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Purification of Oat and Rye Phytochrome[1]

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ABSTRACT

A purification procedure employing normal chromatographic techniques is outlined for isolating phytochrome from etiolated oat (*Avena sativa* L.) seedlings. Yields in excess of 20% (25 milligrams or more) of phytochrome in crude extract were obtained from 10- to 15-kilograms lots. The purified oat phytochrome had an absorbance ratio ($A_{280\ nm}/A_{665\ nm}$) of 0.78 to 0.85, comparable to reported values, and gave a single major band with an estimated molecular weight of 62,000 on electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. A modification of the oat isolation procedure was used to isolate phytochrome from etiolated rye *Secale cereale* cv. Balbo) seedlings. During isolation rye phytochrome exhibited chromatographic profiles differing from oat phytochrome on diethylaminoethyl cellulose and on molecular sieve gels. It eluted at a higher salt concentration on diethylaminoethyl cellulose and nearer the void volume on molecular sieve gels. Yields of 5 to 10% (7.5–10 milligrams) of phytochrome in crude extract were obtained from 10- to 12-kilogram seedling lots. The purified rye phytochrome had an absorbance ratio of 1.25 to 1.37, significantly lower than values in the literature and gave a single major band with an estimated molecular weight of 120,000 on electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. It is suggested that the absorbance ratio and electrophoretic behavior of rye phytochrome are indices of purified native phytochrome, and that oat phytochrome as it has been described is an artifact which arises as a result of endogenous proteolysis during isolation. A rationale is provided for further modifications of the purification procedure to alleviate presumed protease contaminants.

Since the initial isolation of phytochrome (4), several problems have persisted in its purification and characterization. First, a number of partial purifications have been reported from a variety of plant sources (2, 15, 29, 35–39), but highly purified phytochrome has been obtained only from etiolated oat (13, 20) and rye (7) seedlings. Second, despite the use of similar purification procedures, there are marked differences between the product isolated from oats (13, 20) and rye (7). Finally, there appears to be some variability in the characteristics of the rye product despite an apparently uniform procedure (7). Thus, considerable doubt exists as to what criteria should characterize a purified preparation of phytochrome, and the source of variability in the protein's *in vitro* behavior has remained obscure.

Most attempts at phytochrome purification have been based on modifications of procedures established by Siegelman and Firer (33). They achieved a partial (60-fold) purification of phytochrome from etiolated oats, utilizing ion exchange and gel filtration chromatography, as well as ammonium sulfate fractionation. Mumford and Jenner (20) were the first to report a highly purified (750-fold) oat preparation. They modified Siegelman and Firer's procedure by adding a continuous flow electrophoretic fractionation and reported a product which had an absorbance ratio (A_{280}/A_{660}) of 1.07 and a molecular weight of 55,000 to 62,000 on gel filtration. Hopkins and Butler (14) subsequently obtained similar preparations with an absorbance ratio between 0.78 and 0.92, with the same molecular weight (13). Utilizing modifications of Siegelman and Firer's procedure and working under green safelights to maintain phytochrome as Pr, the more stable form (2, 5), they reported a final yield of 1 mg/kg of etiolated oat tissue (10% yield), which is higher than the 3% yield reported by Mumford and Jenner.

In contrast, Correll *et al.* (7) reported yields of up to 25% and a 1200-fold purification in isolating phytochrome from etiolated rye. Their preparations were achieved also through modification of the Siegelman and Firer procedures, mainly by an early, improved ammonium sulfate fractionation. The reported product, however, had an absorbance ratio between 1.75 and 3.5. Moreover, it had a molecular weight of 180,000 on equilibrium sedimentation and a value of 9S on velocity sedimentation with some indications of polydispersity (8).

Despite the apparent differences in purified oat and rye phytochrome, there is evidence that the initial chromatographic behavior of the two proteins is quite similar. Briggs *et al.* (2) examined fresh extracts of oat phytochrome by gel filtration and reported two distinct chromatographic species with molecular weights roughly estimated as 80,000 and 180,000. Recently, Correll and Edwards (6) confirmed the presence of two gel filtration species in partially purified oat phytochrome and showed two similar species in rye extracts, although they made no estimate of molecular weight. Finally, Briggs *et al.* (1) and Gardner *et al.* (10) have shown that the larger molecular weight species can be enzymatically converted to the smaller using a variety of endoproteases including one from etiolated oat seedlings (22).

The present work was undertaken for two purposes. The first aim was to study in parallel the behavior of oat and rye phytochrome during purification, develop techniques to obtain purified phytochrome from both plants, and establish more definitive criteria for purified phytochrome. The second aim was to complement evidence presented elsewhere (1, 10) that a source of variability in molecular weight in phytochrome isolated by different laboratories using different techniques is endogenous proteolysis during isolation.

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MATERIALS AND METHODS

Plant Materials. Oats (*Avena sativa* L., USDA C12020, 1965, 1968, and 1969 harvests) were planted on one layer of moist cellulose packing material (Kimpak 6223, Kimberly-Clark) in plastic refrigerator boxes (7½ × 11 inches), using 30 g of seeds and 150 ml of tap water per box. About 10 kg of seeds were sown in a single planting. When necessary, additional seeds were planted on Kimpak in sixteen 18- × 26-inch aluminum baking trays, using 200 g of seeds and 900 ml of tap water per tray. The trays were placed in a modified baking cabinet (33). Both boxes and cabinet were placed in a 25 C constant temperature, 80% constant humidity room and allowed to grow in total darkness for 4.5 days. Prior to harvest the plant material was transferred to a 4 C cold room and allowed to chill overnight in total darkness.

Rye (*Secale cereale* L. cv Balbo), Robson Quality Seeds, Inc., purchased in 1970, was handled in a similar manner. About 20 kg of seeds were sown in a single planting, using 75 g of seeds and 200 ml of tap water per box or 250 g of seeds and 1200 ml of tap water per tray. The plants were grown 4 days prior to chilling for harvest.

Harvest. Under the growth conditions employed, the plant roots form a solid mat within the Kimpak. The root mats were pressed onto beaverboard containing a series of 1-inch wood screws, the board was held vertically, and the plants were cut with either an electric hedge trimmer or electric carving knife. A single planting of oats yielded 6 to 8 kg, and rye yielded 10 to 12 kg of coleoptile and leaf material. The tissue was weighed into 500-g lots, wrapped in foil, and placed on ice in a cold room until used. Rye was extracted the subsequent day. A second planting and harvest was carried out for oats. The first harvest tissue was kept close to 0 C by daily changes of ice (6–8 days). No loss of activity was detectable during storage. Harvesting, extraction, and all subsequent manipulations were carried out under dim green safelights at 4 C.

Buffers. All tris buffers were adjusted to the proper pH with HCl. All phosphate buffers were made either from potassium salts or sodium salts in the proper proportion to give the desired pH (11). Final pH adjustment was made at 4 C with a Corning (Model 12) pH meter equipped with a temperature compensator. All stock solutions were 1 M with the exception of EDTA which was 0.1 M (as the disodium salt). 2-Me[3] was purchased from Eastman Distillation Products. Ammonium sulfate was purchased from Mann (ultrapure, enzyme grade). A saturated stock solution was made by dissolving 708 g/l and titrating to pH 7.8 at 4 C with solid tris (7) or ammonium hydroxide.

