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Characterization of a Molecular Modification of Phytochrome That Is Associated with Its Conversion to the Far-Red-Absorbing Form[1]

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ABSTRACT

Phytochrome that has been photoinduced to pellet by irradiation of intact oat (cv. Garry) shoots and recovered from a pellet obtained by centrifugation of crude extracts exhibits modified behavior when compared to soluble phytochrome isolated from shoots that had never been irradiated. This modified behavior includes retarded mobility during sodium dodecyl sulfate polyacrylamide gel electrophoresis (Boeshore ML, LH Pratt 1980 Plant Physiol 66: 500–504). The electrophoretic mobility of several different kinds of phytochrome preparations were examined to study how this modification might arise.

Phytochrome that was extracted in the pelletable condition from red-, far-red-irradiated tissue, but without added divalent cation so that it did not pellet, did not exhibit an altered electrophoretic mobility. Hence, this modification of phytochrome is not required for the expression *in vitro* of pelletability induced *in vivo*. Phytochrome that was extracted in the pelletable condition and in the far-red-absorbing form, but without added divalent cation so that it did not pellet, and phytochrome in the far-red-absorbing form that remained in the supernatant after collection of pellets containing pelleted phytochrome both electrophoresed with reduced mobility. Thus, this modification does not arise as a consequence of phytochrome having been pelleted. Differential sensitivity of phytochrome to different handling conditions also is not the cause of this modification since the far-red-absorbing form of phytochrome, which was extracted in the pelletable condition but by the same protocol used to extract soluble phytochrome, also exhibited reduced mobility. Furthermore, the reduced electrophoretic rate is not due to a simple differential lability of the far-red-absorbing form of phytochrome to extraction conditions, since partially purified soluble phytochrome that was exposed in the far-red-absorbing form to the isolation and extraction conditions used for preparation of soluble phytochrome did not exhibit the alteration.

The data are instead consistent with the more complex interpretation that phytochrome is modified *in vitro* if two conditions are met: (a) that phytochrome is extracted in the far-red-absorbing form or is converted to the far-red-absorbing form in the crude extract soon after extraction and (b), that phytochrome remains in the far-red-absorbing form in the crude extract for at least a brief period.

The possibility that the phytochrome modification studied here might have arisen because of a change in carbohydrate content was tested by periodic acid Schiff staining of sodium dodecyl sulfate polyacrylamide gels. No carbohydrate was detected in any of the phytochrome preparations that were examined. This inability to detect carbohydrate is in direct contrast to the report of Roux *et al.* (1975 Physiol Plant 35: 85–90).

The *in vivo* process that leads to photoinduced phytochrome pelletability is potentially related to initial events in the mechanism of action of phytochrome (13). Satter and Galston (15) and Marmé (8), among others, have proposed that phytochrome pelletability might be the *in vitro* expression of a significant biological interaction between phytochrome and a proposed membrane-bound receptor. No direct experimental evidence has been presented to validate this hypothesis, however.

The induction of R[3]-enhanced pelletability is itself a classical, photoreversible phytochrome-mediated response which occurs within 5 s at 25 C (10, 12, 13). Even though phytochrome pelletability induced *in vivo* might not represent a biologically important interaction between phytochrome and a membrane or membrane-bound receptor, the phenomenon is of interest because it is closely related in time to the primary action of phytochrome.

Earlier we confirmed and extended data (3, 17, 18) that documented a change in the properties of phytochrome that had been associated with and released from a particulate fraction as Pfr (1). This change in phytochrome, which is most easily seen as reduced mobility during SDS polyacrylamide gel electrophoresis, might occur prior to, during, or after the association of phytochrome with particulate material. Thus, phytochrome modification might be required before pelletability can be expressed *in vitro*, or the modification might be generated while phytochrome is associated with the particulate fraction. Alternatively, pelletability and the modification might not be causally related. More trivial explanations of the modification include differential sensitivity of phytochrome to different extraction environments and a differential reactivity of Pr and Pfr to extraction conditions.

An operational description of phytochrome pelletability as induced *in vivo* leads to the definition of multiple phytochrome "species" (Fig. 1). Phytochrome as synthesized by the cell behaves as a soluble protein upon extraction (Pr^{sol}). Upon irradiation of tissue with R, phytochrome is converted to Pfr which, if extracted immediately, also behaves as a soluble protein (Pfr^{sol}) even in the presence of added divalent cation (12, 13). In a rapid (complete within 10 s at 25 C), nonphotochemical process that is dependent upon temperature and phosphorylative energy (12, 13), phytochrome as Pfr is then converted *in vivo* to a form that is potentially pelletable. If tissue containing potentially pelletable phytochrome is extracted in the absence of added divalent cation, Pfr is found in the supernatant after centrifugation (pelletable Pfr or $Pfr^{p'le}$). If, however, this potentially pelletable phytochrome is extracted in the presence of added divalent cation (*e.g.*, 20 mM Mg^{2+}; see ref. 12), most of the phytochrome is found associated with a particulate fraction upon centrifugation (pelleted Pfr or $Pfr^{p'ed}$). The phytochrome that does not pellet in the presence of added divalent cation is then termed supernatant Pfr (Pfr^{sup}). If tissue containing

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[3] Abbreviations: R, red light; FR, far-red light, PAS, periodic acid Schiff.

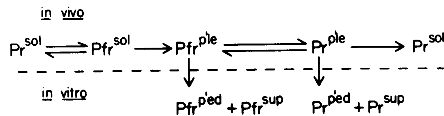

FIG. 1. Operational description as described in text of phytochrome pelletability that is induced *in vivo*.

potentially pelletable Pfr is irradiated with FR and then extracted immediately, the Pr produced is also potentially pelletable. If extracted in the absence of added divalent cation, it does not pellet upon centrifugation (pelletable Pr or $Pr^{p'le}$). If extracted in the presence of added divalent cation, two pools of Pr are obtained. The Pr that is pelleted upon centrifugation is called pelleted Pr ($Pr^{p'ed}$) and that which remains in the supernatant is called supernatant Pr (Pr^{sup}). Following photoconversion of potentially pelletable Pfr to potentially pelletable Pr *in vivo*, Pr is converted back to a form that upon extraction again behaves as a soluble protein, even in the presence of added divalent cation, and is thus called soluble Pr ($Pr^{sol'}$). This nonphotochemical conversion back to a form that behaves as a soluble protein upon extraction occurs with a half life of about 100 min at 4 C (12). $Pr^{sol'}$ is not necessarily identical to Pr^{sol}.

