

Characterization of the Destruction of Phytochrome in the Red-absorbing Form¹

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ABSTRACT

Both the red-absorbing (Pr) and far red-absorbing (Pfr) forms of phytochrome undergo destruction, defined as the loss of photoreversibly detectable chromoprotein following actinic irradiation of dark-grown tissue, in 4-day-old etiolated oat seedlings. Pr and Pfr destruction follow the same time course, exhibit the same time delay after actinic irradiation when the plants are grown in sealed containers, result in a loss of antigenically detectable phytochrome, as determined by radial immunodiffusion assay, equal to the loss of spectrophotometrically detectable phytochrome, and have the same sensitivity to 2-mercaptoethanol and azide. We suggest that Pr destruction is a consequence of the same mechanism that is responsible for Pfr destruction.

Phytochrome destruction, defined as the loss of spectrophotometrically detectable phytochrome following actinic irradiation of dark-grown tissue, has generally been considered specific for Pfr (10, 15). Early reports indicated that, at least in *Avena*, Pr may also undergo destruction following a R,³ FR irradiation cycle (1, 2). This apparent destruction of Pr in *Avena* has been confirmed by Mackenzie *et al.* (8) who demonstrated that about 35% of the spectrophotometrically detectable phytochrome in dark-grown oat seedlings was lost following sequential R and FR irradiations. They also demonstrated that FR light alone had no effect on subsequent spectrophotometrically detectable phytochrome levels, verifying that Pr destruction required prior cycling of the chromoprotein through the Pfr form.

It is not yet known whether Pr and Pfr destruction result from operation of the same mechanism. The experiments reported here are designed to test this question.

MATERIALS AND METHODS

Oats (*Avena sativa* L., cv. Garry) were germinated and grown for 4 days in darkness at 25 ± 1 C on moist cellulose packing material (Kimpak 6234, Kimberly Clark) on open cafeteria trays (35 × 45 cm) (100 g oats) or in plastic buckets (27 × 30 × 12 cm) (75 g oats). Humidity in the growth room was kept near saturation and, except for actinic irradiations, plants were exposed only to green safelights (12).

Actinic R light was obtained from Gro-lux fluorescent lamps (Sylvania) as described previously (17). FR actinic light was obtained by filtering the output of 28 30-w prefocused tungsten

lamps through 3.2 mm of Plexiglas FRF-700. The bulbs were evenly spaced over a 0.38 m² area and were 10 cm above the seedlings. Phytochrome photoequilibrium, determined by direct spectrophotometric assay, was reached in about 5 s with the R source and 90 s with the FR source.

Phytochrome was assayed spectrophotometrically with a custom-built, dual wavelength spectrophotometer (5, 17) at 666 versus 727 nm. Within an experiment, the same number of shoots were used to prepare each 0.6-g sample to minimize biological variability.

Regression lines, standard errors, and tests of the null hypothesis that two regression coefficients are estimates of a common slope were computed as described by Steel and Torrie (16).

Whole seedlings were infiltrated with reagents by immersion in a buffer prepared by mixing 25 mM *N*-morpholino-3-propane sulfonic acid with 25 mM Tris to obtain a pH of 8.0 at 23 C. The buffer contained the desired reagent as indicated. Tissue was vacuum-infiltrated for two 2-min periods using a Welch Duo-Seal two-stage pump. Seedlings after infiltration were returned to moist cellulose pads on plastic cafeteria trays for subsequent irradiations and incubations.

Radial immunodiffusion assays were performed as described previously (13) utilizing crude extracts, clarified by centrifugation for 15 min at 40,000g, of 4-day-old shoots. Petri dishes were prepared with a 1-mm-thick layer of 1% agar in 0.15 M NaCl, 0.02% (w/v) NaN₃, and 0.01 M K-phosphate (pH 7.7), containing rabbit antidegraded-phytochrome serum (antiserum W, *cf.* ref. 12) at a 200-fold dilution. (Antiundegraded-phytochrome serum was not used for reasons presented elsewhere [13].) Serum was added after cooling the agar medium to 55 C. Each 2-mm-diameter agar well was filled twice with 3- μ l aliquots. The plates were incubated for 3 days at 2 C and then washed for 2 days in 0.17 M NaCl. Precipitin rings were stained with Coomassie blue, visibility of the rings was enhanced by immersion into 95% ethanol for 1 h (18), and their diameters were measured.

