Nonphotochemical Transformations of Phytochrome in Vivo[1]

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Introduction

Many aspects of plant growth and development are controlled by red and far-red light. The photoreceptor which effects control over these various processes is a reversible, photochromic pigment, which exists in 2 interconvertible forms.

$$P_{660} \underset{730\ m\mu}{\overset{660\ m\mu}{\leftrightarrows}} P_{730}$$

Some of the photochemical properties of this pigment, which has been dubbed phytochrome, have been described previously (5, 7, 8). The present paper is concerned with the nonphotochemical transformations of phytochrome in vivo.

Dark transformations of phytochrome were indicated from studies of the germination of lettuce seeds (2) and the flowering of photoperiodic plants (3). The far-red absorbing form of phytochrome, P_{730}, was found to revert to the red-absorbing form, P_{660}, in darkness. This dark reaction was postulated to be the basis of the time-sensing mechanism of photoperiodism (4). The photometric measurement of phytochrome (7) permits the dark conversion of P_{730} to P_{660} to be measured in vivo. In dark-grown seedlings, this dark conversion is accompanied by another dark reaction which results in a loss of reversible phytochrome. It is not known whether this loss is due to a destruction of phytochrome or merely to a loss of photoreversibility. The present paper reports on these 2 nonphotochemical processes which appear to involve phytochrome as a reactant.

Materials and Methods

Maize seedlings (U.S.D.A. 13) were grown in plastic boxes on moistened cellulose pads in darkness for 6 to 7 days at 25°. Samples were prepared for spectroscopic examination by cutting the shoots into small segments 3 to 5 mm long under dim green light. In some cases only the coleoptiles and enclosed primary leaves were used in the sample; in other cases, only the first internodes were used. A 3-g sample was pressed firmly into a cell giving a sample thickness of about 1.5 cm. A fresh 3-g sample was harvested and prepared immediately before each experimental determination. Measurements on cauliflower were made on 3-g samples taken from the outer portion of the head.

Relative amounts of phytochrome were determined with a dual-wavelength photometer (fig 1) which measured the optical density difference between 660 and 730 mμ, $\Delta\ OD = OD_{660} - OD_{730}$. The sum of P_{660} plus P_{730}, referred to as total reversible phytochrome, was indicated by the change in the optical density difference reading following irradiation of the sample with actinic sources of red and far-red light which drove the photoreaction to completion in the appropriate direction, $P_{660} + P_{730} = K(\Delta\ OD_{\text{far-red irrad.}} - \Delta\ OD_{\text{red irrad.}})$ where K is a constant of proportionality. The relative amount of either P_{660} or P_{730} could be determined by measuring the Δ OD before the actinic irradiation and noting the change of the Δ OD reading due to the first irradiation; e.g., $P_{730} = K(\Delta\ OD_{\text{far-red irrad.}} - \Delta\ OD_{\text{initial}})$. The Δ OD scale has an arbitrary zero adjustment so that the values are not absolute. The changes in the Δ OD values, referred to as Δ(Δ OD), were calibrated against known optical density changes obtained by introducing a neutral density screen into one of the measuring beams so that the Δ(Δ OD) values are absolute. The irradiation sequence and a typical set of instrument readings are shown in table I for a sample of tissue in which protochlorophyll, P_{660} and P_{730} are present.

$$\Delta(\Delta\ OD)_{P_{730}} = 0.080 - 0.070 = 0.010$$

$$\Delta(\Delta\ OD)_{P_{660} + P_{730}} = 0.040 - 0.005 = 0.035$$

Table I

Optical Density Difference Readings of Dual-wavelength Photometer Following Irradiation of the Sample with Actinic Sources of Red and Far-red Light

Irradiation sequence	Δ OD		
	initial	after far-red irrad.	after red irrad.
1st	0.070	0.080	0.005
2nd	...	0.040	0.005
3rd	...	0.040	0.005

[1] Received Jan. 28, 1963.