Extraction. Batches of 1 kg of oat tissue were ground briefly (30–45 sec) in a Waring Blendor with 1 liter of 0.05 M tris, pH 8.5, containing 0.1 M 2-Me. As necessary, 1 N NaOH was added to keep the pH above 7.2 during grinding. The resulting brei was filtered through two layers of cheesecloth, using a hand wine press for maximal extraction of liquid. The crude extract was centrifuged at 16,300*g* for 30 min to remove cell debris. The supernatant was brought to 10 mM $CaCl_2$ with a 1 M stock solution, stirred for 20 to 30 min, readjusted to pH 7.2 with 1 M tris, and recentrifuged to remove the precipitated pectic compounds. The final supernatant was brought to 1 mM EDTA by addition from an 0.1 M stock solution. The final pH was 7.2 ± 0.2.

Rye extraction was performed as with oats with the following buffer modifications: 0.05 M tris near pH 10.0 (untitrated) was used, and the addition of NaOH was deleted; 5% (v/v) glycerol was added. All subsequent buffers were 5% (v/v) in glycerol, and 2-Me was omitted following brushite fractionation.

Column Chromatography. Brushite chromatography for oats and rye was carried out by a modification of the procedure of Miller *et al.* (18) and is similar to that adopted by Hopkins and Butler (14). Brushite ($CaHPO_4/2H_2O$) was precipitated at room temperature in 4-mole batches by a modification of the Siegelman *et al.* procedure (34). The reaction was carried out by transferring 4 liters of 1 M $CaCl_2$ and 4 liters of 1 M K_2HPO_4 to a 16-liter polyethylene drum at a flow rate of 3 ml/min (by means of a peristaltic pump [Buchler]). The mixture was stirred throughout the precipitation, then allowed to sit in the mother liquor for 7 days. The brushite was decanted 7 times with distilled water, then stirred overnight in a 12-gallon polyethylene drum in a 40-liter solution of 10 mM K_2HPO_4. The solution was decanted, and the gel was washed in 10 mM KPB, pH 7.8, and stored at 2 C in an 8-liter polyethylene bottle until used. The pH of the stored brushite remained 6.6 to 6.8.

Two Plexiglas columns (12 × 60 cm) were prepared giving 6 liters of packed bed volume of gel per column. Prior to use, each column was equilibrated with 10 mM KPB, pH 7.8, containing 0.1 M 2-Me until the column eluate reached pH 7.2 (generally two column volumes). The crude extract (15–25 liters) was passed in parallel through the two brushite columns (approximately 1 liter gel per kg tissue). Each column was subsequently washed with about 6 liters of 10 mM KPB, pH 7.8, made 0.1 M in 2-Me, followed by about 6 liters of 15 mM KPB, pH 7.8, made 14 mM in 2-Me. Phytochrome was step eluted from the column with 0.62 M KPB, pH 7.8, made 14 mM in 2-Me. The brushite columns were not reused. Active fractions were pooled and then precipitated with solid ammonium sulfate. Following collection by centrifugation at 16,300*g*, the precipitate was solubilized in the equilibration buffer of the next column. Where dialyses were performed, the dialysis tubing was prepared by treating briefly with boiling 10 mM EDTA and storage in 1 mM EDTA at 2 C until use.

DEAE-chromatography for oats was carried out with two 2.5 × 45 cm columns run in parallel. Cellex D (0.7 meq/g) was purchased from Calbiochem. The exchanger was acid-base cycled according to the manufacturer's instructions, titrated with 1 N HCl to pH 7.4 in 10 mM tris, 10 mM KCl (designated tris-KCl) and stored at 2 C until used. Two 2.5 × 45 cm columns were poured to 220 ml of packed bed volume of exchanger per column. Prior to use each column was equilibrated with tris-KCl until the eluate stabilized at pH 7.4 (generally two column volumes). Columns were not reused; the exchanger was recycled and repoured prior to each use.

Following addition of the sample (40–80 ml per column), each column was washed with a volume of equilibration buffer equal to the sample volume. The flow rate was established at 3 to 4 ml/min and elution was achieved with a convex gradient of 250 ml of 10 mM tris-KCl with 500 ml of 10 mM tris 0.3 M KCl, pH 7.4, as the limit buffer. All buffers were made 14 mM in 2-Me. Analysis of the salt gradient was done with a cation selective electrode (Beckman, Model 39137) attached to a Corning (Model 12) pH meter. Active fractions were pooled (100–200 ml) and concentrated by precipitation with an equal volume of saturated ammonium sulfate. The solution was centrifuged at 16,300*g*, and the pellet was solubilized in the next column buffer.

Chromatography for rye was carried out with an altered buffer system. A solution of 10 mM KPB, pH 7.8, containing 0.1 mM EDTA was substituted for tris buffer, pH 7.4. Elution was carried out with the same convex KCL gradient.

CM-Sephadex chromatography for oat purification was

[3] Abbreviations: KPB: potassium phosphate buffer; NaPB: sodium phosphate buffer; DEAE: diethylaminoethyl; CM: carboxymethyl; HA: hydroxylapatite; R: red light; FR: far red light; SDS: sodium dodecyl sulfate; 2-Me: 2-mercaptoethanol; PMSF: phenylmethyl sulfonylfluoride.

carried out as described by Hopkins (13). CM-Sephadex (C-50, 4.5 ± 0.5 meq/g, 40–120 μ) was purchased from Pharmacia Fine Chemicals. The exchanger was acid-base cycled according to the manufacturer's instructions and titrated with 1 N KOH to pH 6.0 in the presence of 0.07 M KPB pH 6.0. The exchanger was then transferred to the same buffer and stored at 2 C until used. A column 2 × 20 cm with a 60-ml bed volume was poured and equilibrated with the same buffer until the eluate stabilized at pH 6.0 (generally five to six column volumes).

Following ammonium sulfate precipitation, the sample was redissolved in 0.07 M KPB, pH 6.8, and dialysed against this buffer overnight. It was then dialysed for 4 hr against the pH 6.0 equilibration buffer (longer dialysis caused substantial precipitation of the pigment). The sample was then applied to the column, washed with an equivalent amount of equilibration buffer, and the bound protein eluted with 0.07 M KPB, pH 7.8, 14 mM in 2-Me. The flow rate was about 1 ml/min. Active fractions were pooled (about 25 ml), and the sample was concentrated by ammonium sulfate precipitation as before. The precipitate was solubilized in 0.1 M NaPB, pH 7.8.

HA chromatography was substituted for CM-Sephadex for rye purification. Hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) was prepared according to the procedure of Siegelman *et al.* (34). One mole of freshly prepared brushite was transferred to a glass jar, and an equivalent volume of distilled water was added. The solution was stirred, and 1 mole of KOH was added slowly as a 45% (w/v) solution. This solution was stirred an additional hr then stoppered and allowed to stand at room temperature for 3 days. It was then decanted and washed five times with distilled water to remove the fine crystals formed and stored at 2 C until used. The pH of the washed slurry was 8.1 and remained stable. Prior to use the slurry was washed two times with 10 mM KPB, pH 7.0, and a column (2 × 40 cm) with a packed bed volume of 120 ml was poured. Equilibration was continued with the same buffer until the eluate approached pH 7.0. (Generally, columns were run when pH 7.2 was reached.)

Following addition of the sample (30 ml), the column was washed with about 100 ml of sample buffer (10 mM KPB, pH 7.0). Elution was carried out with a linear buffer gradient, 250 ml of 10 mM KPB, pH 7.0, and 250 ml of 0.25 M KPB as the limit buffer (28). Flow rate was 15 ml/hr. Active fractions were pooled and solubilized in 0.1 M NaPB, pH 7.8, 0.1 mM EDTA.

