reverting form of phytochrome.

During the longer period that the reversion rate increases, *i.e.* virtually doubles, the amount of photoreversible phytochrome decreases to half of that initially present. The actual amount of reversion taking place stays reasonably constant, but when reversion is expressed as a percentage of the total photoreversible phytochrome present at that time, it appears to increase. If partially denatured P_{FR} were more sensitive to complete denaturation which resulted finally in loss of optical reversibility, one should observe a loss of P_{FR} capable of dark reversion accompanying the loss in photoreversible phytochrome. If the rate of reversion actually increases, the rate of denaturation reactions leading to a dark reversible form of P_{FR} must exceed the rate of reactions leading to complete loss of optical reversibility. This possible explanation is not helped by the surprising degree of consistency in the reversion of crude or purified isolates produced in this work in a number of different ways.

The potential for reversion of phytochrome isolated from various plants and plant parts does appear to be markedly different. Phytochrome purified from light grown *Mesotaenium* (23) showed little or no reversion over a 4 hour period at 1°. Phytochrome isolated from oats reverted slowly (50 % in 110 hr at 1°), while phytochrome from parsnip leaves reverted quickly (50 % in 1½ hr at 1°) and isolates from other sources fell between these extremes. The absorption maxima of the red and far-red absorbing forms of the pigment isolated from etiolated oats and peas are similar but phytochrome isolated from parsnip leaves and roots, dark grown *Sphaerocarpos* and light grown *Mesotaenium* (23) have absorption maxima which are 10 to 20 nm toward shorter wavelengths. Denaturation can cause a shifting of the absorption maxima to shorter wavelengths, but other characteristics of the phytochrome alter at the same time. Phytochrome isolated from *Mesotaenium* (23) showed good stability during storage. The ratio of optical density changes at peak wavelengths and quantum efficiencies for photoconversion were also similar to those reported for oat phytochrome which is assumed to be nondenatured (6, 21). Techniques used in the isolation of phytochrome from parsnip roots and leaves were similar to those employed in the *Mesotaenium* work and phytochrome from the parsnip tissues did not appear to be denatured since it was reasonably stable and the ratio of optical density changes during photoreversals was close to unity. Whether the phytochromes are denatured or not, one cannot extrapolate directly from this *in vitro* data to an *in vivo* situation. In intact etiolated tissues P_{FR} decays rather than reverting (4) and rates of reversion observed *in vitro* do not approach those observed *in vivo*. A tentative P_{FR} half-life of 2.5 hours at 25° has for example been reported in oat mesocotyl (8) while the half-life of oat phytochrome reverting *in vitro* has been calculated to be 9 hours at 25° (18). Similarly the rate of reversion of P_{FR} in parsnip roots at 23° (13) is 5 to 10 times faster than that observed *in vitro* at 13°. Nevertheless the dark reversion reaction undergone by parsnip root phytochrome *in vitro* does appear mechanistically similar to that occurring *in vivo* since both proceed in at least 2 distinct phases. The rate of the dark reversion process in intact green leaves could be quite rapid therefore, since phytochrome isolated from parsnip leaves reverts much more rapidly than that isolated from parsnip roots. This assumption is strengthened by physiological data suggesting a P_{FR} reversion half-life of only 15 minutes in chrysanthemum leaves (2).

It has been noted that the reversion process both *in vivo* and *in vitro* appears to proceed in at least 2 distinct phases. This may mean that there are at least 2 naturally occurring forms of phytochrome. There is some physiological and direct spectrophotometric evidence which may support this. Briggs and Chon (7) report that dosages of red light sufficient to saturate the phototropic sensitivity of corn coleoptiles are 100 times too small to induce measurable phytochrome transformations. This and the rapid decay of the bulk of P_{FR} in etiolated seedlings have given rise to the concept of active and inactive phytochrome fractions (14). If phytochrome isolated from light grown parsnip leaves is indeed "active" phytochrome, its pattern of reversion although more rapid is still basically similar to that of "inactive" phytochrome obtained from etiolated peas. Of more direct interest is the observation by Spruit (22) that a different form of phytochrome exists in the leaves of etiolated peas than in the internode immediately below the leaves. These forms detected by difference spectroscopy differ in the position of their absorption maxima and in the ratio of optical density changes at their absorption maxima during phototransformations. It seems likely that different forms of phytochrome exist in some plant species and possibly within the same plant. These differences most obviously being in the rate of dark reversion of P_{FR} to P_R and in the position and magnitude of the absorption maxima of the 2 forms.