In this paper we examine the different phytochrome preparations defined above to study the cause of the previously documented (1) modification of phytochrome. In particular, we ask whether there is a causal relationship between phytochrome pelletability induced *in vivo* and this phytochrome modification. Since Roux *et al.* (14) reported that phytochrome contains 4% carbohydrate, and since covalently attached carbohydrate residues are known to alter electrophoretic mobilities significantly (2), we also consider the possibility that this phytochrome modification might be related to a change in carbohydrate content.

MATERIALS AND METHODS

Oats, cv. Garry, were grown in darkness at 25 C for 4 to 5 days at near-saturating humidity (9). Freshly harvested shoots were extracted to obtain all labeled phytochrome preparations. Fresh tissue was also extracted to obtain all unlabeled phytochrome preparations, except that frozen tissue was used occasionally for preparation of Pr^{sol}.

Actinic Irradiations. The R source for *in vivo* irradiations is the same as that used previously for irradiation of tissue (1). Tissue was irradiated at room temperature with FR by filtering the output of 30 w prefocused tungsten bulbs (Sylvania, Danvers, MA) spaced 13 cm apart through a 3.2 mm thick broad band filter (Plexiglas FRF-700; Westlake Plastic Co., Lenni, PA) with a short wavelength cutoff at about 700 nm. Photostationary equilibrium was reached in 90 s with the FR source. R and FR for *in vitro* irradiation of phytochrome was obtained by filtering the output of a tungsten lamp (Unitron LKR microscope illuminator, Unitron Instruments Inc., Plainview, N.Y.) through a 660-nm, Balzer B-40 interference filter or through a piece of the Plexiglas filter described above. All preparations were converted to Pr prior to use in all assays except that prior to SDS polyacrylamide gel electrophoresis this conversion to Pr was occasionally not made.

Phytochrome Preparations. Phytochrome was labeled *in vivo* by applying radioisotopes ([^{3}H]leucine and [^{35}S]SO_4^{-2}) to seedlings as described earlier (1).

The extraction medium used for preparation of Pr^{sol}, Pfr^{sol}, $Pfr^{p'le}$, $Pr^{p'le}$, and $Pr^{sol'}$, which does not permit enhanced phytochrome pelletability, is 50 mM Tris-Cl (pH 8.5 at 25 C), 0.2 M 2-mercaptoethanol. For preparation of $Pfr^{p'ed}$, $Pr^{p'ed}$, and Pfr^{sup} the extraction medium is 25 mM *N*-morpholinopropane sulfonic acid-Tris, 14 mM 2-mercaptoethanol, 20 mM $MgCl_2$ (pH 7.5) (1). The addition of $MgCl_2$ permits optimal levels of enhanced pelletability (12). Pr^{sup} was not studied.

Pr^{sol} was prepared by brushite chromatography followed by 0 to 33% ammonium sulfate fractionation as described before for "control phytochrome" (1). Inasmuch as only 10 to 50 g of tissue was extracted for labeled Pr^{sol} preparations, small brushite columns with bed volumes adjusted for the amount of tissue extracted were prepared in 50-ml syringe barrels.

Pfr^{sol} was obtained from tissue that was irradiated with R for 4 s at 0 C in the extraction vessel which was already in place on the homogenizer, and then was extracted immediately for 5 s at 0 C (12). For this irradiation a 15-ml Corex (Corning Glass Works, Corning, NY) centrifuge tube was placed in a plastic ice-bath. Three slide projectors (E. Leitz Inc., Rockleigh, NJ) with 500 w light sources and one Unitron LKR microscope illuminator were placed around the ice bath about 10 cm from the extraction vessel. The outputs of these sources were filtered through Balzers (Liechtenstein) 660-nm B-40, 667-nm B-20, 663-nm B-40, and 664-nm B-40 interference filters. For each extraction procedure 2.5 ml of the extraction medium, prechilled to 0 C, was added to 1.5 g chopped oat shoots. Instantaneously after R irradiation the tissue was extracted with an Ultra Turrax (Tekmar Co., Cincinnati, OH) homogenizer. After homogenization the extract was chromatographed through brushite and fractionated with ammonium sulfate as for Pr^{sol}. Actinic FR was given either before or after the ammonium sulfate fractionation. We observed no difference due to the variability in the time at which FR was given.

$Pfr^{p'le}$ and $Pr^{p'le}$ preparations were obtained from fresh shoots using the same extraction and isolation conditions as those used for Pr^{sol}. Tissue was irradiated for 3 min with R before extraction of $Pfr^{p'le}$ and for 3 min with R followed by 3 min with FR before extraction of $Pr^{p'le}$.

$Pr^{sol'}$ was extracted and purified as for Pr^{sol} from tissue that was first irradiated for 3 min with R followed by 3 min with FR and then incubated for 4 h in darkness at 4 C (12).

$Pfr^{p'ed}$ was released from the particulate fraction obtained by centrifugation of crude extracts of R-irradiated shoots in the presence of $MgCl_2$ as described earlier (1). $Pr^{p'ed}$ was extracted and isolated in the same way except that tissue was given 3 min FR immediately after R treatment and before extraction.

