IN VIVO MEASUREMENTS OF THE PHYTOCHROME PHOTOSTATIONARY STATE IN FAR RED LIGHT*

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Abstract—The in vivo photostationary state, \( \varphi_r = ([P_{fr}]_{fr}/[P]) \), of phytochrome in far red light has been determined in mustard seedling cotyledons by three different methods. The \( \varphi_r \) is a function of the length of time of etiolation (\( t = 36 \) hr, \( \varphi_r = 0.14; t = 72-120 \) hr, \( \varphi_r = 0.075 \)). The calculated \( \varphi_r = 0.8 \). The amount of \( P_{fr} \) is strongly dependent on the time of onset of far red light. These data imply that it would be almost impossible to maintain a constant level of \( P_{fr} \) in mustard cotyledons over a considerable period of time.

INTRODUCTION

The absorption spectra of both forms of phytochrome (\( P_r \) and \( P_{fr} \)) exhibit considerable overlap [1]. Therefore, saturating light doses of most wavelengths establish photostationary states of these two forms. For the calculations of the photostationary states between 300 and 800 nm, Butler et al. [1] used first order phototransformation kinetics and the in vitro absorption spectra of oat phytochrome with the assumption that the absorption of \( P_r \) is zero for wavelengths longer than 725 nm.

Hartmann [2] calculated the wavelength-dependent photostationary states from the in vitro absorption spectra of oat \( P_r \) and \( P_{fr} \) as published by Butler et al. [1, 3]. He assumed, further, that for the wavelength \( \lambda = 650 \) nm, a photostationary state (\( ([P_{fr}]_{650}/[P]) = \varphi_{650} = 0.8 \pm 0.02 \)) and a wavelength-independent ratio of the quantum yields \( \varphi_r/\varphi_{fr} = 1.23 \pm 0.23 \). With these assumptions he calculated that for \( \lambda = 717 \) nm, \( \varphi_{717} = 0.03 \), and for wavelengths greater than 725 nm, \( \varphi_{\lambda > 725} = 0 \).

Using in vivo first order phototransformation kinetics of corn coleoptiles, Pratt and Briggs [4] obtained for the wavelength interval 720 to 743 nm: \( \varphi_{720-743} = 0.01 \). These values for the far red region (\( \lambda > 717 \) nm), \( 0 \leq \varphi_{\lambda > 717} \leq 0.03 \), are often used to correlate the content of active phytochrome (\( P_{fr} \)) with physiological responses. A comparison of the difference-spectra for phytochrome in vivo and in vitro shows that the main absorption peaks are shifted in vivo towards longer wavelengths [5]. Detailed studies on phototransformation kinetics of pumpkin and mustard in vivo [6, 7] showed that the kinetics could not be interpreted by a single first-order reaction. These data suggest that correlations of physiological responses and photostationary states calculated from in vitro data are not valid, because the assumptions made for the in vitro calculations do not apply in vivo.

*Abbreviations: \( P = P_{fr} + P_r \) = total phytochrome; \( r = \) red; \( fr = \) far red; \( \varphi_r = ([P_{fr}]_{fr}/[P]) \) = photostationary state of phytochrome at wavelength \( \lambda \); \( \varphi_r = \) quantum yield at wavelength \( \lambda \); \( k_0 = \) zero order rate constant of \( P_r \) synthesis; \( k_1 = \) first order rate constant of \( P_r \) destruction; \( \tau^d_{1/2} = \) half-life of \( P_r \) destruction; \( \tau^d_{1/2} = \) half-life of \( P \) disappearance under continuous far red light.

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In this paper three methods are presented by which phytochrome photostationary states in vivo may be estimated.

MATERIAL AND METHODS

Plant material. Mustard seedlings (Sinapis alba L., 1969) were grown in the dark at 25°C following a standard procedure[8]. The cotyledons were dissected on ice with a razor blade under a dim green safelight.

