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Particle-Bound Phytochrome: A Function of Light Dose and Steady-State Level of the Far-Red-Absorbing Form

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Summary. The binding of phytochrome to a particulate fraction from maize coleoptiles has been examined as a function of the level of P_{fr}[1] offered *in vivo*. Further evidence is provided that the degree of binding is a function of both the form of the phytochrome and the state of the binding sites; and that P_{fr} induces a change in the state of the binding sites such that the subsequent affinity for P_r is enhanced. Increasing the steady-state level of P_{fr} offered *in vivo* results in a subsequent binding curve for P_r that is suggestive of cooperativity. However, increasing both time and irradiance parameters of the light dose while holding the steady-state P_{fr} level constant results in increased binding of P_r up to a saturation level. This indicates that the response is the product of both the steady-state P_{fr} concentration and the light dose and is determined therefore ultimately by the cycling rate. The system appears to respond therefore to the total number of P_{fr} molecules integrated over time rather than simply to the steady-state concentration of P_{fr}.

We recently reported the presence of significant levels of particle-bound phytochrome in extracts from maize coleoptiles and pumpkin hooks [9]. The key to this question was the observation that irradiation with red light enhances the binding of phytochrome to the particulate fraction. Subsequent irradiation with far-red light does not reduce this level to that observed prior to red irradiation. This enhanced level of P_r-binding decays exponentially in the dark reapproaching the control level with a half life of 50 min in maize. The cycle is repeatable. These data are taken to indicate that the degree of phytochrome binding is a function of both the form of the pigment and the state of the binding sites; that P_{fr} induces a change in the state of the binding sites such that the subsequent affinity for P_r is enhanced;

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1 Abbreviations: P_r and P_{fr} = red- and far-red-absorbing forms of phytochrome, respectively; P_{tot} = total detectable phytochrome.

that this 'enhanced affinity' state of the binding sites reverts to the pre-irradiation state with a half life of 50 min; and that the P_{fr}-induced changes in the state of the binding sites are repeatable. Further investigation of the pumpkin system has resulted in separation of the binding structures from cytochrome *c* oxidase activity.[2] These structures appear as vesicles in negative stain and are capable of binding phytochrome *in vitro*.

On the premise that phytochrome might be a ligand capable of interaction with a membrane matrix [9], we have proceeded to examine binding as a function of the level of the putative effector molecule P_{fr} *in vivo*. In this approach we have been guided by the procedures and nomenclature employed by Changeux and associates [2, 3, 4] for analyzing such phenomena. We report now on the influence of both light dose and photosteady-state P_{fr} concentration on the binding process in maize coleoptiles.

Materials and Methods

The apical 1 to 2 cm of 5-day-old dark-grown (25 °C) corn (*Zea mays, L.*, cv. WF-9 × M-14) coleoptiles were used in all experiments. Harvesting and all subsequent manipulations were carried out under green safelight. All irradiations were performed at 25 °C on the isolated coleoptile tips maintained in a moist atmosphere. Wavelengths between 660 and 756 nm were obtained using Schott D.I.L. interference filters and a Leitz Prado 500 projector as a light source. Irradiation intensity was varied using neutral glass. Phytochrome ($P_{tot}, \Delta(\Delta A)$) was measured at 0 °C with a modified Ratiospect [8]. $CaCO_3$ was used as a scattering agent for all *in vitro* measurements [1]. *In vivo* determinations were performed on fifteen 3-mm coleoptile tip segments packed into a 6-mm internal diameter cuvette.

For routine preparations of particulate fractions, prechilled tissue was chopped with a razor blade and homogenized in isolation medium in a mortar and pestle. In early experiments a solution/tissue ratio of 1:1 (v/w) and a medium containing 0.6 M sucrose, 5 mM $MgCl_2$, 14 mM 2-mercaptoethanol and 65 mM MOPS (N-morpholino-3-propansulfonic acid), pH 8.5, were used. In later experiments a solution/tissue ratio of 4:1.5 (v/w) and medium containing 0.25 M sucrose, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol and 35 mM MOPS, pH 7.9, were used. In both cases the pH in the final homogenate was between 7.2 and 7.4. The homogenate was squeezed through nylon cloth and precentrifuged at 500 to 1,000 × *g* for 10 min. The supernatant was then recentrifuged at 20,000 × *g* for 30 min. The pellet was resuspended in 25 mM MOPS, 14 mM 2-mercaptoethanol, pH 7.3 and P_{tot} measured in either the 1,000 × *g* supernatant and the 20,000 × *g* pellet or the 20,000 × *g* supernatant and the 20,000 × *g* pellet. The percent pelletable phytochrome was then expressed either as