Pfr^{sup} was obtained from the soluble phytochrome fraction remaining in the supernatant following extraction of R-irradiated shoots and centrifugation of the extracts in the presence of $MgCl_2$. Pfr^{sup} in the supernatant of this first centrifugation after extraction

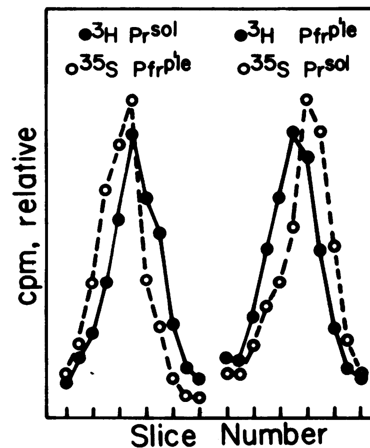

FIG. 2. Radioactivity profiles of Pr^{sol} and $Pfr^{p'le}$ monomer bands taken from two cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 6000 cpm of each isotope were applied to each gel. Slices were 0.6 mm thick. In both cases the origin is to the left. Radioactivity in peak slices was as follows: 262 cpm for [^{3}H]Pr^{sol} and 378 cpm for [^{35}S]$Pfr^{p'le}$ in profiles at left; 172 cpm for [^{3}H]$Pfr^{p'le}$ and 277 for [^{35}S]Pr^{sol} in profiles at right.

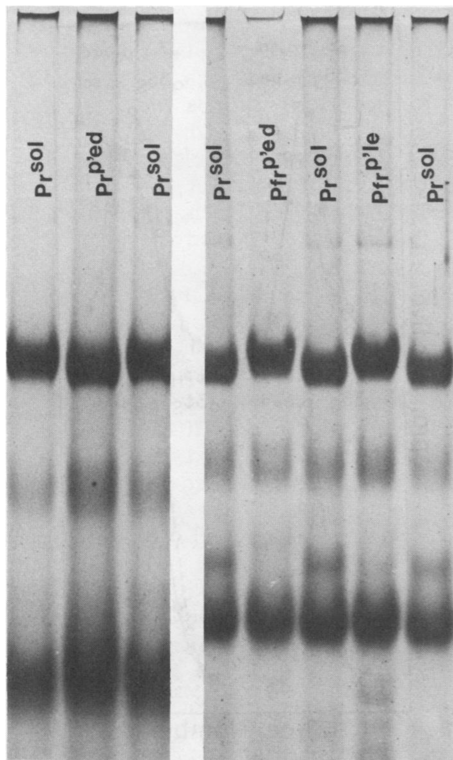

FIG. 3. Photograph of SDS 5% polyacrylamide gel slabs after electrophoresis of immunoprecipitates of Pr^{sol}, $Pfr^{p'ed}$, $Pfr^{p'le}$, and $Pr^{p'ed}$. The 118,000-dalton phytochrome monomer is located near the middle, the 50,000-dalton immunoglobulin heavy chain near the bottom. The origin is at the top. The first three lanes are from one slab and the remaining five lanes are from a second slab.

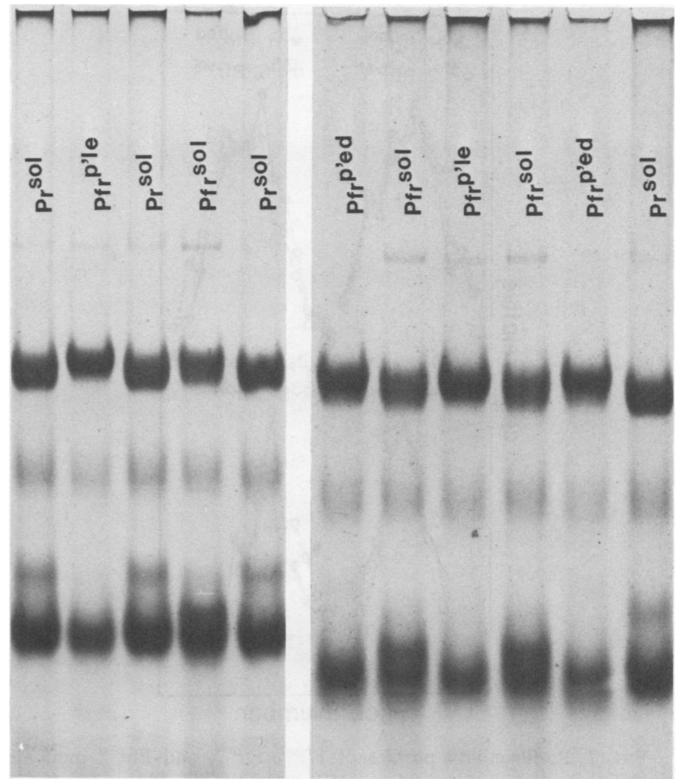

FIG. 5. Photograph of SDS 5% polyacrylamide gel slabs after electrophoresis of immunoprecipitates of $Pfr^{p'ed}$, $Pfr^{p'le}$, Pfr^{sol}, and Pr^{sol}. The 118,000-dalton phytochrome monomer is located near the middle, the 50,000-dalton immunoglobulin heavy chain near the bottom. The origin is at the top. The first five lanes are from one slab and the remaining six lanes are from a second slab.

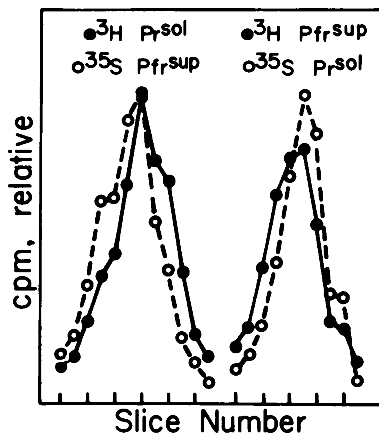

FIG. 4. Radioactivity profiles of Pr^{sol} and Pfr^{sup} monomer bands taken from two cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 6000 cpm of each isotope were applied to each gel. Slices were 0.6 mm thick. In both cases the origin is to the left. Radioactivity in peak slices was as follows: 216 cpm for $[^3H]Pr^{sol}$ and 177 cpm for $[^{35}S]Pfr^{sup}$ in profiles at left; 126 cpm for $[^3H]Pfr^{sup}$ and 304 cpm for $[^{35}S]Pr^{sol}$ in profiles at right.

