Comparative Immunochemistry of Phytochrome

Received for publication July 5, 1972

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ABSTRACT

Partial purified high molecular weight preparations of phytochrome, estimated to be close to 440,000 molecular weight based upon chromatography through a calibrated Bio-Gel P-300 column, were obtained from Garry and Newton oats (Avena Sativa L., cv. Garry and cv. Newton), rye (Secale cereale L., cv. Balbo), barley (Hordeum vulgare L., cv. Harrison), and pea (Pisum sativum L., cv. Alaska) by a sequence of three chromatographic steps: brushte, diethylaminoethyl cellulose, and Bio-Gel P-300. No significant differences were observed between these preparations during purification or subsequent handling. In addition, a low molecular weight form of phytochrome was purified from Garry oats. Two specific antisera against a low molecular weight form of phytochrome (60,000 molecular weight) obtained from etiolated Garry oat seedlings are characterized and used to compare the phytochrome preparations. Double diffusion assays indicated antigenic identity between all preparations except that pea phytochrome yielded a spur when compared to oat phytochrome. Micro complement fixation assays yielded complete identity between Garry and Newton oat phytochrome, reduced activity with rye and barley phytochrome, and a complete lack of activity with pea phytochrome at the serum dilutions assayed. Immunozonelectrophoretic assays indicated that all high molecular weight phytochrome preparations were homogeneous by this criterion and that there were only slight differences between the preparations in electrophoretic mobility. Large and small forms of phytochrome isolated from Garry oats were found to be very similar antigens when tested with the anti-small phytochrome sera, although the small form was observed to electrophorese at a much slower rate than the large.

Several laboratories have isolated and characterized phytochrome preparations from a variety of etiolated plant tissues (7, 13, 15, 18, 22) since the early report of Siegelman and Filer (21). However, no molecular function for this morphogenic chromoprotein has yet been established and, except for recent use of specific antisera against phytochrome, the only quantitative and qualitative assay for its presence has been derived from its unique photoreversible absorption characteristics (5). Recently, however, immunological techniques have been used to detect protein conformational differences between Pr and Pfr (13), to localize the chromoprotein in situ by immunocytochemical techniques (17), to examine the relationship between two molecular weight forms of the pigment isolated from Garry oats (10), and to compare the antigenic properties of oat, rye, corn, and pea phytochrome by double diffusion assays (19). I began the work described here to extend the use of specific antisera against phytochrome so that they may serve as an assay system to supplement the information obtained through the use of the spectral assay.

In addition to the development of a second molecular assay for phytochrome, it seemed desirable to attempt to resolve some of the discrepancies regarding the properties of phytochrome in vitro (see ref. 2 for discussion) resulting from published reports from a number of laboratories (4, 6, 9, 15, 18, 22, 23), each of which has generally used a different purification protocol and often a different etiolated plant material for extraction. Hence, the second purpose of this paper is to characterize phytochrome preparations from a variety of etiolated plant tissues prepared by the same protocol in the same laboratory and to use specific antisera against phytochrome to compare these preparations immunologically. Since it is now clear that phytochrome is quite susceptible to proteolysis during the course of purification and that this susceptibility has led to many of the reported discrepancies (11), I have characterized the preparations used in this work in detail so that the form of phytochrome with which I am working will be well defined.

MATERIALS AND METHODS

Preparation of Antisera. Two antisera ("G" and "W") against a low molecular weight form of Garry oat (Avena sativa L., cv. Garry) phytochrome were prepared by injecting two rabbits with highly purified preparations of an approximately 60,000 molecular weight form of the chromoprotein as described earlier (17). The initial injections were given under dim green safelights with one rabbit receiving Pr ("G") and the other Pfr ("W").

Preparation of Phytochrome. All etiolated tissue used for phytochrome extraction was grown at 25 C and near saturating relative humidity on pads of moist cellulose packing material in plastic cafeteria trays. Garry oat, Newton oat (Avena sativa L., cv. Newton), rye (Secale cereale L., cv. Balbo), and barley (Hordeum vulgare L., cv. Harrison) seedlings were harvested on the 4th day by cutting off the shoots just above the seeds with Disston cordless electric grass shears. Pea (Pisum sativum L., cv. Alaska) shoots were harvested on the 6th day, and additional distilled water was then added to the cellulose packing material to permit further growth and a second crop was harvested on the 9th day. The harvested tissue was frozen in 200-g lots and stored at -20 C until extracted.