RESULTS

Time courses for Pr and Pfr destruction indicate that both begin without delay and both occur at the same rate ($t_{1/2}$ = about 95 min) for the first 50 min after irradiation when using plants grown on open cafeteria trays (Fig. 1). The two slopes during the first 50 min are not statistically different (t_{18} = 0.985; 0.3 < P < 0.4). Destruction of phytochrome as Pr ceases abruptly 50 min after irradiation, resulting in a loss of about 35% of the initial level of photoreversibly detectable phytochrome.

When oats are grown in closed containers, a time delay of about 40 min is observed between actinic irradiation and onset of Pfr destruction. This delay apparently results from ethylene accumulation (17). A comparable delay between a R, FR irradiation sequence and the onset of Pr destruction is also seen using plants grown in the same way (Fig. 2). Linear regression analysis of points beyond 50 min for plants grown in closed containers yields

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³ Abbreviations: R: red; FR: far red.

a line that extrapolates back to 100% photoreversibility at 40 min with $t_{1/2}$ of 170 min.

A radial immunodiffusion standard curve (Fig. 3) was prepared by using dilutions of a crude, phytochrome-containing extract prepared from unirradiated tissue. Crude extracts were also prepared 180 min after irradiation of tissue with 1 min R or with 1 min R followed by 2 min FR light and assayed both spectrophotometrically and immunochemically. Phytochrome photoreversibility detected in R-irradiated shoots after the 180-min incubation was 26% as much as that observed in the dark control when measured *in vivo* and 20% as much when measured *in vitro*. Phytochrome detected antigenically *in vitro* was 32% as much. Comparable values for the R, FR-irradiated shoots were 58, 61, and 58%, respectively.

We were unable to confirm the previously reported (4) inhibition of phytochrome destruction by EDTA (Table I). However, both Pr and Pfr destruction exhibited indistinguishable sensitivity to 2-mercaptoethanol and azide (Table I).

DISCUSSION

Both the occurrence in oats of Pr destruction (1, 2), which occurs only after Pr has been cycled through Pfr, and the time course for Pr destruction (8) are confirmed here (Fig. 1).

The data presented here support the hypothesis that both Pr

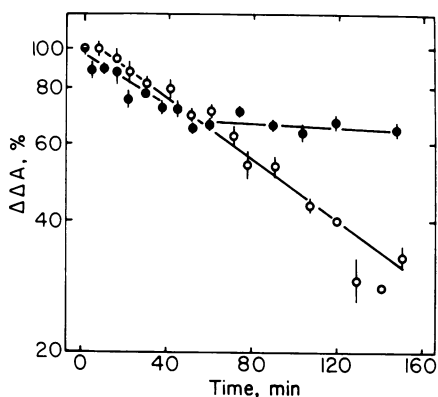


FIG. 1. Phytochrome photoreversibility (100% = about 0.05 A) as a function of time in darkness at 25 C after irradiation of oat seedlings with 4 min R (○) or 4 min R followed by 3 min FR (●) light. Data are averages \pm standard errors of seven (○) and five (●) experiments.

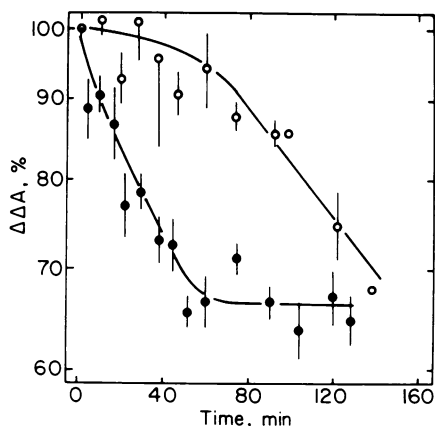


FIG. 2. Phytochrome photoreversibility (100% = about 0.05 A) as a function of time in darkness at 25 C after irradiation of oat seedlings with 1 min R followed by 2 min FR light. Plants were grown either on open cafeteria trays (●) or in buckets tightly covered with aluminum foil (○). Data are averages \pm standard errors of five (●) or three (○) experiments.

