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# "Disaggregation" of Phytochrome *in Vitro*—A Consequence of Proteolysis[1]

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GARY GARDNER, CARL S. PIKE,[2] HARBERT V. RICE,[3] AND WINSLOW R. BRIGGS
*The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138*

## ABSTRACT

**The relationship between a large molecular weight (9S) and a small molecular weight (4.5S, 60,000 molecular weight) species of phytochrome was examined to determine if the larger species was an aggregate of the smaller. Alterations of pH, salt concentration, or phytochrome concentration did not cause any observable formation of the large form from the small form. However, in partially purified phytochrome extracts from *Secale cereale* L. and *Avena sativa* L., the large form was converted to the small form over time at 4 C in the dark. This breakdown was inhibitable by the protease inhibitor phenylmethanesulfonyl fluoride. When highly purified large molecular weight rye phytochrome was incubated with a neutral protease isolated from etiolated oat shoots, the large phytochrome was converted to the small form without qualitative visible absorbancy changes. The effect of the oat protease could be mimicked by a wide variety of commercial endopeptidases, including trypsin. Examination of the trypsin-induced breakdown on sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that as the size of the photoreversible unit changes from large to small, the size of its constituent polypeptide chains is reduced from 120,000 to 62,000 molecular weight. These experiments provide evidence that the endogenous breakdown observed in extracts is a result of contaminant protease and, consequently, that the small molecular weight species of phytochrome is an artifact due to proteolysis.**

Since the early work of Siegelman and Firer (41), several laboratories have attempted to characterize purified phytochrome. Mumford and Jenner (29) first reported a very highly purified photoreversible preparation from etiolated oat seedlings. This material had a molecular weight of about 60,000 as determined by gel chromatography. Subsequently, other laboratories have isolated oat phytochrome of this same molecular weight (17, 34, 37, and L. H. Pratt, personal communication). In contrast, Correll *et al.* (13) characterized native phytochrome from etiolated rye shoots in the analytical ultracentrifuge and found two boundaries with sedimentation values of approximately 9S and 14S. After storage or handling, only the 9S material was observed. Other biochemical differences were also reported between their analysis of rye phytochrome and Mumford and Jenner's analysis of oat phytochrome.

Shortly thereafter, Briggs *et al.* (8) found that fresh preparations of partially purified oat phytochrome demonstrated two peaks of photoactivity on gel chromatography. The molecular weights of these components were roughly estimated as 180,000 and 80,000. The larger component disappeared if the preparation was allowed to stand in the cold for a day. Correll and Edwards (11) also found two components after gel chromatography of partially purified oat and rye phytochrome, but they failed to utilize marker proteins to calibrate their column.

The current work was begun in order to expand the observations of Briggs *et al.* (8) with the prejudice that the phytochrome from closely related plants such as oat and rye was probably not very different. We became concerned about the problem of proteolysis as a result of the work of Pringle (35, 36). The following experiments support the view that the phytochrome molecules from oat and rye are similar in terms of molecular weight and that the 60,000 mol wt photoreversible component is produced from the 9S component by endogenous proteolysis. Breakdown similar to that which occurs endogenously can be catalyzed by a protease isolated from etiolated oat shoots as well as by a wide variety of commercial endopeptidases. A brief account of this work appears elsewhere (6).

## MATERIALS AND METHODS

**Phytochrome Purification and Measurement.** Phytochrome was isolated from etiolated grass seedlings by slightly modifying a procedure described in detail elsewhere (37). Shoots of 5-day-old etiolated oat seedlings (*Avena sativa* L. cv. Victory, USDA CI2020, 1965, 1968, and 1969 harvest; or cv. Garry) or 4.5-day-old winter rye seedlings (*Secale cereale* L. cv. Balbo) grown at 25 C were chilled and harvested, and then extracted in a Waring Blendor in 50 mM tris (untitrated) containing 0.7% (v/v) 2-ME.[4] One liter buffer was used for each kilogram fresh weight of tissue. After centrifugation the supernatant was made 10 mM in $CaCl_2$. The calcium pectate precipitate was removed by centrifugation and the supernatant was applied to a brushite (calcium phosphate gel [37, 42]) column which had been equilibrated with 2 column volumes of 10 mM KPB, pH 7.0, and 2 additional volumes of the same buffer containing 0.7% 2-ME. Approximately 1 liter of brushite was used per

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[2] Present address: Department of Biology, Franklin and Marshall College, Lancaster, Pa. 17604.

[3] Present address: New England Aquarium, Boston, Mass. 02110.

[4] Abbreviations: 2-ME: 2-mercaptoethanol; KPB: potassium phosphate buffer; NaPB: sodium phosphate buffer; DEAE: diethylaminoethyl; SDS: sodium dodecyl sulfate; PMSF: phenylmethanesulfonyl fluoride.

kilogram fresh weight of tissue. After the sample was applied, the column was washed with 1 column volume of 10 mM KPB, pH 7.0, containing 0.5% 2-ME, and 1 volume of 15 mM KPB, pH 7.0 (without 2-ME). The 2-ME was absent from all further buffers in the procedure. The phytochrome was step-eluted with 62 mM KPB, pH 7.8, and precipitated by slowly adding solid ammonium sulfate (Schwarz/Mann Ultrapure) until a concentration of 33% saturation (20 g/100 ml) was reached. The precipitate was centrifuged, resuspended in a small amount of buffer, and dialyzed against buffer for 2 hr to remove residual ammonium sulfate. Phytochrome samples at this stage of purity are referred to as "partially purified."

Rye phytochrome of higher purity was obtained by three additional steps of column chromatography. The resuspended ammonium sulfate pellet was dialyzed against 10 mM KPB, pH 7.4, and applied to a DEAE-cellulose column equilibrated in the same buffer. The column was then washed with a volume of equilibration buffer equal to that of the sample volume. The phytochrome was eluted with a convex gradient consisting of 500 ml of 300 mM KCl in 10 mM KPB, pH 7.4, into 250 ml of 10 mM KPB, pH 7.4. The active fractions were concentrated by making them 40% saturated in ammonium sulfate using a saturated stock solution of ammonium sulfate adjusted to pH 7.8. The precipitate was centrifuged, resuspended in a small volume of buffer, and dialyzed against 10 mM KPB, pH 7.5, for 1 to 2 hr.