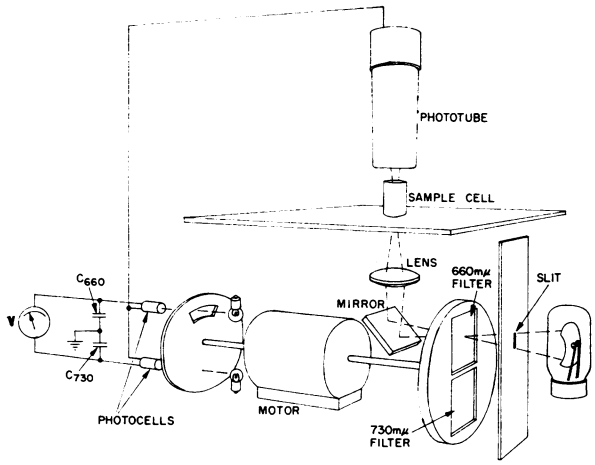


FIG. 1. Schematic diagram of 2-filter difference photometer. Monochromatic beams of 660 mμ and 730 mμ, obtained from the rotating filter wheel, are alternately incident on the sample. The voltage on the multiplier phototube, operating in a constant-current circuit (11), is nearly proportional to the logarithm of the light intensity transmitted through the sample. The small lamps, photoconductive cells, and slotted wheel on the other end of the motor shaft serve as a synchronous switch. The condenser, C_{660}, is charged through the illuminated photoconductive cell to the voltage of the multiplier phototube when the 660 mμ interference filter is in the measuring beam. The condenser, C_{730}, is similarly charged when the 730 mμ interference filter is in the beam. Each condenser is charged when its associated photoconductive cell is in the illuminated, low-resistance state and the charge is maintained while the photoconductive cell is in the dark, high-resistance state. The vacuum tube voltmeter, V, measures the difference between the voltage on C_{660}, which is proportional to the optical density of the sample at 660 mμ, and the voltage on C_{730}, which is proportional to the optical density at 730 mμ, Δ OD = $OD_{660} - OD_{730}$. The intensity of the lamp is sufficiently low that the measuring beams do not change the phytochrome. External actinic sources of red and far-red light are used to drive the phytochrome completely to the P_{660} and P_{730} forms.

The first irradiation with far-red converts the P_{730} present to P_{660}. The next irradiation with red converts protochlorophyll to chlorophyll and P_{660} to P_{730}. Both processes contribute to the change of the Δ OD value. The protochlorophyll-chlorophyll transformation is irreversible, however, and subsequent irradiation sequences involve only phytochrome transformations.

The source of red actinic radiant energy was a tungsten lamp with a 650-mμ interference filter. The far-red actinic source was a tungsten lamp with 2 layers of red and 4 layers of blue cellophane. The radiant energy in the latter source was limited to wavelengths longer than 730 mμ.

The intensities of the 660- and 730-mμ measuring beams of the photometer were reduced sufficiently to prevent significant photoconversion of phytochrome during the photometric measurement.

Results

Concentration of Phytochrome in Dark-Grown Seedlings. The average concentration of phytochrome in plant tissue can be estimated from the reversible optical density changes.

$$\Delta\ (\Delta\ \text{OD}) = 2\ \beta\ \alpha\ c\ l$$

c is the average concentration including the air space between seedling segments in mole/liter and l is the sample thickness in centimeters. The factor of 2 is present because the $\Delta(\Delta$ OD), which is due to the sum of the reversible optical density changes at 660 and 730 mμ, measures the total amount of reversible P_{660} plus the total amount of reversible P_{730}. The light scatter in the sample makes the optical pathlength greater than the sample thickness so that the optical density changes are enhanced (6). The factor of enhancement, β, can be reasonably estimated to be between 5 and 10. The extinction coefficients of $\alpha_{660,\,max} = \alpha_{730,\,max} = 2 \times 10^4$ 1/mole/cm (5). These values assume that the quantum yield for the photoconversion of P_{660} to P_{730}, ϕ_{660}, is unity. If ϕ_{660} is less than one, the extinction coefficients are correspondingly greater and the concentrations are correspondingly less. A typical $\Delta(\Delta$ OD) value for a 1.5 cm thick sample of dark-grown seedlings is 0.03. For such a sample $c = 5 \times 10^{-8}$ to 10^{-7} M.

