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Evidence for Bound Phytochrome in Oat Seedlings[1]

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Abstract. **Phytochrome is consistently observed in pellets centrifuged from homogenates of etiolated, 5-day-old oat seedlings. The majority of pigment associated with the pellet cannot be removed by buffer washes, nor can appreciable quantities of additional phytochrome be adsorbed onto the sedimented material. Over 70 % of phytochrome in the pellet is released by 1 % Triton X-100.**

Storage at 0°, irradiation by white light, and Triton treatment all cause much greater loss of photoreversibility in pelleted phytochrome than in supernatant phytochrome. We conclude that the phytochrome in the 1500 to 40,000*g* (30 min) pellet is distinct from the soluble phytochrome in the supernatant.

Interpretations of the mechanism of phytochrome responses in etiolated seedlings are complicated by the fact that only a portion of the total phytochrome in these plants appears to mediate some of their responses to red and far-red light (2, 5, 8). As a result, it has been suggested that a small active fraction of phytochrome may be present, distinct from the majority of phytochrome (2). Evidence that more than 1 species of phytochrome exists *in vivo* may be adduced from the spectrophotometric data of Spruit (12), the kinetics of *in vitro* dark reversion (13), and the kinetic analyses of reversibility by Purves and Briggs (10).

The possibility that a species of cellular phytochrome exists in association with membranes has been reviewed by Hendricks and Borthwick (7). These authors point out that the previously used procedures for phytochrome isolation have in no way ruled out the existence of a membrane associated phytochrome. We have searched for such a membrane associated phytochrome which differs from the soluble phytochrome most commonly investigated. We will show that pellets obtained after centrifugation at various speeds contain an associated phytochrome, that the pigment cannot be easily washed off sedimented material, and that this bound phytochrome differs in susceptibility to denaturation compared to soluble phytochrome.

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Materials and Methods

After saturating vermiculite with tap water, seeds of *Avena sativa* L. var. California Red were sown on the surface, covered with dry vermiculite, and grown at 28° in the dark. After 5 days, the exposed tissue was cut about 1 cm above the vermiculite and chilled at 4° in darkness for at least 2 hr. Light treatments consisted of exposing plants to 1500 ergs $\times$ cm^{-2} $\times$ sec^{-1} of red light for 5 min.

The seedlings were ground, under fluorescent lighting, in buffer composed of 0.1 M tris-HCl (pH 8.0), 70 mM mercaptoethanol, 2 mM EDTA, 0.5 M sucrose, and 2 % (w/v) insoluble polyvinylpyrrolidone (PVP). For each 100 g tissue, 75 ml buffer were used. The homogenate was strained through 4 layers of cheesecloth before centrifugation. Resuspension of centrifugal pellets was achieved in a tight fitting glass homogenizer in grinding buffer containing 5 % (w/v) soluble PVP (MW 40,000) and, where noted, sucrose was omitted. All the above manipulations were performed at 0 to 4°. In experiments where a mortar and pestle were used, the grinding buffer contained 5 % soluble PVP; sand was added to facilitate grinding.

Phytochrome was analyzed at room temperature on a Cary 14 recording spectrophotometer equipped with a 0 to 0.1 slide wire; a Model 1462 Scattered Transmission Accessory, modified to allow actinic illumination of the sample cell at 660 nm and 725 nm from a Bausch and Lomb grating monochromator; and a high intensity light source. The monochromator was supplemented by appropriate Corning cut-off filters. One cm path length cells were used. From the average of at least 4 difference spectra, $\triangle(\triangle OD)$ values were calculated (13). For each

sample these values varied less than 10^{-3} $\triangle(\triangle OD)$ units. Accuracy of the method allowed detection of OD changes to 5×10^{-4}.

To determine the accuracy of $\triangle(\triangle OD)$ measurements in scattering samples the following experiment was done. The $\triangle(\triangle OD)$ of the 105,000*g* (120 min) supernatant and the 1500 to 40,000*g* (30 min) pellet were measured. The pellet was then resuspended in the supernatant and the $\triangle(\triangle OD)$ measured. The $\triangle(\triangle OD)$ of the combined supernatant was only 15 to 20 % higher than the sum of their $\triangle(\triangle OD)$'s measured separately. Therefore pellet $\triangle(\triangle OD)$'s are reported as measured.

Results and Discussion

The possibility that phytochrome exists in other than soluble forms was examined by centrifuging the crude homogenate at various speeds and analyzing the resuspended pellets for phytochrome content. The data, expressed as a percent of the pigment in the 1500*g* (15 min) supernatant, show varying amounts of phytochrome in all pellets (table I).

