Plant Physiol. (1967) 42, 762–766

Isolation of Phytochrome from the Alga Mesotaenium and Liverwort Sphaerocarpos[1]

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Received December 5, 1966.

Summary. Phytochrome has been isolated from the green alga *Mesotaenium* and the liverwort *Sphaerocarpos*. The *Mesotaenium* pigment had absorption peaks at 649 and 710 nm for the P_R and P_{FR} forms, respectively. Corresponding difference spectrum maxima for the *Sphaerocarpos* pigment were at 655 and 720 nm. While the absorption maxima differ, the reversibility and efficiency with which red and far-red light transform the *Mesotaenium* pigment are very similar to that reported for phytochrome isolated from etiolated seedlings of higher plants. Methods are described which allow efficient separation of phytochrome from highly pigmented light-grown material.

Investigations of Haupt and co-workers have shown that the low intensity induced phototaxis of the chloroplasts of *Mougeotia* and *Mesotaenium* has an action spectrum and a red, far-red reversibility which implicate phytochrome as the photoreceptor (7). The primary biochemical involvement of phytochrome has not been established in any plant. In biochemical investigations of phytochrome action it is of advantage to have unicellular material with a rapid response devoid of the complexities of growth and developmental processes that are usually used as indicators of phytochrome action in higher plants. We are assuming that the sooner a response becomes evident, the nearer is its biochemical mechanism to the original triggering reaction. In view of the potential usefulness of these algae it seemed important to confirm Haupt's conclusions by actual isolation and some characterization of the photoreceptor.

Sphaerocarpos is the liverwort in which Miller and Machlis (12) have found a promotion of growth by low energies of red light, presumably acting through phytochrome. It was included in this work because it can be grown heterotrophically in complete darkness in the manner of a tissue culture (11), a procedure which could also facilitate certain experimental approaches to phytochrome action.

The results reported here confirm the existence of phytochrome in lower plants through direct isolation and measurement of the pigment. It is further established that substantial differences exist in the absorption spectrum of the pigment in plants widely separated phylogenetically.

Materials and Methods

Isolation from Mesotaenium. Pure cultures of *Mesotaenium caldariorum*, strain No. 41, were obtained from the University of Indiana Algal Culture Collection. They were grown in liter flasks in Sager and Granick (13) medium I, enriched with 1 % glucose and 2 g per liter each of yeast extract and sodium acetate to improve their growth rate. Growth was not maintained in complete darkness, so cultures were kept under fluorescent light (100 ft-c) at 18°. These algae settled rapidly, however, so during final stages of growth of the cultures, little light would actually reach most of the cells. Cells were collected by centrifugation (5000 × *g*), resuspended once in 0.5 M potassium phosphate buffer (pH 7.8), recentrifuged, and allowed to cool overnight to 2° in a refrigerator. Subsequent steps were done in a coldroom at 2°. The cells were disintegrated in a prechilled French press in an equal volume of 0.2 M buffer. This buffer and all subsequent ones used throughout the following procedures was 0.5 M potassium phosphate pH 7.8 containing 29 mM 2-mercaptoethanol and 1 mM K^+ EDTA. The dark-green effluent from the press was immediately frozen as a 2 to 4 mm film in petri dishes embedded in dry ice. This was thoroughly lyophilized at − 15°. Dry material was quickly ground in a mortar and pestle at 2°. The resulting powder was dispersed twice into 1 liter volumes of acetone at −19° and collected in a Buchner funnel. The powder was then dispersed in 1 liter of peroxide free ether at −19° and collected in a Buchner funnel. The powder was

[1] Supported in part by N.S.F. Grant GB2123 and by a Sir Walter Mulholland Fellowship awarded by the New Zealand Meat Board to A. O. Taylor.

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finally spread onto aluminum foil and stored under vacuum over P_2O_5 at 2°.