P-150 chromatography for oats was carried out as described by Mumford and Jenner (20) with the substitution of NaPB for KPB to allow SDS polyacrylamide electrophoretic analysis of the column fractions. Bio-Gel P-150 (100–200 mesh, Cal Biochem), was swollen in 0.1 M NaPB, pH 7.8, and stored 3 to 4 days at 2 C prior to use. A column (2 × 90 cm) with a bed volume of 280 ml was poured and equilibrated with the same buffer until used (generally 5 to 7 days). When rechromatography was required, a second column 1.2 × 100 cm with a packed bed volume of 110 ml was employed. The equilibration routine was the same. Both columns used a gravity flow system with a 25-cm hydrostatic head from a 2-liter reservoir equipped as a Marriot flask. Sample volumes were 1 to 5 ml in 0.1 M NaPB and column flow rates were maintained at 2 to 3 ml/hr. Purified fractions were either analyzed immediately or stored at −20 C wrapped in foil. The protein was stored as Pr.

For rye purification, Bio-Gel A 1.5 M (8% agarose, 100–200 mesh) filtration was substituted for Bio-Gel P-150. A column (2.5 × 90 cm) with a 440-ml bed volume was poured and equilibrated with 0.1 M NaPB, pH 7.8. EDTA at 0.1 mM was added to the buffer system. When rechromatography was required, a 1.2 × 100 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, 40–120 μ) was prepared in the same way as for Bio-Gel P-150. In early experiments, Bio-Gel P-300, prepared in the same way, was occasionally used. Sample volumes were 5 to 10 ml and the flow rate was about 15 ml/hr for Bio-Gel chromatography. Sample volumes were 2 to 3 ml, and flow rates were 4 to 6 ml/hr for G-200 chromatography. Void volume determinations were made on some columns. In these cases a sample volume equivalent to the preparative sample volume was made 0.1 mg/ml in blue dextran (Pharmacia Fine Chemicals), and the column was rerun. Blue Dextran was monitored at 600 nm, and fractions were measured volumetrically.

Final column fractions were assayed both for activity and by SDS polyacrylamide electrophoresis. Unused fractions were frozen immediately in liquid nitrogen and stored at −20 C wrapped in foil.

Assay. Phytochrome activity was measured in crude and brushite fractions with a 2-wavelength difference spectophotometer (Ratiospect, Model R2, Agricultural Specialties Co.). A 5-cm path length was used for crude fractions and a 1-cm path length for brushite fractions. The Ratiospect actinic light source was that described by Purves and Briggs (25), using Baird-atomic 665-nm and 730-nm interference filters. Activity is expressed as $\Delta(\Delta A_{665-730})$/ml $[\Delta(\Delta A)]$ using 2-min irradiations with R and FR. In stable preparations, $\Delta(\Delta A) \simeq A_{665}$ following irradiation with FR. Thus, A_{665} may be used as a convenient method for monitoring column fractions provided no other red absorber is present. All columns following brushite fractionation were monitored in this fashion. Following saturating irradiation with FR (2-min Ratiospect actinic source) A_{665} was measured wth a Zeiss PMQ II spectrophotometer (1-cm path length).

Protein was routinely estimated in fractions by measuring a corrected A_{280}/A_{260} and using the table provided by Layne (16) to obtain a corrected A_{280}. This was taken as mg/ml for a 1-cm path length. In some purified samples, protein was measured by the Lowry (16) procedure using bovine serum albumin (Mann) as a standard. Specific activity is expressed as $\Delta(\Delta A)/A_{280}$ corrected for a 1-cm path length. The ratio A_{280}/A_{665} is used as an index for purified preparations.

Absorption spectra were determined in a Cary 14R spectrophotometer at 25 C with 0.1 M NaPB, pH 7.8, as the standard buffer. Some spectra were also recorded at 4 C using a chilled sample block (Cary Instrument Co.) connected to a constant temperature bath (Neslab Instrument Co.). The Ratiospect actinic light source was used with the samples being transferred to the sample compartment following irradiation. Five-minute irradiations were given for both R and FR. In some instances, a portable 500-w incandescent slide projector (Airequipt Co.) was used. FR was obtained with Corning glass filter 7-69 and measured 289 ergs/cm^2·sec at 10 cm with Corning filter 7-56 used to correct for infrared. R was obtained with the same Baird-Atomic filter (665 nm) used in the Ratiospect source and measured 770 ergs/cm^2·sec at 10 cm. Five-minute irradiations were given for both R and FR.

SDS polyacrylamide electrophoresis (31) was utilized as a routine assay for purified fractions and as a test for protein stability as described by Pringle (23, 24). Acrylamide (for electrophoresis), methylenebisacrylamide and N,N,N′,N′,-tetramethylethylenediamine were purchased from Eastman Distillation Products. Bromphenol blue and Coomasie brilliant blue (R-250) were purchased from Mann, as were guanidine-HCl (ultrapure), urea (ultrapure), and iodoacetamide. Ammonium persulfate and sodium lauryl sulfate were purchased from Fisher. The SDS was recrystallized from ethanol before use. Buffer salts, glacial acetic acid, and methanol were reagent

grade. Samples were prepared, using the modifications of Weber and Osborn (40), with the exception that they were initially brought to 100 C for 4 min prior to 37 C incubation. Heat treatment is a method suggested by Pringle (23, 24) to inhibit proteolysis. Some samples were denatured in 6 M guanidine-HCl buffered at pH 8.5 with 0.1 M tris and 0.14 M in 2-Me. The sample was brought to 100 C for 4 min, incubated overnight in the dark at room temperature, then alkylated with 0.3 M iodoacetamide according to the procedure of Sela *et al.* (30). Following alkylation, reagents were removed by overnight dialysis against 8 M urea. The sample was then dialyzed into 1% SDS (6–8 hr) and diluted into the Weber and Osborn (40) sample buffer (0.1% SDS, 10 mM NaPB, pH 7.2). Electrophoresis was performed in 10% gels 0.6 × 10 cm at 6 ma/gel. Running time for 9-cm migration of the tracking dye was 7 to 8 hr.

RESULTS

Oat Purification. A rapid (5–7 days) and reproducible procedure for the extraction and purification of phytochrome was achieved with Victory oat seedlings. A purification of 16 kg of tissue is summarized in Table I. The over-all purification is 2890 times, each step providing a yield of at least 70%; the over-all yield was 32%. The latter is somewhat better than normal, but yields in excess of 20% have been achieved consistently. For purposes of comparison, the final sample pool had an A_{280}/A_{665} value of 0.83. Samples generally have ranged from 0.78 to 0.85.

In the purification procedure, ease of handling large volumes (up to 24 liters) of crude extract is facilitated by applying the extract directly to two brushite columns. Normally the tissue is only blended at sufficient time intervals (4 kg every 2 hr) to maintain a constant column flow rate of approximately 1 l/hr. Phytochrome is fully bound on the brushite column at 10 mM KPB. Following washes with 10 mM and 15 mM KPB, a step elution at 62 mM KPB forms a blue to blue-green band of phytochrome, which can be seen even under green safelight conditions. Some yellow colored material remains bound to the column following elution, occupying about 50% of the total column volume.

After solid ammonium sulfate fractionation, 240 g/l (0–40%) and overnight dialysis, the sample solution is a clear blue. Absorption spectra show only phytochrome spectra above 500 nm, indicating the absence of any major red-absorbing contaminant. DEAE-fractionation can be followed visually, as can all subsequent column fractionations. An elution profile of one of the two DEAE-columns is shown in Figure 1. The bulk of the phytochrome is eluted before the main protein peak at less than 0.2 M KCl. There is some indication of a second component eluting at a salt concentration higher than 0.2 M KCl.