$$= \frac{P_{tot} \text{ in } 20{,}000 \times g \text{ pellet}}{P_{tot} \text{ in } 1{,}000 \times g \text{ supernatant}} \cdot 100$$

or

$$= \frac{P_{tot} \text{ in } 20{,}000 \times g \text{ pellet}}{P_{tot} \text{ in } 20{,}000 \times g \text{ supernatant} + P_{tot} \text{ in } 20{,}000 \times g \text{ pellet}} \cdot 100.$$

2 D. Marmé, J. Boisard, J. Mackenzie and W. Briggs *(manuscript in preparation)*.

Results

The photosteady-state levels of P_{fr} established by wavelengths between 660 and 730 nm were determined *in vivo* in coleoptile tips following saturating light doses (Fig. 1). On this basis the wavelengths used in subsequent experiments were selected. Similarly, *in vivo* measurements of the time-course of photoconversion were performed to determine the time required for saturation. At an intensity of 300 erg cm^{-2} sec^{-1} the half time for the conversion of P_{fr} to P_r by a wavelength of 724 nm was 20 sec. Thus, within 2 min at this intensity photoconversion was more than 98% complete.

Initially, to examine the influence of the level of P_{fr} on phytochrome binding, coleoptile tips were irradiated for 3 min with wavelengths between 660 and 730 nm and the percent pelletable phytochrome determined (Fig. 2).

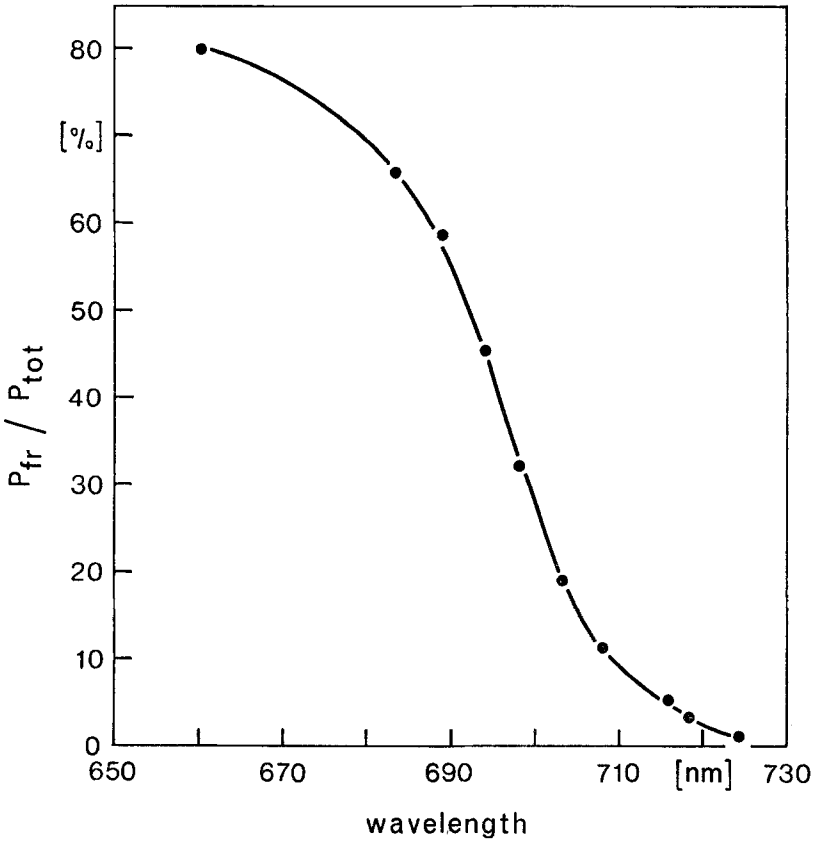


Fig. 1. *In vivo* photoequilibrium levels of P_{fr} in maize coleoptile tips following saturating irradiations with wavelengths between 660 and 727 nm. Fifteen 3-mm coleoptile tips were packed into a 6-mm internal diameter cuvette, irradiated for 1 min at 0 °C with the wavelength designated and the proportion of the total phytochrome in the P_{fr} form determined