All handling of the tissue and subsequent phytochrome-containing extracts was done under dim green light obtained by wrapping green fluorescent tubes with two layers of dark green plastic (Cinemoid No. 24, Kiegl Bros., New York). Whenever possible, the extracts were left in total darkness even though the green safelight did not produce detectable levels of Pfr even after long exposures.

Frozen plant tissue was extracted and chromatographed through a brushite column as described by Hopkins and Butler.
The precipitate was removed from the column by adjusting the Tris-Cl buffer to pH 8.6, then dissolving the precipitated material with a final concentration of 0.1M sodium phosphate buffer, pH 7.8. The sample was maintained at 3 liter/hr in order to obtain maximal yields of the high molecular weight form of phytochrome (6). Phytochrome-containing fractions were concentrated and purified by precipitating with either 200 g/l or 240 g/l ammonium sulfate (see Table 1) while maintaining the pH near 7.8 with 3M tris. The precipitate was dissolved in 10 mM tris-Cl, pH 7.4, with 10 mM KCl and then frozen overnight at −20 °C. Total elapsed time to this stage of purification was usually about 10 to 12 hr.

On the 2nd day, the sample was clarified by centrifugation, desalted by passage at a flow rate of 300 ml/hr through a Sephadex G-26 column (2.4 cm × 55 cm), previously equilibrated with 10 mM tris-Cl, pH 7.4, containing 10 mM KCl, and loaded onto and eluted from a DEAE-cellulose (1 meq/g) column (2.4 cm diameter, length = 7 cm/kg tissue initially extracted) as described by Rice et al. (20), except that mercaptoethanol was not used. The 240 g/l ammonium sulfate precipitate was further purified by precipitating with either 200 g/l or 240 g/l ammonium sulfate (see Table 1) while maintaining the pH near 7.8 with 3M tris. The precipitate was dissolved in 10 mM tris-Cl, pH 7.4, with 10 mM KCl and then frozen overnight at −20 °C. Total elapsed time to this stage of purification was usually about 10 to 12 hr.

The agar medium was 10 mM potassium phosphate buffer, pH 7.7, with 0.15 N NaCl, which was made 1.0% (w/v) with Noble agar and 0.02% (w/v) with sodium azide. Holes were 2 mm diameter with centers 7 mm apart. Since the plates were approximately 1 mm thick, a volume of 3 µl was required to fill each well. Antigen was added at a concentration of 0.2 to 0.4 units/ml, and antiserum was added undiluted. The plates were incubated overnight at 4 °C. Excess protein was removed by diffusion into 0.15 N NaCl, and the precipitin bands were stained with Coomassie blue (24).

Immuno-electrophoresis. Immuno-electrophoresis was performed with the LKB immunoelectrophoresis apparatus (LKB 6800A) as described in their accompanying operating manual. Agarose (1% w/v) plates were prepared from a diethylbarbiturate buffer (pH 8.6, ionic strength 0.1) containing 50 mM sodium acetate and 0.02% sodium azide (w/v). Electrolysis of the antigens (approximately 1.5 µl at a concentration of 1 to 15 µl/ml) was performed at 350 volts (125 cm) for 2 hr at 4 °C. After 10 min, the solution was added under green light with diffusion taking place in darkness at 4 °C. The plates were washed with free of excess protein by diffusion into 0.15 N NaCl and then stained with Coomassie blue (24).

Micro Complement Fixation. Micro complement fixation assays were performed as described by Levine and Van Vunakis (14). All handling of the assay mixtures from the addition of phytochrome to the final centrifugation of unlysed erythrocytes was done under green light.

RESULTS

Characterization of Phytochrome Preparations. Phytochrome preparations typically exhibited two peaks of activity when chromatographed through Bio-Gel P-300 (Fig. 1). Estimation of the molecular weights of these two fractions from a standard curve prepared by plotting log molecular weights of ferritin, catalase, γ-globulin, and bovine serum albumin as a function of Ks, yields values of approximately 440,000 for the high molecular weight fraction (large phytochrome) and approximately 240,000 for the low molecular weight fraction. The absorbance ratio (A450 nm/Asm), with phytochrome present as Pr will be used as an estimate of purity. All absorbance measurements were made with a Shimadzu MPS-50L recording spectrophotometer using a 10 mm cuvette cooled with circulating ice water or, for absorbance values in excess of 3, with an uncooled 2 mm pathlength cuvette.