Following concentration (by ammonium sulfate precipitation and resuspension) dialysis at 0.07 M KPB, pH 6.0, a protein precipitate is normally formed, and centrifugation is required to clarify the solution. A small amount of the phytochrome activity remains in the pelletable fraction and is not readily solubilized even in alkaline buffers. Fractionation on CM-

Table I. *Yield and Purification of Phytochrome Isolated From 16.6 Kg of Oat Tissue (9/69)*

Fraction	Volume	Pooled Activity[1]	Activity[1]	Protein[2]	Specific Activity[2]	Yield	Purification	A_{280}/A_{665}
	ml		*per ml*			%	*fold*	
Crude	24,000	199.5	0.007	20.40	0.000344	100		
Brushite	1,500	165.0	0.117	3.19	0.0368	82.5	107	
DEAE[3]	180	84.5	0.47	2.21	0.212	43	615	
CM	25	71.3	2.85	5.35	0.534	35	1,580	1.68
P-150	10	64.8	6.48	6.15	1.050	32	2,890	0.835

[1] Expressed as $\Delta(\Delta A)$ units and assuming $\Delta(\Delta A) \simeq A_{665}$ for a 1-cm path length after FR irradiation.

[2] Protein expressed as A_{280} corrected for A_{280}/A_{260}; specific activity expressed as $\Delta(\Delta A)$/corrected A_{280} for 1-cm path length.

[3] Includes 0 to 40% ammonium sulfate fractionation of brushite pool.

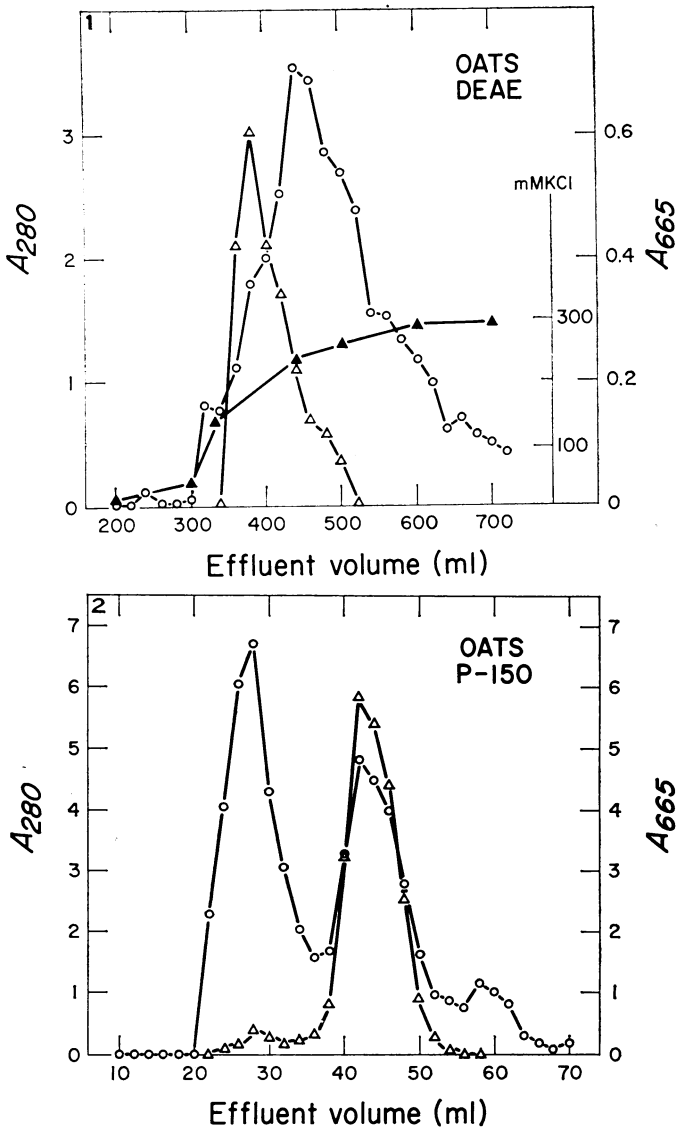

FIG. 1 (upper). DEAE-cellulose (2.5 × 40 cm) elution pattern for oat phytochrome. Sample volume was 60 ml, containing phytochrome of specific activity 0.056 and 1.15 g protein. The column was initially washed with 60 ml of 10 mM tris-KCl, pH 7.4. Elution was carried out with a convex gradient of KCl from 10 mM to 30 mM KCl with a starting buffer of 250 ml of 10 mM tris-KCl and 500 ml of 30 mM KCl in 10 mM tris, pH 7.4. All buffers were 14 mM in 2-Me. The flow rate was 3.3 ml/min, and 10-ml fractions were collected. ○: Absorbance at 280 nm; △: absorbance at 665 nm of Pr following 2-min irradiation with FR; ▲: KCl gradient.

FIG. 2 (lower). Bio-Gel P-150 column (2 × 90 cm) elution pattern for oat phytochrome. The sample volume was 8 ml, containing phytochrome of specific activity 0.530 and 130 mg of protein. The column was eluted with 0.1 M NaPB, pH 7.8. The flow rate was 2 ml/hr and 2-ml fractions were collected. ○: Absorbance at 280 nm; △: absorbance at 665 nm of Pr following 2-min irradiation with FR.

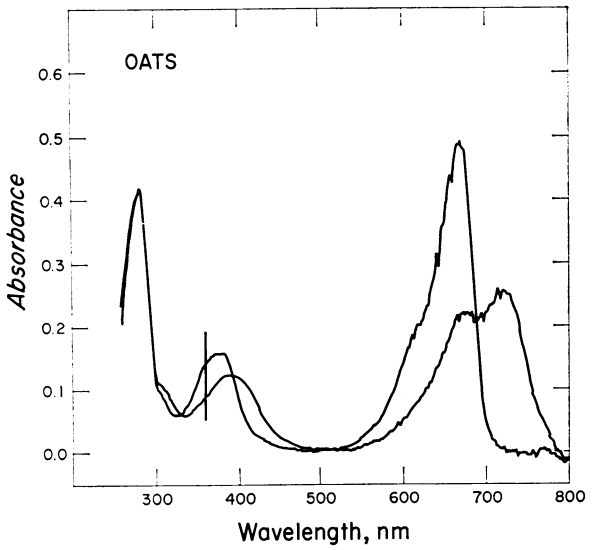

FIG. 3. Absorption spectra of purified oat phytochrome, specific activity 1.050 and A_{280}/A_{665} 0.83, in 0.1 M NaPB, pH 7.8, 25 C after 5-min R irradiation and 5-min FR irradiation.

Sephadex is accomplished by binding at pH 6.0 and step-eluting at pH 7.8. Recovery of the active fractions usually exceeds 80%.

Final chromatography of the concentrated, dialyzed sample on Bio-Gel P-150 is shown in Figure 2. The majority of the phytochrome is retarded relative to the main protein peak (a void volume fraction); a minor peak of activity is sometimes present near the main protein peak. Both peaks showed normal photoactivity. Pooling of fractions with A_{280}/A_{665} of 1.0 or less from the major peak gives a sample with a ratio of 0.83 and a specific activity of 1.090.

Rechromatography of the sample (Fig. 2) gave no appreciable gain in the A_{280}/A_{665} ratio. The most active fraction had a value of 0.75 and a specific activity of 1.190. For most purification purposes, a single gel filtration was usually sufficient.