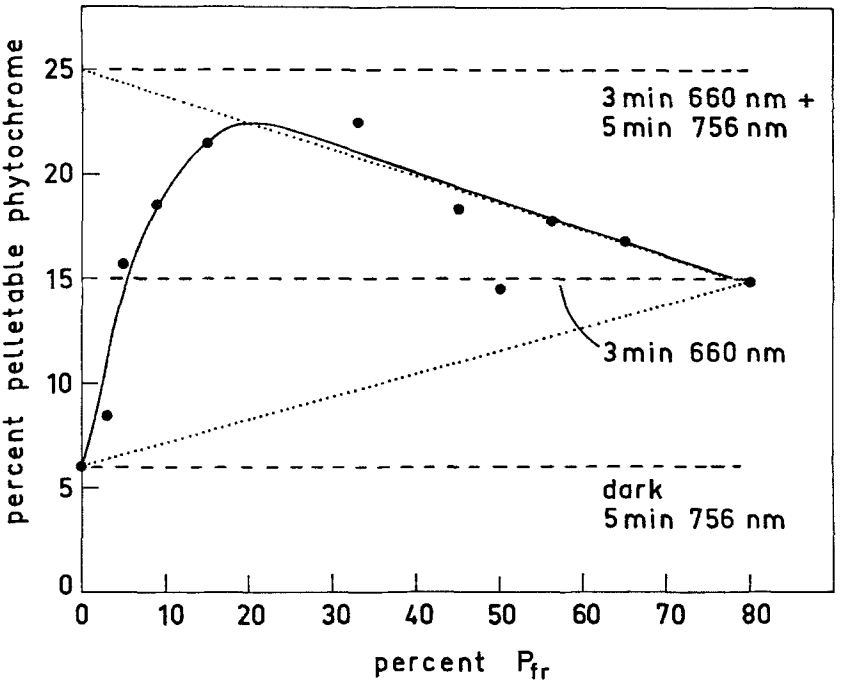


Fig. 2. Percent pelletable phytochrome in extracts from maize coleoptile tips following 3-min irradiation at 25 °C *in vivo* with wavelengths between 660 and 730 nm. Percent P_{fr} refers to the photosteady-state concentration of P_{fr} established by the various wavelengths. The horizontal dashed lines represent the percent pelletable phytochrome from dark- and 756 nm-irradiated controls (no detectable P_{fr}); following 3 min 660 nm (80% P_{fr}); and following 3 min 660 nm plus 5 min 756 nm (no detectable P_{fr}). For these extractions a medium containing 5 mM Mg^{2+} and a solution/tissue ratio of 1:1 (v/w) were used

If binding were a simple function of the relative proportions of P_r and P_{fr} in the extract, the dotted line running diagonally from lower left to middle right would be expected. However, it has been observed [9] that 3 min 660 nm light (80% P_{fr}) induces an enhanced affinity of the binding sites subsequently for P_r. Therefore, if P_{fr} at *any* level induced a maximum transformation of the binding sites, the dotted line running diagonally from upper left to middle right would be expected.

For P_{fr} levels above 20 to 25% the observed binding (closed circles, Fig. 2) is consistent with the concept of maximum binding-site transformation whereas below 20% P_{fr} the data suggest partial transformation. This indicates that binding is not a simple function of the relative proportions of P_r and P_{fr} in the extract but also of the degree of P_{fr}-induced, enhanced binding of P_r. The data also imply that relatively low steady-state levels of

