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Immunochemistry of Phytochrome[1]

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ABSTRACT

Rabbit antibody was elicited against purified oat phytochrome polypeptides. Immunodiffusion and immunoelectrophoresis indicated the antibody elicited was predominantly a single precipitin system. No antigenic difference was detected between red-absorbing phytochrome and far red-absorbing phytochrome. Crude preparations of rye and corn phytochrome showed a line of identity when cross-reacted with oat polypeptide phytochrome; pea phytochrome showed a line of partial identity. Precipitin reactions with purified rye phytochrome analyzed with sucrose density gradient centrifugation and immunodiffusion confirmed that the same class of determinants was available to the antibody when the protein was known to be in a state which had not undergone extensive proteolytic attack.

Since the initial detection of phytochrome (11), attempts to investigate differences between phytochromes from different higher plants have been limited mainly to spectral and kinetic measurements of reversion (18). Phytochrome in etiolated grass seedlings does not show reversion *in vivo* (10, 18). *In vivo* reversion has been detected in some dicots (15, 20), but not all (22). Phytochrome from etiolated grass seedlings is reported to revert slowly *in vitro* (1, 14, 26, 37), whereas that from dicots reverts more rapidly (37). However, recent work (28) has shown substantial differences in the reversion behavior of 9S rye phytochrome and a 4 to 5S phytochrome produced from the 9S material by mild proteolysis. Thus, differences in the reversion behavior of phytochromes isolated from different species may reflect only differences in degradation following initial extraction, rather than fundamental differences between the phytochromes themselves.

Immunochemical techniques have proved useful in assessing dissimilarities among related proteins (21). Immunodiffusion has been used extensively by Berns (3, 4) and others (5, 38) in investigating differences among the biliproteins phycocyanin and allophycocyanin. The present work undertakes to describe an immunochemical assay for phytochrome based on rabbit antibody elicited against purified oat phytochrome polypeptide(s). Serological comparisons of oat, rye, corn, and pea phytochrome are also described. In addition, an attempt has been made to detect differences between Pr and Pfr using immunodiffusion and immunoelectrophoretic procedures. It is known that Pr and Pfr show disparities in lability towards protein denaturants (7, 12, 16, 35). More recent evidence derived from ultraviolet difference spectra, complement fixation, and circular dichroic measurements has also suggested differences which might be ascribed to different protein structure between the two forms (19).

MATERIALS AND METHODS

Preparation of Phytochrome. Oat seeds (*Avena sativa* L. cv Victory USDA C12020) were dry sown on moist cellulose packing material (Kimpak 6223, Kimberly-Clark) and grown for 5 days in the dark, as described previously (32, 34). Rye seeds (*Cereale secale* L. cv Balbo, Robson Quality Seeds, Inc.) were grown in a similar manner for 4 days. Pea seeds (*Pisum sativa* L. cv Alaska, W. Atlee Burpee Co.) were soaked for 4 hr in tap water before sowing and were grown in the dark for 7 days. Corn seeds (*Zea mays* L. strain B14ATmg × FR37, lot No. 5370542, Illinois Foundation Seed Inc.) were soaked overnight (16 hr) in running tap water, wet sown, and grown in vermiculite for 5 days in the dark.

Harvesting and handling of tissue material was performed as reported for oat and rye phytochrome (34). Oat phytochrome polypeptide(s) used for immunization was purified as described (32, 34). This purification included calcium phosphate (brushite) chromatography, 0 to 40% ammonium sulfate fractionation, DEAE[3]-cellulose, CM-Sephadex, and Bio-Gel P-150 chromatography. Rye phytochrome used in immunodiffusion studies was also prepared as described (32, 34). This procedure included brushite chromatography, 0 to 33% ammonium sulfate fractionation, DEAE-cellulose, HA, Bio-Gel A 1.5 M, and Sephadex G-200 chromatography. Partially purified samples of rye, corn, and pea phytochrome were prepared by using the first two steps of the oat and rye procedures (brushite and ammonium sulfate 0 to 40% fractionation). Such partially purified samples have been designated brushite phytochrome. Corn phytochrome did not bind fully to the brushite, and both wash and bound fractions were combined for ammonium sulfate fractionation. Purified oat phytochrome polypeptide(s) and phytochrome samples used in immunodiffusion were dialyzed overnight against 0.15 M sodium phosphate buffer, pH 7.4, (designated NaPB) or 0.15 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.4, (designated NaPB-saline) before use. Generally, two changes of 200 volumes of buffer were used. All manipulations of phytochrome prior to use were made at 4 C under dim green safelights to ensure maintenance of the pigment in the more stable Pr form (12). All buffer salts

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[3] Abbreviations: DEAE: diethylaminoethyl; CM: carboxymethyl; HA: hydroxylapatite; SDS: sodium dodecyl sulfate; R: red light; FR: far red light; PC: phycocyanin; APC: allophycocyanin; NaPB: sodium phosphate buffer; NaPB-saline: sodium chloride in sodium phosphate buffer.

were reagent grade. Ammonium sulfate was purchased from Mann (ultrapure).