Absorption spectra for a purified oat sample of specific activity 1.050 and A_{280}/A_{665} 0.83 are shown in Figure 3. Equal gain and loss of absorbance are evident following R and FR irradiation at 665 and 730 nm. Pr λ_{max} is 665 to 667 nm; Pfr λ_{max} is 725 nm. Lowry determination of this sample shows 0.05 mg/ml with bovine serum albumin as a standard. $A_{280}^{0.1\%}$ is calculated as 0.87 and $A_{665}^{0.1\%}$ is $\simeq$ 1.0. Freezing of the pigment (by liquid nitrogen) in 0.1 M NaPB, pH 7.8, at protein concentrations greater than 1 mg/ml and storage at −20 C in the dark does not alter or cause a loss of spectral activity.

Analysis of this sample in 0.1% SDS, 10% polyacrylamide electrophoresis is shown in Figure 4. The Coomasie blue-stained gel shows a single major band, with indication of a minor band. Subsequent analysis (27) gives a molecular weight estimate of 62,000 for the major band.

Rye Purification. Attempts to purify rye phytochrome with the oat procedure were initially frustrated by the appearance of two components eluted from DEAE-cellulose and gel filtration columns, since photoactivity readily partitioned over large elution volumes with concomitant losses in yield per column. Table II summarizes the purification of phytochrome from 10.5 kg of rye tissue, using a revised procedure (5 days). The over-all purification is 3250 times. Each step yields about 50% or better; the over-all yield is 5%. The latter is somewhat low, and smaller purifications (2 kg tissue) have yielded 10% final yield. The final sample pool had an A_{280}/A_{665} value of 1.3. Samples generally have ranged from 1.25 to 1.37.

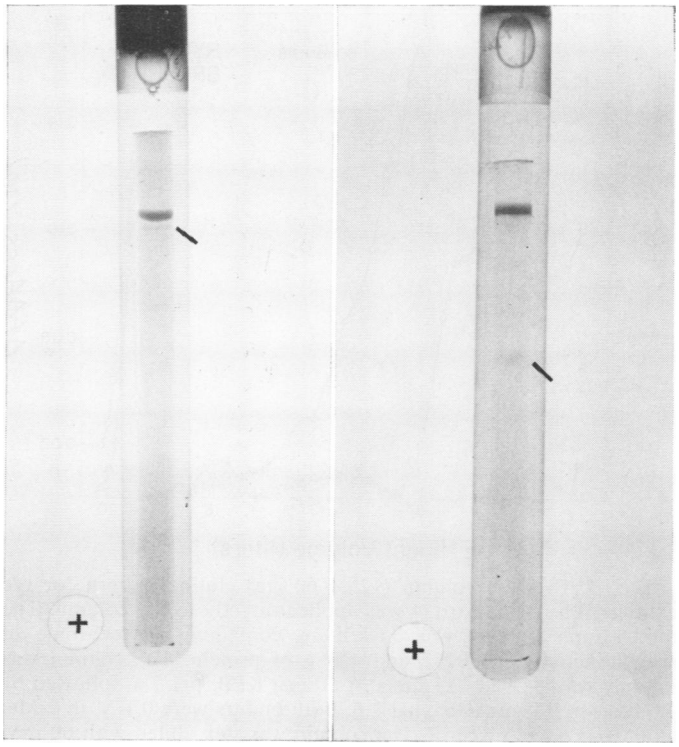

FIG. 4. Stained 0.1% SDS, 10% polyacrylamide electrophoretic pattern of purified oat and rye phytochrome. Samples (10 μg) were reduced and alkylated in guanidine-HCl. Direction of migration is toward anode (+). Black lines indicate presence of minor components. Left, oat; right, rye.

The initial brushite fractionation was similar to that for oats. Figure 5 shows the elution profile for one of two columns run. Phytochrome was not quite fully bound at 10 mM KPB, and activity was lost in both buffer washes reducing the over-all column yield to about 60%. During elution, a blue to blue-green band was formed, and chromatography could be followed visually. Yellow-colored material occupied approximately 75% of the total column volume after elution.

The resulting brushite pool was fractionated with 0 to 33% solid ammonium sulfate (200 g/l). Table III indicates the recovery of activity at this step compared to 0 to 40% fractionation for an equivalent sample. Recovery of activity has consistently been 70% or less for 0 to 33% fractionation. The gain in specific activity (0.065 compared to 0.057) does not

Table II. *Yield and Purification of Phytochrome Isolated From 10.5 Kg of Rye Tissue (8/70)*

Fraction	Volume	Pooled Activity[1]	Activity[1]	Protein[2]	Specific Activity[2]	Yield	Purification	A_{280}/A_{665}
	ml		*per ml*			*%*	*fold*	
Crude	14,000	152.0	0.0108	50.5	0.000214	100		
Brushite	1,700	93.0	0.055	3.42	0.0161	61	75	
DEAE[3]	225	43.5	0.192	1.17	0.164	28.6	755	
HA	22	26.0	1.18	4.07	0.290	17.1	1,350	
Agarose	21	15.2	0.435	0.770	0.565	10.0	2,620	1.6
G-200[4]	8	7.7	0.96	1.38	0.695	5.1	3,250	1.3

[1] Expressed as $\Delta(\Delta A)$ units and assuming $\Delta(\Delta A) \simeq A_{665}$ for 1-cm path length after FR irradiation.

[2] Protein expressed as A_{280} corrected for A_{280}/A_{260} and specific activity expressed as $\Delta(\Delta A)$/corrected A_{280}.

[3] Includes 0 to 33% ammonium sulfate fractionation of brushite pool.

[4] Only 10 A_{665} units placed on column from agarose pool.

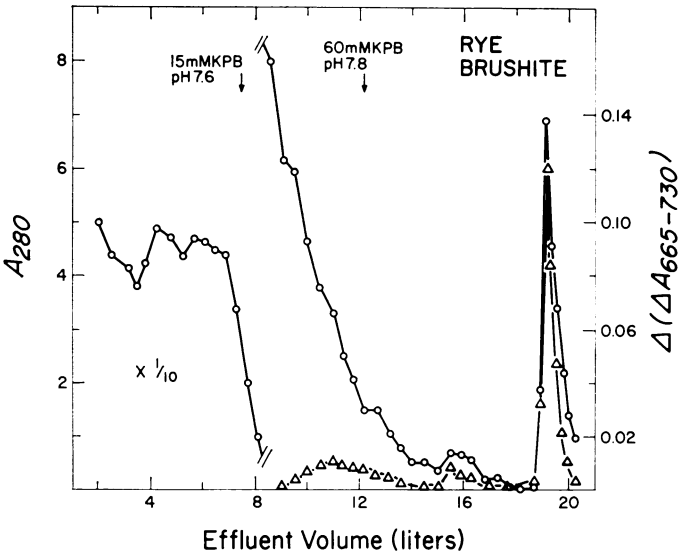

FIG. 5. Brushite column (12 × 60 cm) elution pattern for rye extracts. The crude extract was applied directly to the column. The total sample volume was 8.67 liters, containing phytochrome of specific activity 0.000182 and 475 g of protein. The column was initially washed with 7.5 liters of 10 mM KPB, pH 7.4, followed by 4.5 liters of 15 mM KPB, pH 7.6. Both buffers were 0.1 M in 2-Me, and 5% (v/v) in glycerol. The column was step eluted with 60 mM KPB, pH 7.8, 14 mM in 2-Me, and 5% (v/v) in glycerol. The flow rate was maintained at 750 to 800 ml/hr and 20-ml fractions were collected. Effluent volume is the volume from the beginning of the wash. ↓: Application of buffer; ○: absorbance at 280 nm; △: phytochrome activity/ml.

compensate for the lower yield (65%). The reason for using 0 to 33% fractionation is that a single phytochrome peak is obtained on subsequent DEAE-chromatography. Absorption spectra taken after the ammonium sulfate cut show that phytochrome is the only absorber above 500 nm. Subsequent column analyses of phytochrome absorbance were therefore monitored at 665 nm.