This sample was then applied to an hydroxylapatite (37, 42) column which had been equilibrated with 10 mM KPB, pH 7.5, until the column effluent reached pH 7.5. The column was then washed with a volume of the equilibration buffer equal to that of the sample and eluted with a linear gradient from 10 mM KPB, pH 7.5, to 200 mM KPB, pH 7.5. The active fractions were concentrated as described for the DEAE-cellulose fractions and dialyzed for 1 to 2 hr against 0.1 M NaPB, pH 7.8. The sample was then layered on a column of Bio-Gel A1.5m (8% agarose, 200–400 mesh) equilibrated in 0.1 M NaPB, pH 7.8, and eluted in the same buffer. The most active fractions, referred to as "agarose-purified" phytochrome, were used in the exogenous enzyme experiments described below. These fractions were generally 70 to 90% pure, as defined below. All manipulations were carried out at 4 C under dim green light.

Phytochrome activity was determined with a Ratiospect Model R-2 dual wavelength difference spectrophotometer (Agricultural Specialties Co., Beltsville, Md.). Activity is expressed as $\Delta(\Delta OD)$ at 665 and 730 nm following successive irradiations with red and far red light (7).

Protein was estimated in fractions by measuring $A_{280}/A_{260}$ and using the table provided by Layne (22) to obtain a corrected $A_{280}$. This value was taken as milligrams per milliliter for a 1 cm path length. The ratio $A_{280}/A_{665}$ was used as an index of purity. This ratio is about 1.3 for pure phytochrome (37). These measurements were made with a Zeiss PMQ II spectrophotometer.

Absorption spectra of phytochrome samples in the visible (500–800 nm) region were obtained with the Biospect Model 61 single beam spectrophotometer (Agricultural Specialties Co.). The samples were always kept at 0 C, and the Ratiospect actinic light source was used to phototransform the phytochrome.

**Enzymes and Reagents.** Trypsin, chymotrypsin, papain, carboxypeptidase A, and leucine aminopeptidase were purchased from Worthington. The trypsin used (code TRTPCK) had been treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone to inhibit contaminant chymotryptic activity (40). The carboxypeptidase A (COADFP) had been treated with di-isopropylfluorophosphate to eliminate trace tryptic and chymotryptic activity. Ficin and subtilisin (Protease, Bacterial Type VII) were purchased from Sigma. The carboxypeptidase and leucine aminopeptidase samples were assayed by standard methods to insure that they had the appropriate exopeptidase activity.

The use of PMSF as a protease inhibitor was suggested to us by Pringle (35, 36). A stock solution of 0.2 M PMSF (Schwarz/Mann) was prepared in 95% (v/v) ethanol and was diluted, while stirring, into the sample buffer to a final concentration of 5 mM.

**Preparation of Oat Protease.** Oat protease was isolated from 5-day etiolated shoots of *Avena sativa* L. cv. Garry. The partial purification procedure was as described elsewhere (6, 31, C. S. Pike and W. R. Briggs, unpublished). Over-all purification of the protease preparation used was estimated at about 200-fold by a modification of the hemoglobin digestion assay of Anson (3, 18, 31, C. S. Pike and W. R. Briggs, unpublished). Routine proteolytic assays were performed using the insoluble, hydrophilic substrate Azocoll (Calbiochem) (27, 31). One Azocoll unit of activity was defined as the quantity of enzyme which, when incubated in a 2-ml reaction volume and 8 mg Azocoll at 37 C for 1 hr, gave an $A_{580\,nm}$ of 0.001.

**Sucrose Gradient Centrifugation.** The size distribution of each phytochrome preparation was determined by ultracentrifugal analysis on sucrose gradients. Solutions of 5% (w/v) and 20% (w/v) sucrose (Mann Ultrapure) were prepared in the sample buffer (either 0.1 M NaPB, pH 7.8, or 62 mM KPB, pH 7.8). Linear gradients of 12 ml were formed from 5 to 20% sucrose in thin walled polypropylene tubes, 14.5 × 96 mm. One milliliter of sample (or marker protein solution) was layered on each gradient, and the gradients were centrifuged in the SB-283 swinging bucket rotor of the International B-60 ultracentrifuge at 40,000 rpm for 20 hr at 4 C. After centrifugation, a hole was punched in the bottom of each tube, and the gradients were assayed on a Gilford Model 2000 spectrophotometer equipped with a flow-through cell. An Isco Model A fraction collector and drop counter were used to collect 24-drop fractions from each gradient (usually 22–24 fractions per gradient). Each fraction was assayed on the Ratiospect to determine phytochrome activity.

An estimate of the sedimentation coefficients of the classes of phytochrome was determined by the method of Martin and Ames (26). Beef liver catalase (Worthington), rabbit muscle aldolase (Worthington), human γ-globulin (Mann), and bovine serum albumin (Mann) were used as marker proteins.

**SDS-Polyacryamide Electrophoresis.** In order to assay the polypeptide products of mild proteolytic treatment, SDS-polyacrylamide electrophoresis (39, 45) was employed. Samples in 0.1 M NaPB, pH 7.8, normally were prepared by addition of SDS and 2-ME to a final concentration of 1% (w/v) each and heating to 100 C for 4 to 5 min (35), followed by incubation at 37 C in stoppered tubes for 4 to 6 hr. Aliquots were then added directly to the gel sample buffer (0.1% [w/v] SDS, 10 mM NaPB, pH 7.2), generally in a 10:1 dilution, giving 5 to 10 μg of protein per gel. Total sample volume was 50 μl. Electrophoresis was performed in 10% gels 0.6 × 10 cm at 6 ma/gel. Running time for 9-cm migration of the bromphenol blue tracking dye was 7 to 8 hr. Staining with Coomassie brilliant blue (R-250, Mann) and destaining were carried out as described by Weber and Osborn (45). A description of the reagents used, and a detailed calibration of the gel system are found elsewhere (37).

