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Partial Characterization of Oat and Rye Phytochrome[1]

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ABSTRACT

Purified oat and rye phytochrome were examined by analytical gel chromatography, polyacrylamide gel electrophoresis, N-terminal, and amino acid analysis. Purified oat phytochrome had a partition coefficient on Sephadex G-200 (σ_{200}) of 0.350 with an estimated molecular weight of 62,000; sodium dodecyl sulfate polyacrylamide electrophoresis gave an equivalent weight estimate. Purified rye phytochrome had a σ_{200} value of 0.085 with an estimated molecular weight of 375,000; sodium dodecyl sulfate electrophoresis gave a weight estimate of 120,000, indicating a multimer structure for the nondenatured protein. Comparative sodium dodecyl sulfate electrophoresis with purified phycocyanin and allophycocyanin gave a molecular weight estimate of 15,000 for allophycocyanin, and two constituent classes of subunits for phycocyanin with molecular weights of 17,000 and 15,000. Amino acid analysis of oat phytochrome confirmed a previous report; amino acid analysis of rye phytochrome differs markedly from a previous report. Oat phytochome has four detectable N-terminal residues (glutamic acid, serine, lysine, and leucine, or isoleucine); rye phytochrome has two detectable groups (aspartic and glutamic acids). Model experiments subjecting purified rye phytochrome to proteinolysis generate a product with the characteristic spectral and weight properties of oat phytochrome, as it has been described in the literature. It is concluded that the structural characteristics of purified rye phytochrome are likely those of the native protein.

Characterization of phytochrome *in vitro* has consisted largely of spectral studies. Extensive information on phototransformation intermediates has been gathered from low temperature (20, 45, 46), flash photolysis (33, 34), and other transformation studies (3, 10, 11, 14, 22, 37, 39, 42, 47) performed on oat phytochrome preparations of varying purity. Recently, circular dichroic measurements (4, 28, 31) have been made on more highly purified oat phytochrome. The only nonspectral characterization of oat phytochrome, however, has been an amino acid analysis reported by Mumford and Jenner (38).

As noted previously (51, 53), rye preparations reported by Correll *et al.* (19) differ in several respects from oat phytochrome. A molecular weight of 150,000 to 190,000 has been estimated for rye compared with 58,000 to 62,000 for oat (38). Correll *et al.* (19), in addition, have characterized the rye protein as a tetramer with a molecular weight of 160,000, composed of a unit polypeptide of 42,000, whereas there is apparently no unit with a molecular weight less than 60,000 in oat phytochrome (F. E. Mumford, personal communication). An amino acid analysis of rye phytochrome (19) also shows marked differences in several residues, including the absence of half-cystine compared with oat phytochrome. These differences in molecular weight, apparent aggregation state, and amino acid composition, led Correll *et al.* (19) to suggest that the two proteins might be quite dissimilar.

In the present work, a direct comparison is made of amino acid composition, N-terminal amino acid residues, and behavior on calibrated Sephadex G-200 columns between purified oat and rye phytochrome. Serological characteristics are compared elsewhere (52). In addition, an attempt is made to characterize more clearly the basic structure of rye phytochrome. This analysis, using SDS[3]-polyacrylamide gel electrophoresis, has been extended to include comparison of two other biliproteins, phycocyanin and allophycocyanin.

Evidence has been presented elsewhere (12, 24, 51, 53) that apparent differences between purified oat and rye phytochrome are at least partly a consequence of proteolysis during the isolation and purification of oat phytochrome. Further evidence favoring this thesis is provided in the present work by additional comparison of oat phytochrome with rye phytochrome subjected to mild proteolysis, either with commercial proteases or with an oat shoot protease described by Pike and Briggs (43). A brief account of portions of this work has appeared elsewhere (12).

MATERIALS AND METHODS

Reagents. Guanidine hydrochloride (ultrapure), urea (ultrapure), and iodoacetamide were purchased from Mann. Freshly prepared urea solutions were treated (5 g/100 ml) with BioRad XG501 ion exchange resin (Calbiochem) and filtered through Whatman No. 1 paper before use to remove cyanate ions. SDS was obtained from Fisher and was purified by recrystallization from ethanol. DTT was purchased from Calbiochem; 2-Me from Eastman. Dialysis tubing (Fisher) was treated with hot 0.01 M EDTA before use. All buffer salts were reagent grade.

Proteins. Cytochrome *c* (horse heart), myoglobin (sperm whale), γ-globulin (human), catalase (beef liver), and apoferri-

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[3] Abbreviations: SDS: sodium dodecyl sulfate; PC: phycocyanin; APC: allophycocyanin; DEAE: diethylaminoethyl; CM: carboxymethyl; HA: hydroxylapatite; R: red light; FR: far red light; DTT: dithiothreitol; 2-Me: 2-mercaptoethanol; TEMED: tetramethylethylenediamine; MBA: methylenebisacrylamide; FMN: flavin mononucleotide; BPB: bromphenol blue; DNFB: dinitrofluorobenzene; DNP: dinitrophenyl; NaPB: sodium phosphate buffer.

tin (bovine) were purchased from Mann. Ribonuclease A (beef pancreas), aldolase (rabbit muscle), phosphorylase A (rabbit muscle), and β-galactosidase (*E. coli*) were purchased from Worthington. Glutamate dehydrogenase and glyceraldehyde phosphate dehydrogenase (rabbit muscle) were from Calbiochem. Papain was from Nutritional Biochemicals, and bovine fibrinogen from Sigma.

Purified *Venus* paramyosin was a gift from L. Riddiford. *E. coli* ribonucleic acid polymerase was a gift from R. Losick, Harvard University. PC and APC from *Plectonema boryanum* (Indiana Culture Collection, No. 581) and PC and APC from *Anacystis nidulans* (Indiana Culture Collection, No. 625) were gifts from A. Bennett, Harvard University. Partially purified oat protease isolated from dark-grown oat seedlings (*Avena sativa* L. cv. Garry, Desert Seed Co., El Centro, Calif.) was a gift from C. Pike.

Phytochrome Preparation and Assay. Oat phytochrome was isolated and purified from oat seedlings as described (53). The procedure included extraction in tris buffer, calcium phosphate (brushite) chromatography, 0 to 40% ammonium sulfate fractionation, DEAE-cellulose, CM-Sephadex, and Bio-Gel P-150 chromatography. Rye phytochrome was isolated and purified as described (53). This procedure included extraction in tris buffer, brushite chromatography, 0 to 33% ammonium sulfate fractionation, DEAE-cellulose, HA, Bio-Gel A 1.5 M, and Sephadex G-200 chromatography. In some cases, partially purified rye fractions were used, and these fractions are designated by their relative stage in the isolation procedure.

Phytochrome absorbance and activity were measured either in a Zeiss PMQ II spectrophotometer or a Ratiospect R-2 spectrophotometer (Agricultural Specialties Co., Beltsville, Md.), as described elsewhere (53). Activity ($\Delta(\Delta A)$) and specific activity ($\Delta(\Delta A)/A_{280}$) are expressed as described previously (53). The ratio A_{250}/A_{665} following saturating FR irradiation is also given when useful. Protein was determined by the Lowry procedure (32) using bovine serum albumin (Mann) as a standard.

Absorption spectra were determined in a Cary 14R spectrophotometer with 0.1 M sodium phosphate, pH 7.8, buffer as the standard buffer. Spectra were recorded at 4 C using a chilled sample block (Cary Instrument Co.) connected to a constant temperature bath (Neslab Instrument Co.). The actinic source used was the Ratiospect source, and 5-min irradiations were given for R and FR.

Proteolysis. Purified or partially purified samples of rye phytochrome in NaPB were subjected to mild proteolysis for 20 to 48 hr in the dark at 4 C. The protein was reacted as Pr. A sample generally 0.5 to 1.0 mg/ml in phytochrome was made 0.075 to 0.1% (w/w relative to phytochrome) with a commercial protease, or 50% (w/w) with a partially purified oat protease. Aliquots were taken at 0, 20, and 48 hr, and, unless analyzed spectrally, they were denatured by boiling for 4 min in 1% (w/v) SDS, 1% (v/v) 2-Me (48, 49) to inhibit proteolysis, followed by incubation at 37 C for 4 to 20 hr in stoppered tubes.

The commercial proteases were purchased from Worthington, and all units of activity are those provided by Worthington: bovine pancreas trypsin (TRTPCK, 220 units/mg), chymotrypsin (CSD, 48 units/mg), and carboxypeptidase A (COA, 35 units/mg). The oat protease preparation had 500 units/mg as measured by a dye-bound collagen (Azocoll) assay (36, 43). Initial tests showed these enzymes were inhibited by the treatment of boiling for 4 min in 1% SDS, 1% 2-Me.

Analytical Gel Filtration. Sephadex G-200 (Pharmacia Fine Chemicals, 100–200 μ) was allowed to swell at 4 C in 0.1 M NaPB, pH 7.8, for 5 days before use. A column (1.2 × 95 cm, bed volume 110 ml) was poured following the procedure of Andrews (5). The column reservoir was fitted as a Marriot flask, and the liquid level of the hydrostatic head was 25 cm above the column outlet. The column was equilibrated 2 to 3 days in NaPB prior to use. The flow rate was 2 to 4 ml/hr. Samples were dissolved in equilibration buffer (1.5 ml) and layered with a syringe under the solution present at the top of the column. Effluent was collected in 2-ml fractions. Elution volumes (V_e) were then determined by pooling fractions following analysis. All elutions were performed at 4 C.