Following solubilization and centrifugation, direct application of the ammonium sulfate sample to DEAE gives an elution profile as shown in Figure 6. Omission of dialysis and the consequent presence of ammonium sulfate causes some loss of resolution in the column. The activity elutes as a broad peak at a concentration greater than 0.2 M KCl.

After DEAE-fractionation, attempts to bind rye phytochrome to CM-Sephadex were unsuccessful. In the case of oats, a small fraction (about 5%) was lost in centrifugation when

Table III. *Ammonium Sulfate Fractionation of Brushite Phytochrome from Rye*

Sample	Sample Specific Activity[1]	Fraction	Supernatant Activity[2]		Recovered Activity[2]		Fraction Specific Activity[1]
				%			
A[3]	0.014	0–40% (240 g/l)	3.04	2.7	102	91	0.057
B[4]	0.016	0–33% (200 g/l)	8.10	6.9	76	65	0.065

[1] Protein expressed as corrected A_{280} and specific activity as $\Delta(\Delta A)$/corrected A_{280}.

[2] Expressed as $\Delta(\Delta A)$.

[3] Sample A was 112 $\Delta(\Delta A)$ total activity and 5.2 mg/ml protein.

[4] Sample B was 117 $\Delta(\Delta A)$ total activity and 5.6 mg/ml protein.

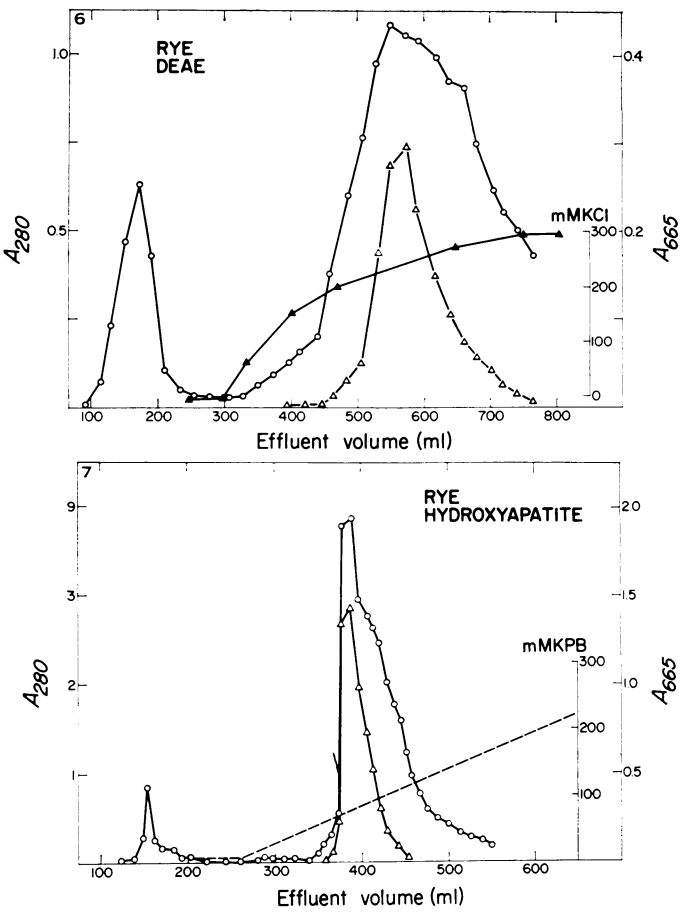

FIG. 6 (upper). DEAE-cellulose column (2.5 × 40 cm) elution pattern for rye phytochrome after 0 to 33% ammonium sulfate fractionation. Sample volume was 80 ml containing phytochrome of specific activity 0.051 and 750 mg of protein. The column was initially washed with 80 ml of 10 mM KPB, pH 7.8. Elution was carried out with a convex gradient 250 ml 10 mM KPB and 500 ml 10 mM KPB 0.3 M KCl as the limit buffer. All buffers contained 5% (v/v) glycerol and 0.1 mM EDTA. A flow rate of 2 ml/min was maintained and 11-ml fractions collected. ○: Absorbance at 280 nm; △: absorbance at 665 nm of Pr following 2-min FR irradiation; ▲: KCl gradient.

FIG. 7 (lower). Hydroxylapatite column (2 × 35 cm) elution pattern for rye phytochrome. Sample volume was 30 ml, containing phytochrome of specific activity 0.164 and 260 mg protein. The column initially was washed with 120 ml 10 mM KPB, pH 7.0. Elution was carried out with a linear gradient of 200 ml of 10 mM KPB and 200 ml of 0.25 M KPB, pH 7.0, as the limit buffer. All buffers contained 5% (v/v) glycerol. A flow rate of 15 ml/hr was maintained and 3-ml fractions were collected. ○: Absorbance at 280 nm; △: absorbance at 665 nm of Pr after 2-min irradiation with FR; – – –: buffer gradient.

the pH was dropped to 6.0. This fraction became as much as 35% for rye, and binding attempts above pH 6.0 proved unsuccessful. Figure 7 shows the elution profile of the HA column which was adopted as a substitute step. Full binding of the phytochrome was obtained at pH 7.0 (0.01 M KPB) and elution achieved at the same pH with a linear gradient (0.01 M-0.25 M KPB). Phytochrome activity eluted at approximately 0.1 M KPB, and the bulk of the contaminant protein trails phytochrome activity. The column recovery is 60%.

When the concentrated, solubilized HA sample is applied to an 8% Agarose column, an elution profile such as that shown in Figure 8 is obtained. A broad single peak of activity is partially retarded on the gel. There is some indication of

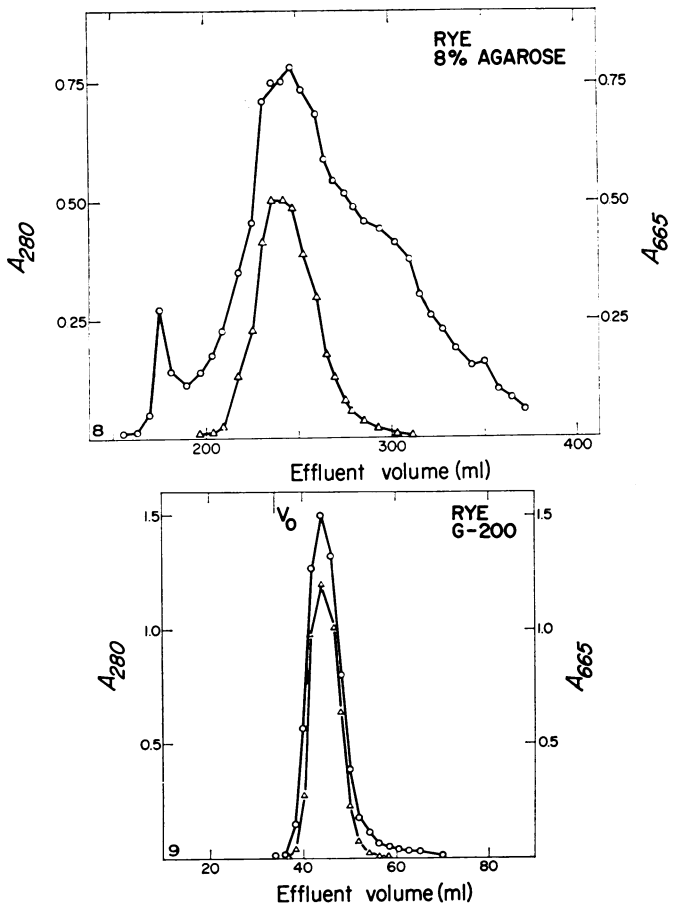

FIG. 8 (upper). Bio-Gel A 1.5 M Agarose column (2.5 × 90 cm) elution pattern of rye phytochrome. The sample volume was 5 ml, containing phytochrome of specific activity 0.290 and 89 mg of protein. The column was eluted with 0.1 M NaPB, pH 7.8, containing 5% (v/v) glycerol and 0.1 mM EDTA. The flow rate was 17.5 ml/hr and 3.5-ml fractions were collected. ○: Absorbance at 280 nm; △: absorbance at 665 nm of Pr following 2-min irradiation with FR.