For extraction, dry algal acetone powder (76 g) was stirred rapidly into 300 ml of 0.15 M buffer, and the slurry allowed to stand on ice for 10 minutes. It was centrifuged at 9000 × *g* for 15 minutes yielding a dark-green supernatant (170 ml). The pellet was stirred again with 50 ml of buffer and centrifuged yielding 50 ml of supernatant. The successive extracts were combined and passed through a 4 × 56 cm Sephadex G-50 column, equilibrated with extraction buffer. The first 250 ml of eluate containing high molecular weight compounds was immediately passed through a 5.5 × 63 cm DEAE-cellulose (0.3 meq/g exchange capacity) column pre-equilibrated with the same buffer. Phytochrome isolated from peas is slightly retarded by but not adsorbed to DEAE-cellulose under these conditions. A UV monitor was used to record the elution of 254 nm absorbing components. The effluent was collected as the first proteins came from the column, and was stopped after 320 ml had been pooled. An equal volume of 80 % saturated $(NH_4)_2SO_4$ (adjusted to pH 7.8 with NH_4OH) was rapidly stirred into this pooled fraction, and the resulting protein precipitate sedimented at 30,000 × *g*. The precipitate was resuspended in 6 ml of 0.15 M phosphate buffer. The resulting bright yellow solution showed optical density changes typical of phytochrome (8) after irradiating with red and far-red light. The sample was still contaminated with chlorophyll, however, so its phosphate molarity was lowered by passing it through a 1.1 × 27 cm column containing 0.03 M buffer equilibrated Sephadex G 50. It was then adsorbed to the top of a 2 × 25 cm DEAE-cellulose (0.3 meq/g exchange capacity) column equilibrated with the same buffer. This column was eluted using a linear gradient mixer containing 0.04 M starting buffer (110 ml) and 0.6 M limit buffer (110 ml). Flow rate (50 ml/hr) was kept constant with a pump and 8 ml fractions were collected. Fractions containing phytochrome were pooled (57 ml) and precipitated by the addition of an equal volume of 80 % saturated $(NH_4)_2SO_4$ (pH 7.8). The small greyish precipitate collected by centrifugation was resuspended in 5 ml of 0.1 M buffer and recentrifuged to remove scattering debris. This sample was used in all subsequent spectral analyses.

Isolation from Sphaerocarpos. Plant material for this work was kindly supplied by David H. Miller, Department of Botany, University of California, Berkeley. It consisted of small dark-grown plants from shake cultures in a mineral salts medium (11) modified by using 1.5 % sucrose as the carbon source and raising the nitrate concentration to 0.01 M.

Success with the *Mesotaenium* isolation prompted a similar approach to extraction from *Sphaerocarpos*. The starting material was only 15 g dry weight after lyophilization so the columns and extractions were scaled to a third of their previous size. Resuspension of the pellet from the first $(NH_4)_2SO_4$ precipitation gave a sample (5 ml) that was sufficiently pure to obtain a reasonable difference spectrum between 600 and 800 nm. Further purification did not seem worthwhile because of the small amount of phytochrome present and the losses incurred in steps involving gradient elution from DEAE-cellulose. Data in figure 2 are obtained from the sample without further purification.

Measurement of Photoeffectiveness of Pigment Conversion. The use of the rate equations for transformations of P_R and P_{FR} to calculate limits for the extinction coefficients of the 2 forms is discussed in Hendricks, et al. (8). These general methods were used here with the exception of modifying the equations by a factor of 0.43 to use the conventional definition of a first order rate constant. No corrections (4) were made to account for the overlapping absorption of the 2 pigment forms and the resulting photostationary states which are at less than complete conversion. There was enough extraneous absolute absorption in the samples to preclude accurate application of the correction factors which depend upon optical purity of the sample.

The rate equations for transformation of P_R and P_{FR} by monochromatic light of wavelength λ can be transformed to:

$$\frac{0.43\ K}{E\lambda} = \frac{\varepsilon_{fr}\lambda\ \phi_{fr}}{(P_{R\infty})\lambda} = \frac{\varepsilon_r\lambda\ \phi_r}{(P_{FR\infty})\lambda}$$

$(P_{R\infty})\lambda$ and $(P_{FR\infty})\lambda$ are the mole fractions of P_R and P_{FR} at the photostationary state ($t = \infty$) in monochromatic light of wavelength λ, E is the intensity of monochromatic light (einstein cm^{-2} sec^{-1}), $\epsilon_r\lambda$ and $\epsilon_{fr}\lambda$ are the extinction coefficients of P_R and P_{FR} at λ (cm^2 $mole^{-1}$, to the base 10) and ϕ_r and ϕ_{fr} are the quantum yields for the transformations of P_R and P_{FR} (moles $einsteins^{-1}$). E and K are determined.