Molecular Weight Estimation with Bio-Gel P-300. Blue dextran (Pharmacia) and tryptophan were added to phytochrome solutions before placing them on the P-300 column so that accurate determinations of the relative elution volumes (Vs) of active peaks, relatively independent of bed geometry, could be determined (25). The column was calibrated by determining Ks values for horse spleen ferritin (GBC), catalase (Calbiochem 21901), human 7S γ-globulin (Calbiochem 345872), and bovine serum albumin (Sigma A-4378), again using blue dextran as a measure of Vs and tryptophan as a measure of Vs. Molecular weights were taken to be 67,000 for a bovine serum albumin; 160,000 for γ-globulin; and 240,000 for catalase. Since ferritin elutes at virtually the same volume as apoferritin (1), its molecular weight is plotted as 450,000.

Ouchterlon Double Diffusion. Agar plates were prepared with LKB immunoelectrophoresis equipment (LKB 6800A) as described in their accompanying operating manual. The agar medium was 10 mM potassium phosphate buffer, pH 7.7, with 0.15 N NaCl, which was made 1.0% (w/v) with Noble agar and 0.02% (w/v) with sodium azide. Holes were 2 mm diameter with centers 7 mm apart. Since the plates were approximately 1 mm thick, a volume of 3 µl was required to fill each well. Antigen was added at a concentration of 0.2 to 0.4 units/ml, and antiserum was added undiluted. The plates were incubated overnight at 4 °C. Excess protein was removed by diffusion into 0.15 N NaCl, and the precipitin bands were stained with Coomassie blue (24).

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Micro Complement Fixation. Micro complement fixation assays were performed as described by Levine and Van Vunakis (14). All handling of the assay mixtures from the addition of phytochrome to the final centrifugation of unlysed erythrocytes was done under green light.

Abbreviation: DEAE: diethylaminoethyl.

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90,000 for the low molecular weight fraction (small phytochrome) (Fig. 2).

Absorption spectra between 550 and 800 nm of the two molecular weight species from Garry oat seedlings in the red-absorbing form are indistinguishable. Absorption maxima for Pr are at 667 nm for oat and pea preparations and 664 nm for rye and barley preparations.

Although not apparent in Figure 1 because of absorption at 667 nm by blue dextran, I have repeatedly observed a third small peak or shoulder of phytochrome activity even closer to the void volume on the P-300 column than that resulting from large phytochrome (Fig. 3). This shoulder or peak of activity close to V₀ has been verified to be phytochrome by measurements of photoreversibility at 667 nm and 723 nm using a dual wavelength difference spectrophotometer.

A summary of the purification and yield data for the phytochrome preparations used in these immunological assays is presented in Table I. Both the total number of units recovered following each chromatographic step and the absorbance ratio of each pool are tabulated. In addition, the concentration of ammonium sulfate used in the preliminary concentration step following brushite chromatography is given along with the relative yields of the two sizes of the chromoprotein separated on the P-300 column. Several entries are estimated values because the active fractions from the P-300 column were not pooled. Values were estimated by averaging the measurements of individual fractions. No significant differences were observed during the course of purification in the behavior of phytochrome extracts from the different plant sources used. The Kₐr for the first phytochrome peak was the same regardless of the source of the phytochrome; accurate measurements of Kₐr values for the second peak could not be made in most cases because there was very little photoactivity present.

Double Diffusion Assays. The specificity of both antisera against small phytochrome is evident in Figure 4a in which Garry oat phytochrome preparations of different purities but equal concentrations are diffused against the two sera. Only single, confluent precipitin bands are seen indicating that the sera recognize only phytochrome in the plant extracts. Figure 4a also demonstrates that the sera recognize both large and small Garry oat phytochrome with complete identity since no spur is visible.

