Plant Physiol. (1968) 43, 1259–1263

# Kinetically Distinguishable Populations of Phytochrome[1]

William K. Purves[2] and Winslow R. Briggs
The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Received April 25, 1968.

*Abstract.* **Two or more kinetically distinguishable populations of phytochrome molecules were observed in living tissues of oat, pea, maize, and cauliflower, as well as in extracts of oat. At least 3 different populations occurred in cauliflower florets, while 2 were observed in each of the other species. In extracted oat phytochrome, the relative proportions of the 2 forms remained constant during successive stages of purification. The physiological significance of this multiplicity of forms remains unclear.**

One of the more frustrating aspects of phytochrome physiology has been the difficulty of correlating the state of phytochrome measured spectrophotometrically with the physiological responses of tissues to light. For example, pea epicotyl segments which possess no spectrally detectable $P_{FR}$ may show typical growth responses to low dosages of far red light (7). The phytochrome-mediated alteration of phototropic sensitivity in maize coleoptiles is saturated by red light dosages so low as to cause no measurable phytochrome transformation (1). Results such as these have led to the suggestion that not all of the phytochrome in a cell is active (1, 5, 7). That is, there may be a separation of phytochrome into a small "active" population and one or more "inactive" populations.

We have studied phytochrome both in living tissues and in extracts, seeking evidence for the existence of distinguishable phytochrome populations which might represent the active and inactive forms. In each of the species tested, it has been possible to demonstrate the occurrence of 2 or more phytochrome populations which differ with respect to the kinetics of their phototransformations by red and far red light. However, the relationship of these populations to the hypothetical active and inactive fractions remains as yet unresolved.

## Materials and Methods

*Phytochrome Extraction and Purification.* Phytochrome was extracted from oat seedlings and partially purified by a modification of the methods described by Siegelman and Firer (11). Seeds of *Avena sativa* L. cv. USDA CI-2020 were sown on moist cellulose packing material (Kimpak 6223, Kimberly-Clark) and grown for 5 days in the dark at 25° in plastic boxes. On the day of harvest, the seedlings were cooled to 4° in the dark before they received any light; and all further steps were carried out at that temperature, in the dark or under dim green illumination. Shoot tissue (*ca.* 700 g) was harvested and ground in 0.05 M tris-HCl buffer, pH 8.1, containing 0.7 % (v/v) mercaptoethanol. The extract was filtered, centrifuged, treated with $CaCl_2$ (final conc 10 mM) to remove pectic substances and soluble polyuronic acids, centrifuged, passed through a Sephadex G-50 column, and chromatographed on calcium phosphate (brushite). Active fractions from the brushite column were pooled, giving the Stage I preparation referred to in the next section. Stage I phytochrome was precipitated with $(NH_4)_2SO_4$ and redissolved in 1 mM phosphate buffer, pH 7.8, yielding the Stage II preparation. Such preparations were passed through a Sephadex G-200 column and the active fractions pooled to give the Stage III preparation. Purifications were approximately 15, 25, and 40 fold for the 3 respective steps.

*Measurements of Phytochrome Transformation.* These were performed with a 2-wavelength difference spectrophotometer (Ratiospect Model R2, Agricultural Specialties Co., Beltsville, Md.) fitted with an actinic light source. This source was a General Electric DFA 150-watt incandescent projection lamp mounted approximately 36 cm from the sample. Red actinic light was obtained by interposing a 662 nm interference filter, and far red with a 725 nm interference filter (both were Baird Atomic type B-2 filters). Total phytochrome [$\triangle(\triangle OD)$] in a sample was determined by conventional methods (2). For dose-response curves, the sample was first saturated with either red or far red light and then exposed to a series of brief irradiations with the actinic source operating at a reduced intensity (55 % line voltage). After each irradiation, the

[1] Supported by National Science Foundation Grant GB-6683 and a grant from Research Corporation to W. R. Briggs.

[2] National Science Foundation Senior Postdoctoral Fellow. Permanent address: Department of Biological Sciences, University of California, Santa Barbara, California 93106.

difference in optical density between 660 and 730 nm was determined. This procedure was continued until all of the phytochrome in the sample had been transformed. In the figures, data are presented as $\log_{10}$ (% phytochrome not transformed) versus light dosage (seconds of actinic irradiation); the curves are log "decay" plots. It had previously been established that the changes in $OD_{660}$ and $OD_{730}$ were equal during the phototransformations. Except for cauliflower florets, all samples were kept at 0° during irradiations and measurements. Cauliflower florets were at room temperature.