FIG. 9. (lower). Sephadex G-200 column (1.2 × 90 cm) elution profile of rye phytochrome. The sample volume was 1.5 ml, containing phytochrome of specific activity 0.565 and 15.5 mg of protein. The column was eluted with 0.1 M NaPB, pH 7.8, containing 5% (v/v) glycerol and 0.1 mM EDTA. The flow rate was 4 ml/hr and 2-ml fractions were collected. ○: Absorbance at 280 nm; △: absorbance at 665 nm of Pr following 2-min irradiation with FR. V_o indicates void volume determined in a separate elution.

trailing on the smaller side of the peak. Rechromatography of the heart fractions on Sephadex G-200 is shown in Figure 9. The elution profile again shows only a single peak partially retarded relative to the void volume (V_o). There is no indication of a markedly retarded peak such as that seen in Figure 2. Pooling of the peak fractions gives a sample with an A_{280}/A_{665} ratio of 1.3 and a specific activity of 0.695. In small scale isolations (2 kg of tissue), two gel chromatography steps (Agarose and G-200) have not been required and equivalent samples ($A_{280}/A_{665} = 1.25$) have been obtained with a single G-200 filtration.

Absorption spectra of a purified rye sample of A_{280}/A_{665} 1.27 are shown in Figure 10. Scans were made at 4 C rather than 25 C. Initial attempts to record at 25 C were frustrated by rapid dark reversion of Pfr to Pr which gave incomplete measurements at 725 nm (see ref. 21). The ratio of loss and gain at 665 nm and 725 nm following R and FR irradiation is 1.15; Pr λ_{max} is 665 to 667 nm, Pfr λ_{max} is 730 nm. Lowry determination of this sample shows 1 mg/ml with bovine serum albumin as a standard. $A_{280}^{0.1\%}$ is 0.74 and $A_{665}^{0.1\%}$ is 0.55. Samples of rye phytochrome have proved less stable to freeze-thawing in 0.1 M NaPB than oat phytochrome at protein concentrations greater than 1 mg/ml. Freezing by liquid nitrogen with 5% (v/v) glycerol in the sample followed by storage at −20 C wrapped in foil is sufficient to preserve spectral activity without appreciable loss.

Analysis of the sample in Figure 10 in 0.1% SDS, 10% polyacrylamide electrophoresis is shown in Figure 4. The Coomasie blue-stained gel shows a single major band, with indication of a minor band. Subsequent analysis (27) gives a molecular weight estimate of 120,000 for the major band.

Tests of the stability and variation in the purified rye phytochrome were conducted using SDS polyacrylamide electrophoresis as an assay system. Samples of differing specific activity were heat denatured in SDS (time zero, control). Equal aliquots were allowed to remain at 25 C in the dark as Pr for approximately 40 hr, then heat denatured in SDS. Polyacrylamide gels were then run and the Coomasie blue-stained gels scanner densitometrically with a Gilford 2000 spectrophotometer. A sample with a specific activity of 0.720 showed 95 to 97% of the stain in the major band both before and after the 40 hr incubation; a sample with a specific activity of 0.695 showed 92% stain in the major band before and after incubation, indicating no significant alteration during the 40-hr incubation at 25 C for samples of phytochrome with specific activities of 0.69 to 0.72.

DISCUSSION

The purified oat phytochrome preparations reported here, A_{280}/A_{665} of 0.78 to 0.85, are similar to those of Mumford and Jenner (20) and Hopkins and Butler (14). The actual preparative procedure resembles that of Hopkins (13); however, the over-all yields of 20 to 30% are substantially higher than previously reported for oat phytochrome. SDS polyacrylamide analysis, showing a single major band of 62,000 mol wt strongly suggests a preparation very close to that of Mumford and Jenner.

It is clear, however, that an initial larger component is present in oats (2, 6, 26), although only a smaller species is obtained in purification (Fig. 2). Briggs *et al.* (2) reported that a larger species of phytochrome present in partially purified oat extracts was readily lost with time and handling, and only a smaller species was retained. In contrast, the larger species present in rye extracts appears more stable and is readily fractionated on molecular sieve gels. For the two phytochromes examined here, the presence of a weakly bound DEAE-fraction (eluting at less than 0.2 M KCl) generally is associated with the smaller species during subsequent gel filtration (26). On the other hand, the presence of a tightly bound DEAE-fraction (greater than 0.2 M KCl) generally is associated with a larger species of phytochrome on subsequent gel filtration.

Rather than reflecting disaggregation, the two species observed in gel filtration probably reflect proteolysis; the smaller species of the purified oat type may be a proteolytic artifact. Work by Pringle (23, 24) first suggested that proteolysis might account for the discrepancies observed in the various measurements of phytochrome molecular weight. In purifying malate dehydrogenase from yeast, he noticed two species on Sephadex G-200 chromatography. The less retarded protein, when concentrated and rechromatographed, gave rise to the more markedly retarded species. PMSF, an agent known to inhibit serine proteases and at least one sulfhydryl protease (9, 41), prevented this breakdown.

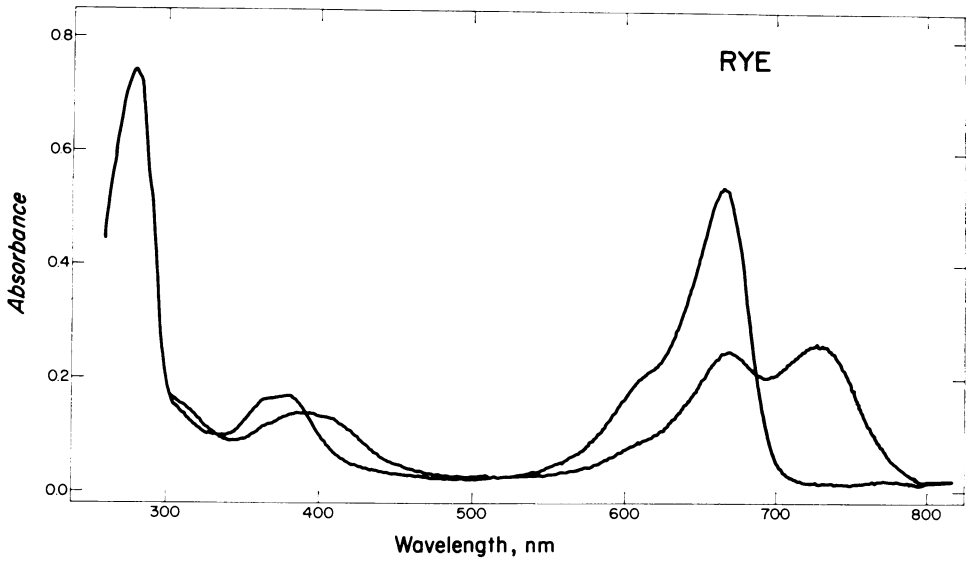

FIG. 10. Absorption spectra of purified rye phytochrome, specific activity 0.720, A_{280}/A_{665} 1.27, in 0.1 M NaPB, pH 7.8, 5% (v/v) glycerol, 4 C, after 4-min R and FR irradiation.