The remaining plates in Figure 4 compare large molecular weight Garry oat phytochrome with large phytochrome from other plant sources; complete identity is seen in all assays except for that with pea phytochrome where an obvious spur is seen (Fig. 4e). I have also compared Pr and Pfr (both large

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Table I. Summary of Phytochrome Purification from a Variety of Plant Tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Garry oats</th>
<th>Garry oats</th>
<th>Newton oats</th>
<th>Rye</th>
<th>Barley</th>
<th>Pea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue extracted (kg)</td>
<td>5.8</td>
<td>4.0</td>
<td>2.0</td>
<td>5.0</td>
<td>6.0</td>
<td>8.3</td>
</tr>
<tr>
<td>Concentration of first (NH₄)₂SO₄ cut (g/l)</td>
<td>240</td>
<td>200</td>
<td>240</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Brushite pool</td>
<td>102/0.036¹</td>
<td>48/0.056</td>
<td>18/0.019</td>
<td>25/0.022</td>
<td>8.0/0.0013</td>
<td>42/…</td>
</tr>
<tr>
<td>DEAE pool</td>
<td>54/0.091</td>
<td>17/0.167</td>
<td>7.3/0.044</td>
<td>8.5/…</td>
<td>4.7/0.014</td>
<td>18/0.084</td>
</tr>
<tr>
<td>P-300, large phytochrome pool</td>
<td>14.3²/0.125</td>
<td>3.9²/0.445</td>
<td>3⁴/0.13³</td>
<td>4.3/0.159</td>
<td>1.5/0.071</td>
<td>12/0.179</td>
</tr>
<tr>
<td>P-300, small phytochrome pool</td>
<td>8.3²/0.135</td>
<td>0.8³/ND³</td>
<td>0.5³/0.03⁴</td>
<td>&lt;0.5³/ND</td>
<td>0.5³/ND</td>
<td>0.5³/ND</td>
</tr>
<tr>
<td>Per cent phytochrome eluted as larger form</td>
<td>63</td>
<td>80</td>
<td>86⁴</td>
<td>&gt;90⁴</td>
<td>75⁴</td>
<td>&gt;90⁴</td>
</tr>
</tbody>
</table>

¹ The numerator represents total phytochrome units pooled following the indicated step; the denominator is the absorbance ratio.
² Only 37.5 units from the DEAE-column were applied to the P-300 column.
³ Only 9.7 units from the DEAE-column were applied to the P-300 column.
⁴ Estimated values (see “Results”).
⁵ Not determined.
Fig. 4. a-e: Precipitin patterns on double diffusion plates following incubation at 4°C overnight. The wells were filled once as indicated with antiserum G (G), antiserum W (W), purified large Garry oat phytochrome (L) (absorbance ratio = 0.125), purified small Garry oat phytochrome (S) (absorbance ratio = 0.135), crude Garry oat phytochrome (C) (absorbance ratio = 0.036), large Newton oat phytochrome (N) (absorbance ratio = 0.13), large rye phytochrome (R) (absorbance ratio = 0.159), large barley phytochrome (B) (absorbance ratio = 0.071), and large pea phytochrome (P) (absorbance ratio = 0.179).

Fig. 5. a-e: Immunoelectrophoretic patterns of phytochrome preparations from different plant tissues. Conditions were as described in “Materials and Methods.” Antigen wells were filled as indicated (symbols are the same as those defined in Fig. 4). Antiserum G was placed in the troughs following electrophoresis.

and small Garry oat phytochrome in all possible combinations) on double diffusion plates and have never detected a spur.

Immunoelectrophoresis. Immunoelectrophoretic assays of both large and small phytochrome yield a single precipitin band again indicating that the antiserum used is specific for phytochrome (Fig. 5a). Electrophoresis of large phytochrome yields a single precipitin band which indicates that this form of the antigen is relatively homogeneous by this criterion. However, electrophoresis of small phytochrome yields a precipitin band which suggests the presence of at least two electrophoretically distinct but immunochemically indistinguishable forms of the chromoprotein (Fig. 5a). Comparison of the two precipitin bands also demonstrates that the larger form of the chromoprotein electrophoreses more rapidly than the smaller form under these conditions.