Phytochrome activity of crude and brushite fractions was measured in a Ratiospect R-2 spectrophotometer (Agricultural Specialties, Co., Beltsville, Md.). Activity of purified fractions was measured in a Zeiss PMQ II spectrophotometer, using a 1-cm path length. Activity is expressed as $\Delta(\Delta A_{665-730})$ indicated as $\Delta(\Delta A)$ following saturating R (2 min) and FR (2 min) irradiation. The actinic source was a Ratiospect 500 w incandescent source (31) fitted with Baird Atomic 665 nm and 730 nm interference filters. Specific activity is expressed as $\Delta(\Delta A)/A_{280}$ corrected (32, 34), where corrected A_{280} absorbance for a 1-cm light path is obtained from A_{280}/A_{260} and the table provided by Layne (23). Amounts of purified phytochrome protein are calculated from $A^{0.1\%}_{655}$ 1.0 for oat and $A^{0.1\%}_{655}$ 0.55 for rye (32, 34). Where useful for comparative purposes, the ratio A_{280}/A_{665} following saturating FR irradiation is also given for purified samples.

Heterologous Proteins. Purified samples of *Plectonema boryanum* (Indiana Culture Collection, No. 581) PC and APC, and *Anacystis nidulans* (Indiana Culture Collection, No. 625) PC and APC were gifts from A. Bennett, Harvard University. Prior to use in immunodiffusion, they were dialyzed overnight against NaPB (two changes of 200 volumes each).

Sucrose Gradient Centrifugation. Sucrose was purchased from Mann (ultrapure). Gradient centrifugation (25) was performed in a SB-283 rotor in an IEC B-60 ultracentrifuge. Five to 20% sucrose gradients (11 ml) were formed in NaPB, pH 7.4. Samples were prepared by dialysis (4 hr, about 200 volumes) against the same buffer. Where precipitation reactions were monitored, whole sera (0.5 ml) were layered on the gradient, and phytochrome (0.5 ml) was layered on the sera. The gradient tube was then supported at the base on ice and the antigen-antibody reaction allowed to proceed for about 1 hr. Controls were treated in the same manner, substituting unchallenged sera (time zero). Gradients were run 20 hr at 40,000 rpm at 4 C. Phytochrome was monitored as Pr (2-min FR saturating irradiation Ratiospect actinic source) at 667 nm in a Gilford Model 2000 multiple sample absorbance recorder equipped with a flow cell, and roughly 0.5-ml fractions were collected. Phytochrome activity was then read in each fraction with the Ratiospect R-2 spectrophotometer. Marker proteins were run in separate gradients and monitored at 280 nm in the Gilford spectrophotometer. Bovine serum albumin (1 mg/ml) was purchased from Cal Biochem, human γ-globulin (1 mg/ml) from Mann.

Absorption Spectra. Absorption spectra of phytochrome and phytochrome immunoprecipitates were made in a Biospect 61 spectrophotometer (Agricultural Specialties Co., Beltsville, Md.), a single beam scanning spectrophotometer adapted for light-scattering samples. Immunoprecipitates were prepared by layering a phytochrome solution in NaPB-saline on an equal volume of whole sera in a 12-ml glass centrifuge tube. The reaction was allowed to proceed for 2 hr at 25 C, the solution was chilled to 4 C and centrifuged at 12,000g for 20 min. After being washed twice with NaPB-saline, the precipitate was recentrifuged and dispersed in 1 ml of the same buffer. Phytochrome was reacted as Pr, and all manipulations were carried out under green safelights. The actinic light source used was the Ratiospect source.

Preparation of Antisera. The antisera used in these studies were produced in prebled rabbits (New Zealand white males) using oat phytochrome with an A_{280}/A_{665} index of 0.85. The animals were injected under fluorescent lights and maintained under their normal daily light regime. Weekly subcutaneous injections of 4 mg of phytochrome in a 1:1 emulsion with Freund's Complete Adjuvant (Difco Laboratories) were given for 4 weeks. After 2 weeks, 1.5 mg was given intravenously without adjuvant, and the sera was taken 12 days later by bleeding the ear. Globulins were separated from the whole sera by fractionation with 0 to 50% ammonium sulfate at room temperature (13). Following resuspension in NaPB-saline and dialysis (200 volumes), the globulin volume was adjusted to that of the original sera. Titers were determined by ring test (8). A stock antigen solution (1 mg/ml) in NaPB-saline was serially diluted, and 0.1 ml was layered over 0.1 ml of sera or globulin solution in 6- × 15-mm glass tubes. End point precipitates were measured within 2 hr.

Ouchterlony Double Diffusion Plates. Double diffusion plates were prepared by modification of the procedure of Ouchterlony (27). Photographic cover slides (3¼ × 4 inch, Kodak) were coated with 1% (w/v) Agarose (Mann, special grade) in NaPB-saline (10 ml per plate), and wells were cut (6.5 mm diameter for antigen wells and 10.0 mm diameter for antibody well). In some studies, NaPB was used without any indication of different precipitin behavior. Reactions were run by placing 0.1 ml of phytochrome or heterologous protein and 0.15 ml of sera (full strength) or globulin fraction (equivalent strength) in adjacent wells. Concentration effects were investigated by serial dilution of the antigen. Normally plates were prepared under fluorescent lights, but where reactions of Pr and Pfr were investigated, all manipulations were carried out under dim green safelights. Reactions were run at room temperature (25 C) for 36 to 48 hr in the dark. The plates were examined, washed for 24 hr in NaPB-saline, then rinsed in distilled water, and dried for 14 hr at 37 C. Semipermanent mounts for photography were prepared by staining the dried slides in 0.5% Amido Schwarz, 7.5% acetic acid, and 5% methanol for 10 to 15 min. After washing in acetic acid-methanol and distilled water, the slides were redried for 14 hr at 37 C.