Blue dextran 2000 (Pharmacia Fine Chemicals) was used to determine the column void volume (V_o). The sample contained 0.1 mg of blue dextran, and column fractions were monitored at 620 nm with a Zeiss PMQ II spectrophotometer. Phenol red (0.05 mg/sample) was used to determine the internal volume (V_i) and was monitored at 500 nm. Proteins (2–7 mg/sample) were estimated at 280 nm, with the exception of catalase which was monitored at 420 nm. Phytochrome was measured at 280 nm and at 665 nm following FR irradiation. Activities were also determined with the Ratiospect R-2 spectrophotometer when necessary. Phytochrome chromatography was performed under dim green safelights, with the protein in the Pr form as described previously (53).

Generally three proteins were chromatographed at a single time. When possible a single protein marker was run with phytochrome samples. Volume elution (V_e) is taken as the fraction volume showing highest absorbance. V_e is expressed as the partition coefficient, σ, described by

$$\sigma = \frac{V_e - V_o}{V_i - V_o}$$

as suggested by Ackers (1, 2) for small zone elution chromatography.

The symbol σ_{200} refers to the partition coefficient for Sephadex G-200. Molecular weights for marker proteins are those used by Andrews (5), unless otherwise indicated.

SDS-8% Agarose-Gel Filtration. Bio-Gel A 1.5 M (Calbiochem, 100–200 mesh) was washed twice with 0.1 M NaPB, pH 7.8, containing 1% SDS and 10 mM DTT. A column (2 × 75 cm, bed volume 230 ml) was poured following the procedure of Andrews (5). The column reservoir was fitted as a Marriot flask, and the liquid level of the hydrostatic head was 25 cm above the column outlet. The column was equilibrated 3 days in the same buffer prior to use, and the flow rate was 4 ml/hr. The sample, dissolved in equilibration buffer (2 ml), was layered on top of the column, washed with an equivalent amount of buffer, and elution continued with the gravity flow system. Effluent was collected in 2-ml fractions. All manipulations, including chromatography were performed under dim green light and at room temperature, 25 C.

Partially purified rye phytochrome (Agarose fraction) with a specific activity of 0.455 and containing 22.5 mg of protein (Lowry) was denatured by treatment with 1% SDS in 0.1 M NaPB, 10 mM DTT at 100 C for 5 min. The sample was then kept at 37 C in a stoppered tube for 4 hr in the dark and dialyzed against 0.1% SDS and 1 mM DTT (two changes of 20 volumes each) overnight in the dark, then lyophilized and stored at −20 C in a foil-covered tube until ready for use.

Column fractions were monitored at 280 nm with the Zeiss PMQ II spectrophotometer, and absorption spectra were obtained with the Cary 14R spectrophotometer. Spectra were made at 25 C using the 0.0 to 0.1 A slide wire and a 1-cm path length. Selected fractions were analyzed by SDS polyacrylamide electrophoresis (see following section) by diluting the column fraction with the electrophoretic sample buffer (0.1% SDS 0.01 M NaPB, pH 7.2). Finally, after electrophoretic

analyses, peak fractions were pooled on the basis of spectra and electrophoretic pattern.

SDS-Polyacrylamide Electrophoresis. Reagents, polymerization, and buffer conditions for SDS-polyacrylamide electrophoresis were those described previously (53). Both 5 and 10% polyacrylamide gels were run (59, 65). Gel size was uniformly 0.6 × 10 cm for both gel systems. Electrophoresis was performed at 4 to 6 ma/gel constant current (about 140 v). Running time for 9-cm migration of the tracking dye in the 10% gel was 7 to 8 hr, and in the 5% gel, 4 to 6 hr.

Samples (in 0.1 M NaPB pH 7.8) normally were prepared by addition of SDS to 1% (w/v) and 2-Me to 1% (v/v) and heating to 100 C for 4 to 5 min (48, 49), followed by incubation at 37 C in stoppered tubes for 4 to 6 hr. Aliquots were then taken and added directly to the gel sample buffer (0.1% SDS 0.01 M NaPB, pH 7.2), generally in a 10:1 dilution, giving 5 to 10 μg of protein/gel. Total sample volume was 50 μl. In some cases, denaturation was performed with hot 6 M guanidine hydrochloride (100 C, 4–5 min), followed by alkylation with iodoacetamide as described (53, 58). Dialysis was performed against 8 M urea, then 1% SDS, and aliquots were added directly to the gel sample buffer. Crude fractions of phytochrome, PC, and APC were routinely treated in this manner.

Molecular weight estimations in the gels were calculated according to the procedure of Shapiro *et al.* (59) where electrophoretic mobility is calculated as:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before destaining}}{\text{distance of dye migration}}$$

Molecular weight standards are those of Weber and Osborn (65), Burgess (13), and McKee *et al.* (35).

Urea Polyacrylamide Electrophoresis. Reagents for electrophoresis were those previously described (53). Electrophoresis in 8 M urea was performed at pH 8.7 and pH 3.5 in 7.5% acrylamide gel. The two electrophoretic systems are modifications (6, 13) based on the procedures of Davis (21).

Gels (pH 8.7) were photopolymerized at 1 to 2 inches from an F30 ww fluorescent light (Sylvania) for 30 min. The separating gels were 10 cm long, the stacking gel 1 cm, in a 0.6-cm tube. Samples were prepared either by heating (100 C for 4 min) in 8 M urea, 0.01 M DTT, followed by incubation for 4 to 6 hr at 37 C in a stoppered tube, or by reduction and alkylation in guanidine hydrochloride as described previously (53). Sample solutions (50–100 μl) containing 6 to 20 μg of protein were layered on top of the gel in 8 M urea with 0.01 M DTT and 0.0001% BPB. Electrophoresis was performed at 2 ma/tube constant current (200–300 v) at 4 C for 4 to 6 hr.

Gels were 10 × 0.6 cm for the pH 3.5 system; no stacking gels were used. Photopolymerization was carried out as for the pH 8.7 system. Samples were prepared in the same way and were layered on top of the gel (50–100 μg of protein) in 8 M urea 0.01 M DTT. Electrophoresis was performed at 2 ma/tube (120 v) for 16 hr at 4 C. Staining (Coomassie brilliant blue R-250) and destaining were carried out as described for SDS electrophoresis (65).

End Group Analysis. End group analysis was performed with a micro-modification (13) of the Sanger DNP method (55). Samples (7–10 mg of protein) were dialyzed overnight against 0.02 M sodium bicarbonate (two changes, 400 volumes each), then lyophilized. The lyophilized protein was taken up in 200 to 400 μl of 0.02 M sodium bicarbonate, 1% (w/v) SDS and placed in a 12- × 100-mm Pyrex tube for acid hydrolysis. DNFB (Mann) as a 10% solution in ethanol was added in two 50-μl aliquots to the reaction mixture. The pH was adjusted to 8.8 with 0.1 N sodium hydroxide, and the tube was covered with foil and placed in a shaking water bath (37 C). The pH was checked and adjusted to 8.8 with 0.1 N sodium hydroxide every 30 min, and the reaction was allowed to proceed to 3.5 hr. SDS was then removed by adding prechilled acetone (80–90%, v/v) and centrifuging at 1500 rpm in a Sorvall SS-34 rotor for 30 min at 4 C. The pellet was then extracted two more times with 2 ml of 90% cold acetone and dried under a stream of nitrogen. The dried pellet was dissolved in 0.5 ml of 5.7 N HCl, lyophilized for 15 min, and sealed under a vacuum. The contents were then hydrolyzed at 115 C for 8 to 10 hr. After hydrolysis, DNP-amino acids were removed by fresh anhydrous ethyl ether extraction four times to a total volume of 2 ml of ether. The ether extracts were washed with 0.5 ml of 1 N HCl, vortexed, ether phase removed, and dried in a stream of nitrogen. The sample was taken up in 20 to 40 μl of methanol.

Two-dimensional chromatography was performed according to the procedure of Wang and Wang (64), using 20 × 20 cm polyamide thin layer chromatographic plates (Brinkmann polyamide MN-6). Solvent I was benzene-glacial acetic acid (80:20, v/v). Solvent II was formic acid-water (50:50, v/v). Normally the solvent front was run 12 to 15 cm (25 min for system I; 50 min for system II). DNP-amino acid standards (Calbiochem) were cochromatographed on the same plate. Bovine serum albumin (4 mg) gave a single DNP product identified as DNP-asp (63) when used as a test for the chromatographic system.

Amino Acid Analysis. Amino acids other than tryptophan were determined by the method of Spackman *et al.* (61). Before analysis, the phytochrome sample was dialyzed overnight against either 0.005 NaPB, pH 7.8, or distilled water (1000 volumes) to remove ammonium sulfate carried over from precipitation steps. The dialyzed solution was divided into aliquots and distributed to Pyrex tubes; each aliquot was mixed with an equal volume of concentrated HCl (to 5.7 N final N), lyophilized for 10 to 15 min, and sealed under a vacuum. The tubes were kept at 110 C for 24, 48, and 72 hr. Following hydrolysis, the samples were dried *in vacuo* in a rotary evaporator with a water bath (40–50 C). Aliquots of hydrolysate equivalent to about 50 μg of protein in sodium citrate buffer, pH 2.2, were then analyzed with a Beckman-Spinco Model 120B amino acid analyzer. Norleucine (Calbiochem) was used as an internal standard. Calibration standards (Calbiochem) were run with each set of analyses.

Half-cystine and methionine were determined as cysteic acid and methionine sulfone by the performic acid method of Hirs (27). A mixture of 1.0 ml of 30% hydrogen peroxide and 9.0 ml of 88% formic acid was allowed to stand at room temperature for one hr. Samples (0.1 ml) were then oxidized on ice 2 to 4 hr with 2 ml of the performic acid solution. Following oxidation, samples were diluted to 12 ml with distilled water, frozen, and lyophilized overnight. The residue was then taken up in 5.7 N HCl, 2-ml aliquots distributed to Pyrex tubes and sealed under a vacuum by the usual procedure. Hydrolysis was for 20 to 24 hr at 110 C. A single analysis for acidic and neutral amino acids was then made in the Beckman-Spinco analyzer.