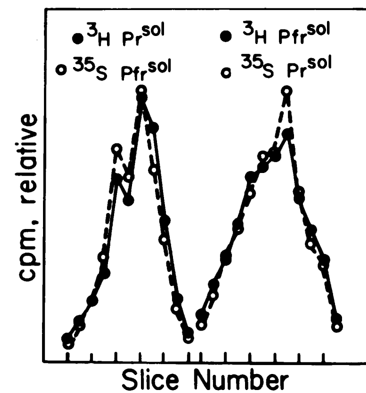

FIG. 6. Radioactivity profiles of Pr^{sol} and Pfr^{sol} monomer bands taken from two cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 6000 cpm of each isotope were applied to each gel. Slices were 0.6 mm thick. In both cases the origin is to the left. Radioactivity in peak slices was as follows: 195 cpm for $[^3H]Pr^{sol}$ and 203 cpm for $[^{35}S]Pfr^{sol}$ in profiles at left; 122 cpm for $[^3H]Pfr^{sol}$ and 130 cpm for $[^{35}S]Pr^{sol}$ in profiles at right.

was precipitated by addition of solid ammonium sulfate to 33% saturation. The pellet was collected by centrifugation at 16,000g for 15 min and dissolved in 25 mM Tris-Cl, 0.11 M 2-mercaptoethanol, 5 mM K_3PO_4, 5 mM EDTA (pH 7.8 at 25 C) at a ratio of 1 ml buffer/gram of tissue extracted. The dissolved pellet was clarified at 16,000g for 15 min, chromatographed through brushite, and fractionated by 0 to 33% saturation ammonium sulfate.

To obtain Pr^{sol} that was irradiated *in vitro*, 5 g of shoots (chopped into 1-cm segments) were extracted with 7 ml extraction medium in a 10-ml Waring Blendor (Waring Products Div., New Hartford, CT). The output of two Unitron LKR microscope illuminators, which were mounted above the extraction vessel, was filtered through Balzers 663-nm B-40 and 664-nm B-40 interference filters. Samples were irradiated for 15 s, during which time photoequilibrium was reached. The R-irradiated Pr^{sol} (now Pfr) was chromatographed though brushite as for unirradiated Pr^{sol} above,

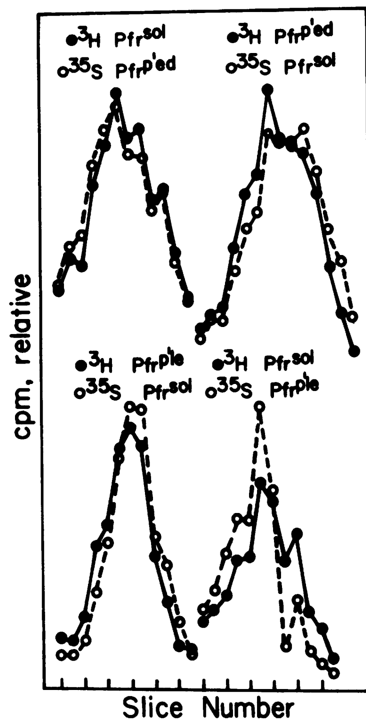

FIG. 7. Radioactivity profiles of Pfr^{sol}, $Pfr^{P'le}$, and $Pfr^{P'ed}$ monomer bands taken from four cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 6000 cpm of each isotope were applied to each gel. Slices were 0.6 mm thick. In all cases the origin is to the left. Radioactivity in peak slices was as follows: 129 cpm for $[^3H]Pfr^{sol}$ and 95 cpm for $[^{35}S]Pfr^{P'ed}$ in profiles at upper left; 294 cpm for $[^3H]Pfr^{P'ed}$ and 117 cpm for $[^{35}S]Pfr^{sol}$ in profiles at upper right; 173 cpm for $[^3H]Pfr^{P'le}$ and 183 cpm for $[^{35}S]Pfr^{sol}$ in profiles at lower left; 137 cpm for $[^3H]Pfr^{sol}$ and 139 cpm for $[^{35}S]Pfr^{P'le}$ in profiles at lower right.

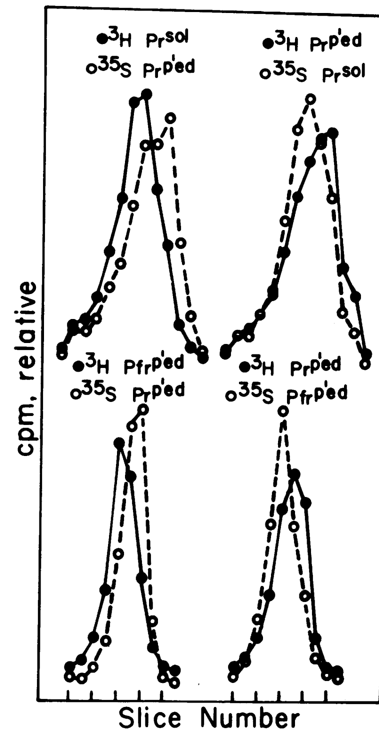

FIG. 8. Radioactivity profiles of Pr^{sol}, $Pr^{P'ed}$ and $Pfr^{P'ed}$ monomer bands taken from four cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 6000 cpm of each isotope were applied to gels at top. Slices were 0.6 mm thick. About 3000 cpm of each isotope were applied to gels at bottom and slices were 1.1 mm thick. In all cases the origin is to the left. Radioactivity in peak slices was as follows: 223 cpm for $[^3H]Pr^{sol}$ and 197 cpm for $[^{35}S]Pr^{P'ed}$ in profiles at upper left; 250 cpm for $[^3H]Pr^{P'ed}$ and 263 cpm for $[^{35}S]Pr^{sol}$ in profiles at upper right; 189 cpm for $[^3H]Pfr^{P'ed}$ and 293 cpm for $[^{35}S]Pr^{P'ed}$ in profiles at lower left; 187 cpm for $[^3H]Pr^{P'ed}$ and 205 cpm for $[^{35}S]Pfr^{P'ed}$ in profiles at lower right.

irradiated with FR, and then fractionated as above with ammonium sulfate.