Electrophoresis. Immunoelectrophoresis was performed by modification (27) of the Grabar and Williams procedure. Plates were prepared by pipeting hot (60 C) 1% agarose in 0.05 M tris-barbital, pH 8.6, (Gelman high resolution buffer) onto microscope slides (2 ml per 26- × 76-mm slide). The agarose was allowed to solidify in a humid chamber and wells (1.5 mm o.d.) and trough (2 mm width) cut with an LKB punch (LKB Instrument Co., Model 51449). The discs of gel were removed from the wells, but the rectangle in the trough remained through electrophoresis. Antigen (10 μl/well) was placed in each well, and electrophoresis was performed in the same buffer system, using a Gelman DC power supply and Gelman electrophoresis chamber (Model 51170). Electrophoresis was continued for 1.5 hr at a constant voltage of 300 v and an average of 3 ma/slide. Following electrophoresis, the trough gel was removed, and whole sera were added. The plates were allowed to incubate in a humid chamber in the dark for 18 to 24 hr. Semipermanent mounts for photography were made by drying and staining with Amido Schwarz as in the case of immunodiffusion plates.

SDS polyacrylamide electrophoresis (36) was used as an assay for phytochrome stability during immunodiffusion reactions. Samples were prepared according to the modifications of the techniques of Weber and Osborn (39) and Pringle (30), described previously (32, 34).

RESULTS

Absorption Spectra and Immunodiffusion. Oat phytochrome proved quite immunogenic in eliciting rabbit antibodies. Titers of 1:3000 to 1:4000 were obtained. Subsequent challenges

have produced second-course sera with titers up to 1:6000. Unchallenged sera showed no precipitation reaction in a ring test. That antibody is directed against oat phytochrome polypeptide(s) can be demonstrated by absorption spectrophotometry. Figure 1 shows the absorption spectra of oat phytochrome polypeptide(s) (left) and of the resulting immunoprecipitate following 2 hr reaction with whole sera. The washed precipitate (right) still shows photoactivity, although the Pfr peak at 725 nm is suppressed and the Pr peak at 667 nm has been shifted to 660 nm.

The precipitate lines observed in a double diffusion agarose plate with serially diluted oat phytochrome show a predominately single precipitin system (Fig. 2). Secondary lines are present at higher antigen concentrations, but at equivalence (wells 8, 16) a single line is present. Precipitin lines observed with oat phytochrome at equivalent concentration but varying in specific activity from 0.023 to 1.10 show a major single component without spurs (Fig. 3), indicating that the antibody is directed against the phytochrome in these preparations. Secondary lines are present in the two least pure preparations indicating some heterogeneity in the antigen-antibody reaction.

The phytochrome in Figures 2 and 3 diffused as a mixture of Pr and Pfr, a photostationary state (under fluorescent light) of the same type used in eliciting antibody. Figure 4 represents an attempt to demonstrate differences between Pr and Pfr. The two forms were allowed to diffuse toward common antisera. The presence of a single confluent line without spurs indicates that no class of antibody detectable by immunodiffusion was directed solely against Pr or Pfr polypeptide(s). Figure 5 represents an attempt to show differences in lability between glutaraldehyde-treated Pr and Pfr. Samples of the two forms were reacted with 0.1% (v/v) glutaraldehyde (Fisher, 50%) in 0.1 M NaPB, pH 8.2, at 4 C for 30 min, followed by overnight dialysis against NaPB (400 volumes). Control (untreated) samples were allowed to diffuse as a mixture of Pr and Pfr. The precipitin reaction is diminished in both cases, and presence of a single confluent line indicates no class of antibody was directed against a glutaraldehyde-modified Pr or Pfr.

The immunodiffusion pattern of serially diluted brushite rye phytochrome reacted with oat antibody is shown in Figure 6. A predominantly single precipitin system is present, with a minor interior and exterior line present at equivalence (wells 8, 16). The pattern of cross-reactivity with purified oat phytochrome is shown in Figure 7. Confluence of the major precipitin line suggests a high degree of partial identity, if not identity, between the two phytochromes. Serially diluted pea phytochrome shows a reduced reactivity towards oat antibody (Fig. 8). When cross-reacted with purified oat phytochrome, the precipitin line shows confluence, interference, and the presence of an attenuated spur, indicating partial identity between the two antigens (Fig. 9). Brushite corn phytochrome exhibits a high degree of identity when cross-reacted with oat phytochrome polypeptide(s). Figure 10 shows a cross-reaction with purified oat phytochrome polypeptide(s). The precipitin lines are confluent.