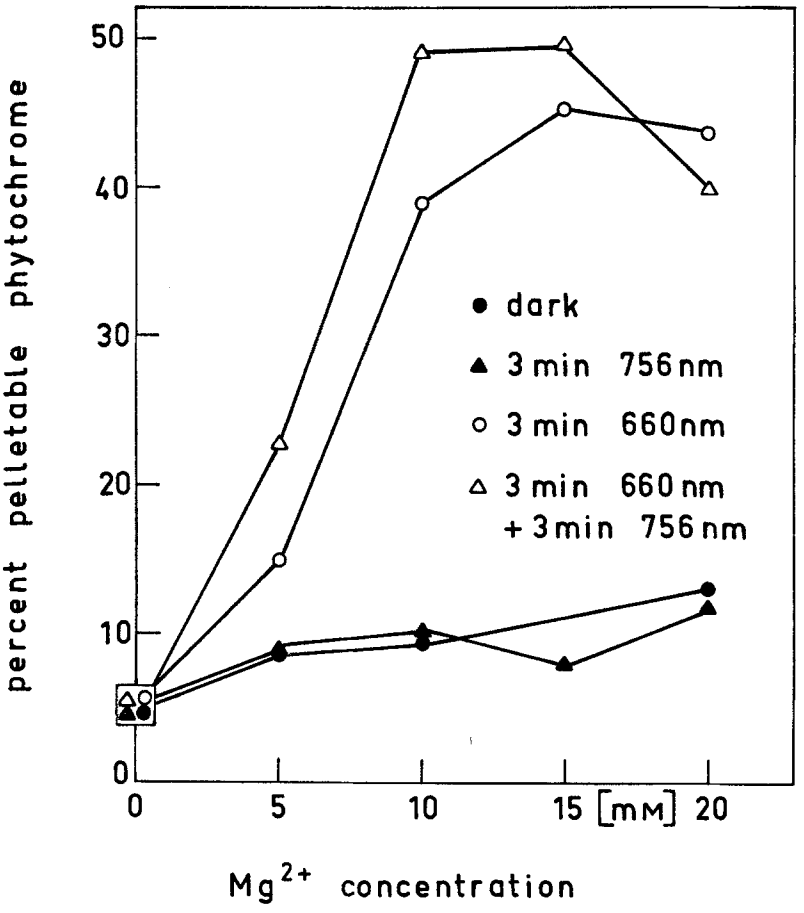


Fig. 3. Percent pelletable phytochrome as a function of Mg^{2+} concentration in the extraction medium and of *in vivo* preirradiation. 1.5 g lots of maize coleoptile tips were irradiated at 25 °C as indicated and extracted in a medium containing Mg^{2+} at 0 to 20 mM using a solution/tissue ratio of 4:1.5 (v/w)

P_{fr} elicit the maximum response. However, it was not possible to determine a simple binding curve [2, 4] for P_{fr} because of the differential binding of P_r and P_{fr}.

Following these experiments a methodological advance increased the resolution of the binding assay. It was observed in the pumpkin system that by increasing the level of Mg^{2+} in the extraction medium from the 5 mM previously used [9] to 10 mM, the level of pelletable phytochrome was increased considerably.[1] Similar results were obtained for maize (Fig. 3). Increasing the Mg^{2+} concentration above 5 mM resulted in substantial increases in pelletable phytochrome in the irradiated samples while the

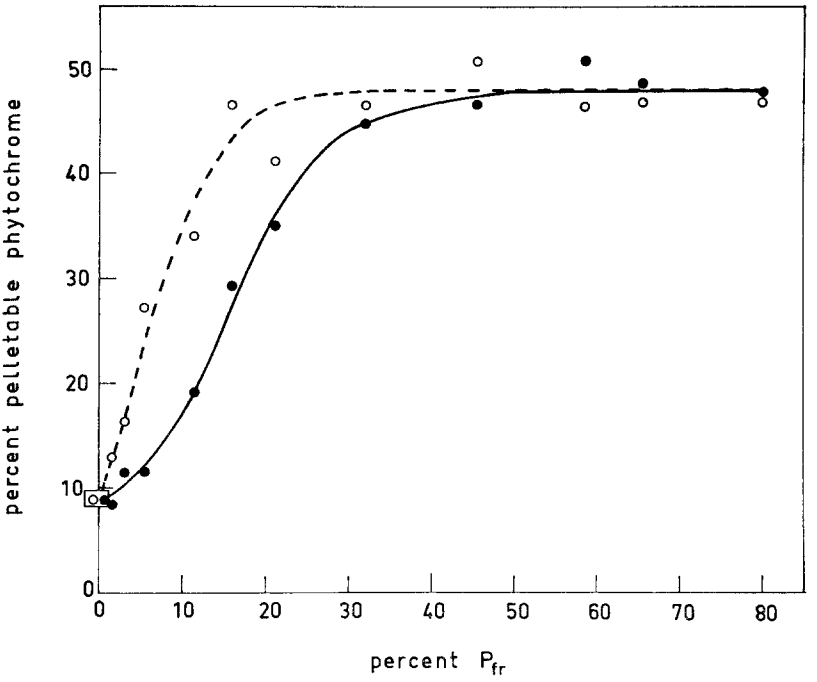


Fig. 4. Percent pelletable phytochrome in extracts from maize coleoptile tips following irradiations at 25 °C *in vivo* of 3 min with wavelengths between 660 and 730 nm followed immediately in all cases with 5 min 756 nm before extraction. Percent P_{fr} refers to the photosteady-state level of P_{fr} established *in vivo* during the initial 3-min irradiation. (•——•) irradiations of approximately 500 erg cm^{-2} sec^{-1}; (○----○) irradiations of approximately 3,000 erg cm^{-2} sec^{-1}. The extraction medium contained 10 mM Mg^{2+} and a solution/tissue ratio of 4:1.5 (v/w) was used