RESULTS

Absorption Spectra. Absorption characteristics of oat and rye phytochrome are quite similar (53). A qualitative similarity of absorption spectra also exists between rye phytochrome and rye phytochrome samples which have been subjected to mild

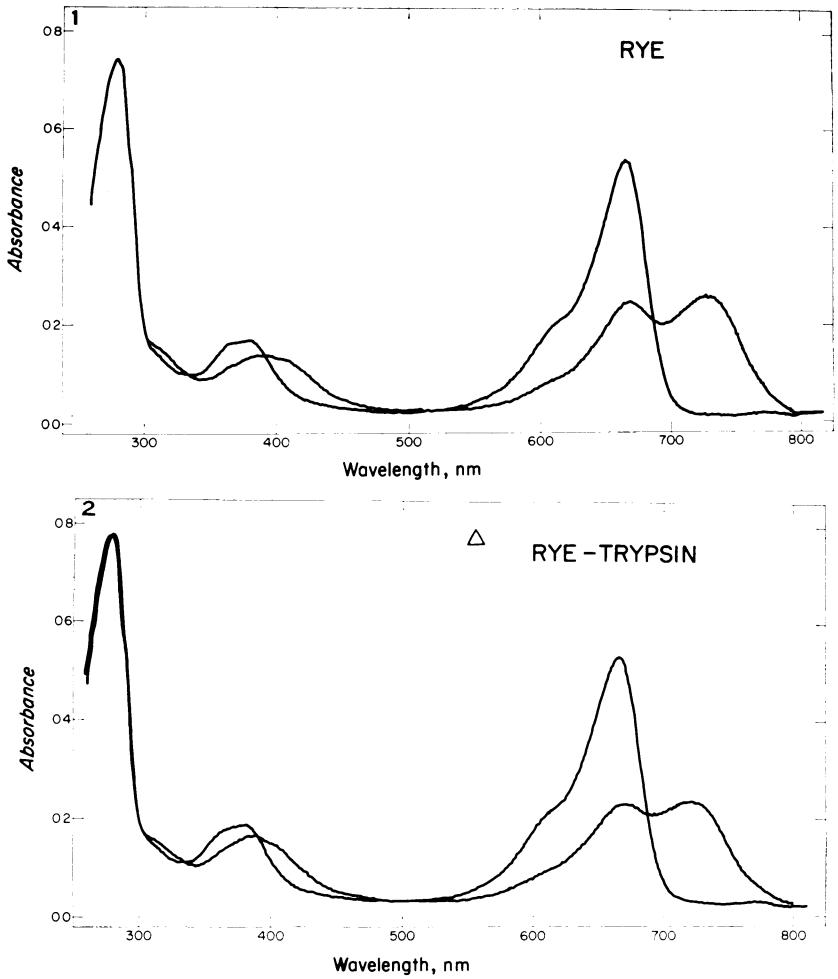


FIG. 1. Absorption spectra of purified rye phytochrome (1 mg) with specific activity of 0.720 in 0.1 M NaPB, pH 7.8, 5% (v/v) glycerol, at 4 C after 5-min R and FR irradiation.

FIG. 2. Absorption spectra of an equal aliquot of rye phytochrome from the sample in Figure 1, treated for 20 hr at 4 C with 0.1% (w/w) trypsin (2 units). Sample is in 0.1 M NaPB, pH 7.8, 5% (v/v) glycerol at 4 C. Spectra are after 5-min R and FR irradiation.

proteolysis. Figure 1 shows the absorption spectra of a sample of purified rye phytochrome. Figure 2 shows the absorption spectra of an equal aliquot treated for 20 hr as Pr at 4 C with 0.1% (w/w phytochrome) trypsin. There is little difference between the spectra in these two figures. The control sample had an A_{280}/A_{665} value of 1.27; the trypsin treated sample, 1.47. The most pronounced change is seen in the relative absorbance of the Pfr form in the rye-trypsin sample (Fig. 2). Here the 730 nm peak has undergone a shift to 725 nm and there is a loss of absorbance. This is seen in the ratio ($Pr_{665} - Pfr_{665}$/ $Pfr_{730} - Pr_{730}$) which is 1.5 as opposed to the control value of 1.2. The Pr peak at 665 to 667 nm is the same in both control and trypsinized samples; both samples were also stable to repeated (three to four) photoconversions.

Analytical Chromatography. Although there is a qualitative similarity between absorption spectra of rye and trypsinized rye phytochrome, there is marked dissimilarity in their gel filtration behavior. Figure 3 shows the Sephadex G-200 elution profiles for purified rye (Fig. 1) and trypsinized rye (Fig. 2). The two separate profiles are normalized to V_o. Rye has a σ_{200} value of 0.085, whereas this peak is lost in the trypsinized rye and an elution peak is seen which has a σ_{200} value of 0.351. No photoactivity was measurable (by the Ratiospect R2) in fractions corresponding to the rye peak in the trypsinized rye elution, although some 280 nm-absorbing material was present between the void volume and the peak. No photoactivity was measurable in the purified rye elution at fractions corresponding to the trypsinized rye peak. The protein load of the purified rye was 1.35 times the rye-trypsin sample. Column recoveries of photoactivity were comparable: 83% for rye, 86% for trypsinized rye. The ratio A_{280}/A_{665} was 0.82 in the trypsinized sample, and the photoconversion ratio was 1.2 with higher values for trailing fractions.

Figure 4 shows the elution profile of purified oat phytochrome normalized to the same V_o as shown in Figure 3. Recovery of photoactivity on the column was 91%, A_{280}/A_{665} for the peak fraction was 0.88, and the σ_{200} was 0.350. A plot of σ against log molecular weight is shown in Figure 5 for one set of phytochromes and marker proteins. The molecular weight estimate for rye is 375,000; for oat and trypsinized rye, 62,000. Maximal deviation for replicates, including markers in separate runs, was ± 0.01 for σ.

Electrophoretic Analysis. Parallel experiments utilizing SDS-polyacrylamide electrophoresis as an assay system show that rye products arising from proteinolysis of purified rye phytochrome have a mobility similar to oat phytochrome. Figure 6 shows the electrophoretic pattern of purified oat phytochrome (gel A) compared with rye phytochrome (gel B) treated with three proteases: partially purified oat protease (gel D); trypsin (gel E); and chymotrypsin (gel F). Gel C is the oat protease pattern alone at 1.3 times the concentration present in gel D, the treated sample. The major band of oat phytochrome has an

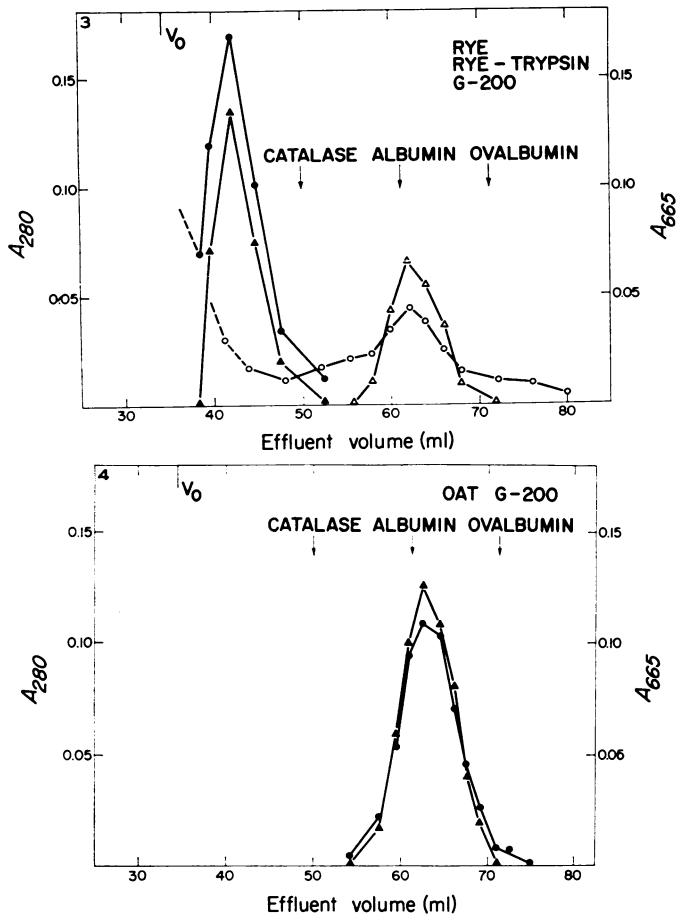


FIG. 3. Sephadex G-200 column (1.2 × 95 cm) elution patterns of rye phytochrome (1.35 mg) of specific activity 0.720 and trypsinized rye phytochrome (1.0 mg). Sample volumes were 1.5 ml. The column was eluted with 0.1 M NaPB, pH 7.8, at 4 C. Flow rates were 4 ml/hr and 2-ml fractions were collected. V_o indicates the position of the void volume marker (blue dextran 2000). Elution volumes from the separate samples are normalized to the void volume. ↓: Relative positions of marker proteins: beef liver catalase, bovine serum albumin, and ovalbumin determined in separate elutions. ●: Rye absorbance at 280 nm; ○: trypsinized rye absorbance at 280 nm; ▲: rye absorbance 665 nm; △: trypsinized rye absorbance at 665 nm; – – –: 280 nm absorbance contributed by blue dextran. Phytochrome absorbance in region of overlap was determined by photoactivity with $\Delta(\Delta A)$ assumed equal to A_{665}.