Tests for Heterogeneity. Immunodiffusion plates were run with PC and APC against oat antibody as a test for cross reactivity with a heterologous protein. Tests with PC from both *Plectonema* and *Anacystis* at concentrations up to 0.6 mg/ml were negative, as were tests with APC (to 0.3 mg/ml) from the same organisms.

The presence of minor precipitin lines in some immunodiffusion plates suggested heterogeneity in the antigen-antibody system. Immunoelectrophoresis of brushite and purified oat phytochrome polypeptide(s) gave a major and a minor arc confirming heterogeneity in the precipitin reaction. Displacement

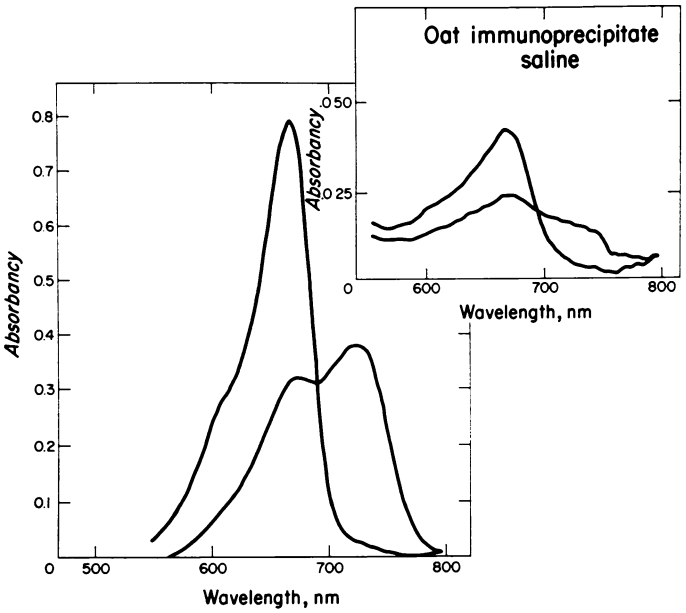

FIG. 1. Absorption spectra of oat phytochrome polypeptide(s) and oat phytochrome immunoprecipitate after saturating (5 min) irradiation with R and FR. Left: before reaction; phytochrome is 0.8 mg/ml with A_{280}/A_{665} index of 0.83. Right: washed immunoprecipitate after reaction of 2.5 ml whole sera with 2 ml of phytochrome solution.

of the arc indicated that it was not a secondary duplication of the major precipitin line. Tests with Pr and Pfr samples gave the same pattern seen indicating no detectable class of determinants specific for Pr or Pfr.

The oat phytochrome used to elicit antibody has been considered a mixture of polypeptide(s) retaining chromophore activity (6, 17, 32–34). The conditions under which immunodiffusion plates were run (25 C, 36–48 hr, partially purified preparations) all favor proteolytic activity. It is not clear, therefore, what the precipitin properties are of the higher molecular weight species known to be present initially in oat, rye, and pea phytochromes. Figure 11 shows the reactivity of brushite rye phytochrome with oat antibody as examined in a sucrose gradient. Two parallel gradients were monitored after reactions with challenged and unchallenged sera. The difference indicates the degree of reaction with antibody. The main peak (9S) reacts with antibody as does the polypeptide (4–5S) fraction which appears as a shoulder near the position of the bovine serum albumin marker. Figure 12 shows the immunodiffusion pattern of purified rye phytochrome (9S) cross-reacted with oat phytochrome (4–5S). A confluence of the two lines indicates that the class of antibodies elicited from the oat preparation is available in the 9S species. Replicate samples of the rye phytochrome sample were examined by SDS polyacrylamide electrophoresis after 43 hr at 25 C. No indication of proteolytic activity was evident.

It is possible to prepare trypsin-treated rye samples which have similar chromatographic and sedimentation (4–5S) properties to purified oat phytochrome. Partially purified rye phytochrome was treated with 0.075% (w/w phytochrome) trypsin (Worthington) for 20 hr at 4 C, as Pr and isolated on a sucrose gradient (17). Figure 13 shows the immunodiffusion pattern of this rye fraction reacted with oat phytochrome polypeptide(s), and Figure 14 shows the immunodiffusion pattern of the 4 to 5S rye fraction reacted with 9S rye phytochrome. In Figures 12, 13, and 14, confluence of the precipitin lines indicates a