For *in vitro* irradiation of $Pfr^{P'le}$ with FR, R-irradiated (3 min) oat shoots were extracted as for unirradiated $Pfr^{P'le}$ as explained. The extract was immediately given saturating 15 s FR by filtering the output of two Unitron LKR microscope illuminators through the plastic broad band filter described above.

To expose exogenous Pfr to the extraction conditions used for preparation of $Pfr^{P'le}$, the following procedure was used. Exogenous Pfr was obtained by irradiating with R undegraded oat phytochrome, which had been partially purified as Pr^{sol} by brushite chromatography and 0 to 33% saturation ammonium sulfate fractionation. Immediately after irradiation, about 1.1 mg (10) of this Pfr (about a 10-fold excess over endogenous phytochrome) was added to extraction medium, which was then used immediately for homogenization of 5 g of R-irradiated (3 min) oat shoots. Phytochrome in the crude extract was then partially purified as for Pr^{sol}, with an actinic FR irradiation given after the ammonium sulfate fractionation. Since final recoveries of phytochrome were substantially greater than 10-fold more than were obtained when exogenous phytochrome was not added, we conclude that these preparations contained insignificant quantities of endogenously derived phytochrome.

Gel Electrophoresis. Prior to SDS polyacrylamide gel electrophoresis all phytochrome preparations, except for some used for carbohydrate assay (see below), were immunoprecipitated and prepared for electrophoresis as described earlier (1). Electrophoresis was performed by a modification (1) of the procedure of Weber and Osborn (16). Coelectrophoretic experiments utilizing cylindrical gels were carried out by mixing, prior to electrophoresis, aliquots of immunoprecipitates that had been prepared for electrophoresis. After electrophoresis, gels were cut, slices were dissolved, and radioactivity was counted by liquid scintillation as before (1). Radioactivity profiles of phytochrome monomers taken from SDS polyacrylamide gels are presented as the percentage in each slice of the total radioactivity in each peak.

Micro Complement Fixation. Micro complement fixation assays were performed as described by Levine and Van Vunakis (7).

Analytical Gel Filtration. Phytochrome preparations were chromatographed through a Sephadex G-200 column (2.4 × 98 cm) with 0.1 M Na-phosphate (pH 7.8). A void volume marker, blue dextran, and a total volume marker, tryptophan, were added to each sample before application to the column (9).

Carbohydrate Assay. Phytochrome preparations that were tested for carbohydrate content by PAS staining of SDS polyacrylamide gels after electrophoresis of phytochrome were prepared as follows. Immunopurified undegraded oat phytochrome was prepared as described by Hunt and Pratt (4). Barley (*Hordeum vulgare* L., cv. Harrison), rye (*Secale cereale* L., cv. Balbo), and pea (*Pisum sativum* L., cv. Alaska) phytochrome was conventionally purified (9) and subsequently immunoprecipitated. Oat $Pfr^{P'le}$ was obtained as described above. Phytochrome immunoprecipitates were made and prepared for electrophoresis as described earlier (1). Electrophoresis was carried out in cylindrical 5% polyacrylamide SDS gels as above.

After electrophoresis, gels were fixed overnight in 12% (w/v) TCA and stained with Coomassie brilliant blue R or by a modification of the PAS procedure described by Zacharius *et al.* (19).

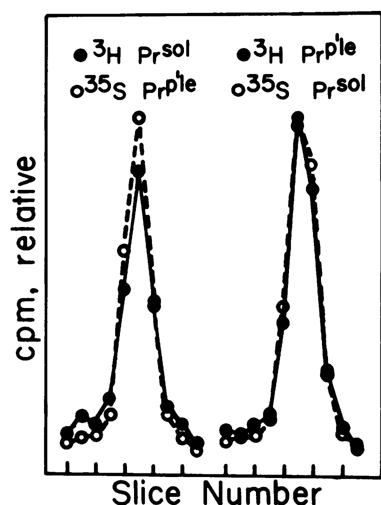

FIG. 9. Radioactivity profiles of Pr^{sol} and $Pr^{p'le}$ monomer bands taken from two cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 3000 to 3500 cpm of each isotope were applied to each gel. Slices were 1.1 mm thick. In both cases the origin is to the left. Radioactivity in peak slices was as follows: 188 cpm for [^{3}H]Pr^{sol} and 238 cpm for [^{35}S]$Pr^{p'le}$ in profiles at left; 213 cpm for [^{3}H]$Pr^{p'le}$ and 156 cpm for [^{35}S]Pr^{sol} in profiles at right.

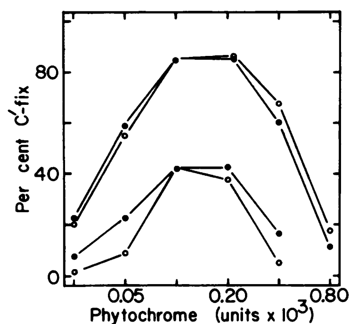

FIG. 10. Micro complement fixation assay of Pr^{sol} (○) and $Pr^{p'le}$ (●) at a 325-fold (lower curves) and at a 275-fold (upper curves) antiserum "W" (6) dilution. One unit of phytochrome equals about 1.7 mg (7).

For the PAS stain, gels were washed after fixation for 1 to 2 days in a gel destaining unit (Model 172A Diffusion Destainer, Bio-Rad) with several changes of distilled H_2O. They then were immersed in 1% (w/v) periodic acid (dissolved in 3% (v/v) acetic acid) for 1 h. After washing for about 4 h with several changes of distilled H_2O in the same destaining unit, gels were submerged in Schiff Reagent (Fisher Scientific Co., Pittsburgh, PA) for 1 h at 25 C. Gels then were photographed immediately.