Comparisons of the electrophoretic mobilities of large phytochrome preparations from the different plant sources used indicate only minor differences. Repeated observations verify that rye, barley, and Newton oat phytochrome all electrophorese at slightly faster rates than Garry oat phytochrome (Fig. 5, b–d), whereas pea phytochrome electrophorese at about the same rate (Fig. 5e).

Micro Complement Fixation. A comparison of the complement fixing activity of large and small Garry oat phytochrome (Fig. 6) indicates that higher concentrations of the large form of the chromoprotein, at least in terms of absorbance units, are required in order to get maximal activity. However, no significant difference in maximal activity of the two forms has been observed.

Large Garry oat and large Newton oat phytochrome are indistinguishable antigens in the micro complement fixation assay, while both large rye and large barley phytochrome exhibit decreased activity, although they both have maximal activity at the same ratio of antibody to antigen (Fig. 7). Only in the case of pea phytochrome is there no evidence for com-
plement fixing activity (Fig. 7), even though as much as $50 \times 10^4$ units of phytochrome and a doubled concentration of antiserum have been assayed (results not shown).

In order to determine whether there are immunoglobulins which recognize determinants on oat phytochrome which are absent from pea phytochrome, an excess of the latter (0.01 units) was added to the assay mixture 1 hr prior to the addition of Garry oat phytochrome. The resulting complement fixation curve (Fig. 8) verifies that there are immunoglobulins present in the antiserum which do not bind or which bind very weakly with pea phytochrome and which yield complement fixing activity when oat phytochrome is added. Comparable assays performed in the presence of excess rye and barley phytochrome exhibit no activity (Fig. 8).

**DISCUSSION**

Since recent reports have suggested both that phytochrome may be significantly degraded by proteases without any apparent loss of activity as measured spectrophotometrically (11) and that a protease is present in crude phytochrome-containing extracts of grass seedlings (16), the protocol used to obtain purified solutions of phytochrome for this study incorporates several suggestions made by Gardner et al. in order to minimize proteolytic degradation during the course of purification (11). The use of mercaptoethanol was omitted after the brushite step, a lower concentration of ammonium sulfate was used in the initial ammonium sulfate fractionation (200 g/l instead of 240 g/l), and a general decrease in the time required to carry out the protocol was effected. With respect to the latter point, specific changes included operation of the brushite column at a flow rate of 3 liters/hr instead of the previously used rate of 1 liter/hr, replacement of dialysis by rapid passage through Sephadex G-25, and storage at $-20$ C overnight, again instead of extended dialysis. None of the latter alterations were observed to change the yield or degree of purification.

Comparison of the data for two Garry oat preparations utilizing different ammonium sulfate concentrations (Table I) verifies the marked increase in absorbance ratio of the large phytochrome fraction which may be obtained using the lower salt concentration (6, 11). In addition, relatively high yields of large Garry oat phytochrome may be obtained if sufficient speed is used during isolation so that relatively pure preparations of the large form of the chromoprotein, hopefully undergraded by a protease, may be obtained, even with the same oat cultivar which has previously been used to obtain highly purified low molecular weight phytochrome preparations (13, 15, unpublished).

An examination of the results obtained during the purification of phytochrome from several sources using the same protocol (Table I) does not reveal any significant qualitative differences between the different preparations. The comparable behavior of the preparations at each step indicates that they have chemically similar protein moieties and their identical elution volumes from the P-300 column indicate that they are quite similar in size. The low yield of phytochrome from the brushite column starting with rye and barley shoots is apparently a reflection of the low activities in the initial extracts resulting from a different distribution of phytochrome throughout the shoot than is the case with oat seedlings. The highest phytochrome concentrations in oat shoots are found near the shoot apex and near the tip of the coleoptile (3, 17), whereas the highest concentration in rye and barley shoots is at the shoot apex (3, 8, Pratt and Coleman, manuscript in preparation). In the case of oat shoots, the apex is well separated from the scutellum and both regions of high phytochrome concentration are thus readily harvested while the shoot apex in rye and barley seedlings is very close to the scutellum and thus not readily harvested (3, and Pratt and Coleman, manuscript in preparation).