Irradiations and spectrophotometric measurements. The far red light was obtained from a far red standard field[9], which has been used for photomorphogenic research[8]. The other irradiations were performed with AL interference filters from Schott, Mainz, Germany (658 nm filter and 2 mm Plexiglass PG 501 from Röhm und Haas, Darmstadt, Germany; 756 nm filter and 3 mm RG 9 from Schott, Mainz, Germany), or the filter combination of our standard far red sources[9]. The light source was a Leitz Prado 500.

The phytochrome content of the samples was measured at 0°C using a modified Ratiospect R-2[10]. For each sample 36 cotyledons were used. The measuring light was obtained through Schott DIL interference filters at 659 and 728 nm (Figs. 2, 3 and 5), 728 and 805 nm (Fig. 4), and the actinic light through Schott DIL interference filters at 656 and 733 nm. With the technique of repeated irradiations and measurements as described by Marme[9], interference with the protochlorophyll–chlorophyll system has been avoided. Each point in the figures represents the mean of at least 10 parallel measurements with brackets equal to twice the standard error.

RESULTS AND DISCUSSION

A kinetic model as shown in Fig. 1 accounts for the main phytochrome reactions of the mustard cotyledons in darkness or under continuous far red irradiation. In this case one need not take into account the existence of two phytochrome populations[6, 7], a transient form of \( P_{fr} (= P_{pT}) \), the lag phase of the dark destruction[11], and the red pretreatment-dependent synthesis of \( P_r \)[9, 13], which can be detected after a saturating dose of red light.

The simplified model is characterized by a wavelength-dependent photostationary state, \( \varphi \), which is established within about 1 min for the quantum flux densities used, and two dark reactions, the de novo synthesis \( P_r \xrightarrow{k_d} P_r \) and the destruction \( P_{fr} \xrightarrow{k_d} P_{fr} \).

The time courses of change of the total phytochrome in the dark, in continuous far red light, and in the dark after 48 hr continuous far red and a saturating dose of 756 nm are shown in Fig. 2. The kinetics of \( P_{tot} \) change after the far red light pretreatment suggests that the de novo synthesis depends neither on the total amount of \( P_r \) nor on the

\[
\begin{align*}
& P_r \xrightarrow{k_d} P_{fr} \\
& P_r \xrightarrow{k_s} P_r
\end{align*}
\]

Fig. 1. Simplified model of phytochrome in mustard cotyledons. \( P_r \xrightarrow{k_d} P_{fr} \) represents the de novo synthesis; \( P_r \xrightarrow{k_s} P_r \) represents the light reaction under steady state conditions; \( P_{fr} \xrightarrow{k_d} P_{fr} \) represents the dark destruction.
Phytochrome photostationary state

preirradiation. During the time interval 48–60 hr after sowing both dark curves can be fitted by a zero order reaction with a rate constant $k_s = 3 \times 10^{-5} \Delta (\Delta OD) \text{ min}^{-1}$. The $P_{fr}$ destruction can be described by a first order reaction $P_{fr} \to P_{fr}$. The rate constant $k_d$ does not depend on the photostationary state[12] and the quantum flux density[9, 13], but is strongly dependent on the time after sowing:

\[ k_d(t = 36 \text{ hr}) = \frac{\ln 2}{45 \pm 7} \text{ min}^{-1} \text{ (Ref. [9])}; \quad k_d(t = 48 \text{ hr}) = \frac{\ln 2}{34 \pm 4} \text{ min}^{-1} \text{ and} \]

\[ k_d(t = 72 \text{ hr}) = \frac{\ln 2}{24.5 \pm 4} \text{ min}^{-1} \text{ (Ref. [11])}. \]

With these assumptions the mathematical treatment of the model yields the following equation for the time course of the total phytochrome,

\[ P_{tot}(t) = \frac{0k_s}{1k_d\varphi_\lambda} + \left( P_{tot}(t = 0) - \frac{0k_s}{1k_d\varphi_\lambda} \right) e^{-1k_d\varphi_\lambda t} \tag{1} \]

\[ t = \text{time after onset of light, which establishes the photostationary state } \varphi_\lambda \text{ virtually immediately}. \]