RESULTS

To minimize repetition with the "Discussion", we will present the results here without attempting to make clear in each case why the experiments were performed.

$Pfr^{p'le}$, like $Pfr^{p'ed}$ (1), exhibits a distinctly reduced mobility when coelectrophoresed with Pr^{sol} (Fig. 2). If Pr^{sol} and $Pfr^{p'le}$ are electrophoresed in adjacent tracks in SDS polyacrylamide gel slabs, the reduced mobility of $Pfr^{p'le}$ is readily apparent (Fig. 3). Co-electrophoresis of Pr^{sol} and Pfr^{sup} shows that Pfr^{sup} also has a slower mobility (Fig. 4).

Pfr^{sol} electrophoreses only marginally more slowly than Pr^{sol} as shown by observation of polyacrylamide slab gels (Fig. 5) and by comparison of isotope distributions (Fig. 6). Compared to both $Pfr^{p'le}$ and $Pfr^{p'ed}$, however, Pfr^{sol} migrates more rapidly (Figs. 5 and 7). Based on quantitative and visual determinations of relative

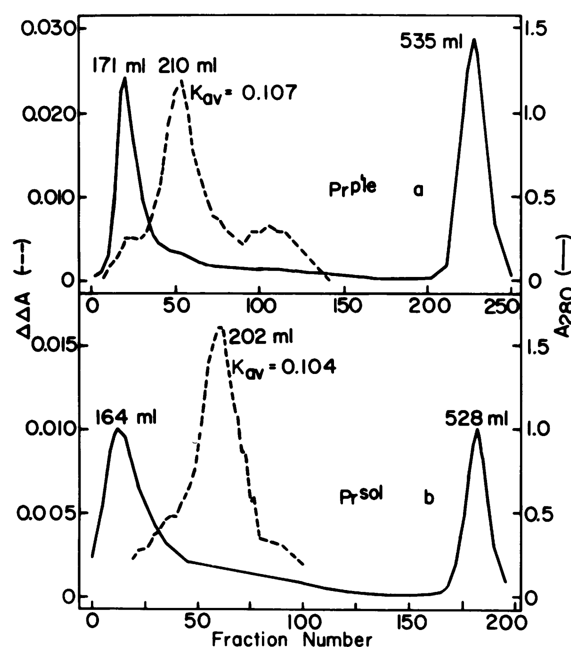

FIG. 11. Elution profiles of $Pr^{p'le}$ (a) and Pr^{sol} (b) preparations from a Sephadex G-200 column. The Pr^{sol} sample contained 1.23 units (7) of phytochrome in 3.2 ml, and the $Pr^{p'le}$ sample contained 2.20 units (7) of phytochrome in 3.0 ml. Blue dextran and tryptophan were added to each sample before chromatography. Columns were eluted at a rate of about 0.4 ml/min. Blue dextran and tryptophan are identified by A at 280 nm. Phytochrome photoreversibility was measured between 667 and 727 nm using an optically clear sample with a 1.0 cm pathlength. The relative elution volume of phytochrome (K_{av}) is calculated from the void volume (V_o), total volume (V_t), and phytochrome elution volume (V_e) by the relationship $K_{av} = (V_e\text{-}V_o)/V_t\text{-}V_o)$.

mobility it appears that Pfr^{sol} electrophoreses with a mobility intermediate between that of Pr^{sol} and $Pfr^{p'ed}$ or $Pfr^{p'le}$ although it appears to be more similar to Pr^{sol}.

Analysis of $Pr^{p'ed}$ preparations show that they electrophorese faster than Pr^{sol} (Figs. 3 and 8). The expected faster mobility of $Pr^{p'ed}$ compared to $Pfr^{p'ed}$ was also obtained (Fig. 8).

During coelectrophoresis with Pr^{sol}, $Pr^{p'le}$ migrates with a mobility indistinguishable from that of Pr^{sol} (Fig. 9). The microcomplement fixation assay, a sensitive indicator of antigenic activity, also did not detect a difference between Pr^{sol} and $Pr^{p'le}$ (Fig. 10). The chromatographic rates of Pr^{sol} and $Pr^{p'le}$ through a Sephadex G-200 column were also indistinguishable (Fig. 11).

Pr^{sol} shows a mobility similar to that of $Pr^{sol'}$ (Fig. 12). The expected reduced electrophoretic rate of $Pfr^{p'ed}$ compared to $Pr^{sol'}$ is also seen (Fig. 12).

Electrophoretic analysis of Pr^{sol} that was given R immediately upon extraction shows that the modification, which leads to a decreased electrophoretic rate, can be induced by irradiation *in vitro*. R-irradiated Pr^{sol} migrated with a slower mobility compared with Pr^{sol} on an SDS polyacrylamide gel slab and with an indistinguishable mobility compared to $Pfr^{p'le}$ (Fig. 13). Conversely, if $Pfr^{p'le}$ is given a saturating dose of FR immediately after extraction, this phytochrome does not show reduced mobility. $Pfr^{p'le}$ + FR migrates at a rate indistinguishable from that of Pr^{sol} and more rapidly than $Pfr^{p'le}$ (Fig. 14).