## RESULTS

**Attempts to Reaggregate Small Oat Phytochrome.** We initially attempted to make large phytochrome (as described in

ref. 8) from small. We followed the hypothesis that the small 60,000 component was simply a disaggregate of the larger component, and that we could determine the proper conditions for aggregation.

We have observed differences in the phytochrome from various seed lots (as have 12, 43, and 44). In our hands, the amount of phytochrome (on a fresh weight basis) did not vary greatly in different seed lots. However, the proportion of the two size classes did vary.

Our first experiments were performed on "partially purified" oat phytochrome (see "Materials and Methods"). A concentrated sample was divided into two or more portions and each portion was dialyzed against a different buffer. The samples were layered on sucrose gradients containing their respective buffers, centrifuged, and assayed for photoreversibility. In Victory oats harvested in 1965, we could not detect the larger form. To attempt reaggregation we assayed a range of pH values including pH 6.0, 6.8, 7.8, and 9.0. In no case was any large molecular weight phytochrome produced. Salts such as 2.0 M KCl or 0.1 M guanidine HCl also had no effect.

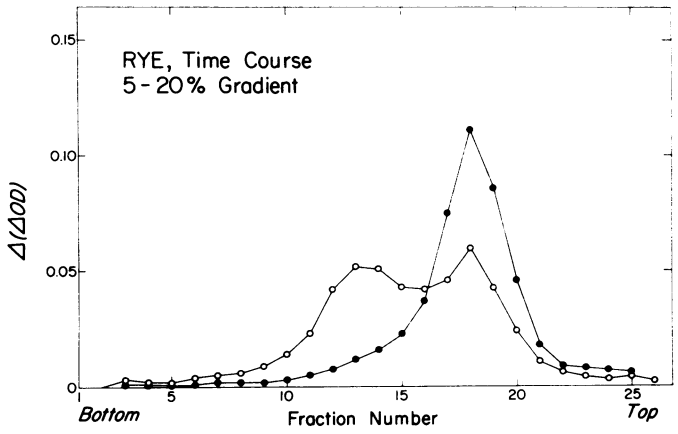


FIG. 1. Endogenous breakdown of partially purified rye phytochrome over time. An aliquot of partially purified rye phytochrome in 62 mM KPB, pH 7.8, was layered on a 5 to 20% linear sucrose gradient and centrifuged as described in "Materials and Methods." Open circles indicate the initial size distribution of the phytochrome. Closed circles indicate the phytochrome size distribution after 100-hr incubation at 4 C. The total photoreversibility of each sample was equivalent.

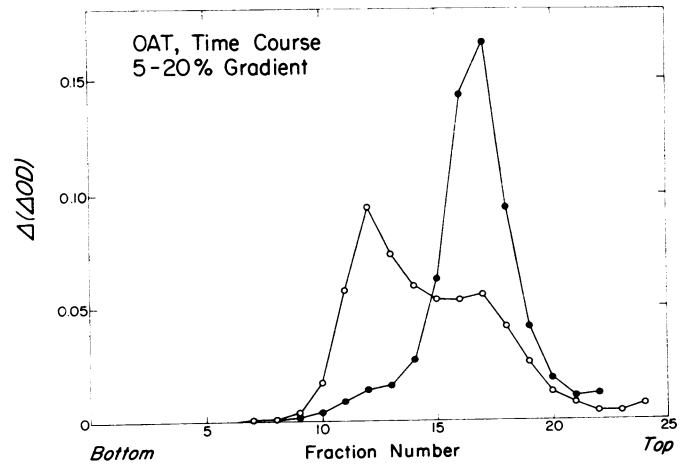


FIG. 2. Endogenous breakdown of partially purified oat phytochrome over time. Open circles: size distribution on a linear sucrose gradient of a partially purified phytochrome sample, in 62 mM KPB, pH 7.8, in an extract of Victory oats; closed circles: size distribution of the same phytochrome extract after 86 hr. The 86-hr sample had 97% of the total photoactivity of the time zero sample.

The 1968 harvest of Victory oats did show a significant proportion of the large molecular weight phytochrome. The salt concentration was varied from zero KCl to 0.5 M KCl (in the presence of 10 mM KPB, pH 7.8), but the proportion of the two sizes remained unchanged. Although salt and pH seemed not to affect the size of the phytochrome, the higher the purity of the preparation, the greater was the proportion of the small as determined on sucrose gradients.

Another possible hypothesis was that these were two distinct molecules, and that we were selectively purifying the smaller. The observation that antibody to small phytochome recognized large phytochrome (37) indicated, however, that the two sizes had in common at least some antigenic determinants.

The 1969 Victory oat harvest contained only a very low amount of the large form. We thus prepared highly purified oat small phytochrome and attempted to achieve reaggregation by varying the phytochrome concentration from 1.8 mg/ml to 15.8 mg/ml. Analytical ultracentrifugation revealed no evidence for the large form.[5]

When Rice (37) examined highly purified oat small phytochrome for amino-terminal amino acids, he found four end groups. If the preparation were truly homogeneous, one would expect only a single end group.

In summary, we were never successful in our attempts to "reaggregate" the small molecular weight oat phytochrome. Rice (37) also found that varying salt concentrations and pH did not appreciably alter the gel filtration behavior of the two species. Because of the apparent low proportion of the large form in our current supply of oats (1969 harvest), and because of the success of Correll *et al.* (12) in isolating large molecular weight phytochome from rye, we then began to study the phytochrome isolated from etiolated Balbo rye seedlings.