If $t$ is sufficiently large, the second term can be neglected and equation (1) can be written as,

\[ P_{tot}(t \to \infty) = \frac{0k_s}{1k_d\varphi_\lambda} \tag{2} \]

Under these conditions, $\varphi_\lambda$ can be calculated if $P_{tot}(t \to \infty)$, $0k_s$ and $1k_d$ are known.
After 48 hr of continuous far red light, $P_{\text{tot}}(t = 48 \text{ hr}) = 1.79 \times 10^{-2} \Delta(\text{OD})$, $\theta_k = 3 \times 10^{-5} \Delta(\text{OD}) \text{ min}^{-1}$ and $k_d = [(\ln 2)/34] \text{ min}^{-1}$ (Ref. [11]). From these values the photostationary state in the standard far red can be estimated as,

$$\varphi_{\text{fr}}(t = 48 \text{ hr}) = 0.083 \pm 0.01$$  \hspace{1cm} (3)

The measurable amount of the total phytochrome under continuous far red light seems to be constant for $t > 48 \text{ hr}$. The de novo synthesis becomes slower and can be described at $t = 72 \text{ hr}$ by $\theta_k = 1.5 \times 10^{-5} \Delta(\text{OD}) \text{ min}^{-1}$ (Fig. 2). The destruction becomes faster: $k_d = [(\ln 2)/24.5] \text{ min}^{-1}$ (Ref. [11]). From equation (2) the calculation can be made that,

$$\varphi_{\text{fr}}(t = 72 \text{ hr}) = 0.03 \pm 0.006$$  \hspace{1cm} (4)

Equation (1) suggests another possibility for determining the photostationary state $\varphi_\lambda$ under continuous irradiation and can be written in the form,

$$P_{\text{tot}}(t) - P_{\text{tot}}(t \to \infty) = [P_{\text{tot}}(t = 0) - P_{\text{tot}}(t \to \infty)]e^{-t \theta_k}$$  \hspace{1cm} (5)

which can be represented as a straight line in a semilogarithmic plot, if $k_d, \theta_k$, and $\varphi_\lambda$ are constant with time. Figure 3 shows the phytochrome destruction under continuous standard far red light, begun at 48 hr after sowing in darkness and plotted as described.

![Fig. 3. Semilogarithmic plot of the difference $P_{\text{tot}}(t) - P_{\text{tot}}(t \to \infty)$ under continuous far red light after 48 hr darkness.](image)
by equation (5). The value \( P_{\text{tot}}(t \to \infty) \) has been taken from the continuous far red curve in Fig. 2. During the first 10-12 hr the expected first order kinetics with a half-life,

\[
\tau_{1/2}^{P_{\text{tot}}}(t = 48 \text{ hr}) = 6.2 \text{ hr}
\]

for the total phytochrome is obtained. For longer irradiations the curve decreases. The main reason for this deviation is the time-dependence of the destruction rate constant: at 72 hr the rate constant is 1.45 times that at 48 hr[11].

From equation (6) and the dark decay of \( P_{fr} \) at 48 hr[11], the calculation may be made that.

\[
\frac{\tau_{1/2}^{P_{fr}}}{\tau_{1/2}^{P_{fr}}(t = 48 \text{ hr})} = \varphi_{fr}(t = 48 \text{ hr}) = 0.091 \pm 0.01
\]

From direct AOD measurements after saturating light doses of various wavelengths, a third method can be derived to determine the photostationary state \( \varphi_\lambda \).

In turbid materials Lambert-Beer's law can be modified to account for the scattering[14].

\[
\text{OD} = \beta \cdot \varepsilon \cdot c \cdot d
\]

\( \beta \): scattering factor
\( \varepsilon \): extinction coefficient
\( c \): concentration of the pigment
\( d \): sample thickness.