A preliminary characterization of the sizes of phytochrome used for this study may be made on the basis of elution values from the P-300 column (Figs. 1 and 3). The large phytochrome fraction has an apparent molecular weight of approximately 440,000, whereas the small phytochrome fraction has an apparent molecular weight of approximately 90,000 (Fig. 2). These two fractions are similar to those reported by Correll and Edwards (6), Briggs et al. (4), Rice and Briggs (18), and Gardner et al. (11). As suggested by Gardner et al., the large phytochrome fraction is probably equivalent to the 9S component of Correll et al. It is possible that the minor component which elutes before the large phytochrome fraction (Fig. 3) is equivalent both to the 14S component of Correll et al. (9) and the higher molecular weight fraction reported by Gardner et al. (11) to be present in very pure rye phytochrome extracts based on preliminary ultracentrifugal studies. This minor component has never been detected in more than trace quan-
tity and its relationship to the other forms of phytochrome is unknown.

In order to determine the relationship between the small phytochrome fraction described here and the 60,000 mol wt preparations described by others (13, 15) and also obtained from Garry oat seedlings in my laboratory (unpublished), I rechromatographed a portion of the small phytochrome pool from the preparation described in Figure 1 on a Bio-Gel P-150 column immediately after calibrating the column with bovine serum albumin, in both cases using blue dextran and tryptophan as markers. Identical Kav values for the two proteins were obtained, thus suggesting that the small phytochrome fraction reported here is not as different as might have been anticipated from that characterized earlier as having a molecular weight of 60,000 based on chromatography through P-150, even though its somewhat anomalous behavior on the two pore size gels remains unexplained. It is possible that the phytochrome had been further degraded by a protease prior to passage through the P-150 column although this possibility was minimized by storage in liquid nitrogen until it was assayed.

Since the antisera described here are quite specific for phytochrome (Figs. 4 and 5), they may be used as a very sensitive assay for phytochrome even in extracts of low specific activity. Using the micro complement fixation assay, maximal activity can be obtained with concentrations of oat phytochrome as low as 0.2 \( \times 10^{-3} \) units/ml (Figs. 6 and 7). Since Hopkins and Butler (13) had previously used this assay to detect a conformational difference between Pfr and Pfr, I attempted to confirm their experiment with both freshly isolated and aged phytochrome preparations using both large and small phytochrome fractions and both serum preparations. These attempts, in addition to repeated double diffusion and immunoelectrophoresis assays, all failed to detect any difference between Pr and Pfr. Rice and Briggs (19) also were unable to detect any difference between Pr and Pfr using double diffusion assays.

As anticipated from the observation of Rice and Briggs (19) that large rye and small oat phytochrome exhibit identity when assayed by double diffusion against antismall oat phytochrome serum, a comparison of large and small Garry oat phytochrome indicates that they are very similar antigens, at least when using antismall phytochrome serum (Figs. 4a and 6). Such a result is not unexpected since small phytochrome is apparently derived from large by cleavage of peptide bonds (11), and hence the same determinants should be present (although not necessarily available to the antibodies) in both forms of the chromoprotein. (Other work to be reported elsewhere [10], however, suggests that the two sizes of phytochrome are distinctly nonidentical antigens when using antilarge phytochrome serum as the assay system, again in agreement with the suggested relationship between the two sizes proposed by Gardner et al. [11].) The slight shift in the complement fixation curve towards higher antigen concentration with large phytochrome (Fig. 6) may be interpreted as a reduced affinity of several determinants for the corresponding immunoglobulins (14) resulting from interference by the nonantigenic primary structure which represents the difference between large and small phytochrome and/or by a general conformational difference between “free” small phytochrome and small phytochrome as incorporated in large phytochrome. It is also possible, however, that the ratio of the number of antigenic determinants to chromophore absorbance is lower for large phytochrome although this seems unlikely since recent evidence suggests that small phytochrome is the only fragment obtained from the proteolytic cleavage of large phytochrome which is associated with a photoreversible chromophore (10).

One distinct difference can be detected between large and small phytochrome immunochromically, although the difference results from electrophoretic rather than immunological properties. Large phytochrome clearly migrates more rapidly when electrophoresed under the conditions used for the immunoelectrophoretic assay (Fig. 5a), thus providing a rather simple, rapid, and sensitive assay for determining the size of phytochrome present in an unknown sample or for verifying that a preparation is indeed large phytochrome throughout the course of an experiment designed to determine its properties and that it has not degraded to the small form. Such an assay should be of great practical value since other methods for measuring size either require highly purified preparations (e.g., SDS-acrylamide gel electrophoresis) or relatively large amounts of material and relatively long periods of time (e.g., gel exclusion chromatography).