**Endogenous Breakdown of Large Molecular Weight Phytochrome.** A partially purified extract of phytochrome was prepared from rye seedlings. A portion of this sample was layered on a sucrose gradient after its activity was determined from visible absorption spectra taken on the Biospect. After centrifugation, fractions were collected and assayed on the Ratiospect for photoactivity. The remainder of the sample was left at 4 C as Pr for 100 hr. Spectra were then taken and this second aliquot was layered on a sucrose gradient and centrifuged. After 100 hr at 4 C photoactivity was not qualitatively changed. The 100 hr sample had 97% of the activity of the initial sample (well within the errors of measurement) and the absorption spectra looked similar (see below for comparable spectra). Figure 1 shows the results of the sucrose gradient assay. The initial sample is indicated by the open circles. The size distribution of the phytochrome clearly falls into two classes of about equal proportion. After 100 hr at 4 C (as indicated by the closed circles) all the phytochrome is in the small molecular weight class. From these data estimates were made of sedimentation coefficients for both size classes. The large molecular weight form is approximately 8S and the small form is 4S.

We then examined the situation in oats. By working very rapidly (the initial sample was placed on the gradient within 15 hr from the time the tissue was ground), we were able to achieve the same results for partially purified Victory oat phytochrome. The open circles of Figure 2 show the initial size distribution. Approximately 60% of the phytochrome ac-

[5] Analytical ultracentrifugal studies on phytochrome will be published elsewhere (G. Gardner and W. R. Briggs). Preliminary values of the sedimentation coefficients obtained are 4.6S for small oat phytochrome and 9.2S for large rye phytochrome.

tivity was in the large form. After 86 hr at 4 C (closed circles) the phytochrome was mostly in the small form. In this case the photoactivity of the two samples was equal. Qualitatively, the same results were obtained with partially purified phytochrome isolated from Garry oats. The breakdown consistently occurred more rapidly in oat extracts than in rye extracts; however, oats and rye both contained these two sizes of phytochrome. Although the proportion of large and small could not be affected by pH, salt concentration, or phytochrome concentration, quantitative conversion of large molecular weight phytochrome to small molecular weight phytochrome did occur over time at 4 C.

**Inhibition of Endogenous Breakdown with a Protease Inhibitor.** The time dependent conversion of large to small suggested the possibility that this degradation occurred as a result of contaminating proteolytic activity. Support for this hypothesis would be obtained if a protease inhibitor also inhibits the conversion of large to small phytochrome. Pringle (personal communication, 35, 36) suggested the use of PMSF, an agent that inhibits some proteases, such as trypsin, known to require serine groups for activity (16); however, this compound is thought to react with a sulfhydryl group in its inhibition of papain (46).

Partially purified phytochrome was extracted from Garry oat seedlings. The ammonium sulfate precipitate was resuspended in a small amount of 62 mM KPB, pH 7.8, and the sample was divided. One portion was dialyzed against 62 mM KPB, pH 7.8, and a second aliquot was dialyzed against the same buffer containing 5 mM PMSF. After 33 hr at 4 C, the samples were layered on separate sucrose gradients and centrifuged. The resultant phytochrome distribution is shown in Figure 3. The open circles show that the untreated phytochrome sample is practically all small molecular weight. Treatment with PMSF (closed circles), however, yielded about one-third of the phytochrome being in the large molecular weight form. No obvious effect of PMSF on phytochrome absorption spectra or photoreversibility was observed. Direct assay of the samples for protease using Azocoll showed significant activity in the untreated control and little activity after PMSF treatment (Table I).

Table I. *Protease Activity in Phytochrome Extracts*

Values for oat extracts are normalized to the phytochrome concentration in the comparable rye extracts. Incubation was carried out for 2.5 hr at 37 C. Absorption at 580 nm of an Azocoll blank under these conditions was 0.030. Values are corrected for absorbancy of phytochrome at 580 nm.

| | Oat Extracts $A_{580\ nm}$ | Rye Extracts $A_{580\ nm}$ |
|---|---|---|
| Control | 0.116 | 0.105 |
| PMSF | 0.057 | 0.080 |

The same type of experiment was performed with partially purified rye phytochrome. In this case the samples were allowed to dialyze for 93 hr at 4 C before centrifugation. Figure 4 shows that the PMSF caused the retention of 50% of the photoactivity in the large molecular weight form, while the control was almost completely converted to the small form. Direct assay for protease activity (Table I) showed the presence of PMSF-inhibitable Azocoll activity in these rye phytochrome samples as well. The amount of control activity seemed less in the rye extract than in the oat.

Thus, PMSF inhibited both protease activity found in extracts of rye and oats and the conversion of large molecular weight phytochrome to the small molecular weight form in these extracts.

**Conversion of Large Molecular Weight Phytochrome to Small by the Addition of Various Proteases.** We wanted also to determine if we could achieve breakdown of large phytochrome by the addition of various proteolytic enzymes. We first used the protease isolated from dark-grown oat shoots (31, C. S. Pike and W. R. Briggs, unpublished). The pH-activity curve of this enzyme is broad in the neutral range, and the enzyme is apparently dependent on the presence of a reduced sulfhydryl group for activity: low concentrations of reductants stimulate it, while inhibition has been obtained with a variety of sulfhydryl antagonists, including mercuric ion and PMSF. Our initial experiments were done on partially purified phytochrome. Because of the probable existence of an

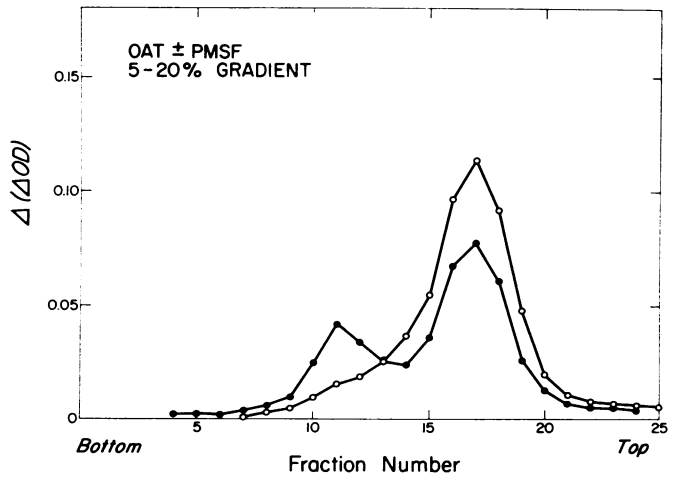


FIG. 3. PMSF inhibition of endogenous phytochrome breakdown in extracts of oats. A partially purified phytochrome extract from Garry oats was divided. One portion was dialyzed against 62 mM KPB, pH 7.8, for 33 hr. A second portion was dialyzed for the same time period against 62 mM KPB, pH 7.8, containing 5 mM PMSF. Open circles: control size distribution; closed circles: size distribution in the PMSF-treated sample. The total photoactivity of each sample was equivalent.