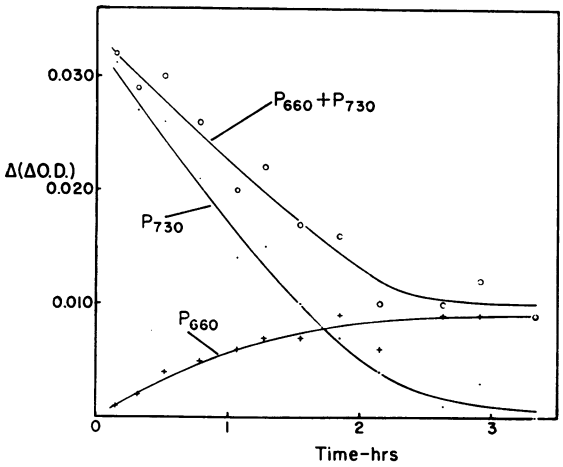
State of Phytochrome in Dark-Grown Seedlings. The amount of P_{730} present in dark-grown seedlings can be determined from the change in the Δ OD reading due to the initial irradiation with far-red light. The photoconversion of P_{730} to P_{660} will cause the Δ OD measurement to increase. When such an experiment is made one finds that the Δ OD reading decreases slightly, which is the action expected from red light. The decrease of the Δ OD value occurs because the phytochrome is entirely in the P_{660} form and the long-wavelength tail of the P_{660} absorption band extends to sufficiently long wavelength that the far-red light converts a small fraction of the P_{660} to P_{730}.

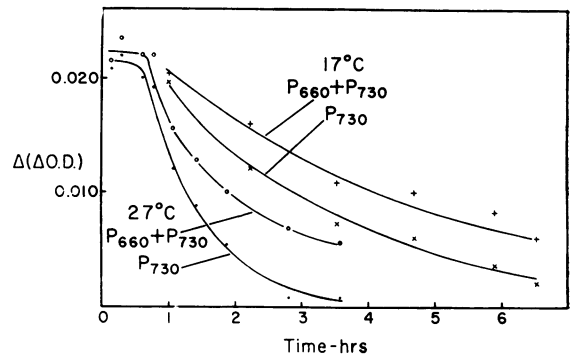
In a later section we will want to know the fraction of phytochrome which is P_{730} at the photostationary state in far-red light. The ratio of P_{730} to P_{660} at the photostationary state in monochromatic light of wavelength λ is given by:

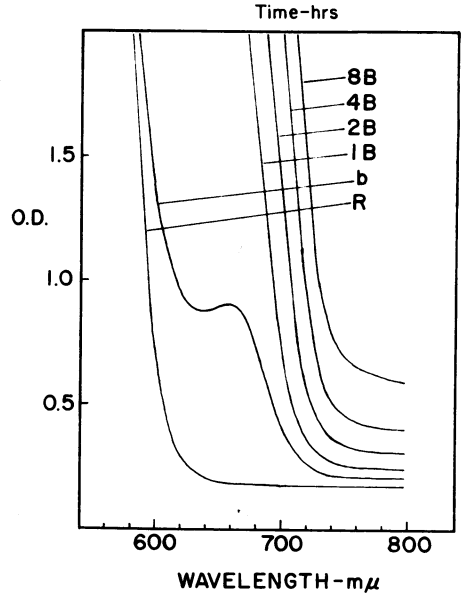
$$\frac{P_{730}}{P_{660}} = \frac{\alpha_{660\lambda}\ \phi_{660}}{\alpha_{730\lambda}\ \phi_{730}}$$

where $\alpha_{660\lambda}$ and $\alpha_{730\lambda}$ are the extinction coefficients of P_{660} and P_{730} at λ and ϕ_{660} and ϕ_{730} are the quantum yields for the photoconversions of P_{660} and P_{730}, respectively. (It was previously shown that $\frac{\phi_{660}}{\phi_{730}} = 4$, (5); more recent work indicates the ratio factor is 3.)

Since the phytochrome in dark-grown plants is entirely P_{660}, we can determine the photostationary state ratio in far-red light by measuring the Δ OD decrease due to the initial irradiation with far-red.







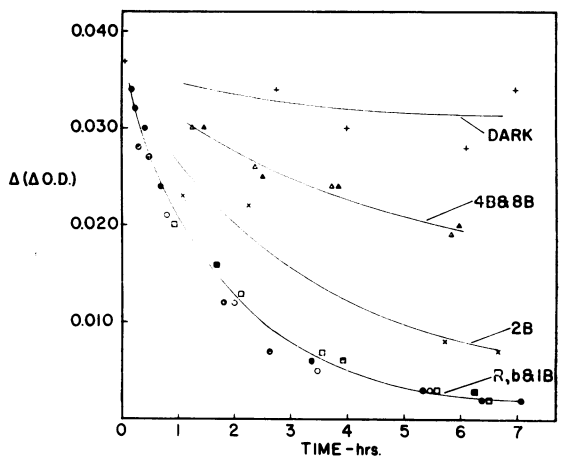


FIG. 2 (*upper left*). Phytochrome changes in the dark at 27° in coleoptiles and primary leaves of 7-day-old, dark-grown maize seedlings following a 2-minute irradiation with red light. ○, $P_{660} + P_{730}$; ●, P_{730}; +, difference between $P_{660} + P_{730}$ and P_{730}.