A direct demonstration that proteolysis occurs during purification has been given by the work of Gardner *et al.* (10). They were able to show that the breakdown of a large (9S) rye phytochrome species was partially inhibited by PMSF. Gardner *et al.* have also shown that the oat protease isolated by Pike and Briggs (22) from dark-grown oat seedlings will catalyse the breakdown of the 9S species present in rye brushite phytochrome to a 4-5S rye species (10).

The notion that the two species of phytochrome detected on gel filtration reflects proteolysis during purification is further supported by a comparison of the SDS polyacrylamide electrophoretic behavior of purified oat and purified rye phytochrome, as shown in Figure 4. If the smaller oat species were a dissociation product of a larger species similar to purified rye, one would expect similar subunit banding on SDS gels. This is not the case, and the molecular weight of the oat band is about half that of the major rye band (27).

In this connection, the recent reports by Walker and Bailey (38, 39) on purified oat phytochrome species with molecular weights of less than 60,000 are probably misleading. The extended duration of their purification procedure and the heterogeneous character of the final products suggests that extensive degradation may have occurred.

The modifications made in the oat purification procedure in order to purify phytochrome from rye seedlings may be understood, then, as attempts to mitigate proteolytic activity of the oat protease type. Rye phytochrome was selected for purification because Correll *et al.* (8) had already reported fairly highly purified preparations with a sedimentation coefficient of 9S, and Pike and Briggs (22), using the protease substrate azocoll (19), showed protease activity to be lower in crude rye extracts than in crude oat extracts. The most important modification has been the substitution of 0 to 33% for 0 to 40% saturated ammonium sulfate fractionation. It is significant that only Correll *et al.* (7) have used 0 to 33% fractionation, and that they have been the only workers to obtain a larger species of phytochrome. The neutral oat protease isolated by Pike and Briggs (22) fractionates at 40 to 70% ammonium sulphate. Substitution of a 0 to 33% fractionation yields a predominantly single peak profile during subsequent DEAE-chromatography, as noted (Fig. 6). Although the fractionation does not offer any appreciable gain in specific activity (Table III), it may partially fractionate protease activity. The observation of a single peak profile on DEAE supports the report of Correll and Edwards (6) who noted that 0 to 33% fractionation of crude rye phytochrome gave a single large species during subsequent Sephadex G-200 filtration. They also reported that 33 to 40% fractionation gave a smaller phytochrome species during Sephadex G-200 filtration. Whether 33 to 40% fractionation separates the two species directly, or reflects partial protease activity, or both, is not clear.

Among the other modifications made, 2-Me was omitted following brushite chromatography to avoid subsequent reduction of active sulfhydryl groups of proteases (22). It had been retained during oat purifications because initial observations indicated a slightly lower absorbance ratio (A_{280}/A_{665}) was obtained in its presence. Dialyses between chromatographic steps were eliminated to cut down handling time of the protein in a partially purified state. Some loss of column resolution may accompany this procedure (Fig. 6). The hydroxylapatite step (Fig. 7) was substituted for the original CM-Sephadex step in the oat procedure. This substitution was necessary, as noted, because rye phytochrome proved relatively insoluble below pH 6.5. Insolubility below pH 6.5 is a characteristic of both crude oat (33) and crude rye phytochrome (8), and the behavior of the purified rye more nearly resembles that of the crude protein than purified oat phytochrome of the Mumford and Jenner (20) type which shows much higher solubility below pH 6.5.

The modified procedure has yielded purified rye preparations with an absorbance ratio of 1.25 to 1.37 which show only a single partially retarded species in Sephadex G-200 gel filtration (Fig. 9). SDS polyacrylamide gel electrophoresis indicates a major and minor band, with the major band having a molecular weight of 120,000 (Fig. 4). Densitometry indicates that in the most purified preparations, 95% of the protein stained is in the major band. The gel chromatographic behavior suggests that these preparations most nearly resemble those of Correll *et al.* (7). The present preparations, however, show a lower A_{280}/A_{665} ratio. Preparations with ratios of 1.7 to 3.5, described by Correll *et al.* (7) as pure, often have yielded double-peak gel filtration profiles and multiple banded SDS polyacrylamide electrophoretic patterns (27). Besides the difference in product, other major differences in the purification procedure reported here and that described by Correll *et al.* are the substantially lower yields obtained during purification

(5–10%, compared to 25%) and the requirement of an extra chromatographic step (HA). The lower yields, however, are consistent with those reported by Correll *et al.* for some seed lots and for some lots subjected to long storage (6 months).

It is suggested here that the variability reported by Correll *et al.* (7) for different seed lots of rye, and the difficulties encountered in heterogenous peak behavior of oat and rye phytochrome on DEAE and gel filtration columns, reflect levels of protease activity. That proteolysis may be occurring has been obscured by the fact that recovery of photoactivity in oat-type preparations can be quite high (as indicated by an over-all yield of 32% in Table I). Comparison of the spectra for purified oat (Fig. 3) and for purified rye (Fig. 10) also indicates no ready qualitative differences. It does, however, suggest that relative absorbance, A_{280}/A_{665}, is by itself no indication of relative purity and may even be quite misleading. On a protein basis (Lowry determination) the oat preparation clearly shows relatively higher absorbance at 665 nm ($A_{665}^{0.1\%} = 1.0$) than that obtained with rye (0.55). SDS-polyacrylamide electrophoresis has been used, therefore, as an adjunct to absorption spectra for deciding purity. A single electrophoretic band of 120,000 containing 95% of the stained protein is taken as the criterion of a purified rye preparation.

Most of the rye preparations have proved stable to incubation at 25 C for 40 hr. Some preparations, however, have shown the presence of multiple minor banding below the 120,000 mol wt. Although this banding pattern was not altered during the 40-hr incubation period, the presence of minor bands of this type is taken as indication (27) that some proteolytic degradation has occurred during the purification. These tests of stability have been of limited duration and type. Only the undenatured protein has been examined. Pringle (23, 24) has shown, however, that even when undenatured malate dehydrogenase did not show breakdown as detected by SDS electrophoresis, proteolytic cleavage of partially denatured enzyme incubated in 1% SDS, 1% 2-Me at 37 C could be detected by SDS electrophoresis.

The purification procedure described here apparently mitigates but does not completely remove proteolytic activity. The procedure should therefore be regarded as provisional and additional precautions are required. The most likely addition is the use of PMSF during purification. It may be possible also to substitute another phenolic inhibitor for 2-Me. In this regard, polyvinylpyrrolidone (17) (PVP_{360}, Sigma) at 2% (w/v) gives 85% activity of that obtained with 2-Me in crude rye extracts. Glycerol at 5% (v/v) has been used here and is known to stabilize some proteins (3), but it is probably not required for phytochrome stability except as an adjunct to freezing (10). Further, any procedure which decreases the time and handling of the partially purified protein is to be recommended.

The major suggestion made here is that the difficulties encountered in phytochrome purification and discrepancies of oat- and rye-type preparations are the result of proteolytic activity. Detailed information is available only on such exotic types as ficin, papain, and some bromelins (12). These higher plant proteases generally appear to be sulfhydryl proteases with rather broad substrate specificity (32). A lack of information on the properties of proteases likely to be encountered in purifying proteins from specific higher plants makes it extremely difficult to obviate their effects. Proteases are ubiquitous, however, in higher plants. It is quite likely that the problems posed for phytochrome purification may be a general hazard in any protein purification and that discrepancies such as those posed by the oat and rye phytochrome preparations indicated here may be a common if unreported occurrence.

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