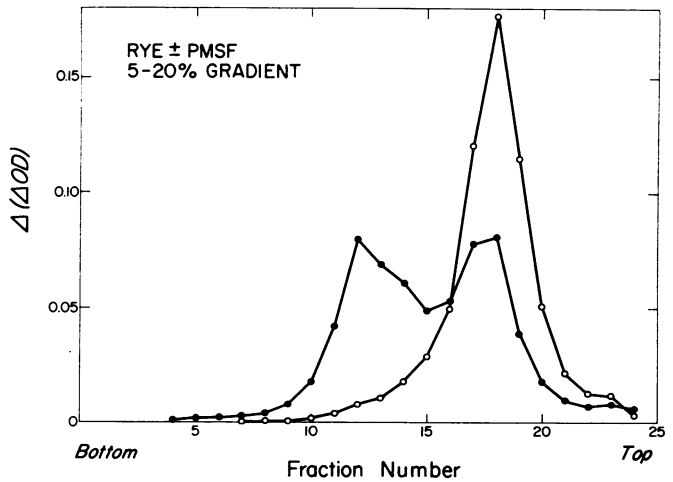


FIG. 4. PMSF inhibition of endogenous phytochrome breakdown in rye extracts. One aliquot of partially purified rye phytochrome was dialyzed against 62 mM KPB, pH 7.8, for 93 hr. A second aliquot of the same sample was dialyzed for the same amount of time against 62 mM KPB containing 5 mM PMSF. Open circles: size distribution in the untreated control; closed circles: size distribution in the PMSF-treated sample. The total photoactivity of the PMSF-treated sample was 102% of the control.

endogenous protease in the partially purified material, we could not exclude the possibility that the added enzyme was acting synergistically to increase the activity of the endogenous contaminant. Therefore, the following experiments were performed utilizing only "agarose-purified" rye phytochrome that did not break down over the time of the experiment.

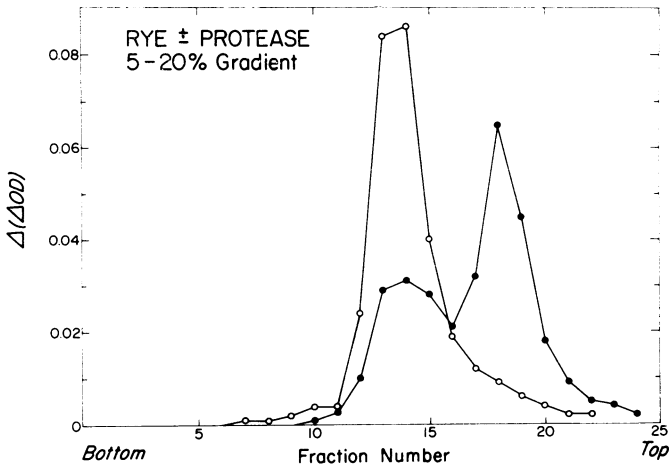


FIG. 5. Effect of an oat protease on phytochrome size distribution. An aliquot of "agarose-purified" rye phytochrome ($A_{280\,nm}/A_{665\,nm}$ = 1.8) was incubated with oat protease for 22.5 hr at 4 C. The phytochrome, in 0.1 M NaPB, pH 7.8, had a final concentration of 0.4 Δ(ΔOD)/ml in the reaction mixture. The oat protease had an initial activity of 690 Azocoll units/2 ml and was diluted 1:5 in the phytochrome. A second aliquot of the same phytochrome sample was incubated with buffer under the same conditions. Open circles: phytochrome distribution in the control; closed circles: effect of protease treatment.

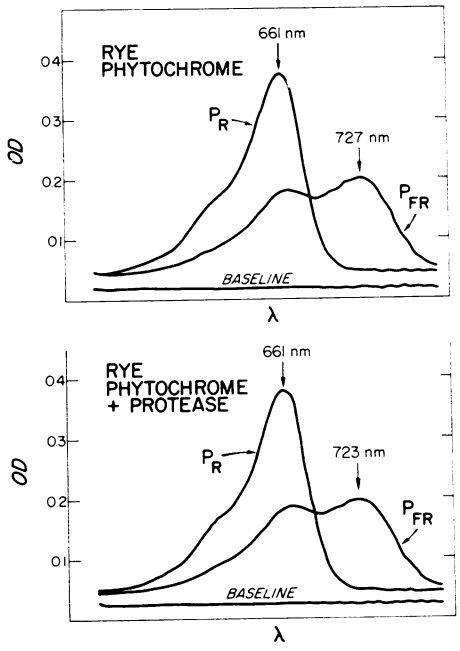


FIG. 6. Effect of an oat protease on visible absorption of phytochrome. Visible absorption spectra were determined for the samples used in Figure 5 after incubation and before centrifugation. Spectra were obtained after 3 min saturating far red light (Pr) and 2 min saturating red light (Pfr). One ml of sample was used for each spectrum, and the baseline was set against 1.0 ml of water. The upper spectra are of the control phytochrome sample. This sample upon subsequent analysis (Fig. 5) was shown to be 88% large mol wt phytochrome. The lower spectra show the protease-treated phytochrome. Sixty-two per cent of this preparation was in the small molecular weight form (Fig. 5). Peak wavelengths are indicated for reference.