FIG. 3 (*upper right*). Phytochrome changes in the dark at 27° and 17° in mesocotyls of 7-day-old, dark-grown maize seedlings following a 2-minute irradiation with red light. ○, $P_{660} + P_{730}$ at 27°; ●, P_{730} at 27°; +, $P_{660} + P_{730}$ at 17°; ×, P_{730} at 17°.

FIG. 4 (*lower left*). Absorption spectra of cellophane filters. R, 2 layers of red cellophane; b, 2 layers of red plus 1 layer of light blue cellophane; 1B, 2 layers of red plus 1 layer of dark blue cellophane; 2B, 2 layers of red plus 2 layers of dark blue cellophane; 4B, 2 layers of red plus 4 layers of dark blue cellophane; 8B, 2 layers of red plus 8 layers of dark blue cellophane.

FIG. 5 (*lower right*). $P_{660} + P_{730}$ in coleoptiles and primary leaves of dark grown seedlings after being placed under continuous irradiation from a tungsten source with the filters shown in figure 4. ○, R; ●, b; □, 1B; ■, 1B plus cheesecloth to reduce intensity in half; ×, 2B; Δ, 4B; Δ, 8B and +, dark control.

The precise determination has proved difficult, however, because very little P_{660} is converted by the far-red light and because other irreversible, light-induced absorbancy changes may also occur. For instance, if protochlorophyll is present in the tissue, irradiation with far-red light may form a small amount of chlorophyll by virtue of the long-wavelength tail of the protochlorophyll absorption band. The measurements indicate, however, that in the order of 1 % or less of the P_{660} in a dark-grown plant is converted to P_{730} by the far-red source used.

Dark Conversion of P_{730} to P_{660} In Vivo. After conversion of P_{660} to P_{730} by a brief irradiation with red light, a dark reversion of P_{730} to P_{660} occurs which can be followed in vivo during the ensuing dark period. The results shown in figure 2 were obtained from the coleoptiles plus primary leaves from 7-day-old, dark-grown maize seedlings and

the results in figure 3 were obtained with the mesocotyls. At zero time, the seedlings were irradiated with red light for 2 minutes and returned to darkness. At various times thereafter, a 3-g sample of tissue was harvested and analyzed for P_{730} and $P_{660} + P_{730}$. With time in darkness, the P_{730} reverted to P_{660} and the total amount of photoreversible phytochrome decreased. After the P_{730} to P_{660} dark conversion was completed, the phytochrome was stable and no further changes occurred. If the seedlings were then given a second brief irradiation with red light, the dark reactions commenced again and the reversible phytochrome decreased still further.

The mesocotyl tissue has less phytochrome per gram fresh weight than the combination of the coleoptiles and primary leaves and shows a more pronounced lag in the time course of disappearance of reversible phytochrome. At temperatures somewhat lower than room temperature, about 22°, the time course for the disappearance of reversible phytochrome from the coleoptiles and primary leaves shows a similar lag phase. At 10° or less, the dark reactions leading to the conversion of P_{730} or to the loss of reversible phytochrome cease so that P_{730} remains in the tissue. Also, if the seedlings are kept at room temperature under N_2, P_{730} is stable in the dark and no loss of phytochrome reversibility occurs. The dark conversion of P_{730} to P_{660} and the accompanying loss of reversible phytochrome have been measured in all dark-grown seedling plants tested; e.g., maize, barley, rye, sorghum, soybean, squash, and peas.

Loss of Photoreversible Phytochrome in Continuous Light. It is apparent in figures 2 and 3 that the dark reactions, which result in the loss of photoreversible phytochrome or in the conversion of pigment form, continue as long as P_{730} is present but cease when P_{730} has reverted entirely to P_{660}. If the seedlings are placed in continuous red light so that P_{660} cannot accumulate, the reversible phytochrome decreases to a very low level at a rate similar to that shown for the decay of P_{730}. This experiment suggested that the rate of disappearance of reversible phytochrome in continuous light should be studied as a function of the steady-state ratio of $P_{730}:P_{660}$ by using sources of different spectral distribution.