The immunochromical differences observed between phytochrome preparations from different sources suggest a direct relationship between phylogenetic distance and immunochromical dissimilarity. The two cultivars of oats examined are immunochromically indistinguishable in both double diffusion (Fig. 4a), and micro complement fixation (Fig. 7) assays, whereas the other two grasses (rye and barley), although they show identity in the double diffusion assay (Fig. 4c, d), show distinctly decreased activity in the micro complement fixation assay without any shift in the peak. Such a change in the latter assay may be interpreted to suggest that rye and barley phytochrome each possess one or a minority of determinants with decreased affinities for the anti-oat phytochrome immunoglobulins, whereas the majority of the determinants remain unchanged (14).

Pea phytochrome is farthest removed from oat phytochrome phylogenetically and also exhibits the greatest immunochromical difference. Double diffusion assays suggest nonidentity by the presence of a spur (Fig. 4e), and the micro complement fixation assay reveals a total lack of activity even though high concentrations of pea phytochrome were tested (up to 50 \( \times 10^{-4} \) units) as well as a greater concentration of antisera (1/32 dilution). The presence of the spur on the double diffusion plate suggests that one or more determinants present on oat phytochrome are either greatly altered in or absent from pea phytochrome. To test this possibility, micro complement fixation activity between antisera and large Garry oat phytochrome was assayed in the presence of excess pea phytochrome (Fig. 8). The complement fixing activity thus obtained confirms the hypothesis that one or more determinants are either missing from pea phytochrome or are altered greatly. Similar competition experiments utilizing excess rye and barley phytochrome exhibit no activity indicating that they possess all of the determinants found on oat phytochrome as anticipated from the absence of spurs in double diffusion assays.

Rice and Briggs (19) have also recently compared phytochrome preparations from a number of sources using double diffusion assays with an antisera prepared against a low molecular weight form of oat phytochrome. As reported here, they also observed identity between preparations from three different grasses (oat, rye, and corn) and, in addition, found that pea phytochrome exhibited only partial identity with grass phytochrome.

The antisera were also used to compare the electrophoretic mobilities of the different phytochrome preparations and obtain an estimate of their homogeneity. Using large Garry oat phytochrome as a reference in each case, it is evident that all preparations appear homogeneous by this criterion (i.e., each precipitin band is a single arc representing the presence of only one electrophoretic species) and that all have similar electrophoretic mobilities (Fig. 5, b–e). Pea phytochrome migrates at
the same rate as Garry oat phytochrome, whereas Newton oat, rye, and barley phytochrome each migrates slightly, but significantly, faster. The only precipitin band which appears to represent a heterogeneous population of antigens is that resulting from small phytochrome (Fig. 5a), which is not unexpected since Rice and Briggs (18) had earlier reported chemical heterogeneity in their small phytochrome preparations when performing end-group analyses.

*In vitro* immunochromical analysis of different phytochrome preparations does suggest a rather severe limitation in the use of antiphytochrome sera obtained against phytochrome isolated from grass seedlings. It appears likely that such antisera will not prove useful with nonmonocotyledenous plant material. As a consequence, repeated attempts to localize phytochrome in etiolated pea seedlings (unpublished) utilizing an indirect antibody labeling technique (17) have provided only slight success with very poor activity. On the other hand, examination of phytochrome localization in several grass seedlings has been successful (Pratt and Coleman, manuscript in preparation) and preliminary efforts to detect phytochrome in dark-grown orchid embryos has also been successful. However, it seems unlikely that present antisera will be of much use in attempting to localize phytochrome in several plants of interest, most notably *Mougeotia*, in which phytochrome is found to be associated with the plasmalemma by indirect physiological measurements (12), and the many dicotyledenous species, such as *Sinapis alba*, whose phytochrome-related physiology has been studied in great detail.

Acknowledgments—I thank Dr. Sidney Harshman for his assistance in the immunization of the rabbits and the collection of the sera as well as for his expert suggestions, advice, and criticism. This research was supported by National Science Foundation Grant GB-17087.

LITERATURE CITED