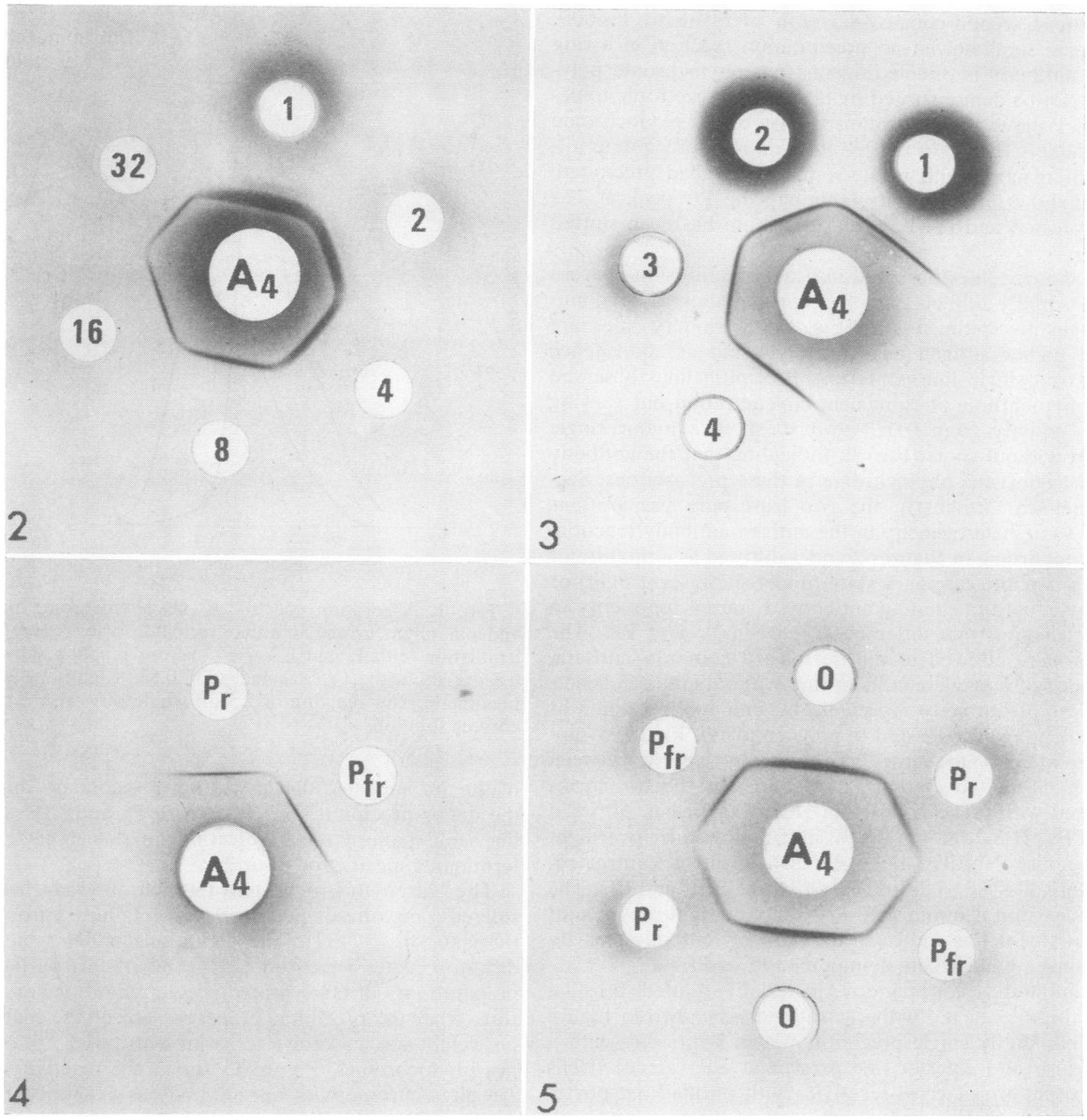

FIG. 2. Stained immunodiffusion plate of serially diluted purified oat phytochrome polypeptide(s). Well numbers indicate dilutions. Concentrations were 5.85 $\Delta(\Delta A)$/ml (1) to 0.183 $\Delta(\Delta A)$/ml (32) with specific activity 1.140. Plate was in NaPB and run for 48 hr. A_4 designates whole sera.

FIG. 3. Stained immunodiffusion plate of oat phytochrome preparations of varying purity. All wells had a phytochrome concentration of 0.5 $\Delta(\Delta A)$/ml. Specific activity was 1.19 (1), 0.33 (2), 0.15 (3), and 0.023 (4). Plate was in NaPB-saline and run for 40 hr. A_4 designates whole sera.

FIG. 4. Stained immunodiffusion plate of purified oat phytochrome polypeptide(s) cross-reacted as Pr and Pfr. Phytochrome concentration was 0.6 $\Delta(\Delta A)$/ml with specific activity 1.190. A_4 designates whole sera.

FIG. 5. Stained immunodiffusion plate of glutaraldehyde-reacted oat phytochrome. Purified oat phytochrome polypeptide(s) (O) were 0.75 $\Delta(\Delta A)$/ml with specific activity 1.090. Pr and Pfr designate samples reacted as Pr and Pfr with 0.1% glutaraldehyde for 30 min. Plate was in NaPB and run for 36 hr. A_4 designates whole sera.

similar class of antibody against rye, as that directed against the oat polypeptide(s), with no detectable difference between 9S and 4 to 5S rye phytochrome.