*Plant Material for Tissue Studies.* Heads of cauliflower (*Brassica oleracea* L.) were obtained from local grocery stores. Florets were harvested with a razor blade and studied in 7- to 15-g lots. In all other cases, seeds were sown on moist cellulose packing material and grown at 25° in the dark in plastic boxes. Seedlings were harvested at 4° under dim green light. Seeds of oat (*Avena sativa* L. cv. USDA CI-2020) were sown dry. Apical 2-mm segments of coleoptiles, without primary leaves, were studied in 0.3-g lots. Seeds of pea (*Pisum sativum* L. cv. Alaska, Burpee Seed Co.) were soaked in tap water for 4 hours in the dark before sowing. The following materials were used for spectrophotometric studies, in 0.3- to 0.6-g lots: 2-mm apical segments of radicles (18 hr seedlings), whole epicotyls (44 hr seedlings), and epicotyl hooks (120 hr seedlings). Seeds of maize (*Zea mays* L. cv. Barbecue, Burpee Seed Co.) were sterilized for 5 minutes in 10% (v/v) commercial hypochlorite (Clorox), soaked 16 hours in running tap water in the dark, and sown. After 90 hours of growth, 3-mm apical segments of coleoptiles (without primary leaves) were harvested and studied in lots of 0.3 to 0.4 g.

## Results

In contrast to a previous report (4), the phototransformations of extracted oat phytochrome (Stage I) did not follow first order kinetics. When the data were plotted as $\log_{10}$ (% untransformed phytochrome) versus light dosage, a straight line was not obtained (fig 1). This curve could be resolved into 2 straight lines ["peeled," (12)] (fig 1), suggesting the presence of 2 populations of phytochrome molecules, 1 rapidly transformed and the other slowly transformed by light. Both the fast and the slow forms were transformed according to first order kinetics. From plots such as that shown in figure 1, it was possible to determine the percent contribution of each form to the total phytochrome in the sample, as well as $\tau$ values (dosage required to convert $1/e$ of each form, $e$ = the base of natural logarithms). In the experiment of figure 1, the slow component made up 76% of the total phytochrome both for the $P_{FR} \rightarrow P_R$ and for the $P_R \rightarrow P_{FR}$ transformation. As long as the phytochrome was tested in 1 mM phosphate buffer at pH 7.8, the percent slow and $\tau_{fast}/\tau_{slow}$ values remained unchanged throughout the purification procedure (table I). At all stages of purification, the $P_R$ form of the pigment had an absorption maximum at 667 nm, as it did *in vivo* (3). The values given in table I were consistent from experiment to experiment; data from 4 separate extractions are reported in the table. As will be seen, the percent slow values for both extracts and tissue samples were roughly equal for red and for far red irradiation. Curves for the transformation of $P_{FR}$ to $P_R$ are the more reliable, since they are not complicated by a significant back reaction, as in the case of $P_R$ to $P_{FR}$ ($P_{FR}$ has significant absorbancy at 667 nm).

To determine whether or not the occurrence of fast and slow phytochrome was an artifact of extraction, we obtained dose-response curves for phytochrome conversion in tissue samples of oat, pea, and maize. In all cases, the curves could be analyzed into fast and slow components, the percent slow and $\tau_{fast}/\tau_{slow}$ values varying with the species tested (table II). A typical dose-response curve for pea epicotyls is shown in figure 2.

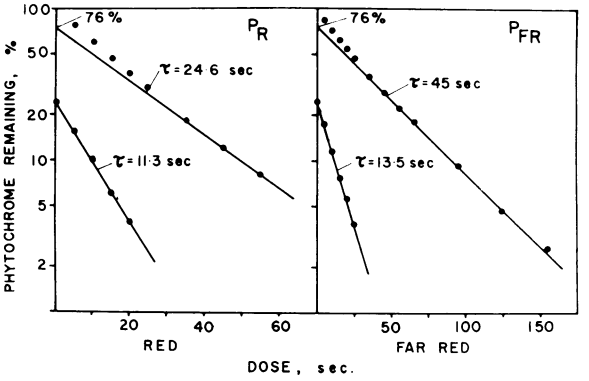


FIG. 1. Dose-response curves for phototransformations of extracted oat phytochrome. Left: $P_R$ conversion; right: $P_{FR}$ conversion. Upper curves are original data; each point represents the average of 2 determinations. Straight lines drawn through the original data were extrapolated to the ordinate to give "percent slow" values. Points on the extrapolated lines were subtracted from the observed values to give the lower (replotted) line, which characterizes the fast component.