FIG. 4. Sephadex G-200 column (1.2 × 95 cm) elution pattern for oat phytochrome (1.72 mg) of specific activity 1.120. Sample volume was 1.5 ml. The column was eluted with 0.1 M NaPB, pH 7.8, at 4 C. The flow rate was 4 ml/hr and 2 ml-fractions were collected. V_o indicates position of blue dextran. ↓: Relative positions of marker proteins determined in separate elutions: beef liver catalase, bovine serum albumin and ovalbumin. ●: absorbance at 280 nm; ▲: absorbance at 665 nm.

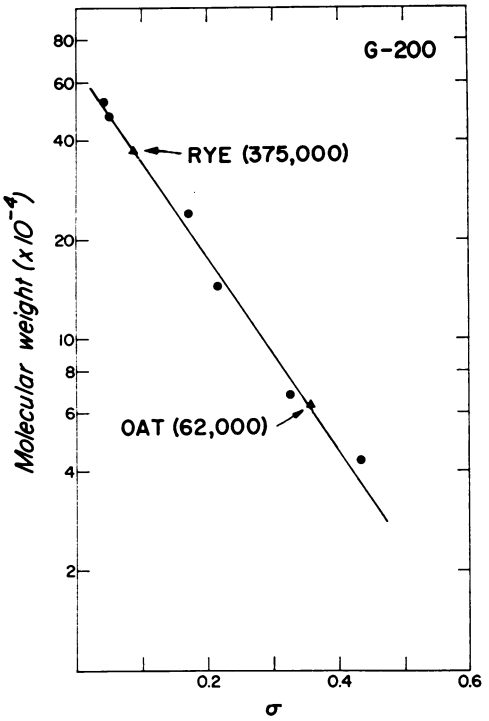


FIG. 5. Plot of distribution coefficient, σ, against log molecular weight for protein markers (●) and phytochrome (▲) on a Sephadex G-200 column (1.2 × 95 cm). The proteins (in increasing mol wt) were: ovalbumin (43,000), bovine serum albumin (67,000), rabbit muscle aldolase (145,000), beef liver catalase (240,000), apoferritin (475,000), *E. coli* β-galactosidase (520,000). Estimated molecular weights for phytochromes are given in parentheses.

estimated molecular weight of 62,000. Both oat protease (D) and trypsin (E) generate a comparable unit, concomitant with the loss of the major band of the purified rye sample (B), estimated at 120,000. A minor band (arrow) is present in the oat protease (C) and rye-oat protease (D) gels, which is present in the purified oat sample (A). Chymotrypsin (F) generates a different product (estimated mol wt of 90,000) without a complete loss of the 120,000 unit. Higher concentrations of chymotrypsin (0.3–0.5%) cause a loss of all major bands with weights above 45,000, in the same time period (23.5 hr).

Figure 7 shows the relative stability of both oat protease and trypsin products after 40 hr. Gel A is the 40 hr rye control; gels B and C are the rye sample treated with oat protease after 23.5 and 40 hr; gels D and E are the trypsin products after the same time periods. The 62,000 unit remains stable, although in the trypsinized rye sample, a second minor band is generated between 32,000 and 42,000 at 40 hr. Treatment with carboxypeptidase A (an exopeptidase) at 0.1 to 0.5% over an equivalent time period (40 hr) failed to alter the 120,000 mol wt unit present in purified rye.

A calibration plot for the 10% gels used in establishing the molecular weights from the gels in Figures 6 and 7 is shown in Figure 8. The molecular weight for purified rye is 120,000; the minor band in rye is 32,000; purified oat as noted is 62,000. The mobility in the latter case was 0.210; the rye-trypsin product was 0.228, and the oat protease-rye product was 0.216. Both fall close to the mobility deviation of oat phytochrome alone (0.210 ± 0.01).

Calibration plots were also run on 5% polyacrylamide gels with a shallower standard curve (51). The calculated molecular weight (120,000) agreed with the 10% gels. The experimental range was 115,000 to 125,000. Where smaller amounts of sample (less than 5 μg/gel) were electrophoresed, a double banding pattern was frequently seen in the 120,000 mol wt unit.

An attempt was made, therefore, to resolve the presence of a second rye component in a second electrophoretic system. Figure 9 shows the electrophoretic pattern of oat phytochrome (A) and rye photochrome (B) in 8 M urea, pH 8.7 (tris-glycine), 7.5% polyacrylamide gels. Oat phytochrome shows a diffuse band; rye phytochrome gives a single electrophoretic band with minor trailing bands which correspond to discontinuities

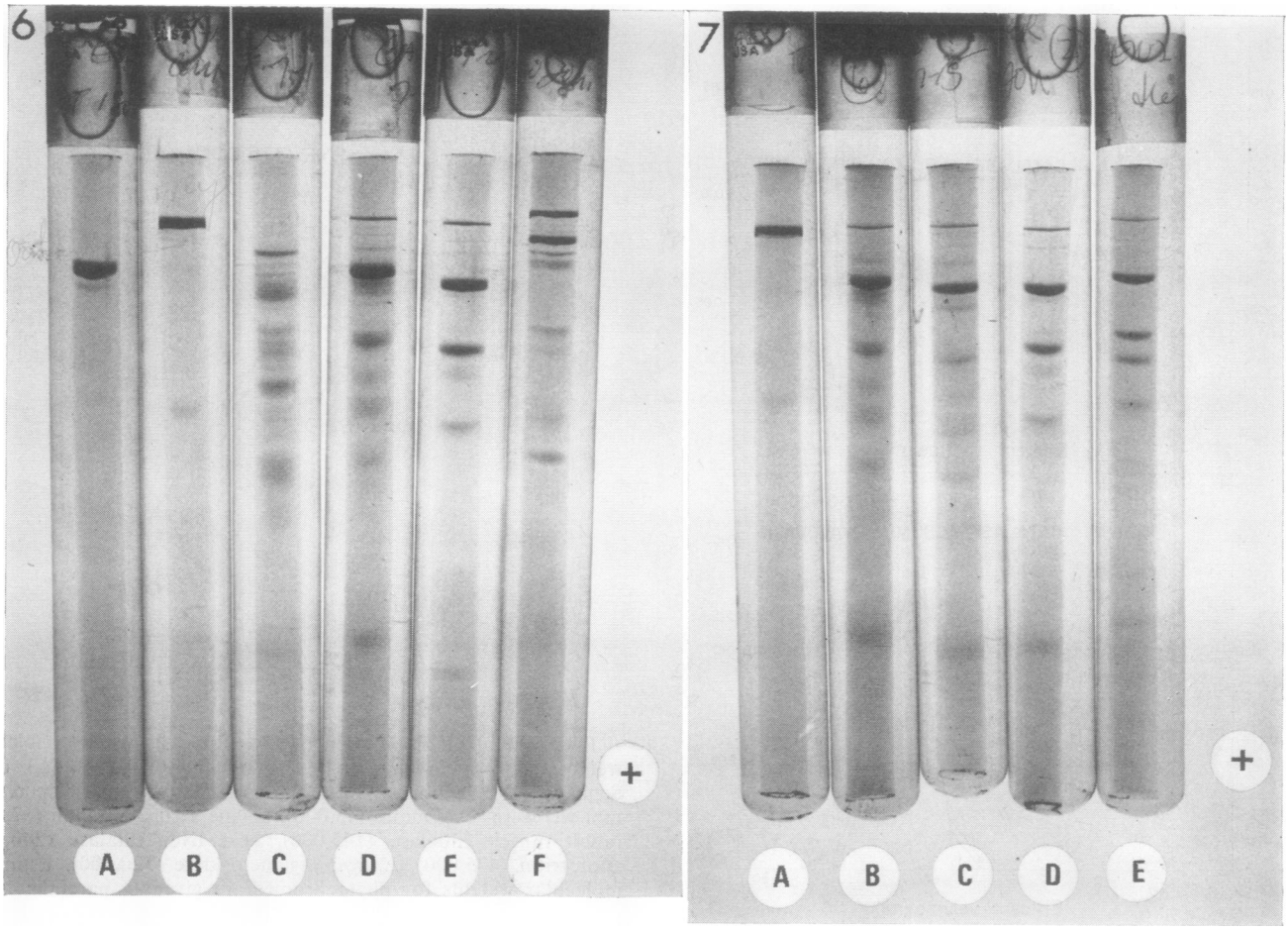


FIG. 6. Stained 0.1% SDS 10% polyacrylamide electrophoretic pattern of purified oat phytochrome (specific activity 1.120) and purified rye phytochrome (specific activity 0.695) subjected to mild proteolysis. Gel A is purified oat phytochrome reduced and alkylated in guanidine hydrochloride. Gel B is purified rye phytochrome control after 23.5 hr at 4 C in 0.1 M NaPB, pH 7.8. Gel C is partially purified oat protease (50 μg, 35 units) after 23.5 hr at 4 C. Gel D is purified rye phytochrome after treatment with 50% (w/w) oat protease (250 units/mg phytochrome) for 23.5 hr at 4 C. Gel E is rye phytochrome after treatment with 0.1% (w/w) trypsin (2 units/mg phytochrome) for the same time. Gel F is rye phytochrome after treatment with 0.1% (w/w) chymotrypsin (0.48 units/mg phytochrome) for the same time. Phytochrome samples in gels were 7.0 μg each. Protein was reacted as Pr. Direction of migration is towards anode (+). Note minor component in oat, just below principal band.