Monochromatic light for conversion was obtained from a projector with interference filters. Routine measurements utilized Baird type B-3 with approximately 25 nm half bandwidth at 660 or 730 nm. For the kinetics of photoconversion narrow band filters with transmission maxima at 652 or 712 nm were added in tandem to the corresponding red or far-red filters above.

Light intensities were measured with a calibrated Eppley thermopile and Kintell electronic galvanometer. Certain of the multilayer interference filters had transmission bands above 900 nm which are not eliminated by 6.6 cm of water. A suitable filter was devised to eliminate these wavelengths from the thermopile measurements. Complexing of 0.072 M nickelous salts with 0.5 M EDTA moves the nickel absorption band from 1180 nm to

1000 nm. Thus the 1 % cut off range of the 6.6 cm pathlength aqueous heat filter was extended down to 900 nm and near infrared eliminated as a contaminant.

Absorption measurements were made at 1° in 10 cm pathlength (0.7 cm inside diameter) cylindrical cuvettes with a Cary 14 recording spectrophotometer.

Results

Mesotaenium. The visible absorption spectrum of the preparation from *Mesotaenium* is shown in figure 1 after saturation with either red or far-red light. A difference spectrum derived from these data is plotted above. The absorption and difference maxima are at 649 for P_R and 710 for P_{FR}, making them quite distinct from higher plant forms of phytochrome as seen in table I. Though the

Table I. *Absorption Maxima of the Red and Far-Red Absorbing Forms of Phytochrome Purified from Various Sources*

Sphaerocarpos figures are estimated from difference spectra (fig 2) and are approximate only. Data on oats are from Siegelman and Firer (14), and those on peas from Bonner (unpublished observations). The cross-over point (isosbestic) is also shown.

	Absorption maxima			
	Meso-taenium	*Sphaero-carpos*	Oat	Pea
	nm	nm	nm	nm
P_R form	649	655	ca 670	ca 667
Isosbestic point	670	680	...	688
P_{FR} form	710	720	ca 725	ca 725

starting material was a deep green-black paste of cells, the chlorophyll contamination was almost entirely eliminated. Residual chlorophyll probably does account for the hump in the 660 to 670 nm region of the spectrum after saturation with red light (fig 1).

The *Mesotaenium* phytochrome was not denatured by 3 weeks storage at 0°. It was repeatedly photoreversible without loss of activity, and the ratio of optical density changes at the peak wavelengths (649 and 710 nm) was close to 1 to 1. The sample was not pure enough to determine a difference spectrum at wavelengths below 450 nm. After saturation with red light, the sample showed little or no dark reversion (1) of P_{710} to P_{649} after 4 hours at 1°.

Sphaerocarpos. A difference spectrum plotted from the absorption spectra after red and far-red saturation of the *Sphaerocarpos* preparation is shown in figure 2. The phytochrome was repeatedly photoreversible and appeared reasonably stable

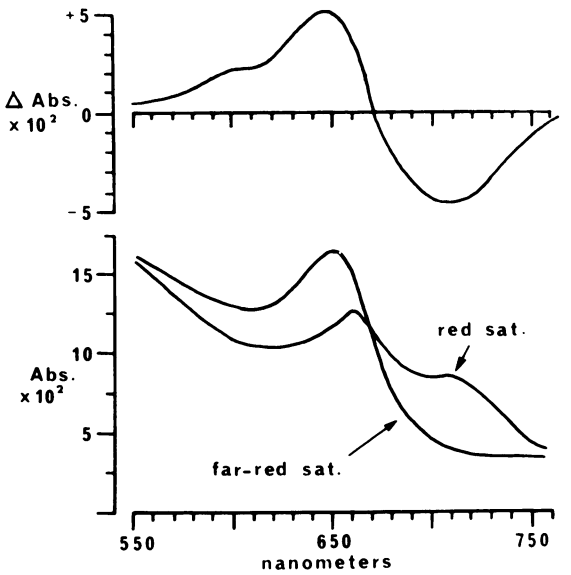

FIG. 1. Absorption spectra of partially purified *Mesotaenium* phytochrome after saturating doses of red (650 nm) and of far-red (712 nm) light (10 cm path length). A difference spectrum taken from these data is plotted above the absorption spectra.