control levels were less dramatically affected. Almost half the total extractable pigment is pelletable at 10 mM Mg^{2+} following red + far-red irradiation. The basis for such effects of divalent cations on membranes has been thoroughly investigated in animal systems and used to advantage for the separation of microsomal and plasma membrane fractions [10, 11]. A concentration of 10 mM $MgCl_2$ was routinely used in the extraction medium in all subsequent experiments.

The binding of P_r alone is a measure of the state of the binding sites [9]. Therefore, the binding of P_r alone following the presentation of varying levels of P_{fr} *in vivo* can be considered a state function [2, 4]. Coleoptile tips were irradiated for 3 min with wavelengths between 660 and 730 nm, followed immediately with a terminal irradiation of 3 min 756 nm to reconvert all the phytochrome to the P_r form before extraction. The percent pelletable phytochrome as a function of the previous, *in vivo*, steady-state P_{fr} concentration is presented in Fig. 4. Initially (closed circles) a state

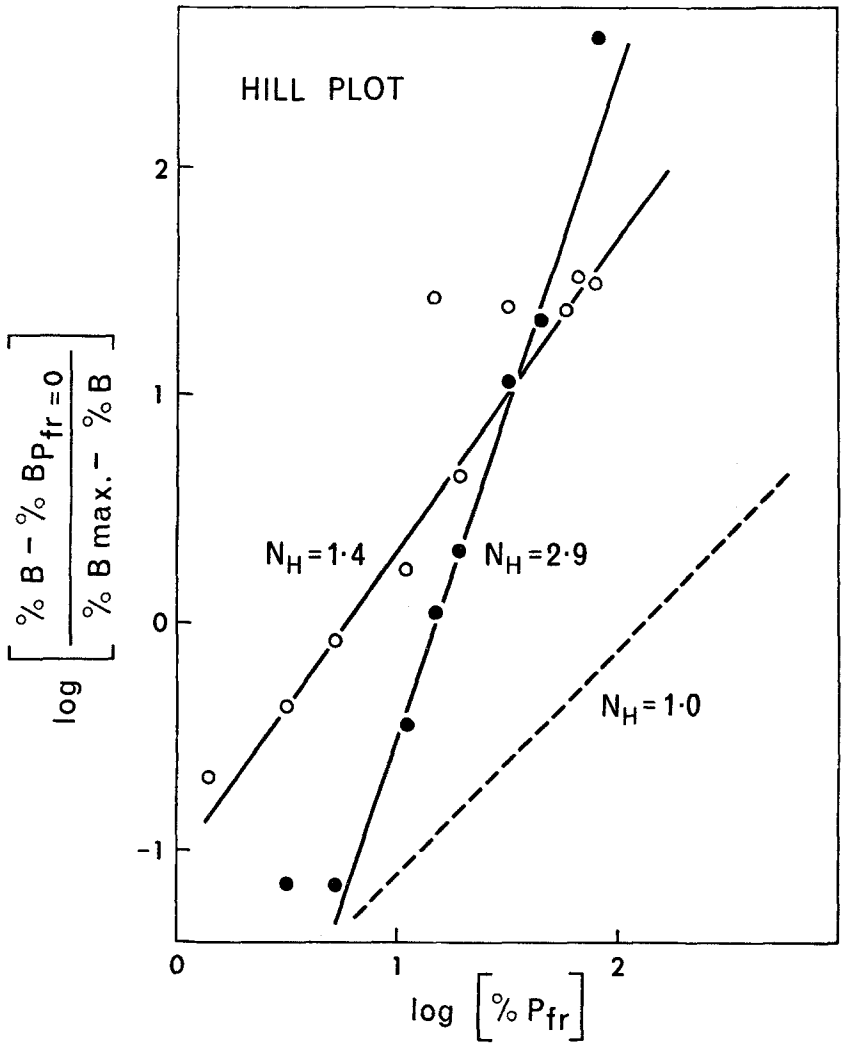


Fig. 5. Hill plot of data from Fig. 4. (•—•) irradiations of approximately 500 erg $cm^{-2}sec^{-1}$; (○—○) irradiations of approximately 3,000 erg $cm^{-2}sec^{-1}$; (----) theoretical; N_H = Hill coefficient