FIG. 7. Stained 0.1% SDS 10% polyacrylamide electrophoretic pattern of purified rye phytochrome (specific activity 0.695) subjected to mild proteolysis. Gel A is rye phytochrome maintained at 4 C in 0.1 M NaPB, pH 7.8, for 40 hr as Pr. Gel B is rye phytochrome after 23.5 hr with 50% (w/w) oat protease (250 units/mg phytochrome). Gel C is after 40 hr with oat protease. Gel D is rye after treatment with 0.1% (w/w) trypsin (2 units/mg phytochrome) for 23.5 hr. Gel E is after 40 hr with trypsin. Phytochrome samples were 7.0 μg. Direction of migration is towards anode (+).

in the separating gel. A minor area of multiple bands (arrow) is seen, but there is no evidence for a second major component. Urea gels (8 M) run at pH 3.5 showed a similar pattern for both proteins, indicating that the mobility of rye relative to oat phytochrome is governed by size rather than charge.

Electrophoretic behavior of rye phytochrome in 0.1% SDS 10% polyacrylamide gels also was compared to the electrophoretic patterns of the biliproteins APC and PC. Figure 10 shows the electrophoretic pattern for PC (A_{250}/A_{620} of 0.2) in gels B to D. It is immediately evident that there is a marked difference when compared with purified rye phytochrome. At least two classes of polypeptides are present. Gel A is the electrophoretic pattern of APC from the same source (*Plectonema*). Here only a single unit is present. The mobility of APC (0.710) is comparable to the faster moving band in PC (gel B, mobility 0.714). Gel C shows the electrophoretic pattern of a reduced alkylated crude soluble fraction, indicating that PC and APC comprise the bulk soluble protein fraction present. Gel D is a second purified sample with ovalbumin as an internal marker (mol wt of the monomer, 43,000; mol wt of the dimer, 86,000). Densitometric scans of gel B, using a Gilford gel scanner with the Gilford 2000 spectrophotometer, showed 57% of the stained material in the light chain and 43% in the heavy chain (25). Both classes of polypeptides can be isolated from the gels on elution in SDS and rerun as single bands in the same system. Figure 11 shows a calibration for the 10% gels seen in Figure 10. A molecular weight estimate of 17,200 is obtained for the heavy band and 15,100 for the light band. A value of 15,300 is obtained for APC.

End Group Analysis. Because of the presence of a minor fraction (32,000 mol wt) of uncertain nature in rye preparations, rye phytochrome was purified on a 1% SDS-8% Agarose column before end group analysis. The sample (agarose fraction, specific activity 0.455) was heat-denatured in SDS before chromatography. The elution profile (Fig. 12) indicates three main fractions. SDS electrophoretic analysis of the major peak fractions is shown in Figure 13. Figure 13A is the electrophoretic pattern of the peak fraction from the preparative agarose column pool used as the sample. The major band is 120,000, two lesser bands (arrows) are 54,000 and 32,000;

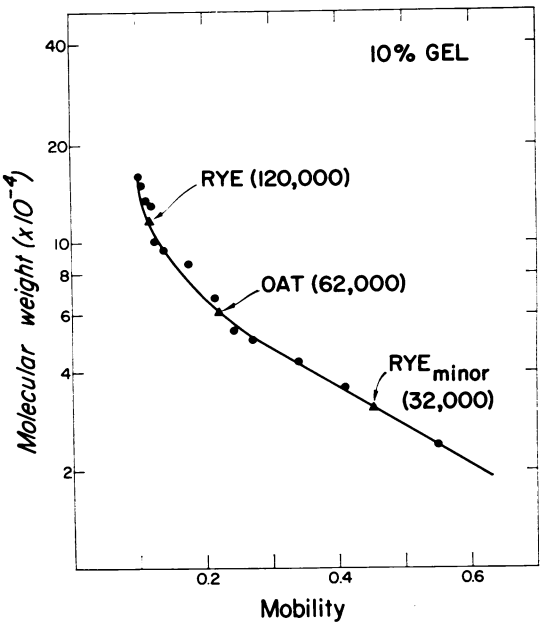


FIG. 8. Plot of mobility against log molecular weights for 10% polyacrylamide gels. ▲: Phytochromes; ●: protein markers. Markers (in increasing molecular weight) were: γ-globulin L chain (23,500); glyceraldehyde phosphate dehydrogenase (36,000); ovalbumin (43,000); γ-globulin H chain (50,000); glutamate dehydrogenase (53,000); bovine serum albumin (67,000); ovalbumin dimer (86,000); phosphorylase A (94,000); paramyosin (100,000); β-galactosidase (130,000); bovine serum albumin dimer (134,000); *E. coli* ribonucleic acid polymerase β and β′ chains (155,000 and 165,000). Estimates of phytochrome molecular weights are given in parentheses. Molecular weight values of the standards are those of Weber and Osborn (65), with the exception of *E. coli* values for polymerase β and β′ chains which were taken from Burgess (24).

two minor bands (single arrow) are less than 15,000. The first column peak (Fig. 12) yields the only 120,000 band. This is seen in gel B which is the fraction at V_e 48 ml. The second column peak is a composite of two lesser bands (54,000 and 32,000) while the trailing peak is associated with the minor bands (less than 15,000). Gel C shows the electrophoretic pattern of the fractions from V_e 46 to 52 ml which were subsequently used for the DNP end group analysis.

Absorption spectra of effluent fractions show visible absorbance above 340 nm associated only with the fraction from V_e 44 to 62 ml. Figure 14 shows the absorption spectrum of the volume fraction at 52 ml. Residual absorbance peaks are present at 655, 590, and 550 nm, a shoulder is present at 410 to 420 nm, and a peak at 345 nm. An absorption spectrum from the volume fraction at 76 ml was superimposable with the baseline scan above 500 nm, and the peak at 345 nm and shoulder at 410 to 420 nm were not evident. Scans of volume fractions 100 to 102 ml were similar to the latter.

DNP end group analyses were performed on the pooled sample (V_e 46 to 52 ml, 7 mg of protein) of the 120,000 mol wt unit. Two-dimensional polyamide TLC of the ether-extracted DNP-amino acids was performed. Two major DNP-residues were present: DNP-glutamic acid and DNP-aspartic acid, together with trace amounts of DNP-glycine and DNP-serine. These may be compared to the DNP-residues of oat phytochrome. An end group analysis of a purified oat phytochrome (5 mg) gave four major DNP products: DNP-glutamic acid, DNP-serine, DNP-bislysine, and DNP-leucine or isoleucine.

Amino Acid Analysis. An amino acid analysis for oat phytochrome is given in Table I. The protein is high in glutamic and aspartic acid, threonine, serine, leucine, and alanine, as well as lysine. Trace amounts of a proline derivative eluting before aspartic acid were detected. No positive identification was made, but its relative elution position corresponds to the probable position of hydroxyproline (D. Lamport, personal communication). Half-cystine was determined as cysteic acid, and methionine as the sulfone by performate oxidation (27). Tryptophan was not determined. A partial specific volume calculated by the method of Cohn and Edsall (16) using the values of Schachman (56) was 0.736 cc/g.

An amino acid analysis for rye phytochrome is given in Table II. Comparison to oat phytochrome may readily be made with the last column where residues are expressed per 62,000. Over-all there is a qualitative similarity to oat phytochrome. The protein is high in glutamic acid, aspartic acid, leucine, alanine, and serine. There are differences, however. Lysine, threonine, and methionine are present in lower amounts; whereas proline, glycine, and alanine are in higher amounts. No proline derivative peak was detectable eluting before the aspartic acid peak. A partial specific volume calculated from the analysis was 0.728 cc/g.

DISCUSSION

The oat preparations used here have been considered by Rice *et al.* (53) as representative of the Mumford and Jenner type (38). As Figures 4 and 5 indicate, Sephadex G-200 gel

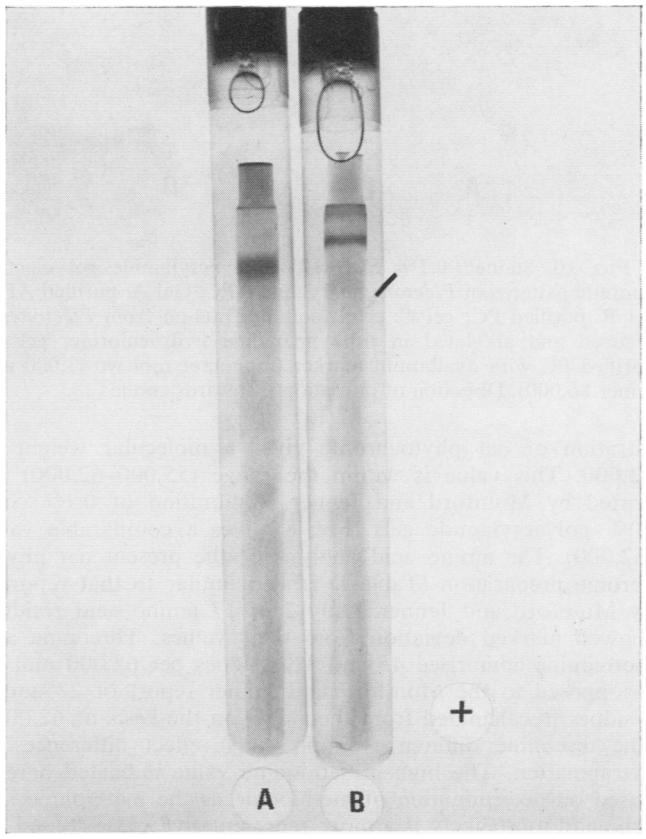


FIG. 9. Stained 8 M urea, pH 8.7, 7.5% polyacrylamide gel (11 cm × 0.6 cm) electrophoretic pattern of purified oat (specific activity 1.120) and rye phytochromes (specific activity 0.695). Rye sample (13.2 μg) was heated to 100 C (5 min) followed by 37 C (6 hr) in 8 M urea 0.01 M DTT. Oat sample (15 μg) was reduced and alkylated in 6 M guanidine hydrochloride, followed by dialysis into 8 M urea. Gels with 1-cm stacking gel were run 4.5 hr at 2 ma/gel (200–300 v). Gel A: oat; gel B: rye. Slanting black line indicates area of minor component in rye. Direction of migration is towards anode (+).