The Ratiospect permits the extinction difference between two wavelengths to be measured. After irradiation with a saturating light dose of the wavelength \( \lambda \), one obtains for the two forms of phytochrome, \( P_r \) and \( P_{fr} \), with the extinction coefficients \( \varepsilon_r \) and \( \varepsilon_{fr} \).

\[
\Delta \text{OD}_\lambda = \beta \cdot c \cdot d \left[ \varphi_\lambda \Delta \varepsilon_{fr} + (1 - \varphi_\lambda) \Delta \varepsilon_r \right]
\]

\( \Delta \varepsilon_{fr, \lambda} = \) difference of the extinction coefficients of \( P_{fr, \lambda} \) at the two measuring wavelengths.

The difference of the \( \Delta \text{OD}_\lambda \) from equation (9) for two wavelengths \( \lambda_1, \lambda_2 \) is.

\[
\Delta(\Delta \text{OD})_{\lambda_1, \lambda_2} = \Delta \text{OD}_{\lambda_1} - \Delta \text{OD}_{\lambda_2} = \beta \cdot c \cdot d \left( \varphi_{\lambda_1} - \varphi_{\lambda_2} \right) \left( \Delta \varepsilon_{fr} - \Delta \varepsilon_r \right)
\]

A value can be estimated for both \( \Delta(\Delta \text{OD})_{\lambda_1, \lambda_2} \) and \( \Delta(\Delta \text{OD})_{\lambda_3, \lambda_4} \) of the same sample after successive irradiation with \( \lambda_3 \) and \( \lambda_4 \), and the ratio [from equation (10)] can be calculated as.

\[
\frac{\Delta(\Delta \text{OD})_{\lambda_1, \lambda_2}}{\Delta(\Delta \text{OD})_{\lambda_3, \lambda_4}} \approx \frac{\varphi_{\lambda_1} - \varphi_{\lambda_2}}{\varphi_{\lambda_3} - \varphi_{\lambda_4}}
\]

This ratio is independent of the total pigment concentration, the scattering, the sample thickness, and the two measuring wavelengths. The independence of the ratio in equation (11) from the two measuring wavelengths was tested by using two different measuring wavelengths pairs: 656/728 nm and 728/805 nm.

Experiments were carried out with \( \lambda_1 = \) standard far red light, \( \lambda_2 = \lambda_4 = 756 \text{ nm} \) and \( \lambda_3 = 665 \text{ nm} = \) standard red light for various periods of etiolation. The result
is shown in Fig. 4. From 36 hr onwards the ratio,
\[
\frac{\Delta(\Delta OD)_{fr,756}}{\Delta(\Delta OD)_{r,756}} = \frac{\phi_{fr} - \phi_{756}}{\phi_r - \phi_{756}}
\] (12)
decreases very rapidly up to 72 hr and remains nearly constant up to 120 hr when measured at 0°C. For 72 hr dark-grown cotyledons this ratio was also determined at 25°C. No significant deviation from the 0°C value could be observed.

At 72 hr, the \(\Delta(\Delta OD)_{fr,756}\) was equal to \(9 \times 10^{-3}\ \Delta(\Delta OD)\). The same value was obtained with an interference filter of 720 nm instead of the standard far red source. \(\phi_{756}\) in equation (12) is, therefore, negligible in comparison to \(\phi_{fr}\) and \(\phi_r\) because the \(\Delta(\Delta OD)_{756,800}\) was a factor of 10 or more smaller than the \(\Delta(\Delta OD)_{fr,756}\) and beneath the limit of detection [\(10^{-3}\ \Delta(\Delta OD)\)].