DISCUSSION

The high titers of rabbit antibody obtained with oat phytochrome polypeptide(s) provide an easy assay for the protein. The detection limit with a direct precipitin reaction (ring test) is about 0.5 to 0.2 μg phytochrome/ml. This assay offers 2- to 5-fold greater sensitivity than the conventional detection of the protein (about 1 μg/ml) with a Ratiospect R-2 spectrophotometer. It is clear, as measured by a direct photoassay of the immunoprecipitate (Fig. 1), that antibody is directed against phytochrome. Recognition is even extended to glutaraldehyde-modified phytochrome, as indicated in Figure 5. The precipitin system examined with immunodiffusion shows mainly a single precipitin line (Figs. 2 and 3), with this class of antibodies directed against both Pr and Pfr (Fig. 4). This observation confirms the report of Hopkins and Butler (19), who also found confluence of lines of Pr and Pfr in double diffusion tests using antibody also elicited against oat polypeptides. They were,

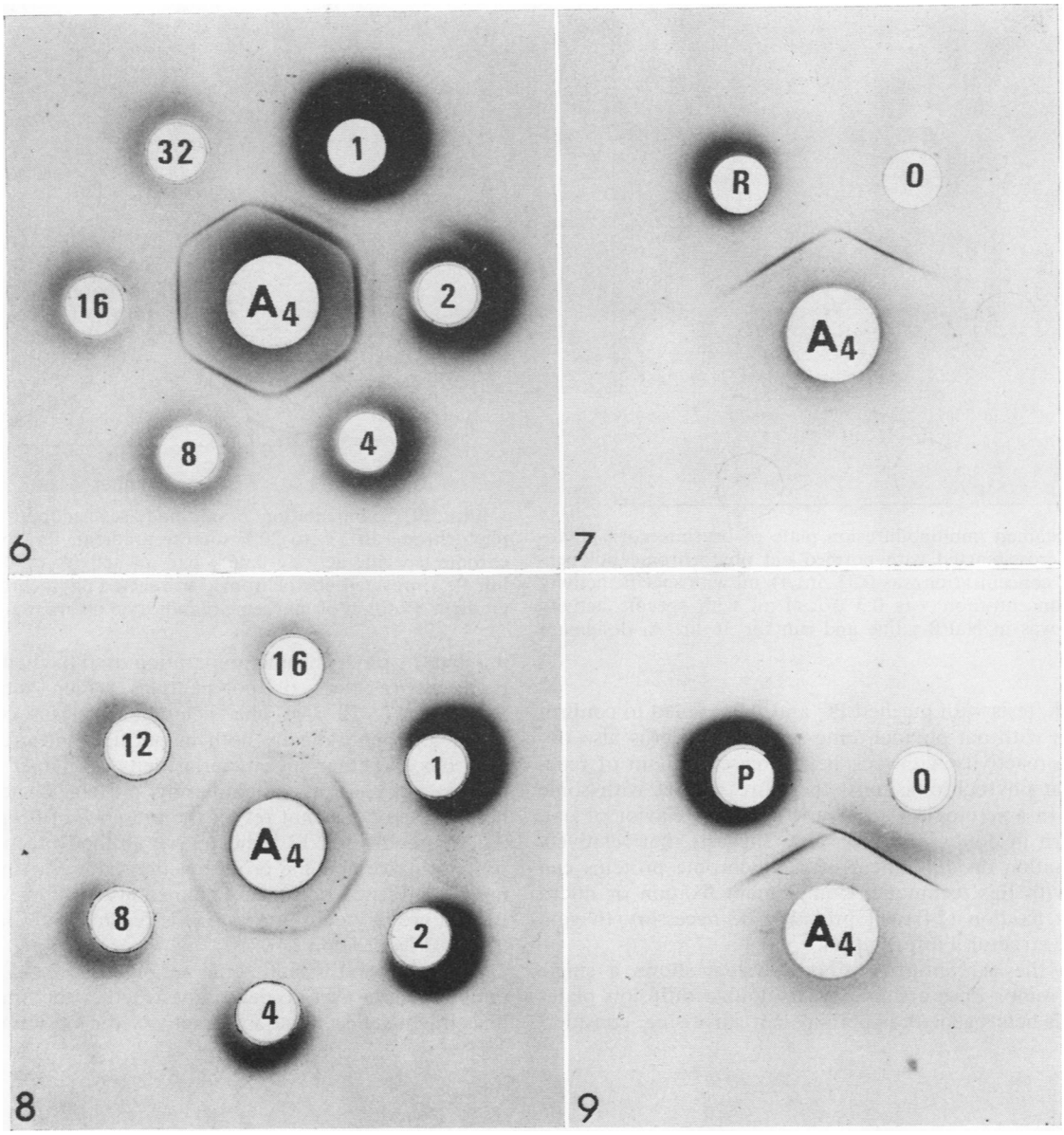

FIG. 6. Stained immunodiffusion plate of serially diluted brushite rye phytochrome. Well numbers indicate dilutions. Concentrations of phytochrome were 3.6 $\Delta(\Delta A)$/ml (1) to 0.11 $\Delta(\Delta A)$/ml (32) with specific activity 0.057. Plate was in NaPB-saline and run 36 hr. A_4 designates whole sera.

FIG. 7. Stained immunodiffusion plate of brushite rye phytochrome (R) cross-reacted with purified oat phytochrome polypeptide(s) (O). R concentration was 0.85 $\Delta(\Delta A)$/ml with specific activity 0.057; O concentration was 0.60 $\Delta(\Delta A)$/ml with specific activity 1.190. Plate was in NaPB-saline and run for 36 hr. A_4 designates whole sera.