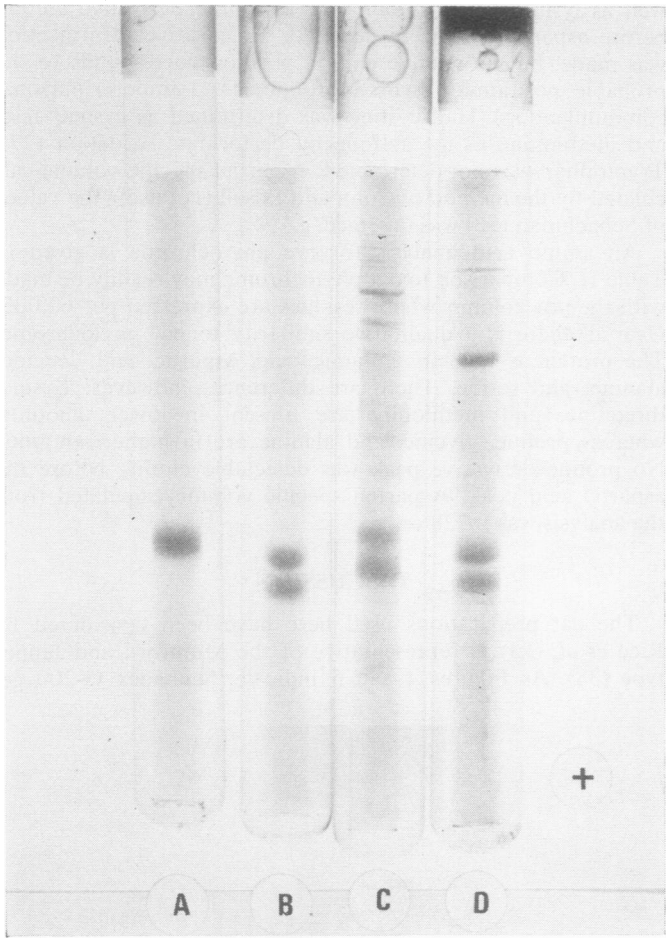
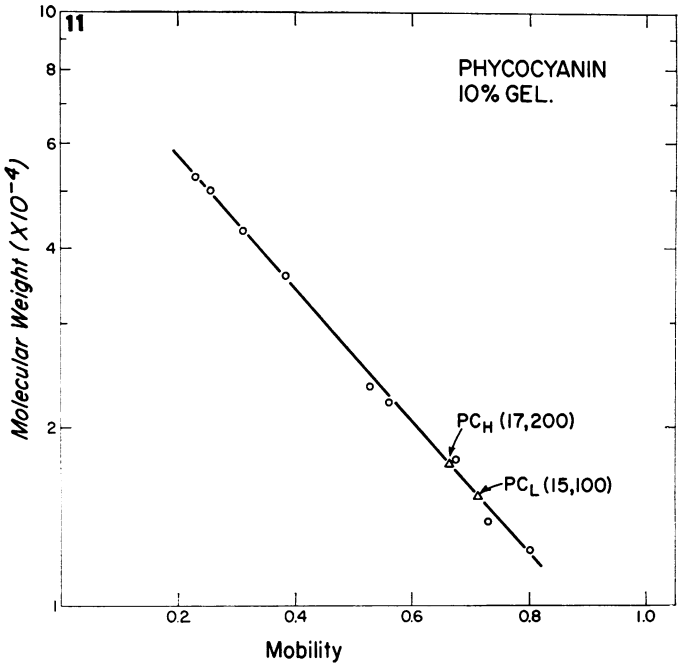


FIG. 10. Stained 0.1% SDS 10% polyacrylamide gel electrophoretic patterns of *Plectonema* PC and APC. Gel A: purified APC; gel B: purified PC; gel C: crude soluble fraction from *Plectonema* reduced and alkylated in 6 M guanidine hydrochloride; gel D: purified PC with ovalbumin marker (monomer mol wt 43,000 and dimer 86,000). Direction of migration is towards anode (+).



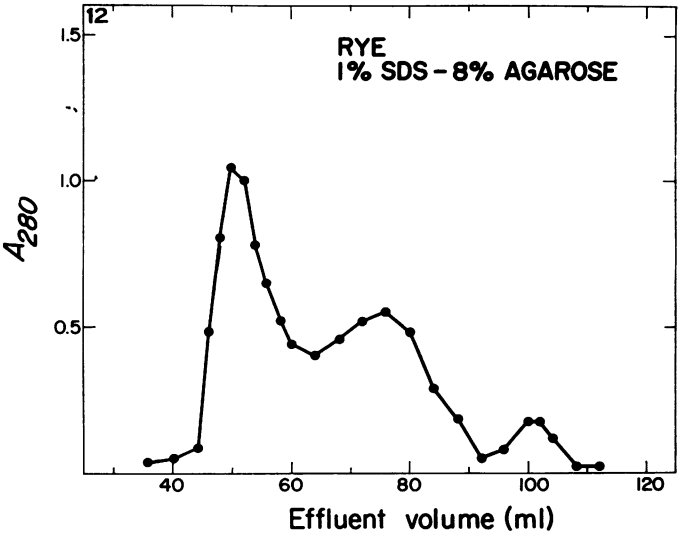


FIG. 11. Plot of mobility against log molecular weight for 10% polyacrylamide gels. ○: Protein markers; △: phycocyanin bands. Markers (in order of increasing molecular weight) are: cytochrome *c* (12,500), RNAase (13,700), myoglobin (17,800), papain reduced and alkylated (22,100), γ-globulin light chain reduced and alkylated (23,000), glyceraldehyde phosphate dehydrogenase (36,000), ovalbumin (43,000), γ-globulin heavy chain reduced and alkylated (50,000), and glutamate dehydrogenase (53,000). Line was drawn with least squares analysis. Estimates of PC heavy and light chains are given in parentheses. Molecular weight values of the standards are those of Weber and Osborn (65).

FIG. 12. Eight per cent agarose (Bio-Gel A1.5M) column (2 × 75 cm; bed volume 230 ml) elution profile of partially purified rye phytochrome (agarose fraction) in 1% SDS, 0.1 M NaPB, pH 7.8, 0.01 M DTT. Sample volume was 2 ml containing 15.5 mg of protein with specific activity 0.455. The column flow rate was 4 ml/hr at 25 C, and 2-ml fractions were collected. ●: Absorbance at 280 nm.

filtration of oat phytochrome gives a molecular weight of 62,000. This value is within the range (55,000–62,000) reported by Mumford and Jenner. Calibration of 0.1% SDS 10% polyacrylamide gels (Fig. 8) gives a comparable value (62,000). The amino acid analysis of the present oat phytochrome preparation (Table I) is also similar to that reported by Mumford and Jenner. Only 2 of 17 amino acid residues showed marked deviation from their values. Threonine and methionine comprised 45 and 31 residues per 62,000 mol wt as opposed to the Mumford and Jenner report of 23 and 4 residues (recalculated from their data on the basis of 62,000). The threonine difference simply may reflect differences in extrapolation. The higher methionine value indicated here is based on determination of methionine as the methionine sulfone and most likely is a more representative value. Based on the gel filtration behavior, electrophoretic properties, and amino acid composition, the oat phytochrome described here is quite similar to that of Mumford and Jenner (38) and other workers (28).

Evidence has been presented elsewhere by Gardner *et al.* (24) and Rice *et al.* (53) that such preparations are the product of proteolytic activity during purification. Several further lines of evidence add support to such a view. Preliminary SDS polyacrylamide gels of oat phytochrome prepared according to the procedure of Shapiro *et al.* (59) gave minor bands with estimated molecular weights of 45,000 and 30,000 in addition to the major band (62,000) and minor band seen in Figure 6,

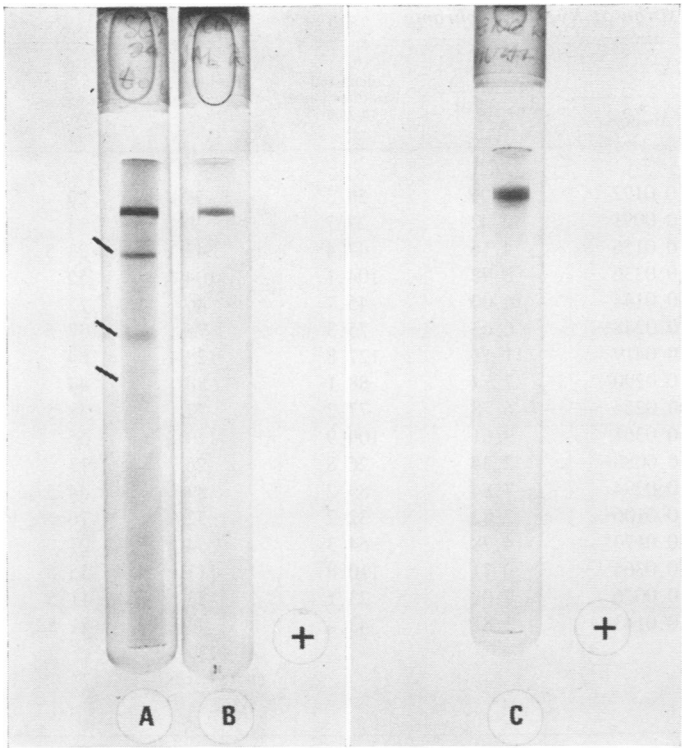


FIG. 13. Stained 0.1% SDS 10% polyacrylamide electrophoretic pattern of rye phytochrome (agarose fraction) isolated from the 1% SDS-8% agarose column in Figure 12. Gel A: sample before fractionation; gel B: fraction from V_e 48 ml; gel C: peak pool from V_e 46 to 52 ml. Samples on the gels were 10, 5, and about 15 μg, respectively. Slanting black lines indicate components less than 120,000 molecular weight. The gels were run in three separate electrophoretic series, and differences in the main band intensity are partially a result of differences in staining. Direction of migration is towards the anode (+).