A 2.0-ml aliquot of "agarose-purified" rye phytochrome was incubated with 0.5 ml of oat protease for 22.5 hr at 4 C. The phytochrome, which was kept as Pr during the experiment, had an activity of 0.416 Δ(ΔOD)/ml in the final mixture, and the protease had 690 Azocoll units of activity in 2 ml. After the incubation period, phytochrome absorption spectra were determined on the Biospect, and the samples were assayed for size distribution on sucrose gradients. The open circles of Figure 5 show the phytochrome distribution of a control sample which was incubated under the same conditions with buffer instead of protease. At least 90% of the control phytochrome was in the large molecular weight form. The closed circles show the effect of protease treatment. Under these relatively mild conditions, protease treatment converted more than 60% of the phytochrome activity to the small molecular weight form. This material sediments in the same position as the material which is produced as a result of endogenous breakdown. An examination of the absorption spectra of the two preparations (Fig. 6) shows that they are qualitatively similar.

Since this oat protease had much the same properties as the endogenous material that caused breakdown (see "Discussion"), we repeated this incubation experiment using several commercially available proteolytic enzymes to find out whether the size of the cleavage product is determined by the protease or by the phytochrome. In each experiment the phytochrome activity was approximately 0.5 Δ(ΔOD)/ml, and the enzyme concentration in the final mixture was 0.75 μg/ml (about 1 part protease per 1000 parts phytochrome). After 20.5 hr at 4 C with trypsin, a phytochrome sample which had been all large was completely converted to small (Fig. 7). As in the case of oat protease treatment, the visible absorption spectra were not qualitatively changed.

Other endopeptidases which were tested included chymotrypsin, papain, ficin, and subtilisin. In each case a predominantly large molecular weight sample was converted to a pre-

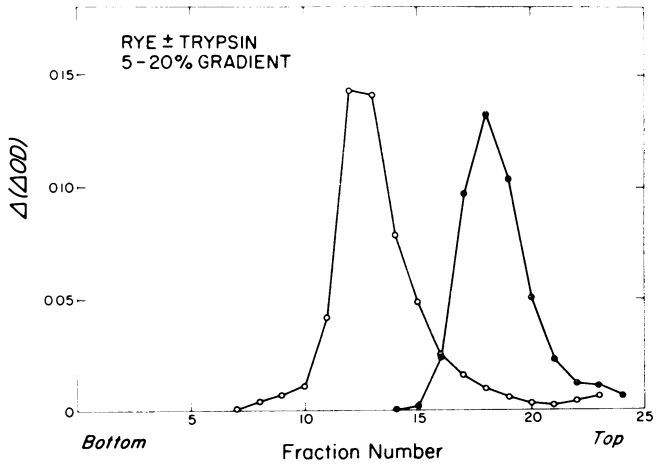


FIG. 7. Effect of trypsin on phytochrome size distribution. An aliquot of "agarose-purified" rye phytochrome ($A_{280\,nm}/A_{665\,nm}$ = 1.6) was incubated with trypsin for 20.5 hr at 4 C. The trypsin concentration in the final reaction mixture was 0.75 μg/ml. A second aliquot of the same sample (in 0.1 M NaPB, pH 7.8) was incubated with buffer under the same conditions. The final phytochrome concentration in each case was about 0.55 Δ(ΔOD)/ml. The open circles show the phytochrome distribution in the untreated control. The closed circles indicate the phytochrome distribution after trypsin treatment. The total photoactivity of the trypsin-treated sample was 92% of the control.

Table II. *Effect of Various Proteases on Phytochrome Size Distribution*

| Treatment | Control | | Experimental | | $\frac{\Delta(OD)_{expt}}{\Delta(\Delta OD)_{control}}$[1] |
|---|---|---|---|---|---|
| | % large | % small | % large | % small | |
| | | | | | % |
| Chymotrypsin | 82 | 18 | 28 | 72 | 95 |
| Papain | 82 | 18 | 0 | 100 | 89 |
| Ficin | 82 | 18 | 20 | 80 | 102 |
| Subtilisin | 88 | 12 | 0 | 100 | 88 |
| Carboxypeptidase A | 82 | 18 | 81 | 19 | 103 |
| Leucine aminopeptidase | 71 | 29 | 68 | 32 | 98 |

[1] Activities are calculated from visible absorption spectra taken immediately before samples were layered on sucrose gradients.

dominantly small sample after 20-hr incubation with the appropriate enzyme at 4 C (Table II). With the exception of the subtilisin product, all the enzyme-produced small forms sedimented to the same location in the gradients. The subtilisin product had an approximate sedimentation coefficient of 3.0S. Ficin treatment did not qualitatively affect the phytochrome visible absorption spectra. Treatment with chymotrypsin, papain, and subtilisin did cause about a 10% reduction in the size of the red Pr peak.

Two exopeptidases were also assayed for their effect on phytochrome size distribution. Both carboxypeptidase A and leucine aminopeptidase had no effect on either size or absorption spectra of phytochrome. In general, then, a wide variety of endopeptidases catalyzed the breakdown of phytochrome at 4 C, although the details of the process may not be the same for each protease tested.

**Electrophoretic Analysis of Phytochrome Breakdown.** The sedimentation data in the above experiments have been expressed solely in terms of photoreversible phytochrome. Nothing has been said to indicate that breakdown of large phytochrome after treatment with proteases is a consequence of the cleavage of covalent bonds. To examine this question, SDS-polyacrylamide electrophoresis was used as an assay of the size of denatured polypeptide chains. A thorough study of the sub-unit structure of phytochrome along with calibration of the gel system appears elsewhere (37).