A preliminary experiment showed that the rate of disappearance of reversible phytochrome from seedlings irradiated continuously with a source which maintained essentially all of the phytochrome as P_{730} was the same as the rate of disappearance under tungsten lamps which maintained $P_{730}:P_{660}$ at a ratio of about 2:1. (In spite of the greater intensity of the far-red wavelengths in a tungsten source, P_{730} predominates at the photostationary state because the quantum yield for the conversion of P_{660} to P_{730} is 3 to 4 times that of the reverse reaction (5).) The tungsten lamps were then used with a series of cellophane filters which maintained phytochrome at different photostationary states. The absorption spectra of these filters are shown in figure 4. The percentage of the total phytochrome which the tungsten source plus these filters maintained as P_{730} at the photostationary state was measured and is shown in table II.

Table II

Photostationary States of Phytochrome

Filter*	R	b	1B	2B	4B	8B
$\frac{P_{730}}{P_{660} + P_{730}} \times 100\,\%$	64	23	10	3	<1	<1

* See figure 4 for filter designation.

The disappearance of photoreversible phytochrome from the coleoptiles and primary leaves which occurs when dark-grown maize seedlings are placed under continuous irradiation with these various filters is shown in figure 5. The tungsten source filtered with 2 layers of red and 1 layer of dark blue cellophane, which would ordinarily be considered a source of far-red radiant energy, maintained enough P_{730} (10 % of the total phytochrome) to saturate the reactions which caused the disappearance of reversible phytochrome. Even the sources which maintained 3 % or less than 1 % of the phytochrome as P_{730} gave measurable rates of disappearance. The intensity of irradiation was not important since the radiation merely maintained a photostationary state. Those seedlings in figure 5 which were covered with cheesecloth lost reversible phytochrome at the same rate as the uncovered plants.

Dark Conversion of Phytochrome in Mature Plants. In spite of the results of figure 5 which show that the photoreversible phytochrome in dark-grown plants decays when the plants are placed in light, physiological evidence demonstrates unambiguously that phytochrome is present and active in mature green plants. The reversible phytochrome does not disappear entirely in the light but decays to a level that it is difficult to detect spectrophotometrically, particularly in the presence of large amounts of chlorophyll. However, phytochrome has been extracted from a number of mature green plants and has been measured in the partially purified solutions (9).

Photoreversible phytochrome can be measured directly in cauliflower heads obtained from the market even though the heads have been exposed to long periods of illumination. Figure 6 shows that in cauliflower tissue P_{730} is also converted to P_{660} in the dark. Samples were taken at various times after the head received a 2 minute irradiation with red light. The rate of dark conversion is comparable to that in etiolated seedlings but no loss of phytochrome reversibility occurs.

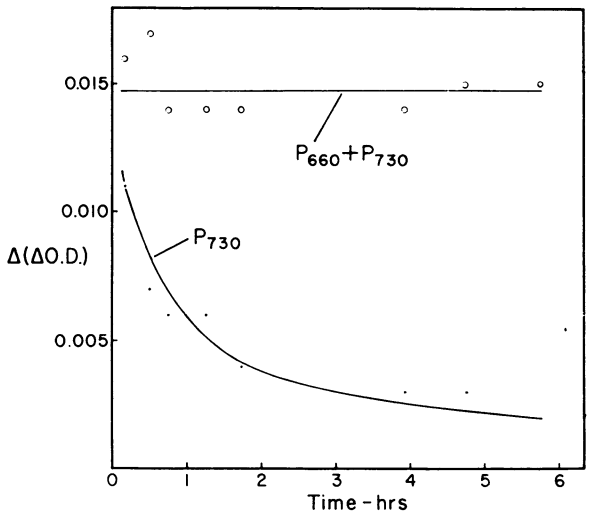


FIG. 6. Phytochrome changes in the dark in a cauliflower head following a 2 minute irradiation with red light. ○, $P_{660} + P_{730}$; ●, P_{730}.

It would be of considerable importance to know how the nondestructible phytochrome differs from the phytochrome in dark-grown plants which loses reversibility in light. We have not detected any difference in the spectral properties between the phytochrome in cauliflower heads or the phytochrome extracted from mature green plants and that present in or extracted from dark-grown plants.

Discussion

The physiological significance of the disappearance of reversible phytochrome, which occurs when dark-grown plants are placed in the light, is not known. It is of practical importance, however, if plants are being grown for the isolation of phytochrome. A few brief periods of illumination during the growth can reduce the amount of phytochrome to a level difficult to detect spectrophotometrically. The dark disappearance of reversible phytochrome and the dark conversion of P_{730} to P_{660} appear to be independent reactions although both involve P_{730}, but not P_{660}. In cauliflower, the dark conversion takes place without the concomitant loss of reversible phytochrome. The same is undoubtedly true in the leaves of mature green plants, although the conversion has not been measured directly in green tissue.