gel A. The procedure of Shapiro *et al.* (59) calls for incubation of the protein at 37 C in 1% SDS and 1% 2-Me for 3 hr, followed by dialysis (16 hr) into 0.1% SDS and 0.1% 2-Me. When oat phytochrome preparations were denatured by heat (100 C, 4 min) either in 1% SDS or in guanidine hydrochloride followed by alkylation, the minor bands were absent, and patterns of the type seen in Figure 6, gel A, were detected. Pringle (49) has described similar behavior in purified yeast malate dehydrogenase and in various purified enzymes from commercial sources and has shown that such an appearance of lower molecular weight bands during the mild denaturating conditions of Shapiro *et al.* is often a consequence of residual protease activity.

Examination of oat preparations by urea polyacrylamide electrophoresis and DNP N-terminal analysis also suggested that such preparations might not be homogeneous. Oat phytochrome electrophoresed in 8 M urea invariably gives a broad diffuse band (Fig. 9) which could reflect heterogeneity, while DNP N-terminal analysis quite clearly indicated four N-terminal residues. The availability of purified rye phytochrome, however, allows a more direct examination of phytochrome lability towards proteolysis.

The absorption spectra of oat and rye phytochromes are qualitatively similar (53), despite the fact that they have markedly different gel filtration behavior (Figs. 3 and 4) and SDS polyacrylamide electrophoretic patterns (Fig. 6, gels A and B). Subjection of purified rye phytochrome to mild proteolysis with trypsin does not appreciably alter its absorption spectra

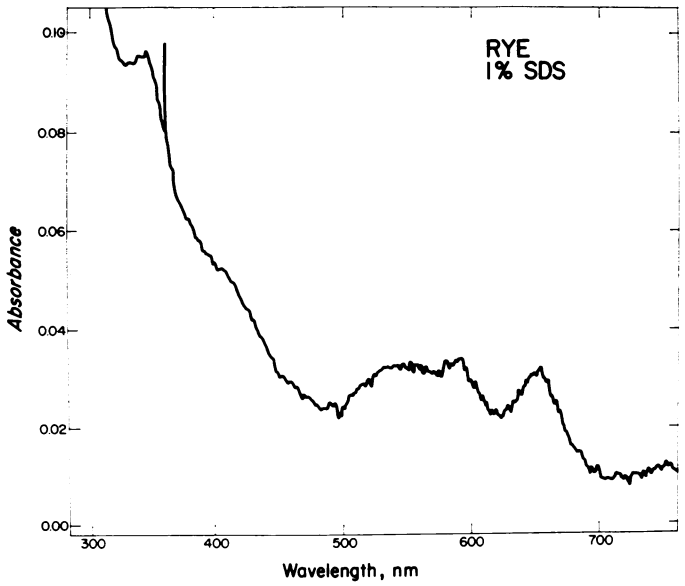


FIG. 14. Absorption spectrum of the volume fraction at V_e 52 ml in Figure 12. Buffer was 1% SDS, 0.1 M NaPB, pH 7.8, and 0.01 M DTT at 25 C.

(Figs. 1 and 2), nor did similar treatment with oat protease (24). Trypsinized rye retains at least 90% of its photoactivity. The most noticeable spectral alteration is seen in a shift of the Pfr peak from 730 to 725 nm with a concomitant loss of some absorbance. The loss of Pfr absorbance is reflected in the relative increase of the spectral ratio (Pr A_{665} − Pfr A_{665}/Pfr A_{730} − Pr A_{730}) from 1.2 for rye to 1.5 for trypsinized rye phytochrome. An increase in the spectral ratio frequently has been taken as an indication of progressive denaturation of oat phytochrome in partially purified preparations (60). Butler *et al.* (15), using much higher concentrations of trypsin and pronase, also have shown that Pfr is more labile to proteolytic attack than Pr. What has proved misleading, however, is that such minor alterations in absorption spectra as seen by comparing

Table I. *Amino Acid Composition of Oat Phytochrome*

Amino Acid	Sample				Total μmoles Recovered	Calculated Residues/62,000[1]	Residues, Nearest Integer
	24 hr	48 hr	72 hr	Average			
	μmole				%		
Lysine	0.01985	0.01900	0.02062	0.0198	6.74	41.0	41
Histidine	0.00854	0.00948	0.00932	0.0091	3.21	18.9	19
Arginine	0.01215	0.01415	0.01161	0.0126	4.34	26.1	26
Aspartic acid	0.02290	0.02485	0.02520	0.0243	8.55	50.2	50
Threonine	0.01635	0.00793	0.00776	0.0215[2]	7.57	44.5	45
Serine	0.01361	0.00700	0.00782	0.0180[2]	6.35	37.2	37
Glutamic acid	0.02590	0.03100	0.02980	0.0304	10.70	62.9	63
Proline	0.01405	0.01490	0.01461	0.0145	5.10	30.0	30
Glycine	0.01328	0.01263	0.01270	0.0129	4.71	26.8	27
Alanine	0.01605	0.02165	0.01995	0.0208	7.32	43.0	43
Half-cystine	0.00504	...	...	0.0050[3]	1.78	10.4	10
Valine	0.01905	0.02018	0.02104	0.0201	7.08	41.7	42
Methionine	0.01480	...	...	0.0148[4]	5.21	30.7	31
Isoleucine	0.01310	0.01369	0.01346	0.0134	4.72	27.3	27
Leucine	0.02700	0.02880	0.02690	0.0276	9.73	57.1	57
Tyrosine	0.00702	0.00711	0.00725	0.0071	2.51	14.8	15
Phenylalanine	0.01153	0.01280	0.01210	0.0121	4.26	24.9	25
Total residues							588

[1] Calculated on the basis of an average residue molecular weight of 104.5.
[2] Values obtained by linear extrapolation to zero time.
[3] Determined as cysteic acid following performic acid oxidation.
[4] Determined as methionine sulfone following performic acid oxidation.

Table II. *Amino Acid Composition of Rye Phytochrome*

Amino Acid	Sample				Total μmoles Recovered	Calculated Residues per 120,000[1]	Residues, Nearest Integer	Integer/2
	24 hr	48 hr	72 hr	Average				
	μmole				%			
Lysine	0.0187	0.0194	0.0195	0.0192	5.09	58.3	58	29
Histidine	0.00904	0.00895	0.00940	0.0091	2.42	27.7	28	14
Arginine	0.0162	0.0151	0.0156	0.0156	4.16	47.4	47	23.5
Aspartic acid	0.0336	0.0331	0.0340	0.0336	8.98	104.1	104	52
Threonine	0.0146	0.0138	0.0148	0.0144	4.00	45.7	46	23
Serine	0.0221	0.0195	0.0194	0.0248[2]	6.63	75.3	75	37.5
Glutamic acid	0.0425	0.0421	0.0410	0.0419	11.15	127.8	128	64
Proline	0.0268	0.0210	0.0181	0.0290[2]	7.74	88.1	88	44
Glycine	0.0278	0.0243	0.0242	0.0254	6.78	77.2	77	38.5
Alanine	0.0362	0.0363	0.0358	0.0361	9.61	109.9	110	55
Half-cystine	0.0088	...	...	0.0088[3]	2.34	26.8	26	13
Valine	0.0269	0.0302	0.0311	0.0294	7.84	89.2	89	44.5
Methionine	0.01055	...	...	0.0106[4]	2.82	32.2	32	16
Isoleucine	0.0171	0.0182	0.0183	0.0179	4.78	54.3	54	27
Leucine	0.0366	0.0361	0.0366	0.0364	9.71	110.8	111	55.5
Tyrosine	0.00778	0.00739	0.00750	0.0076	2.01	23.1	23	11.5
Phenylalanine	0.0146	0.0140	0.0143	0.0143	3.82	43.4	43	21.5
							1139 (total residues)	

[1] Calculated on the basis of μg recovered of each amino acid (—H_2O).
[2] Values obtained by linear extrapolation to zero time.
[3] Determined as cysteic acid following performic acid oxidation.
[4] Determined as methionine sulfone following performic acid oxidation.

Figures 1 and 2 may actually reflect marked alteration in protein structure.

Figure 3 shows that when trypsinized rye phytochrome is fractionated on Sephadex G-200, photoactivity at the elution position of rye phytochrome (σ_{200} 0.08S, 375,000 mol wt) is lost. Instead, trypsinized rye phytochrome shows chromatographic behavior (σ_{200} 0.351, 62,000) similar to purified oat phytochrome (Fig. 4). Moreover, the peak fractions of the trypsinized rye sample show an absorbance ratio (A_{280}/A_{665}) of the type normally associated with purified oat phytochrome (<1.0). The distribution of A_{280} material on the column also indicates that the altered spectral ratio is associated with products which no longer retain chromophore activity.