function indicative of cooperativity was obtained. A Hill coefficient of 2.9 may be calculated from this data (Fig. 5). It was then observed, however, that a sixfold increase in the intensity of the irradiation (from 500 erg cm^{-2} sec^{-1}, closed circles, to 3,000 erg cm^{-2} sec^{-1}, open circles, Fig. 4) resulted in increased binding at P_{fr} levels below 20% with no elevation of the saturation plateau. The Hill coefficient obtained from this data is 1.4 (Fig. 5), a significant erosion of the previously observed, relatively high, apparent cooperativity. This indicates that, in addition to the steady-

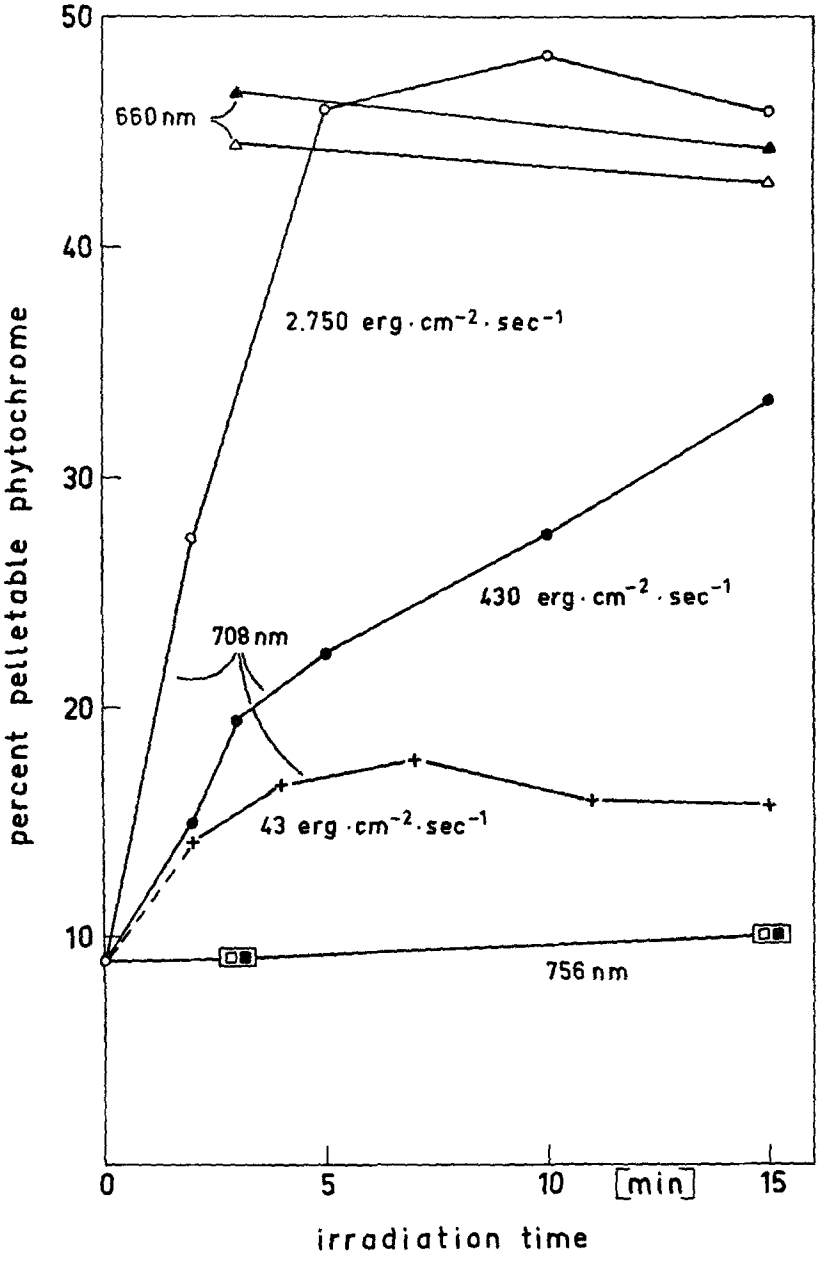


Fig. 6. Percent pelletable phytochrome as a function of time, intensity and wavelength of irradiation. Maize coleoptile tips were irradiated for increasing periods at 25 °C at 756 nm (■ 1,140 erg cm^{-2} sec^{-1}; □ 17,030 erg cm^{-2} sec^{-1}); 708 nm (+ 43 erg cm^{-2} sec^{-1}; • 430 erg cm^{-2} sec^{-1}; ○ 2,750 erg cm^{-2} sec^{-1}); and 660 nm (▲ 1,050 erg cm^{-2} sec^{-1}; △ 3,860 erg cm^{-2} sec^{-1}). In all cases these irradiations were followed by 3 min 756 nm at 25 °C *in vivo* before extraction

state level of P_{fr}, the light dose is an important factor in determining the state transformation.

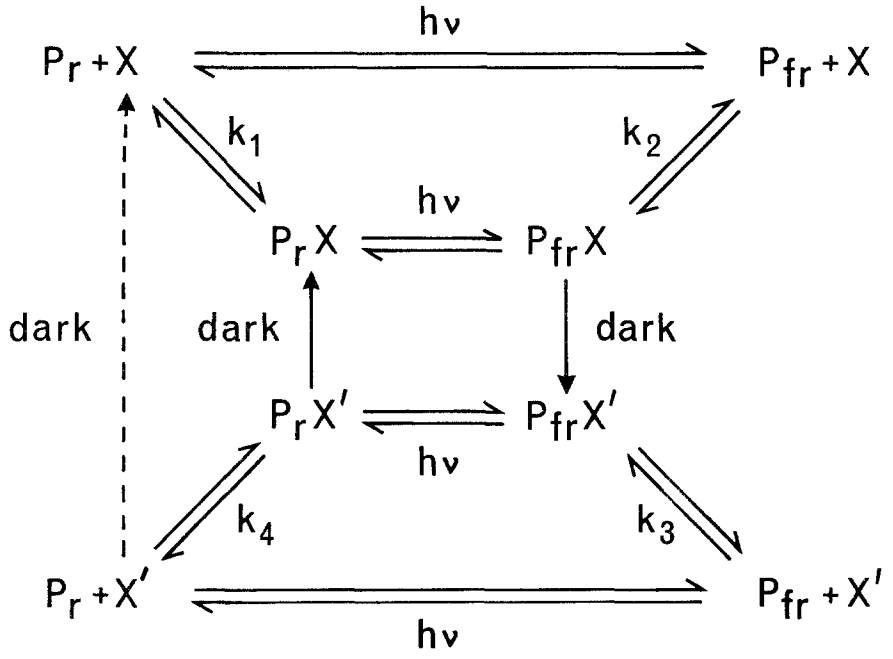