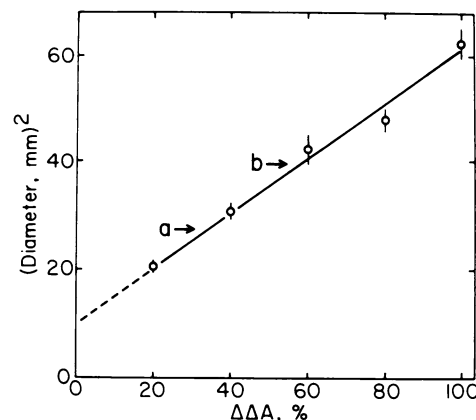


FIG. 3. Radial immunodiffusion assay for phytochrome. The square of the diameter of precipitin rings is given as a function of the amount of phytochrome added. A crude extract of dark control oat shoots was taken as a $\Delta\Delta A$ of 100% ($\Delta\Delta A = 0.0069/\text{cm}$) and diluted appropriately to obtain the other points on the standard curve. Each point is the average \pm standard error of 12 replicates. Unknowns were crude extracts of oat shoots prepared 180 min (at 23–25 C) after irradiation of intact oat seedlings with R (a) or R followed by FR (b) light.

and Pfr destruction result from operation of the same mechanism: (a) both occur at the same rate during the first 50 min after irradiation (Fig. 1); (b) both are equally sensitive to apparent ethylene accumulation resulting from growth of seedlings in closed containers, as demonstrated by the observations that both exhibit an identical time delay under these conditions (Fig. 2 and ref. 17); (c) both result in a loss of antigenically detectable phytochrome that is equivalent to the loss of spectrophotometrically detectable phytochrome (Fig. 3 and ref. 13), indicating that both involve a general degradation of the chromoprotein; and (d) both are equally sensitive to 2-mercaptoethanol and azide (Table I). The experiments testing the effects of various reagents were specifically designed to measure the effects of these reagents on the *rate* of destruction rather than the *extent*. This emphasis on rates was accomplished by measuring destruction after only 60-min incubation following actinic irradiation, at which time Pr destruction (at 23 C) had just reached its maximal extent. Effects of mercaptoethanol and azide seen here are comparable to those reported earlier relative to Pfr destruction (4, 11). It is evident that we have been unable to repeat the previously reported inhibition of destruction by EDTA even though we used up to 20 times more EDTA than that reported by Furuya *et al.* (4) to give a 50% decrease in destruction rate. A possible explanation for this discrepancy arises from the observation that Furuya *et al.* (4) grew their experimental plants in closed containers. As noted elsewhere (17), it is possible that differential handling resulted in a delay prior to the onset of destruction only in those plants treated with EDTA. This delay could appear as an inhibition in destruction rate and is consistent with the observation by Furuya *et al.* that inhibition by EDTA was only transitory.

If, as suggested above, Pr destruction is a consequence of the same mechanism that destroys Pfr, and if destruction occurs in oats by the same mechanism as in other plants, then it is evident that in searching for phytochrome destruction *in vitro* one cannot use specificity for Pfr as a criterion for obtaining destruction as has been done (3, 6).

It seems more reasonable to postulate, based on the data presented here, that phytochrome destruction is specific for the intracellular location of phytochrome or for its association with some other component of the cell rather than for its form. This alternative postulate is consistent with two pieces of correlative evidence, indicating that it deserves further testing. First, following a R, FR irradiation sequence such as that used here, phytochrome

Table I. Sensitivity of Phytochrome Destruction to Various Reagents

Seedlings were vacuum-infiltrated either with buffer alone (-) or with buffer containing the indicated addition (+) and then incubated in darkness for 60 min at 23 ± 2 C after a 1 min red or a 1 min red, 2 min far red irradiation. Values for photoreversibility remaining are averages \pm standard errors of 5 replicates in each of 1 to 3 independent experiments. Photoreversibility measured at $t = 0$, prior to the indicated actinic irradiation, is set at 100% (ca. 0.05 A). Controls were treated as above but not given actinic irradiations. Photoreversibility values for controls had no effect on spectrophotometrically detectable phytochrome levels in the absence of actinic irradiation.

Addition	Red irradiation			Red, far red irradiation		
	Photoreversibility (%)		Inhibition ^a (%)	Photoreversibility (%)		Inhibition (%)
	+	-		+	-	
28 mM S-EtOH ^b (1 ^c)	75 \pm 4	75 \pm 4	NS ^d	81 \pm 2	84 \pm 6	NS
140 mM S-EtOH ^b (2)	85 \pm 2	74 \pm 1	42 \pm 7	89 \pm 3	75 \pm 2	56 \pm 7
0.7 mM EDTA (1)	58 \pm 5	67 \pm 5	NS	80 \pm 4	78 \pm 4	NS
14 mM EDTA (3)	79 \pm 3	80 \pm 1	NS	82 \pm 3	84 \pm 3	NS
1 mM NaN ₃ (3)	67 \pm 2	57 \pm 3	23 \pm 4	75 \pm 2	66 \pm 3	26 \pm 6
5 mM NaN ₃ (2)	88 \pm 4	66 \pm 3	65 \pm 9	93 \pm 3	76 \pm 3	71 \pm 9

^aInhibition = [phytochrome remaining after incubation in the presence of inhibitor (+) - phytochrome remaining after incubation in the absence of inhibitor (-)] / (100 - phytochrome remaining after incubation in the absence of inhibitor).