Some intracellular control of the rate of dark reversion would seem important to the cell. Results obtained in this work are interpreted as indicating that slight changes in the tertiary structure of the protein component can markedly influence the rate of the reversion process. In the intact cell this could be brought about in a number of ways. Mumford (18) has hypothesized that binding of a small molecular weight modifier may control the configuration of the protein. Successive purification steps in the isolation of phytochrome from etiolated tissues do not give any evidence of a compound of this type, since purification does not markedly alter the reversion rate of the phytochrome. Membrane binding of phytochrome could also influence protein configuration and so control the rate of reversion. The turning off of enzyme activity is usually accom-

plished by the regulation of protein synthesis and degradation. Reversion of the active form of phytochrome to an inactive form in darkness is a novel form of regulation. Elucidation of any intracellular control of this reaction would be very worthwhile.

Acknowledgments

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Literature Cited

1. Bonner, B. A. 1962. *In vitro* dark conversion and other properties of phytochrome. Plant Physiol. 37: xxvii.
2. Borthwick, H. A. and H. M. Cathey. 1962. Role of phytochrome in control of flowering of chrysanthemum. Botan. Gaz. 123: 155–62.
3. Borthwick, H. A., S. B. Hendricks, M. W. Parker, E. H. Toole, and V. K. Toole. 1952. A reversible photoreaction controlling seed germination. Proc. Natl. Acad. Sci. U. S. 38: 662–66.
4. Butler, W. L., H. C. Lane, and H. W. Siegelman. 1963. Nonphotochemical transformations of phytochrome *in vivo*. Plant Physiol. 38: 514–19.
5. Butler, W. L. and H. C. Lane. 1965. Dark transformations of phytochrome *in vivo*. II. Plant Physiol. 40: 13–17.
6. Butler, W. L., H. W. Siegelman, and C. O. Miller. 1964. Denaturation of phytochrome. Biochemistry 3: 851–57.
7. Briggs, W. R. and H. P. Chon. 1966. The physiological versus the spectrophotometric status of phytochrome in corn coleoptiles. Plant Physiol. 41: 1159–66.
8. Edwards, J. L. and W. H. Klein. 1964. Relationship of phytochrome concentration of physiological responses. Plant Physiol. 39: 1.
9. Edwards, J. L. and D. L. Correll. 1967. Kinetics of the dark reversion of phytochrome *in vitro*. Plant Physiol. 42: S-10.
10. Furuya, M., W. G. Hopkins, and W. S. Hillman. 1965. Effects of metal-complexing and sulfhydryl compounds on nonphotochemical phytochrome changes *in vivo*. Arch. Biochem. Biophys. 112: 180–86.
11. Furuya, M. and W. S. Hillman. 1966. Rapid destruction of the P_{FR} form of phytochrome by a substance in extracts of *Pisum* tissue. Plant Physiol. 41: 1242–44.
12. Hendricks, S. B., W. L. Butler, and H. W. Siegelman. 1962. A reversible photoreaction regulating plant growth. J. Phys. Chem. 66: 2550–55.
13. Hillman, W. S. 1964. Phytochrome levels detectable by *in vivo* spectrophotometry in plant parts grown or stored in the light. Am. J. Botany 51: 1102–07.
14. Hillman, W. S. 1967. The physiology of phytochrome. Ann. Rev. Plant Physiol. 18: 301–24.
15. Koukkari, W. L. and W. S. Hillman. 1966. Phytochrome levels assayed by *in vivo* spectrophotometry in modified underground stems and storage roots. Physiol. Plantarum 19: 1073–78.
16. Koukkari, W. L. and W. S. Hillman. 1967. Effects of temperature and aeration on phytochrome transformations in *Pastinaca sativa* root tissue. Am. J. Botany 54: 1118–22.
17. Linschitz, H., V. Kasche, W. L. Butler, and H. W. Siegelman. 1966. The kinetics of phytochrome conversion. J. Biol. Chem. 241: 3396–3403.
18. Mumford, F. E. 1966. Studies on the phytochrome dark reaction *in vitro*. Biochemistry 5: 522–24.
19. Siegelman, H. W. and S. B. Hendricks. 1965. Purification and properties of phytochrome: A chromoprotein regulating plant growth. Federation Proc. 24: 863–67.
20. Siegelman, H. W. and W. L. Butler. 1965. Properties of phytochrome. Ann. Rev. Plant Physiol. 16: 383–92.
21. Siegelman, H. W. and E. M. Firer. 1964. Purification of phytochrome from oat seedlings. Biochemistry 3: 418–23.
22. Spruit, C. J. P. 1967. Abnormal phytochrome spectrum in leaves. Biochem. Biophys. Acta 143: 260–62.
23. Taylor, A. O. and B. A. Bonner. 1967. Isolation of phytochrome from the alga *Mesotaenium* and liverwort *Sphaerocarpos*. Plant Physiol. 42: 762–66.