The determination of the state of phytochrome in dark-grown seedlings shows that the equilibrium for the thermal interconversion between P_{660} and P_{730} is entirely to the side of P_{660}. If a small amount of P_{730} were present at thermal equilibrium, the results of figure 5 show that the reversible phytochrome in dark-grown seedlings should slowly decay. Such a decay has not been detected. The prolonged dormancies of light-requiring seeds also indicate that the thermal equilibruim is entirely to the side of P_{660}.

The disappearance of photoreversible phytochrome, which occurs when dark-grown seedlings are placed in continuous light, is worthy of study because it provides a measurable chemical reaction which involves only the P_{730} form of phytochrome. In this respect the chemical reaction is similar to physiological responses in which P_{730} appears to be the active form while P_{660} is quiescent. The loss of reversible phytochrome in vivo was studied in order to draw analogies between the action of P_{730} in this process and the action of P_{730} in physiological responses.

A brief irradiation of dark-grown seedling plants with red light results in a loss of about 70 % of the reversible phytochrome if the plants are assayed several hours later. Most of this loss would have been prevented if the red light had been immediately followed with a brief exposure to far-red light because P_{660} is stable in the dark. Thus, the system demonstrates the repeatable red, far-red reversibility typical of phytochrome-controlled physiological responses. However, a prolonged irradiation with far-red light has the same effect as red light (fig 5). The action of a small amount of P_{730} acting over a long period can be the same as the momentary production of a large fraction of P_{730} followed by the reversion of P_{730} in the dark. The light-induced disappearance of reversible phytochrome shows that the red action of prolonged far-red light is mediated by P_{730} and that the action saturates at relatively low levels of P_{730}. The red action of prolonged far-red cannot be reversed by a subsequent irradiation because physiological processes have gone to completion during long far-red exposure.

The flowering of chrysanthemum shows a similar dependency on red and far-red light (1). Red light, applied as a night-break irradiation, inhibits flowering and a subsequent brief irradiation with far-red, given immediately after the red, restores flowering. A single prolonged (90 min) irradiation with far-red, however, inhibits flowering. Borthwick (1) proposed that the red action of prolonged far-red was due to the small amount of P_{730} maintained during the far-red irradiation, acting over a prolonged period of time. It was recognized, however, that the red action of prolonged far-red might be mediated by an auxiliary, high energy-requiring pigment system which has been postulated to operate in other physiological responses (10). The above experiments on the decay of phytochrome in light provides concrete evidence that the red action of prolonged far-red is due to a low level of P_{730} maintained during the irradiation period.

The direct measurements on phytochrome in vivo have established 2 related phenomena which may be applicable to phytochrome-controlled, physiological responses in general: 1) the action of P_{730} may saturate at low levels of P_{730} and 2) prolonged irradiation periods with far-red light may have the same action as red light.

Summary

Phytochrome was measured spectrophotometrically in dark-grown maize seedlings. Phytochrome in dark-grown seedlings is present entirely as P_{660}. Following a single brief irradiation with red light which converts the P_{660} to P_{730}, P_{730} reverts to P_{660} in darkness and the total amount of photoreversible phytochrome decreases to about 30 % of the original amount. The decay of reversible phytochrome occurs only when P_{730} is present. Both the dark conversion of P_{730} and the loss of reversible phytochrome are prevented if the temperature is lowered or if the plants are placed under N_2.

In continuous irradiation the amount of reversible phytochrome in dark-grown seedlings decreases to a level which is too low to study spectrophotometrically. Heads of cauliflower, however, contain measurable amounts of photoreversible phytochrome which do not decay further in light. In this tissue P_{730} also reverts to P_{660} in darkness, but without any loss of reversible phytochrome.

The decay of photoreversible phytochrome which occurs when dark-grown seedlings are placed in continuous light was studied by using various light sources which maintained different ratios of P_{730}: P_{660} at the photostationary state. The rate of decay saturated when 10 % of the phytochrome was maintained as P_{730}. The decay was still measurable when less than 1 % of the phytochrome was maintained as P_{730}. The results show that far-red light, which maintains a low level of P_{730} by virtue of the long wavelength tail of P_{660}, can produce the same action as red light, particularly if irradiation periods are prolonged.

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