FIG. 8. Stained immunodiffusion plate of diluted brushite pea phytochrome. Dilutions are indicated by the well numbers. Concentrations of phytochrome were 0.8 $\Delta(\Delta A)$/ml (1) to 0.050 $\Delta(\Delta A)$/ml (16) with specific activity 0.0625. Plate was in NaPB-saline and run for 48 hr. A_4 designates whole sera.

FIG. 9. Stained immunodiffusion plate of brushite pea phytochrome (P) cross-reacted with purified oat phytochrome polypeptide(s) (O). P concentration was 0.8 $\Delta(\Delta A)$/ml with specific activity 0.0625; O concentration was 0.75 $\Delta(\Delta A)$/ml with specific activity 1.190. Plate was in NaPB-saline and run for 36 hr. Blur near antigen well is an artifact of washing. A_4 designates whole sera.

however, able to detect differences between the two forms using micro complement fixation (19).

Cross reactions of oat phytochrome polypeptide(s) with partially purified extracts of rye (Fig. 7) and corn phytochrome (Fig. 10) indicate a high degree of similarity of the latter two proteins with oat. Although all three plants are in the Gramineae, they are not closely related genetically (2). It is even more surprising to detect cross reactivity with pea phytochrome (Fig. 9). The reaction here indicates a partial identity of the classical type (27), although the genetic relatedness only extends to the level of class (2). This property is in contrast to proteins from genetically closely related animals and bacteria which often have quite different antigenic properties (9). Phytochrome appears to resemble the biliproteins PC and APC superficially, only with respect to their chromophore structure. Both proteins show a wide genetic range of cross reactivity and APC shows a single precipitin system (4). Berns (4), in examining cross reactivities of PC, reported a reaction of partially purified oat phytochrome with some anti-PC sera and a reaction of some PC with antisera elicited against the oat preparation. In the

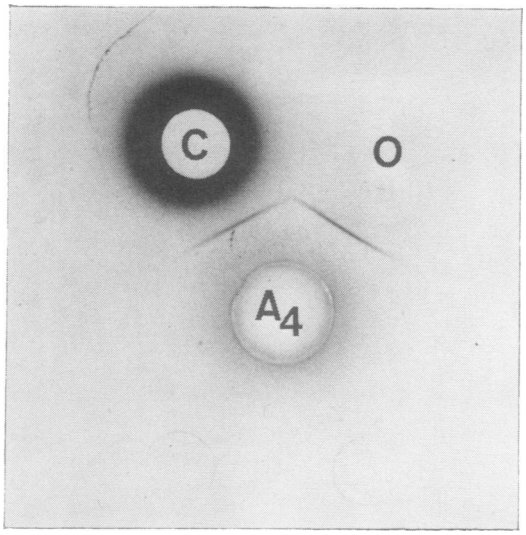

FIG. 10. Stained immunodiffusion plate of brushite corn phytochrome (C) cross-reacted with purified oat phytochrome polypeptide(s) (O). C concentration was 0.33 $\Delta(\Delta A)$/ml with specific activity 0.0058; O concentration was 0.3 $\Delta(\Delta A)$/ml with specific activity 1.190. Plate was in NaPB-saline and run for 36 hr. A_4 designates whole sera.

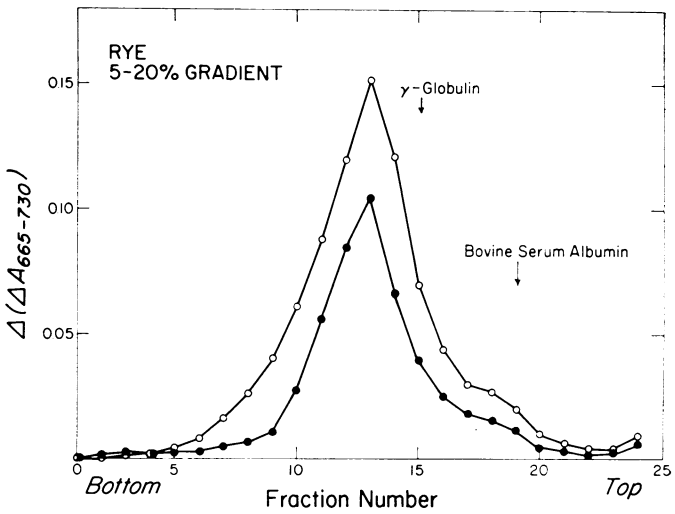

FIG. 11. Sedimentation of antibody-reacted and unreacted rye phytochrome in 5% to 20% sucrose gradient. Rye brushite phytochrome (specific activity 0.068) had an activity of 0.97 $\Delta(\Delta A)$/0.5 ml. ○: Unreacted phytochrome; ●: reacted phytochrome in parallel gradient. Position of markers are indicated by arrows.

present work, tests with purified PC and APC failed to confirm any reaction with oat phytochrome sera. Since Berns also obtained some reactivity with jack bean urease, a claim of reaction with oat phytochrome must therefore be met with some skepticism. In a serological sense, the similar behavior of oat, rye, and corn in double diffusion tests suggests that relatively little information on differences of phytochrome proteins can be gained with this technique. Complement fixation or micro complement fixation (24) will probably be necessary to gain comparative structural information.