Table I. *Rapidly- and Slowly-transforming Components of Extracted Oat Phytochrome*

Samples were dissolved in 1 mM phosphate buffer, pH 7.8.

| Stage of purification | Phytochrome conc $\Delta(\Delta OD)$/ml | Percent slow, starting as $P_R$ | Percent slow, starting as $P_{FR}$ | $\tau_{fast}/\tau_{slow}$, starting as $P_R$ | $\tau_{fast}/\tau_{slow}$, starting as $P_{FR}$ |
|---|---|---|---|---|---|
| I | 0.050 | 76 | 76 | 0.46 | 0.30 |
| I | 0.033 | 71 | 75 | 0.44 | 0.38 |
| I | 0.025 | 72 | 71 | 0.53 | 0.42 |
| I | 0.018 | 75 | 77 | 0.40 | 0.31 |
| I | 0.018 | 79 | 80 | 0.45 | 0.44 |
| II | 0.022 | 79 | 77 | 0.32 | 0.30 |
| III | 0.010 | 67 | 78 | 0.35 | 0.30 |

Table II. *Rapidly- and Slowly-transforming Components of Oat, Pea, and Maize Phytochrome*

| Species | Organ | Phytochrome content, Δ(ΔOD)/g tissue | Percent slow, starting as $P_R$ | Percent slow, starting as $P_{FR}$ | $\tau_{fast}/\tau_{slow}$, Starting as $P_R$ | $\tau_{fast}/\tau_{slow}$, Starting as $P_{FR}$ |
|---|---|---|---|---|---|---|
| Oat | Coleoptile tips | | 60 | 60 | 0.19 | 0.26 |
| | | | 37 | 31 | 0.20 | 0.25 |
| | | | 55 | 52 | 0.27 | 0.29 |
| | | 0.276 | 50 | 49 | 0.22 | 0.25 |
| Pea | Epicotyl hooks | 0.535 | 42 | 37 | 0.33 | 0.27 |
| | Radicles | 0.083 | 48 | 40 | 0.12 | 0.19 |
| | | 0.090 | 40 | 42 | 0.09 | 0.21 |
| | Whole shoots | 0.324 | 40 | 39 | 0.13 | 0.24 |
| Maize | Coleoptile tips | 0.122 | 44 | 55 | 0.20 | 0.21 |
| | | 0.172 | 43 | 49 | 0.22 | 0.30 |
| | | 0.167 | 45 | 41 | 0.24 | 0.26 |

The phytochrome of cauliflower is unique in that it is not "destroyed" during long periods of maintenance in the $P_{FR}$ form (5). In the hope that the phytochrome of this species might exist as a single form only, a dose-response curve was obtained for cauliflower florets (fig 3). Contrary to our expectation, there proved to be more than 2 components. A straight line could be drawn through the later (high dosage) points of the decay curve, indicating the presence of the slowest component. The replotted ("peeled") line was straight over most of its length but passed well below the initial (zero dose) point, suggesting the presence of 1 or more still faster components making up approximately 10 % of the total phytochrome. The low level of phytochrome in cauliflower florets, coupled with the limit of sensitivity of the spectrophotometer, made it impossible to analyze this component further. The results of 5 experiments with cauliflower florets are summarized in table III.

An attempt was made to alter the proportions of fast and slow phytochrome in pea epicotyls. Samples of 44-hour epicotyls were used for dose-response determinations, saturated with red light to convert all of the phytochrome to $P_{FR}$, and kept in darkness

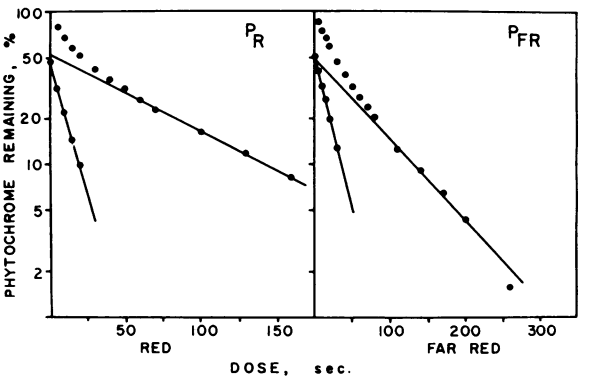


FIG. 2. Dose-response curves for phototransformations of pea phytochrome *in vivo*. Whole shoots of 44-hour seedlings. Left: $P_R$ conversion; right: $P_{FR}$ conversion. Curves as in figure 1.