at 0°. After saturating with red light the sample showed some dark reversion to the red absorbing form over a 4 hour period at 1°. The sample had some light scattering which changed over periods of hours thus precluding the measurement of rates of reversion.

Characteristics of the Photoconversion. The distinctive positions of the absorption maxima of the lower plant phytochrome forms make it desirable to compare other characteristics of a lower plant form with that from higher plants. Without knowledge of the molecular weight or concentration, exact molar extinction coefficients cannot be determined although there are desirable characteristics for use in comparison of pigments. Hendricks, et al. (8) have set lower limits on the extinction

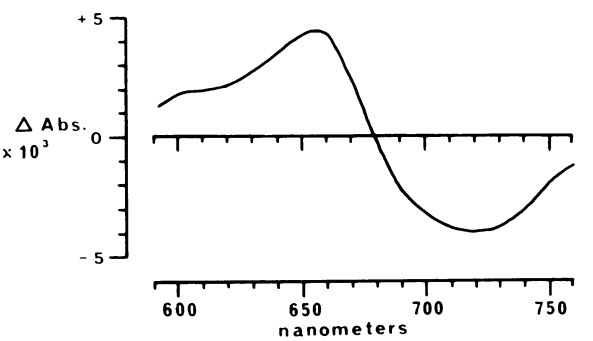

FIG. 2. Difference spectrum of partially purified phytochrome from dark grown *Sphaerocarpos*, after saturating doses of red (660 mμ) and then far-red (730 mμ) light (10 cm path length).

coefficients for P_R and P_{FR} by measuring rate constants for photoconversion with known light fluxes and relating these to the product of extinction coefficient and quantum efficiency for conversion. Knowledge of absolute absorption values and realization that photoconversion is not complete enabled Butler, et al. (4) to further refine the lower limits of the extinction coefficients. In this work the visible absorption spectrum was not completely free of extraneous absorption so corrections for the amount of each pigment form remaining at the photostationary state could not be made. However, comparison of equivalent data for oat and *Mesotaenium* phytochrome show very similar characteristics. The first order rate constant for the net conversion of P_{649} to P_{710} was $K_{650} = 0.045$ sec^{-1} with $E_{650} = 0.77 \times 10^{-9}$ einsteins cm^{-2} sec^{-1} yielding a value of 2.5×10^4 liters cm^{-1} for the product of extinction coefficient and quantum efficiency, $\alpha_{650}\ \phi_{650}$, for P_R. The rate constant for conversion of P_{FR}, $P_{710} \rightarrow P_{649}$, was $K_{712} = 0.0079$ sec^{-1} with $E_{712} = 0.425 \times 10^{-9}$ einsteins cm^{-2} sec^{-1} yielding $\alpha_{712}\ \phi_{712} = 0.8 \times 10^4$ liters cm^{-1} $einstein^{-1}$. These compare with values of 2.0×10^4 and 0.8×10^4, respectively, found for oat phytochrome (4).

Discussion

The phytochrome isolated from *Mesotaenium* has an unexpected absorption spectrum, differing from that of the higher plant pigment in that the absorption maxima are displaced about 15 nm toward the blue. The photoreversibility, the spectral shifts, and the rate constants for photoconversion are those expected of phytochrome. A number of presumed chlorophyll complexes with far-red absorption bands (6), or which produce far-red absorption bands on heating (9) or irradiation (16), have been reported. It seems unlikely that a complex of this type could be confused with phytochrome in the present work.