Although the oat antigen-antibody system shows a single major line, minor lines are present in double diffusion plates (Fig. 3). This heterogeneity is perhaps not surprising, considering that the phytochrome preparation used to elicit the antibody represents a class of polypeptides which retain photoactivity (6, 17, 32–34). This heterogeneity was confirmed immunoelectrophoretically both in purified and crude (brushite) fractions. Whether such heterogeneity is in fact phytochrome heterogeneity or a contaminating protein is not clear. The heterogeneity does not reflect the presence of 9S phytochrome. As Figures 11 and 12 indicate, oat antibody recognizes the 9S protein in rye and the cross reaction shows a single confluent line. Confluence of precipitin lines also occurs when trypsin-treated rye phytochrome (4–5S) is reacted with oat (4–5S) and 9S rye (Figs. 13 and 14).

The immunodiffusion series in Figures 12, 13, and 14 was undertaken, in part, to determine whether the minor precipitin lines might reflect proteolytic activity during immunodiffusion.

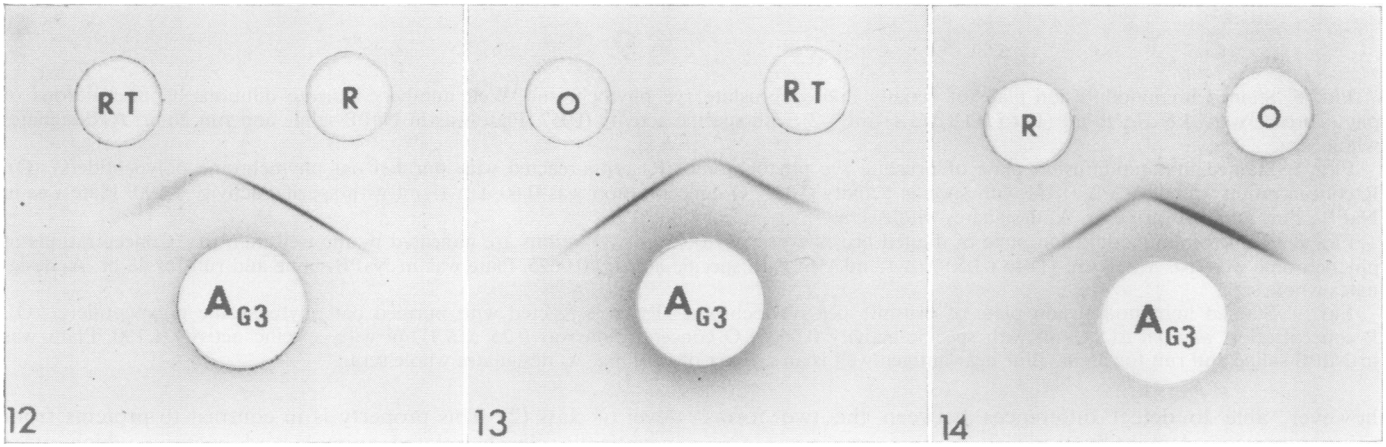

FIG. 12. Stained immunodiffusion plate of purified oat phytochrome polypeptide(s) (O) cross-reacted with purified rye phytochrome (R). O concentration is 1.53 mg/ml with specific activity 1.135. R concentration is 1.82 mg/ml with specific activity 0.695. Plate was run in NaPB-saline for 43 hr. A_{G3} designates globulin fraction of whole sera A_3.

FIG. 13. Stained immunodiffusion plate of purified oat phytochrome polypeptide(s) (O) cross-reacted with sucrose gradient-isolated trypsinized rye polypeptide(s) (RT). O concentration is 0.23 $\Delta(\Delta A)$/ml and RT is 0.1 $\Delta(\Delta A)$/ml. Plate was run in NaPB-saline for 48 hr. A_{G3} designates globulin fraction of whole sera.

FIG. 14. Stained immunodiffusion plate of sucrose gradient-isolated rye phytochrome (R) cross-reacted with gradient-isolated trypsinized rye polypeptide(s) (RT). R concentration is 0.1 $\Delta(\Delta A)$/ml and RT concentration is 0.1 $\Delta(\Delta A)$/ml. Plate was in NaPB-saline and run for 48 hr. A_{G3} designates globulin fraction of whole sera A_3.

Consideration of a multispecific antigen system would suggest that multiple precipitin lines could be detected in immunodiffusion where different polypeptide chains carried two or more separate specificities. Such multiple precipitin lines should show confluence with an undegraded polypeptide chain carrying the separate specificities (27, 29). Such a reaction probably has not occurred in the immunoprecipitin system described here. Where the relative state of the antigens has been carefully monitored, multiple lines have not been detected (Figs. 12–14). It is more likely that multiple precipitin lines in partially purified extracts (Fig. 3) indicate the presence of a heterologous protein in the oat preparations, but proteolytic activity cannot be completely excluded. Proteolysis is not generally discussed (21, 27) as a complicating factor in assessing immunodiffusion patterns, but consideration should be given to its presence in future immunochemical studies of phytochrome. Its possible effects were mitigated by the simple precipitin system which was obtained here with 4 to 5S oat phytochrome polypeptides, but there is no reason to believe such a simple precipitin system will hold for the native 9S phytochrome. Clearly experiments with antibody obtained against 9S phytochrome are highly desirable.

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