As the trypsin experiment described in Figure 7 was being carried out, we removed aliquots at the appropriate times for the SDS-10% polyacryamide gel electrophoretic analysis shown in Figure 8. Gel A is the sample before the incubation with trypsin, and gel B is the control (without trypsin) after 20.5 hr at 4 C as Pr. Gel C shows the sample after 20.5-hr treatment with trypsin at 4 C as Pr. The major band in the control sample (estimated 120,000 mol wt) was nearly lost after the trypsin treatment and was replaced by bands estimated at 62,000 and 42,000 mol wt. The minor band in the control (arrow, 54,000 mol wt) seemed relatively stable to trypsin treatment. (This minor band is probably a contaminant; the "agarose-purified" rye phytochrome used in this particular experiment was about 80% pure.) Gel D is a sample taken from fraction 12 of the control gradient shown in Figure 7. This large molecular weight photoreversible phytochrome shows only one protein band, at 120,000 mol wt. Gel E shows fraction 18 from the gradient containing the trypsin-treated small molecular weight sample (Fig. 7). This sample contains both the 62,000 and 42,000 mol wt bands. Gel F shows that the amount of trypsin used in this experiment was too low to be detected in the SDS gel assay.

Rice (37) has shown that purified small molecular weight oat phytochrome demonstrates a SDS-polyacrylamide gel electrophoretic pattern with a major band of 62,000 mol wt. He has also examined the electrophoretic patterns of rye phytochrome produced by chymotrypsin and by the oat protease used above. The oat protease generated a pattern similar to that produced by trypsin—a 62,000 mol wt band produced as the 120,000 band was lost. Chymotrypsin generated a different product (estimated at 90,000 mol wt) without complete loss of the 120,000 unit.

Thus, as the size of the photoreversible unit changes from large to small, the sizes of its constituent polypeptide chains are also greatly reduced.

## DISCUSSION

As is mentioned above, we were alerted to the possibility of proteolytic contamination by Pringle (35, 36). He encountered losses in activity in attempting to purify malate dehydrogenase from yeast. These losses, at all stages of purification, could be attributed to inactivating attack by one or more PMSF-inhibitable yeast proteases. The inhibitor had no effect on malate dehydrogenase activity but seemed rather specific

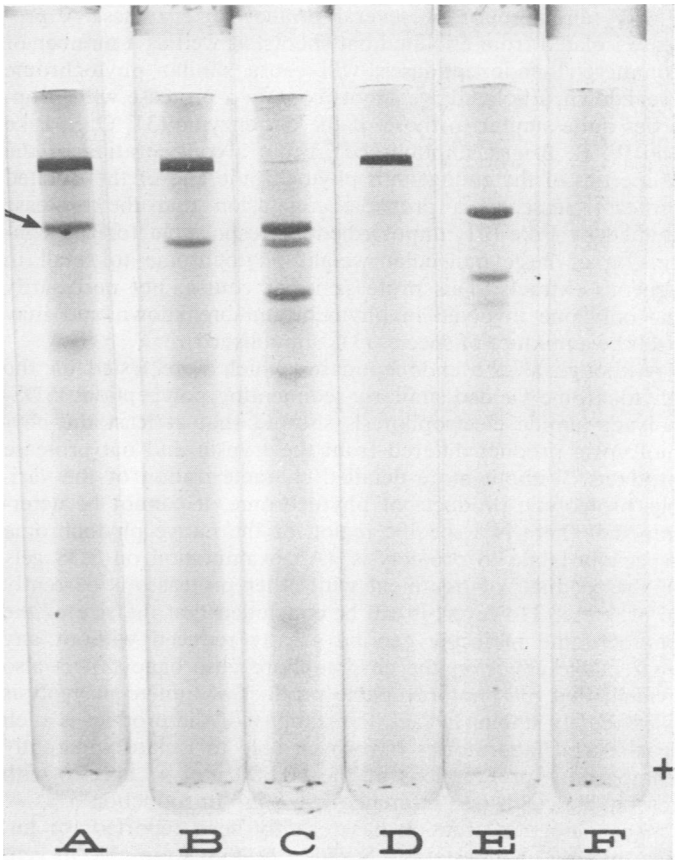


FIG. 8. Electrophoretic analysis of phytochrome breakdown. Stained 0.1% SDS-10% polyacrylamide gel electrophoretic patterns are shown for aliquots taken from the experiment described in Figure 7. Gel A: sample before trypsin treatment; gel B: untreated control after 20.5 hr at 4 C in 0.1 M NaPB, pH 7.8; gel C: after 20.5 hr at 4 C with 0.75 $\mu$g/ml trypsin; gel D: sample taken from fraction 12 of the control gradient (open circles) shown in Figure 7; gel E: fraction 18 from the gradient in Figure 7 with the trypsin-treated sample (closed circles); gel F: sample of trypsin equivalent to that used in sample C. Protein samples in gels A to C were 10 $\mu$g; in D and E, 6 $\mu$g. Direction of migration is toward anode (+).

in its inhibition of the contaminant proteases. Pringle also found (36) that a very small amount of protease would give a detectable effect. Subtilisin at a contaminating level of 0.0002% of the total protein produced major degradation of serum albumin. In order to achieve breakdown of phytochrome, we used protease levels of about 0.1% of the total protein.

More important for the present discussion is Pringle's evidence that modification (with retention of activity) of yeast malate dehydrogenase also occurs as a result of proteolytic attack. Despite the observations that the visible absorption spectra of phytochrome are affected by many factors (10), the fact that the absorption spectra of the large and small forms are very similar does not mean that the protein has not been altered. Considering Pringle's data it is really not surprising that the molecule could be grossly modified without qualitative effects on the absorption spectra. If spectral criteria are to be used as an index of purity of phytochrome (29, 37), they must be coupled with a measure of molecular size of the photoreversible material.

We have shown that conversion of photoreversible phytochrome from a large molecular weight species to a smaller species occurs as a function of time but is independent of several variables which are often factors in aggregating protein systems. This conversion of large to small is inhibited by PMSF, an inhibitor of several proteolytic enzymes. A protease isolated from etiolated oat shoots, as well as a number of commercial endopeptidases, will cause similar phytochrome breakdown. Etiolated rye shoots contain a protease with properties quite similar to those of the oat enzyme (31, C. S. Pike and W. R. Briggs, unpublished). From a consideration of the properties of the endogenous phytochrome and of the isolated protease, it seems a probable conclusion that the protease studied by Pike (31, unpublished) is responsible for the conversion of large molecular weight phytochrome to small in aqueous extracts. This protease is, of course, not necessarily the only one involved in phytochrome breakdown and may itself be a mixture of species (31, unpublished).