The question arises as to whether or not the pigment we isolated matches the action spectra for the chloroplast rotation response. The published action spectra for *Mougeotia* (7) and for *Mesotaenium* (5) are not detailed enough to choose between a 649 and 665 nm P_R peak, although a more detailed action spectrum determined by Corona Machemer (Senior Honors Thesis, Harvard University, 1964) for chloroplast rotation in this strain of *Mesotaenium* shows a maximum at about 650 nm for P_R. Haupt's action spectrum for reversal (P_{FR}) shows maximum activity near 715 nm (7), whereas most phytochrome P_{FR} action spectra peak around 730 nm. It appears that a 715 nm peak in the action spectrum may bear the same relationship to a 710 nm P_{FR} peak that a 730 nm action peak bears to a 725 nm absorption maximum in angiosperms.

Some protein denaturing agents or treatments can cause the absorption peaks of phytochrome isolated from seedlings to undergo shifts to shorter wavelengths. It is pertinent to ask whether the *Mesotaenium* or *Sphaerocarpos* preparations underwent changes in absorption maxima reflecting denaturation during isolation. Denaturation by urea (2, 3), para chloromercuribenzoate (3) or prolonged dialysis against polyethylene glycol (8) causes shifts in the absorption maxima to shorter wavelengths. This is accompanied by a labilization of P_{FR} so that there is a lowered ratio of absorbancy changes at the far-red peak compared to that at the red peak. There is a progressive loss of optically reversible phytochrome under these conditions. Neither of these phenomena was observed with these preparations. The absorbancy changes at the red and far-red maxima were equal and repeatedly photoreversible with no loss of optical activity. The characteristics were checked over several weeks of storage at 1° for the *Mesotaenium* preparation. On the basis of these criteria we conclude that the absorption maxima observed correspond to those of the native state. The differences among the various forms probably reflect changes in amino acid composition around the binding site for the chromophore and would thus be due to varying protein-chromophore interaction. It is also a possibility that structural variations of the tetrapyrrole (15) chromophore can account for the spectral differences.

It is possible to make a rough comparison of pigment concentration in the light grown *Mesotaenium* with that of higher plants. If one assumes fairly complete solubilization of phytochrome from the lyophilized preparation and assumes that the losses during the column purification steps were similar to those of pea phytochrome put through the same procedure, one may obtain a figure of 8×10^{-3} $\Delta\Delta$ OD cc/g dry weight. A figure of 6.3×10^{-3} $\Delta\Delta$ OD cc/g fresh weight can be calculated from Siegelman's data (14) on etiolated oat coleoptiles. If one adjusts the oat figure upwards to convert it to a dry weight basis and downwards to correspond to the drop (approx 90 %) in phytochrome level caused by prolonged red irradiation of etiolated seedlings, the concentrations appear to be of the same order of magnitude in this alga and in some higher plants.

The *Mesotaenium* preparation is considerably cleaner than any other phytochrome solutions prepared from light-grown green material. The spectrum of the *Mesotaenium* preparation has a minor shoulder on the long wavelength side of the P_R peak which is in all likelihood due to a trace of chlorophyll. The dominant peak is due to phytochrome. In the spectra of Lane, et al. (10) of spinach leaf extracts the dominant peak is due to chlorophyll with phytochrome being recognizable only from the difference spectrum.

Several aspects of the isolation and purification

method used here deserve note because of their successful application to green material. Lyophilization of the French press homogenates prior to preparation of acetone powders precluded the formation of large, hard, icy lumps when dispersed into acetone at −19°. This successful use of acetone powders for phytochrome preparation extends a previous application to pea homogenates (unpublished results, B. A. Bonner). Considerable amounts of chloroplast pigments were removed by the acetone and ether steps. The bulk of the remaining chlorophyll was removed by adsorption onto DEAE-cellulose, from which it is not displaced by high molarity buffers (0.2 M phosphate). The early steps were rapid flow-through column operations, designed to remove as many impurities as quickly as possible from the crude isolate. It should be pointed out that there was so much chlorophyll in the initial steps that optical assay of phytochrome activity was out of the question and that most of these procedures were chosen on the basis of our experience with the behavior of phytochrome from peas under similar conditions.

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