Table III. *Multiple Components of Phytochrome in Cauliflower Florets*

Data are from 5 experiments.

| No. | Phytochrome content Δ(ΔOD)/g tissue | Percent slow starting as $P_R$ | Percent slow starting as $P_{FR}$ | Percent medium starting as $P_R$ | Percent medium starting as $P_{FR}$ | Percent fast starting as $P_R$ | Percent fast starting as $P_{FR}$ | $\tau_{med.}/\tau_{slow}$, Starting as $P_R$ | $\tau_{med.}/\tau_{slow}$, Starting as $P_{FR}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.003 | 53 | 53 | 47 | 47 | 0 | 0 | 0.11 | 0 09 |
| 2 | 0.003 | 46 | 40 | 39 | 50 | 15 | 10 | 0.07 | 0.08 |
| 3 | 0.005 | 54 | 53 | 36 | 42 | 10 | 5 | 0.10 | 0.18 |
| 4 | 0.007 | 30 | 36 | 55 | 55 | 15 | 9 | 0.08 | 0.15 |
| 5 | 0.006 | 48 | 42 | 44 | 52 | 8 | 6 | 0.12 | 0.20 |
| Avg | | 46.2 | 44.8 | 44.2 | 49.2 | 9.6 | 6.0 | 0.10 | 0.14 |

Table IV. *Proportions of Phytochrome Components in Pea Epicotyls Before and After Phytochrome "Destruction."*

| No. | Δ(ΔOD)/g Tissue Before | Δ(ΔOD)/g Tissue After | % "Destroyed" | Initial percent slow $P_R$ | Initial percent slow $P_{FR}$ | Final percent slow $P_R$ | Final percent slow $P_{FR}$ |
|---|---|---|---|---|---|---|---|
| 1 | 0.268 | 0.146 | 46 | 52 | 49 | 54 | 55 |
| 2 | 0.380 | 0.193 | 49 | 40 | 42 | 50 | 43 |

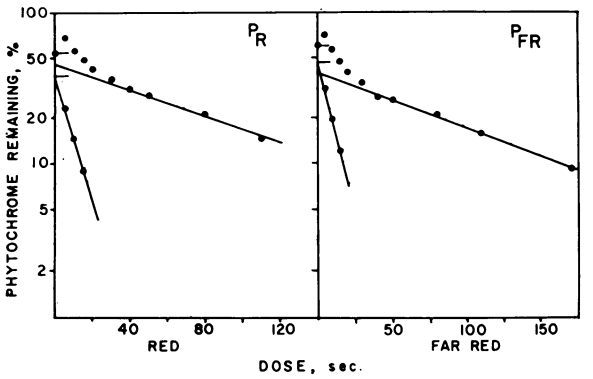


FIG. 3. Dose-response curves for phototransformations of cauliflower phytochrome. Left: $P_R$ conversion; right: $P_{FR}$ conversion. Curves as in figure 1. Note that the lower (replotted) lines pass below the zero dose points (emphasized by horizontal lines), indicating the presence of a third component.

at 25°. After 2 hours, dose-response curves were obtained for the residual phytochrome; approximately half of the original phytochrome had been "destroyed" (8). It was hoped that 1 of the 2 components of the phytochrome would be preferentially "destroyed"; however, no obvious changes in the percent slow values were observed in the 2 experiments (table IV). It is clear that both components of the pigment were partially "destroyed."

## Discussion

It is clear that phytochrome exists as 2 or more distinguishable populations both *in vivo* and in extracts. Similar results have been obtained by Correll *et al.* (6), studying the dark reversion process in phytochrome extracted from rye. They converted the pigment to $P_{FR}$ and monitored changes in optical density at 660 and 730 nm during dark reversion of $P_{FR}$ to a red-absorbing form. Curves similar to our figure 1 were obtained, and these were analyzed to reveal the existence of rapidly- and slowly-reverting components. Whether these correspond to our rapidly- and slowly-phototransforming components remains to be seen, but such correspondence is likely.