^bS-EtOH = 2-mercaptoethanol

^cNumber of independent experiments.

^dNo significant inhibition was observed.

in the same oat cultivar used for these experiments becomes associated with particulate, subcellular material (14). This association was observed in all of 100 plants tested and was slowly reversed at 25 C, with a $t_{1/2}$ for reversal of about 25 min. Second, following the same irradiation sequence and using the same oat cultivar, phytochrome that is detected immunocytochemically is observed in a sequestered condition as though it had migrated within the cell to a limited number of discrete areas (9). This sequestered condition slowly reverses over a period of 1 to 2 h, yielding again a diffuse distribution of the chromoprotein. It thus appears that the Pr destruction described here occurs as long as phytochrome is both pelletable and sequestered. Whether there is a general cause-and-effect relationship between Pr destruction and the other two observations remains to be tested rigorously for two reasons. First, sequestering is either difficult to observe or does not occur in the small number of other plants tested (7). Second, it has not been determined whether Pr destruction can be observed as a general phenomenon.

LITERATURE CITED

1. CHORNEY W, SA GORDON 1966 Action spectrum and characteristics of the light activated disappearance of phytochrome in oat seedlings. *Plant Physiol* 41: 891-896
2. DOOSKIN RH, AL MANCINELLI 1968 Phytochrome decay and coleoptile elongation in *Avena* following various light treatments. *Bull Torrey Bot Club* 95: 474-487
3. FOX LR 1977 The loss of phytochrome photoreversibility *in vitro*. II. Properties of killer and its reaction with phytochrome. *Planta* 135: 217-223
4. FURUYA M, WG HOPKINS, WS HILLMAN 1965 Effects of metal-complexing and sulfhydryl compounds on nonphotochemical phytochrome changes *in vivo*. *Arch Biochem Biophys* 112: 180-186
5. KIDD GH, LH PRATT 1973 Phytochrome destruction: an apparent requirement for protein synthesis in the induction of the destruction mechanism. *Plant Physiol* 52: 309-311
6. LISANSKY SG, AW GALSTON 1976 Phytochrome stability *in vitro*. II. A low molecular weight protective factor. *Plant Physiol* 57: 188-191
7. MACKENZIE JM JR, WR BRIGGS, LH PRATT 1978 Intracellular phytochrome distribution as a function of its molecular form and of its destruction. *Am J Bot* 65: 671-676
8. MACKENZIE JM JR, WR BRIGGS, LH PRATT 1978 Phytochrome photoreversibility: empirical test of the hypothesis that it varies as a consequence of compartmentalization. *Planta* 141: 129-134
9. MACKENZIE JM JR, RA COLEMAN, WR BRIGGS, LH PRATT 1975 Reversible redistribution of phytochrome within the cell upon conversion to its physiologically active form. *Proc Nat Acad Sci USA* 72: 799-803
10. MOHR H 1972 Lectures on Photomorphogenesis. Springer-Verlag, New York
11. PIKE CS, WR BRIGGS 1972 The dark reactions of rye phytochrome *in vivo* and *in vitro*. *Plant Physiol* 49: 514-520
12. PRATT LH 1973 Comparative immunochemistry of phytochrome. *Plant Physiol* 51: 203-209
13. PRATT LH, GH KIDD, RA COLEMAN 1974 An immunochemical characterization of the phytochrome destruction reaction. *Biochim Biophys Acta* 365: 93-107
14. PRATT LH, D MARMÉ 1976 Red light-enhanced phytochrome pelletability: a re-examination and further characterization. *Plant Physiol* 58: 686-692
15. SMITH H 1975 Phytochrome and Photomorphogenesis. McGraw-Hill, London
16. STEEL RGD, JH TORRIE 1960 Principles and Procedures of Statistics. McGraw-Hill, New York
17. STONE HJ, LH PRATT 1978 Phytochrome destruction: apparent inhibition by ethylene. *Plant Physiol* 62: 922-923
18. WIEJA JG, CJ SMITH 1976 Enhanced visibility of precipitin disks in radial immunodiffusion measurements. *Anal Biochem* 74: 636-637