Partially purified phytochrome in the Pfr form which was exposed to the extraction and isolation procedures used to obtain $Pfr^{p'le}$ electrophoresed at a rate indistinguishable from that of exogenous phytochrome added as Pr (Fig. 15). Electrophoresis of this exogenous Pfr in tracks adjacent to those in which $Pfr^{p'le}$ was electrophoresed shows the more rapid mobility expected for ex-

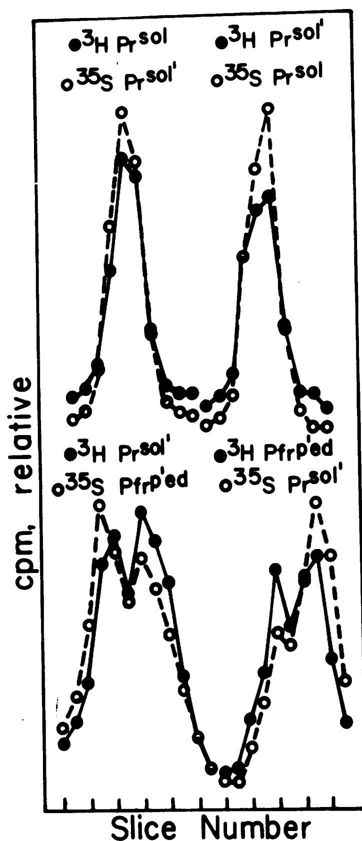

FIG. 12. Radioactivity profiles of Pr^{sol}, $Pr^{sol'}$ and $Pfr^{p'ed}$ monomer bands taken from four cylindrical SDS 5% polyacrylamide gels after coelectrophoresis of immunoprecipitates. Approximately 3000 cpm of each isotope were applied to gels at top. Slices were 1.1 mm thick. To gels at bottom approximately 3500 cpm of each isotope were applied. Slices were 0.6 mm thick. In all cases the origin is to the left. Radioactivity in peak slices was as follows: 138 cpm for $[^3H]Pr^{sol}$ and 252 cpm for $[^{35}S]Pr^{sol'}$ in profiles at upper left; 181 cpm for $[^3H]Pr^{sol'}$ and 544 cpm for $[^{35}S]Pr^{sol}$ in profiles at upper right; 84 cpm for $[^3H]Pr^{sol'}$ and 78 cpm for $[^{35}S]Pfr^{p'ed}$ in profiles at lower left; 150 cpm for $[^3H]Pfr^{p'ed}$ and 103 cpm for $[^{35}S]Pr^{sol'}$ in profiles at lower right. The irregular peaks at bottom result from unequal widths of slices.

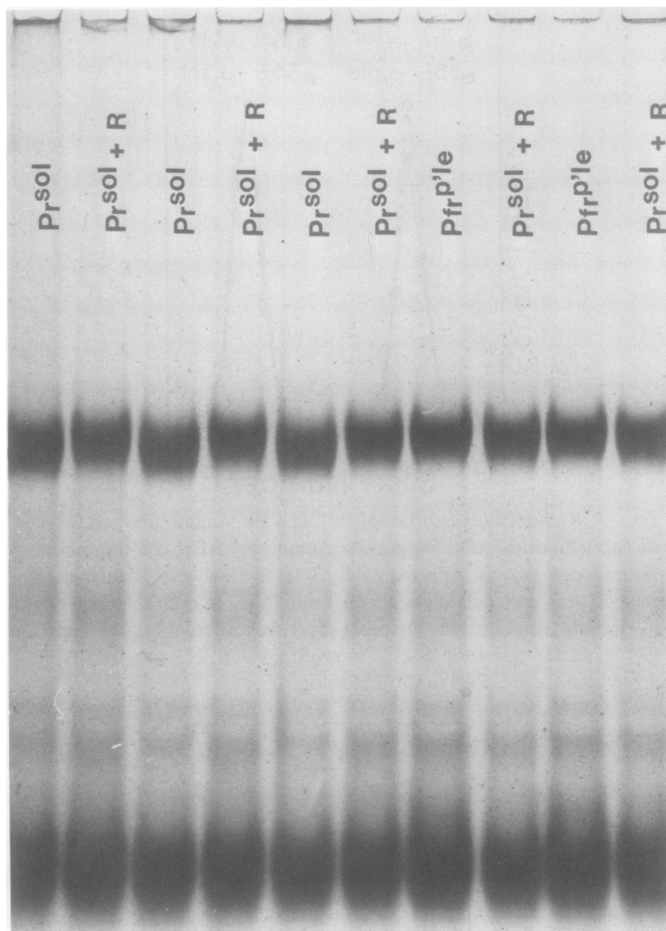

FIG. 13. Photograph of an SDS 5% polyacrylamide gel slab after electrophoresis of immunoprecipitates of Pr^{sol}, R-irradiated Pr^{sol} (Pr^{sol} + R) and $Pfr^{p'le}$. The 118,000-dalton phytochrome monomer is located near the middle, the 50,000-dalton immunoglobulin heavy chain near the bottom. The origin is at the top.

ogenous Pfr (Fig. 15).

PAS staining of SDS polyacrylamide gels after electrophoresis of immunopurified Garry oat phytochrome did not detect any carbohydrate (Fig. 16b). No PAS reaction was obtained for phytochrome after electrophoresis of an immunoprecipitate of $Pfr^{p'le}$ (Fig. 16f), a phytochrome species that has a reduced electrophoretic mobility (Figs. 2 and 3). No sugar was detected on phytochrome in polyacrylamide gels after electrophoresis of immunoprecipitates of pea, rye, barley (Fig. 16c–e) and Newton oat (not shown) phytochrome. Thus, absence of a positive reaction for Garry oat phytochrome is not an isolated observation. Such negative results were obtained with a total of 25 different gels.

Several PAS-stained gels show a carbohydrate-containing band near that of phytochrome (Fig. 16c–f). This band, however, is not phytochrome. Its position corresponds to a size of about 80,000 daltons, significantly removed from the 120,000-dalton phytochrome band. This band has been obtained after electrophoresis of many phytochrome immunoprecipitates and is probably derived from antiserum rather than from phytochrome preparations.

An absorbance scan of one of the Coomassie blue-stained gels (Fig. 16) shows that the expected protein bands may be readily resolved (Fig. 17). Since heavy protein loads were applied the protein bands appear smeared in photographs and are not symmetrical in a gel scan (Fig. 17).

DISCUSSION

Our earlier report (1) documented a change in phytochrome that was pelleted and subsequently released from particulate material in the Pfr form as initially reported by Grombein and Rüdiger (3) and Yu and Carter (17, 18). Compared with Pr^{sol}, $Pfr^{p'ed}$ showed greater microcomplement fixation activity, eluted more rapidly from a Sephadex G-200 column, and electrophoresed more slowly on 5% polyacrylamide SDS gels (1).