Table I. *Centrifugal Fractionation of Phytochrome*

Fraction	Phytochrome[1]
	%
1500*g* (15 Min) supernatant	100
0–1500*g* (15 Min) pellet	1.3
1500-10,000*g* (30 Min) pellet	5.0
10,000–25,000*g* (30 Min) pellet	1.4
25,000–40,000*g* (30 Min) pellet	0.3
40,000–105 000*g* (30 Min) pellet	1.7
105,000 (120 Min) supernatant	66.0

[1] Data expressed as a percent of the phytochrome assaved in the supernatant after centrifugation at 1500*g* (15 Min).

Since the 1500 to 40,000*g* (30 min) pellets contained the largest amount of phytochrome activity, they were combined in subsequent experiments. When the data of 10 different experiments were pooled and analyzed statistically, the quantity of bound phytochrome in the once-washed, 1500 to 40,000*g* (30 min) pellet, was 4.3 ± 1.2 % of the 1500*g* supernatant. Could such an amount be physiologically important? In certain phytochrome-mediated responses, it has been shown that the amount of light needed to saturate a particular physiological response is much lower than that needed to convert all the phytochrome (2, 8). In fact Briggs and Chon (2) have calculated that for the case of *Zea mays* phototropism that only 1 % of the lowest assayable amount of phytochrome need be transformed to saturate the physiological response. If this were also the case for *Avena*, and there are some indications that it is for geotropism (14) and phototropism (1), then the amount of phytochrome found in the pellet could easily participate in certain red light responses of the plant.

The sedimentable phytochrome described above may be a result of A) enclosure of soluble phytochrome within membrane bound organelles and vesicles; B) non-specific adhesion of soluble phytochrome to membranes or other particles; C) the existence of a distinct bound form. The possibility that phytochrome activity of the pellet was due to pigment contained in organelles and vesicles was tested by comparing the supernatants of pellets washed in buffer with or without sucrose. The results (table II) show that the use of a hypotonic resuspending medium, which would be expected to open any membrane enclosed bodies, released no more phytochrome to the supernatant than resuspension in sucrose buffer.

Table II. *Effects of Hypotonic Buffer and Presssure on Removal of Phytochrome Activity From Pellet*

Treatment	Phytochrome activity
	$[\Delta(\Delta OD)] \times ml^{-1} \times 10^3$
Untreated pellet	55
Supernatant of pellet after sucrose wash[2]	7.0
Supernatant of pellet after plain buffer wash[2]	7.1
Supernatant of pellet after passage through French press	16.2

[1] Pellet formed after centrifuging 1500*g* (15 Min) supernatant at 40,000*g* for 30 Min.

[2] Washing performed by resuspending pellet and recentrifuging at 105,000*g* for 30 Min.

Passage through a French press at 6000 psi was also employed to destroy the integrity of the sedimented organelles and vesicles. The loss of phytochrome from the pellet to the supernatant was never large enough to suggest that the bulk of the phytochrome of the pellet was located within membrane-bound organelles (table II). The increase in amount of phytochrome in the supernatant compared to that found in washes with hypotonic buffer may be due to smaller fragments produced by the press which were not sedimented at the speeds used.

The likelihood that the sedimented phytochrome represents only contamination of the pellet by the supernatant was investigated as follows. The 1500 to 40,000*g* pellet was subjected to successive washings. Each wash consisted of resuspension in grinding buffer minus sucrose followed by repelleting at 105,000*g* for 30 min. Table III shows that the supernatant after the first wash contained about 23 % of the original pelletable activity; the second wash released 8 %; and the supernatant of the third wash contained only a trace amount of phytochrome. Thus, almost 70 % of the original activity of the pellet could not be washed off. The washings did.

Table III. *Effect of Successive Washes on Liberation of Phytochrome From Pellet*

Fraction	Phytcchrome activity	Amount of pellet
	[$\Delta(\Delta OD) \times 10^3$]	%
1500–40,000g (30 Min) Pellet	12.8	100
Supernatant after first wash[1]	2.7	23
Supernatant after second wash	1.0	8
Supernatant after third wash	Trace	...

[1] Washing performed by resuspending pellet in buffer and recentrifuging at 105,000g for 30 min.

however, destroy 80 % of the original pellet phytochrome.