To further examine this question, both the time and intensity parameters of the light dose were varied using a 708 nm filter which established a photosteady-state of 11 % P_{fr}. Coleoptile tips were irradiated continuously for increasing periods at three different intensities of 708 nm light followed immediately by a terminal 3 min 756 nm irradiation at the highest intensity before extraction (Fig. 6). Continuous 660 and 756 nm irradiations at the two higher intensities were included as controls. With the lowest intensity (43 erg cm^{-2} sec^{-1}) treatment, the first two minutes of irradiation were performed at 430 erg cm^{-2} sec^{-1} (*see* dotted portion of curve, Fig. 6) to rapidly establish the photostationary state before completing the irradiation at 43 erg cm^{-2} sec^{-1}.

Increasing both time and intensity of irradiation while maintaining a constant P_{fr} concentration results in increased P_r binding (Fig. 6). At the highest intensity, 708 nm, the binding response saturates within 5 min at the level induced by red light. At the lowest intensity no detectable further increase in binding above the preirradiation level is observed during the subsequent irradiation period. Although difficult to estimate accurately, from the limited data a quasi-reciprocity would appear to exist at least for the two higher intensities. This confirms that the state function is dependent on the light dose as well as the steady-state effector P_{fr} concentration. The system would appear therefore to respond (at least in the short term) to the total number of P_{fr} molecules formed integrated over time rather than to the steady-state P_{fr} concentration at any instant.