Although all the endopeptidases which were tested on the phytochrome yielded similarly-sedimenting polypeptides, SDS-polyacrylamide electrophoresis showed that at least the chymotrypsin product differed from the trypsin and oat protease products. Without more detailed characterization of the various proteolytic products of phytochrome, it cannot be determined if there is a specific region of the native phytochrome especially labile to proteolysis. (An examination on SDS gels of the products of treatment with other proteases is currently in progress.) However, it can be concluded that the size of the phytochrome molecule can be greatly reduced without any appreciable effect on the chromophore absorbancy. It is also evident that the photoreversible product of limited proteolysis is extremely stable. In fact, the stability of the product is such that several laboratories have been able to isolate apparently homogenous preparations of the 60,000 mol wt species with reasonable yields (see references cited in "Introduction").

An analogous situation has recently been reported for human plasma cholinesterase. Saeed *et al.* (38) were able to generate several previously reported cholinesterase isoenzymes by treatment of the major plasma cholinesterase fraction with various proteases.

What steps can be taken to prevent proteolysis in the purification of phytochrome? One approach is to use PMSF in a preparative treatment to inhibit the protease. This would at first seem reasonable because of the lack of effect of PMSF on the phytochrome visible absorption spectra. However, PMSF might react with sites on phytochrome which do not directly interact with the chromophore. Any observations on PMSF-treated phytochrome would therefore be suspect until control experiments had been performed to determine the effect of the inhibitor.

Another approach is to alter the purification procedure to minimize proteolytic activity and to purify the phytochrome away from the protease. Since the protease is acting on the phytochrome at 4 C in partially purified material, it is important that the procedure be as rapid as possible. Thus, instead of using one 6-liter brushite column for 6 kg of tissue, we used five smaller brushite columns in parallel and saved approximately 24 hr. Correll and Edwards (11) noted that the large and small forms were separated by DEAE-cellulose chromatography and salted out at different ammonium sulfate concentrations. By using the lower ammonium sulfate concentration (20 g/100 ml), one selectively salts out the large form and leaves behind the small along with a substantial amount of protease (31, C. S. Pike and W. R. Briggs, unpublished). If only the more tightly bound DEAE peak fractions are pooled, a further reduction in protease activity is probably achieved. In any case, it is important not to allow the extract to stand for prolonged periods of time. Dialyses should be limited to 1 to 2 hr rather than overnight.

Further suggestions for phytochrome purification can be obtained by considering the properties of the protease isolated by Pike (31, unpublished). The pH activity curve for that enzyme (broad, maximum around pH 6.5) is similar to the range used in phytochrome extraction. High salt concentrations were shown to be inhibitory to the protease, and it might be advantageous to run the calcium phosphate columns in the presence of high salt (perhaps 100 mM KCl). Finally, low concentrations of nonionic reductants such as 2-ME and dithiothreitol stimulate the protease. We do not use a reductant subsequent to the brushite 10 mM KPB wash step; however, we have still been using 2-ME up to that point. Perhaps an alternative method of removing phenolic compounds such as the use of polyvinylpyrolidone (25) or anion exchange resins (21), should be sought to avoid protease stimulation.

The data in this paper can be used to interpret the wide variance in the literature of estimates for the molecular weight of photoreversible phytochrome. Clearly the two populations of phytochrome discussed in this paper are present in both rye and oats. The more rapid rate of breakdown in oat extracts is probably caused by a higher level of contaminating protease in oat extracts than in rye (31, unpublished). Thus all studies which have been performed on small oat phytochrome have been characterizing a rather large enzymatically produced chromopeptide.

Although Correll and Edwards (11) did not calibrate their Sephadex G-200 columns, their elution profiles seem similar to those obtained in our laboratory (37). They were probably analyzing the same two components discussed in the present work. The large molecular weight phytochrome observed here probably corresponds to the 9S component found by Correll *et al.* (11). The question of the 14S component observed by these workers is not examined in this paper, although ultracentrifugal studies currently in progress have detected a higher molecular weight form in very pure rye extracts. Until these analytical studies are completed, it would be premature to speculate on the relationship between the 9S form and the 120,000 mol wt subunit (G. Gardner, work in progress).

Recent molecular weight estimates by Walker and Bailey (43, 44) are difficult to interpret because, although published in two adjacent papers, they are inconsistent with each other. It is possible that continued proteolysis of the 60,000 mol wt form could result in smaller photoreversible fragments such as they report. Many of their preparations did show altered absorption spectra. Their procedure probably took considerably longer than ours, and thus opportunities for proteolysis were probably

more extensive. In their zeal to maintain the phytochrome as Pr, they ran their DEAE-cellulose chromatography under constant far red irradiation. This treatment would serve to cycle the pigment (9) and would create a constantly changing population of Pfr. Since Pfr is more susceptible to protein denaturation than Pr (10), and a denatured protein is often a better substrate for proteolysis than a native protein (36), any denaturing effect of the DEAE-column as a result of far red irradiation would result in increased proteolysis.

A primary conclusion from this work is that any characterization of 60,000 mol wt phytochrome must be repeated on the native molecule. Studies presented elsewhere (31, C. S. Pike and W. R. Briggs, unpublished) indicated dark reversion properties for the native molecule which are quite different from those of small oat phytochrome as reported by Mumford and colleagues (28–30). Optical rotatory dispersion and circular dichroism studies (2, 17, 19, 20) are currently being reexamined in our laboratory (E. M. Tobin, personal communication). Studies bearing on a possible light-induced protein conformational change (8, 17, 34), intermediates in phototransformation (4, 5, 14, 15, 23, 24, 32—especially those which assume homogeneity in the phytochrome), and thermodynamics of phototransformation (33) all require careful reexamination.

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