Another method of testing for supernatant contamination of the pellet made use of the fact that irradiation of intact plants lowers phytochrome levels in both supernatant and pellet fractions (see table VII). This result enables us to obtain a pellet of low phytochrome activity. If the phytochrome of the pellet were due primarily to binding of supernatant phytochrome, then the activity in the pellet from irradiated plants should be raised to the level of activity of pellets from etiolated plants by its resuspension in supernatant from etiolated plants. Table IV shows that a pellet isolated from light-treated plants contained less than 50 % of the amount of phytochrome in a pellet from etiolated plants. When the pellet from irradiated plants was resuspended in and spun through supernatant from etiolated plants which contained high levels of phytochrome, the amount of phytochrome adsorbed to this pellet was small [6.6 $\Delta(\Delta OD) \times ml^{-1} \times 10^3$] and never approached the level of phytochrome present in the pellet from etiolated plants [37.8 $\Delta(\Delta OD) \times ml^{-1} \times 10^3$].

It is unlikely that the binding of phytochrome to sedimentable particles is due to pH effects. Such an occurrence, discussed by Hillman (9), was reported to be caused by pH values below neutrality and the attachment was reversible. The pH of crude homogenates isolated for this study was always above 7.4 and all pelleted material was washed once in buffer of pH 8.0.

Table IV. *Adsorption of Phytochrome From Supernatant to Pellet*

Fraction	Phytochrome activity
	[$\Delta(\Delta OD) \times ml^{-1} \times 10^3$]
105,000g (120 Min) supernatant from etiolated plants	970
105,000g (120 Min) supernatant from red light-treated plants	620
1500–40,000g (30 Min) pellet from etiolated plants	38
1500–40,000g (30 Min) pellet from red light-treated plants	14
1500–40,000g (30 Min) pellet from red light-treated plants washed in supernatant from etiolated plants	20

Although repeated washes could not remove large amounts of phytochrome from the pellet, treatment for 2 hr at 0° of a once-washed pellet with the nonionic detergent Triton X-100 released a large portion of measurable phytochrome. It can be seen in the last 3 lines of table V that addition of 1 % (v/v) Triton to a resuspended pellet results in a 35 % destruction of reversibility. If the suspension is centrifuged at 105,000g for 45 min, about 75 % of the remaining activity appears in the supernatant.

The data presented so far suggest that phytochrome exists as at least 2 different species in the cell: a soluble form remaining in solution after centrifugation at 105,000g for 120 min and a form which is pelletable. While these data strongly suggest the presence of a distinct pelletable phytochrome,

Table V. *Inhibition and Release by Triton X-100 of Pelletable Phytochrome*

Fraction	Phytochrome activity
	[$\Delta(\Delta OD) \times ml^{-1} \times 10^3$]
105,000g (120 Min) supernatant	90
105,000g (120 Min) supernatant with 1 % Triton	94
1500–40,000g (30 Min) pellet	46
1500–40,000g (30 Min) pellet with 1 % Triton	30
105,000g (45 Min) supernatant of Triton-treated pellet[1]	22

[1] Following addition of Triton, pellet was recentrifuged at 105,000g (45 min) and the resulting supernatant assayed.

they cannot refute entirely the argument that pellet activity is due largely to supernatant contamination. Therefore, attempts have been made to see if there exist distinguishing characteristics between supernatant and pellet phytochrome.

Some evidence that the phytochrome in the pellet may have properties different from that in the supernatant is shown in the first 4 lines of table V. Addition of Triton at the high concentration of 1 % (v/v) had very little effect on phytochrome of the 105,000g (120 min) supernatant, but destroyed the reversibility of the pelletable pigment 35 %. This destruction was greatly accelerated by temperatures above 0°. In contrast, the detergent deoxycholate at 1 % (w/v) completely inhibited phytochrome activity in both supernatant and pellet.

Another characteristic which can be used to separate supernatant from pelletable phytochrome is stability. Figure 1 compares degradation of phytochrome in the 105,000*g* (120 min) supernatant, with that in a resuspended, once washed, pellet. Storage at 4° results in a rapid deterioration of phytochrome in the pellet after only 1 day, while loss of supernatant phytochrome is much slower.