Similar experiments have been performed by Gardner *et al.* (24) using sucrose density gradients as an assay system. A single species of rye sedimenting as 9S generated a 4 to 5S species when trypsinized. Purified oat phytochrome also had an S value of 4 to 5 in the same calibrated system. SDS-polyacrylamide analysis of these gradients (which used partially purified phytochrome) indicated that the 120,000 mol wt band associated with rye phytochrome was lost on trypsinization, and the 4S species gave a band of 62,000 on SDS gels similar to the purified oat band.

The fact that trypsinization generates a 62,000 band of the oat type provides a convenient assay for comparing proteolytic products of rye phytochrome. As Figure 6 indicates, when samples of purified rye phytochrome are incubated with the endopeptidases trypsin, chymotrypsin, and a partially purified oat protease, both trypsin and the protease cause a loss of the 120,000 mol wt band and generate a comparable 62,000 unit together with some lesser molecular weight bands. Under the conditions used, these products are relatively stable over a time period of 40 hr, as seen in Figure 7. The incubation conditions are purely arbitrary (phytochrome as Pr, protease at 0.1% w/w or less, 20–40 hr, 4 C) but they are the type frequently encountered in purification.

The ability of the oat protease fraction isolated from dark-grown oats (43) to shift the rye electrophoretic pattern from 120,000 to 62,000, together with the spectral characteristics, and gel filtration behavior of trypsinized rye is taken as further evidence that oat phytochrome of the Mumford and Jenner type is a mixture of polypeptides resulting from proteolytic attack, but retaining chromophore activity. Such an argument tacitly assumes that purified rye phytochrome is similar to a larger molecular mass phytochrome known to be present initially in oat isolates (for a fuller discussion see ref. 24). Subsequent Sephadex G-200 chromatography by Gardner (personal communication) has shown that brushite oat phytochrome in the presence of the protease inhibitor (23) phenylmethylsulfonyl fluoride has a σ_{200} value comparable to that reported here for rye phytochrome (0.085). Finally, further phenylmethylsulfonyl fluoride inhibition studied by Gardner *et al.* (24) showing that the inhibitor blocks the spontaneous breakdown of 8S oat and rye species into 4S species corroborates the view that oat phytochrome, as it has been described, is an artifact produced during isolation.

Correll *et al.* (19) were the first to attempt to characterize the structure of rye phytochrome and called attention to the differences between their rye preparations and oat preparations of the Mumford and Jenner type (38). They proposed a monomeric structure of 42,000 mol wt with the most stable species (9S) assigned a tetrameric structure of about 160,000 mol wt. The 42,000 value was derived from equilibrium sedimentation in 8 M urea; equilibrium sedimentation gave a molecular weight range of 150,000 to 190,000 for the 9S component, and a labile 13 to 14S component was detected in some velocity measurements. Several discrepancies arise, however, between their description of rye phytochrome and information available

here. Their preparations have shown spectral ratios of A_{280}/A_{665} of 1.7 to 3.5. Where equivalent samples have been examined here, multiple components have been detected either by SDS polyacrylamide electrophoresis (Fig. 13, gel A) or by SDS Agarose gel filtration (Fig. 12). Correll *et al.* (18) have reported retention of residual chromophore absorbancy in their rye phytochrome samples at 350 nm and 580 to 600 nm in the presence of 0.5% SDS. Residual absorbance is confirmed here in the presence of 1.0% SDS. When such samples are fractionated by SDS agarose filtration, however, the visible absorbance peaks (Fig. 14) are associated only with those fractions giving a 120,000 mol wt band on SDS polyacrylamide gels (Fig. 13, gel B). It thus seems possible, although by no means certain, that their samples may have contained varying amounts of extraneous proteins.

A comparison of the amino acid composition given here for rye (Table II) and that reported by Correll *et al.* (19) shows marked dissimilarities. Although there is an over-all similarity (as there is an over-all similarity to oat phytochrome, Table I), 10 of the 17 residues analyzed vary by more than 15% on a weight basis from the values reported by Correll *et al.* Of these ten, three (serine, proline, and tyrosine) represent extrapolated values. Arginine, glycine, and methionine, however, vary by 55, 88, and 68%, respectively. Moreover, the claim that the protein lacked half-cystine is in error. Half-cystine was detected as cysteic acid via performic acid oxidation. Such differences suggest that the rye preparations examined here do not compare exactly with those of Correll *et al.*

Further, we question the subunit structure assigned to phytochrome by Correll *et al.* (monomer mol wt 42,000; tetramer 160,000) on two grounds. First, no provision was made to ensure that proteinolysis had not occurred in their samples. Their value of 42,000 could reflect residual proteinolysis. As Figure 6 indicates, a 42,000 mol wt unit is generated by both oat protease and trypsin attack on rye phytochrome. Moreover, their denaturation conditions (8 M urea, 0.7% 2-Me, 37 C) may not have been rigorous enough to inhibit proteinolysis. As Pringle (49) has shown, both heat (100 C) and guanidine hydrochloride (6 M) are the conditions of choice for denaturing residual proteases. Second, Correll *et al.* (19) have assumed that phytochrome displays the hydrodynamic properties commonly ascribed to globular proteins. This may not be the case. Sephadex G-200 chromatography suggests a multimer molecular weight of 375,000 (Fig. 5). Gardner *et al.* (24), however, using equivalent samples and sucrose density gradient centrifugation report a multimer with an S value of 9 which would correspond to an estimated weight of 180,000.

Such a discrepancy indicates the protein is anomalous in one or both systems. The anomaly could reflect dissociation in the sucrose gradient, or it could indicate that the protein may have nonglobular characteristics. Preliminary equilibrium centrifugation studies (G. Gardner, personal communication) of the predominantly 9S component, utilizing the $\bar{\nu}$ of 0.728 cc/g calculated here from the amino acid analysis, give a weight estimate of roughly 240,000. The multimer value of 160,000 of Correll *et al.* (19) and the assumptions it incorporates thus are questionable.

The greatest discrepancy between the information reported here and that of Correll *et al.* (19) lies in the molecular weight value ascribed to the rye phytochrome subunit or subunits. The value of 120,000 obtained here by SDS-polyacrylamide gel electrophoresis is considerably larger than their 42,000 value. SDS electrophoresis has proved a reliable technique for subunit molecular weight estimates in globular proteins (65). The reliability, as Reynolds and Tanford (50) have shown, arises from the binding of a constant amount of SDS per unit protein (1.4 g SDS/g protein). However, Pitt-Rivers and Impiobato (44) have shown that glycoproteins may bind at less than this ratio. The altered binding causes an overestimate of the molecular weight in the SDS electrophoretic system. Bretscher (9) has recently shown this phenomenon quite clearly for a glycoprotein (over 60% sugar) derived from erythrocyte membranes where a molecular weight estimate of 90,000 was obtained for a protein known to be about 31,000. We assign a molecular weight value of 120,000 to the rye phytochrome subunit on the assumption that it is not a glycoprotein. Two lines of evidence support this assumption. First, Roux (54), studying purified oat phytochrome of the Mumford and Jenner type (mol wt near 60,000) reports a maximum sugar content of 3%. Second, Bretscher (9) has shown that the error in molecular weight estimation for a glycoprotein by SDS gel electrophoresis is not the same for different concentrations of acrylamide, being far greater for lower gel concentrations. In the present work, both 5 and 10% gels gave identical values of 120,000.

Whether there is more than one kind of subunit present is not clear. The N-terminal amino acid analysis would suggest that there is, as would the double banding pattern seen on 5% SDS gels (51). Although the DNFB method of N-terminal analysis has proved reliable with most biliproteins, an exception is known in *Synechoccus* PC (17). In this case, PC proved refractory to DNFB and large amounts of aspartic acid and glutamic acid were detected as N-terminal, whereas the cyanate procedure of Stark and Smyth (62) showed methionine as the N-terminal residue. Therefore, in the absence of clear physical separation of polypeptide chains, the question must be considered unresolved. Even if one could obtain such separation, the possibility that one was simply a slightly degraded version of the other would be difficult to discount. Rice (51) has shown by SDS acrylamide gel electrophoresis that crude rye phytochrome, phytochrome taken after brushite chromatography, and purified phytochrome all show a distinct band of molecular weight 120,000. Evidently, breakdown is not occurring during the purification procedure. However, breakdown during extraction, or minor modification during purification, cannot be discounted by this evidence.

This approach to the subunit structure of phytochrome may seem overly cautious. It appears, however, that phytochrome workers have expended considerable effort in studying protein preparations of dubious value, and it would be unwise to do the same thing in describing the protein's basic structure. For example, considerable effort has been expended in describing the multimer structure of PC in the absence of a clear understanding of its subunit structure. As Figure 10 indicates, there are at least two polypeptide chains present in PC from *Plectonema* as analyzed on SDS gels. The molecular weight values of 17,200 and 15,100 differ markedly from those of Berns (7) and others who have described PC as a single class of polypeptide chains of 28,000 to 30,000 mol wt (7, 30, 40, 57). Still other workers (26, 29) have reported a minimum subunit molecular weight of 46,000, while some (8, 17) have suggested 15,000 to 21,000, but assumed a single polypeptide chain.

Bennett and Bogorad (6) have recently analysed PE and PC and suggested two polypeptide chains for both proteins. The SDS gel values reported here confirm their report. O'Carra (41) has also recently reported SDS polyacrylamide gel values of 20,500 and 18,500 for PC. The situation with APC is less clear. The present work suggests a single peptide chain of molecular weight 17,300. H. W. Siegelman (personal communication) has examined APC in more detail and confirms this suggestion. Clearly although phytochrome, PE, PC, and APC are all biliproteins, SDS gel electrophoresis suggests that the size of the phytochrome subunit(s) is over six times as large as those of the three algal accessory pigments.

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