Discussion

The present data provide further evidence that the level of phytochrome binding is a function of both the form of the pigment and the state of the binding sites; and that P_{fr} induces a change in the state of the binding sites such that the subsequent affinity for P_r is enhanced [9]. The induced change appears to be a function of the total number of molecules transformed to P_{fr} integrated over time.

We previously suggested in simplified form how this interaction of phytochrome with its binding sites might be visualized [9]. A more complete description which includes the possibility of phytochrome behaving as a ligand with specific binding constants is presented below (*see* Scheme 1) where P_r and P_{fr} are the two photoreversible forms of phytochrome, X and X' are two reversible forms of the receptor sites and k_1, k_2, k_3 and k_4



Scheme 1

are the binding constants for the four possible phytochrome-receptor combinations. These binding constants would determine the level of pelletable phytochrome observed in the extracts. In terms of this model the present data are consistent with a quantitative transformation of X to X' in response to the total number of P_{fr} molecules presented to the X sites during the irradiation period.

On the premise that phytochrome might be a ligand capable of interaction with a membrane matrix we have attempted to analyze our data in terms of the concepts developed by Changeux and associates [2, 4] to explain such phenomena. This has been complicated by several unique characteristics of the phytochrome system. First, the putative ligand, as well as the receptor sites is capable of existing in two reversible forms. Second, the $X \rightarrow X'$ transition can be considered virtually irreversible over the short term because of the slow rate of $X' \rightarrow X$ relaxation ($t_{\frac{1}{2}} = 50$ min, ref. [9]) following the reconversion of $P_{fr} \rightarrow P_r$. Third, under photosteady-state conditions the effector population is continually changing (turning over) as a result of cycling even though the absolute P_{fr} level remains constant.

It was not possible from the present data to compute a binding function for P_{fr} because both forms of phytochrome bind (Fig. 2). On the other hand, the induced response $X \rightarrow X'$ could be conveniently measured by the

binding of P_r alone following P_{fr} presentation *in vivo*. The binding of P_r in response to varying levels of P_{fr} previously offered *in vivo* (Fig. 4) can therefore be considered a state function [2, 4]. From these data we hoped to obtain information as to the degree of cooperativity which might exist in the system.

A Hill plot of the state function (Fig. 5) revealed a high degree of apparent cooperativity at medium irradiances of fixed duration but this was dramatically reduced at higher irradiances. Investigation of the effects of the time and irradiance parameters of light dose suggested that the $X \rightarrow X'$ state transformation is a function of the total number of P_{fr} molecules formed integrated over time rather than simply of the steady-state concentration of P_{fr}; i.e., the system appears to 'count' the total number of P_{fr} molecules it has seen. This effect would appear to result from the random cycling of phytochrome between the P_r and P_{fr} forms coupled with the slow reversibility of the P_{fr}-induced $X \rightarrow X'$ transition. This result could be expected whether the system were cooperative or graded where reversibility is slow. It would appear therefore that cooperativity in this case as measured in terms of the steady-state level of P_{fr} is an incomplete description of the system. This is because the response is rather the *product* of both the steady-state P_{fr} concentration and the light dose and depends therefore ultimately on the cycling rate.

In short, the present data appear to be compatible with the following aspects of Changeux's [2, 4] concepts: (a) the capacity of the receptor sites to exist in two reversible conformational states; (b) the capacity of the receptor sites for stereospecific recognition of the excitatory ligand; and (c) the alteration of the affinity of the receptor site towards the corresponding ligand upon the transition from one conformational state to another. The data are suggestive of a certain degree of cooperativity but this alone does not adequately define the phytochrome-receptor site interaction.

The present observations would not appear to be compatible with the 'selective' aspect of Changeux's [2] model; i.e., the *pre-existence* of two reversible conformational states of the receptors. This is because the $X \rightarrow X'$ state transition is not rapidly reversible upon removal of the P_{fr} effector [9]. The data could, on the other hand, be accounted for by an 'induced fit' approach [7] where the $X \rightarrow X'$ transition is induced by P_{fr} subsequent to binding. Removal of P_{fr} could then result in a slow relaxation of the induced state X' back to X as is observed [9].

If phytochrome binding can be shown to have physiological importance, it is possible that the present data could provide an explanation for the almost universally observed absence of a correlation between P_{fr} level and

physiological response [6]; and could provide a molecular basis upon which to explain the high energy response [5].

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