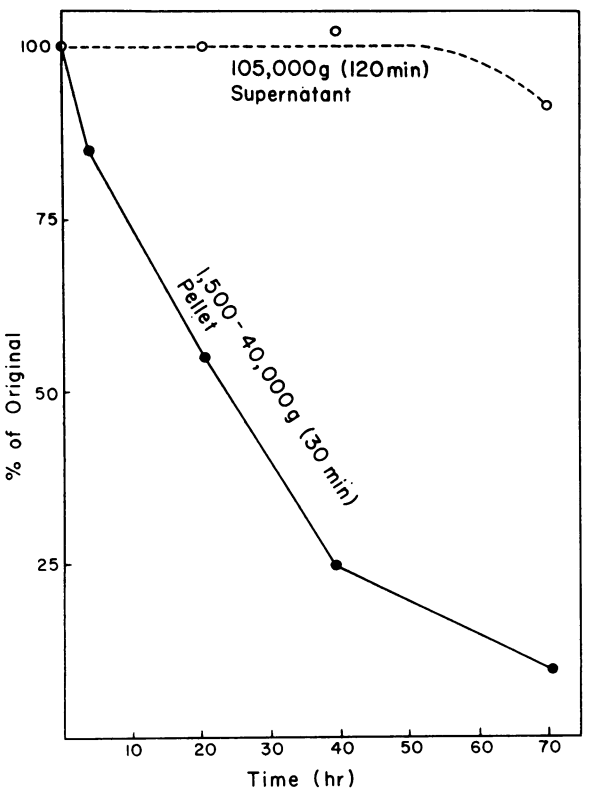

FIG. 1. Stability over time of phytochrome in a resuspended pellet or in the supernatant. The extracts were stored at 4° in fluorescent light of 30 ft-c.

It is possible that the lability differences shown in figure 1 do not reflect inherent differences in the phytochrome molecules themselves but are merely due to concentration of some destructive substance in the pellet. This substance would probably not be the "phytochrome killer" of Furuya and Hillman (6). Their substance was soluble while ours would be restricted to the pellet. Also, they report the "phytochrome killer" to be absent in the plant used for this study, *Avena*. The destructive agent in the present case may be a hydrolytic enzyme. Siegelman and Firer (11) and Butler *et al.* (4) have shown that pronase and trypsin destroy phytochrome photoreversibility. It should be noted, however, that in the present study resuspension of the once-washed 1500 to 40,000*g* pellet in the 40,000*g* supernatant did not accelerate the rate of decay of the supernatant phytochrome. These data speak against the presence of a hydrolytic, phytochrome-destroying substance in the pellet unless this substance acts differently on pelletable phytochrome.

Another difference in lability between pellet and supernatant phytochrome is that only the degradation of pelletable phytochrome appears to be accelerated by light. Table VI compares phytochrome preparations from pellet or supernatant origin which had been stored for 48 hr at 4° in either fluorescent light at 30 ft-c or in the dark. Light had little effect on the activity loss of 105,000*g* (120 min) supernatant but markedly accelerated loss of reversibility of phytochrome found in the pellet.

Irradiation of intact plants with red light results in massive decreases in their phytochrome content, while their sensitivity to red and far-red light is retained (3, 5, 8). We had hoped to show that pelletable phytochrome behaved differently from that in the supernatant with respect to *in vivo* light destruction, but table VII shows that this was not

Table VI. *Light Dependent Destruction of Pelletable Phytochrome*

Preparation	Phytochrome activity		
	Original	After 48 hr dark	light
	[$\Delta(\Delta OD) \times ml^{-1} \times 10^3$]		
105,000*g* (120 Min) supernatant	6.8	6.3	6.5
1500–40,000*g* (30 Min) pellet	6.8	3.8	0.8

Table VII. *Stimulation by Red Light of Phytochrome Loss in Supernatant and Pellet*

Time from light irradiation until harvest	Supernatant[1]			Pellet[2]		
	Light[3]	Etiolated[3]	% Dec. by light	Light[3]	Etiolated[3]	% Dec. by light
hr						
1.5	310	770	61	15	27.5	44
4.0	300	850	65	13	32	61
24.0	170	650	74	7	16	56

[1] Supernatant from centrifugation at 105,000*g* for 120 min.
[2] Pellet after centrifugation of 1500*g* (15 min) supernatant for 30 min at 40,000*g*.
[3] Data expressed as (ΔOD) $\times$ ml^{-1} $\times$ 10^3.

the case. After plants were irradiated, an initial rapid drop in amount of supernatant phytochrome was evident. This level then decreased at a slower rate for 24 hr. If one compares the changes of phytochrome in the once-washed pellet after light treatment, it can be seen that a similar decrease occurs. The decrease in pelletable phytochrome always parallels the decline of phytochrome in the supernatant.

Conclusion

We have presented evidence that a certain portion of cellular phytochrome can be pelleted and is present in amounts which can be of physiological significance. The majority of pelletable phytochrome sediments at intermediate *g*-values, not in the heavier material in the 1500*g* (15 min) pellet nor in the small fragments of the lighter pellets. Phytochrome of the pellet is not removed by repeated buffer washes but is partially released by the detergent Triton X-100. The evidence suggests that pelletable phytochrome may be in some way associated with cell membranes though other explanations may also be applied.

When compared to soluble pigment, pelletable phytochrome shows an enhanced sensitivity to denaturation by detergent action, by storage in the cold, and by white light. We suggest that the differences in abundance and behavior of bound and supernatant phytochrome may be reflected by different physiological roles for the 2 phytochrome types.

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