Other investigators have found that phytochrome exists as a single component showing first order kinetics of phototransformation *in vitro* (4) and *in vivo* (10). Their failure to observe fast and slow components may probably be attributed to an insufficient number of points in their dose-response curves or to limited sensitivity of measuring techniques. The data in figure 6 of the paper by Pratt and Briggs (10), although treated in that paper as a straight line, could probably be "peeled" to reveal a second component. For both $P_R$ and $P_{FR}$ transformation, the early (low dosage) points fall below and the late points above the theoretical straight line. Experiments in this laboratory have shown that certain treatments, such as prolonged exposure to $(NH_4)_2SO_4$ or long periods of time as $P_{FR}$, cause changes in the spectral and kinetic properties of phytochrome extracts [(3) and Rice, Purves and Briggs, unpublished]. In the experiments reported here, all phytochrome extracts showed an absorption maximum at 667 nm in the $P_R$ form.

It is not yet possible to correlate the properties of either of the phytochrome components with the physiological responses of plant tissues. Experiments to test such correlations depend upon the availability of a system which A) possesses sufficient phytochrome for a precise dose-response study and B) shows a large and quantifiable physiological response to red and far red irradiation. The samples used for the physiological and spectrophotometric studies must be identical in all respects and must be irradiated in the same fashion. The most suitable system now available for such a study would appear to be the oat coleoptile tip, which possesses a high level of phytochrome and is promoted in its growth by red irradiation (9). However, preliminary experiments have indicated that this system may not be satisfactory; variability in the different parts of the experiment is too great.

It may be that none of the forms of phytochrome demonstrated here represents the physiologically active form of the pigment. The red light-induced alteration of phototropic sensitivity in maize coleoptiles is saturated by dosages 2 orders of magnitude too small to induce measurable phytochrome transformation (1). It was suggested that the "active" phytochrome in this case was only a fraction of 1 % of the total phytochrome and that its phototransformations were facilitated. A phytochrome population so small would be impossible to detect by our techniques. However, our results do indicate that native phytochrome of maize and other plants does not exist as a single, homogeneous component. The third, very rapidly-transforming component of cauliflower phytochrome may be related to the hypothetical facilitated component of maize phytochrome (1).

## Acknowledgments

We are indebted to Mr. Harbert Rice for valuable discussions and for preparing the phytochrome extracts. Mr. Cecil Jackson provided skillful technical assistance.

## Literature Cited

1. BRIGGS, W. R. AND H. P. CHON. 1966. The physiological versus the spectrophotometric status of phytochrome in corn coleoptiles. Plant Physiol. 41: 1159–66.
2. BRIGGS, W. R. AND H. W. SIEGELMAN. 1965. Distribution of phytochrome in etiolated seedlings. Plant Physiol. 40: 934–41.
3. BRIGGS, W. R., W. D. ZOLLINGER, AND B. B. PLATZ. 1968. Some properties of phytochrome isolated from dark-grown oat seedlings (*Avena sativa* L.). Plant Physiol. In press.

4. BUTLER, W. L., S. B. HENDRICKS, AND H. W. SIEGELMAN. 1964. Action spectra of phytochrome *in vitro*. Photochem. Photobiol. 3: 521–28.
5. BUTLER, W. L. AND H. C. LANE. 1965. Dark transformations of phytochrome *in vivo* II. Plant Physiol. 40: 13–17.
6. CORRELL, D. L., J. L. EDWARDS, AND W. SHROPSHIRE, JR. 1968. Multiple chromophore species in phytochrome. Photochem. Photobiol. In press.
7. HILLMAN, W. S. 1965. Phytochrome conversion by brief illumination and the subsequent elongation of etiolated *Pisum* stem segments. Physiol. Plantarum 18: 346–58.
8. HILLMAN, W. S. 1967. The physiology of phytochrome. Ann. Rev. Plant Physiol. 18: 301–24.
9. HOPKINS, W. G. AND W. S. HILLMAN. 1965. Response of excised *Avena* coleoptile segments to red and far-red light. Planta 65: 157–66.
10. PRATT, L. H. AND W. R. BRIGGS. 1966. Photochemical and nonphotochemical reactions of phytochrome *in vivo*. Plant Physiol. 41: 467–74.
11. SIEGELMAN, H. W. AND E. M. FIRER. 1964. Purification of phytochrome from oat seedlings. Biochemistry 3: 418–23.
12. VAN LIEW, H. D. 1967. Graphic analysis of aggregates of linear and exponential processes. J. Theoret. Biol. 16: 43–53.