With the assumption \(\phi_r = 0.8\) (which is derived from \textit{in vitro} data!), equation (12) is reduced to,
\[
\phi_{fr} = 0.8 \frac{\Delta(\Delta OD)_{fr,756}}{\Delta(\Delta OD)_{r,756}}
\] (13)

The scale on the right of Fig. 4 represents the \(\phi_{fr}\) calculated in this manner for the various periods of time of etiolation,
\[
\phi_{fr}(t = 36 \text{ hr}) = 0.14 \pm 0.01 \\
\phi_{fr}(t = 48 \text{ hr}) = 0.09 \pm 0.01 \\
\phi_{fr}(t = 72 \text{ hr}) = 0.076 \pm 0.008
\] (14)

Fig. 4. Time course of change of the photostationary state \(\phi_{fr}\) in dark-grown mustard cotyledons in far red light.
DISCUSSION

The photostationary state of phytochrome in the far red has been estimated by three different methods. The \( \varphi_{fr} \) of mustard cotyledons which have been grown for 48 hr in the dark (method 2 or 3) or under continuous far red light (method 1), are not significantly different [equations (3), (7), (14)]. This result suggests that the assumption of \( \varphi_r = 0.8 \) (which has been taken from in vitro data) seems reasonable for mustard cotyledons in vivo and that the optical behaviour of the cotyledons is nearly the same for 48 hr dark and 48 hr continuous far red \( (\beta_{48\text{hr}}^{d} \approx \beta_{48\text{hr}}^{fr}) \). The photostationary state at this point seems not to depend on the dark-far red pretreatment. The \( \varphi_{fr} \) of 72 hr old seedlings obtained with method 3 \( [\varphi_{fr} = 0.076 \pm 0.008, \text{ equation (14)}] \) differs significantly from that of method 1 \( [\varphi_{fr} = 0.03 \pm 0.006, \text{ equation (4)}] \). The most probable explanation for the deviation is an increase of the scattering factor \( (\beta_{72\text{hr}}^{d} \approx 2 \beta_{48\text{hr}}^{d}; \beta_{72\text{hr}}^{fr} = 2 \beta_{48\text{hr}}^{fr}) \). The changes in \( P_{tot} \) under continuous far red light (Fig. 2) represent, therefore, only the apparent concentration of phytochrome in comparison with the kinetics in the dark. The real content of total pigment should decrease to about half the value at 72 hr.

In most of the publications dealing with phytochrome-mediated physiological effects [15], the statement is made that under continuous far red light (onset of light at 36 hr after sowing) a low but virtually constant level of \( P_{fr} \) is maintained over a considerable period of time. This statement is based on previous measurements of the total phytochrome content between 36 and 44 hr after sowing (Fig. 9 in [9]). Figure 5 shows the time course of change of total phytochrome under continuous far red irradiation begun at various times after sowing. Dependent on the time of onset of far red light one obtains an increase of the total pigment, followed by an apparent steady state level \( (t = 0 \text{ hr}) \); a nearly constant level with a subsequent decrease followed by the steady

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*\( \beta_{t,d,fr} \) = scattering factor of dark or far red grown cotyledons at time \( t \).
state level \((t = 36 \text{ hr})\); or a rapid decrease which seems to end on the same steady state level \((t = 48 \text{ hr})\). This value is reached at 40 hr under continuous far red, at 50 hr after 36 hr dark followed by continuous far red, and at 70 hr after 48 hr dark followed by continuous far red light. The apparent steady state level is maintained although the *de novo* synthesis (Figs. 2 and 3), the dark destruction [11], and the photostationary state \(\varphi_r\) (Fig. 4) are time-dependent.

Concerning the total phytochrome our results are in accordance with previous data [9]. With the assumption generally made that \(\varphi_s\) does not change with time, these results support the decision previously justified [8] to start physiological experiments in the far red light at 36 hr after sowing if steady state conditions with respect to \(P_r\) are desired [8]. However, the present data suggest that \(\varphi_s\) is not constant but decreases from 0.14 to 0.09 between 36 and 48 hr after sowing (Fig. 4) and that the amount of \(P_r\) in mustard cotyledons which can be maintained over a considerable period of time is neither low (since this depends on the time of onset of far red light, Fig. 5) nor constant (since \(\varphi_r\) changes with time, Fig. 4).

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