The relative electrophoretic mobilities of the preparations outlined in Figure 1 are summarized in Table I. Additional electrophoretic comparisons which are not presented graphically are presented here to show that predicted relative mobilities were experimentally verified. The internal consistency among the relative mobilities of the various phytochrome preparations should be stressed. Each preparation was labeled independently with both ^3H or ^{35}S. Thus, except for some comparisons shown only in Table I, each coelectrophoretic comparison was carried out independently at least twice and the possibility of an isotope effect was simultaneously eliminated.

As pointed out in the introduction, there are several possible explanations for what the modification of $Pfr^{p'ed}$ might mean. One possibility is that the modification and pelletability are causally related. More trivial explanations, such as differential sensitivity

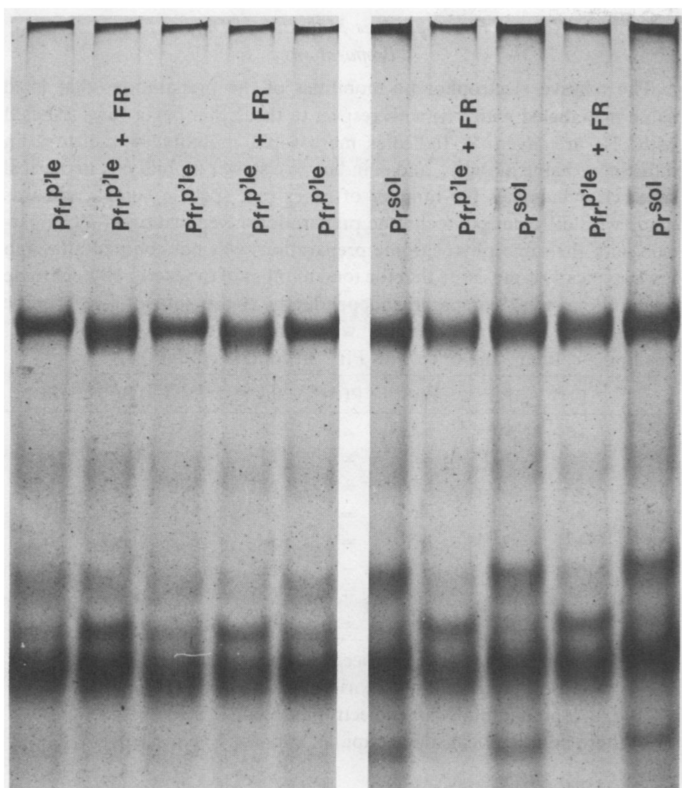

FIG. 14. Photograph of SDS 5% polyacrylamide gel slabs after electrophoresis of immunoprecipitates of $Pfr^{p'le}$ given FR irradiation immediately after extraction ($Pfr^{p'le}$ + FR), $Pfr^{p'le}$, and Pr^{sol}. The 118,000-dalton phytochrome monomer is located near the middle. The origin is at the top. The first five lanes are from one slab and the remaining five lanes are from a second slab.

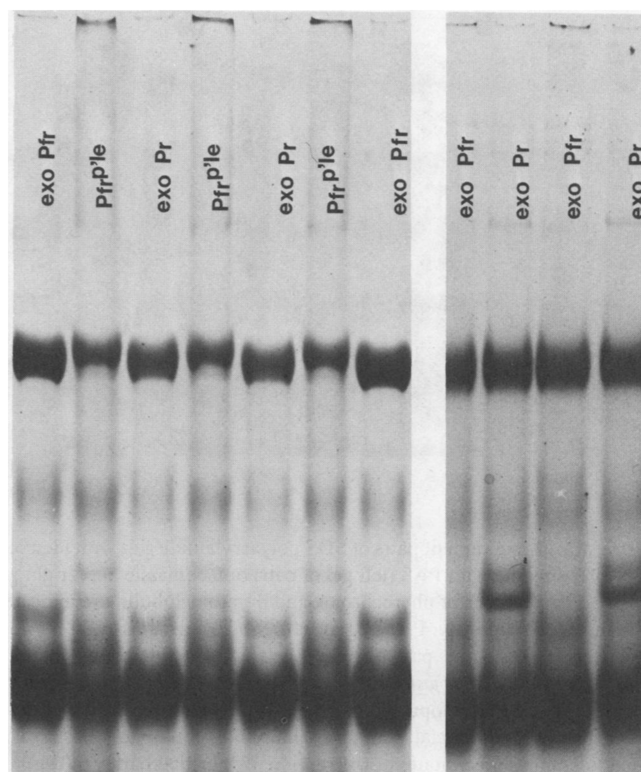

FIG. 15. Photograph of SDS 5% polyacrylamide gel slabs after electrophoresis of immunoprecipitates of Pfr^{ple}, exogenous Pr and exogenous Pfr. Brushite-purified phytochrome, either as Pr or Pfr, was added to the extraction medium before homogenization. Usual phytochrome isolation and immunoprecipitation procedures were then carried out. The 118,000-dalton phytochrome monomer is located near the middle. The origin is at the top. The first seven lanes are from one slab and the remaining four lanes are from a second slab.

of phytochrome to different extraction conditions or a differential reactivity of the two forms, could also account for the alteration seen in $Pfr^{p'ed}$. In the discussion that follows we shall consider these possible explanations for the modification.

Is modification of phytochrome required for *in vitro* expression of pelletability that was induced *in vivo*? If this were the case one would expect $Pr^{p'le}$ to show the modification since this phytochrome was extracted when it was in a potentially pelletable condition (12). Since $Pr^{p'le}$ did not differ from Pr^{sol} during complement fixation (Fig. 10), gel exclusion chromatography (Fig. 11), or SDS polyacrylamide gel electrophoresis (Fig. 9) assays, the modification is apparently not required for the *in vitro* expression of pelletability induced *in vivo*.

Is phytochrome modified as a consequence of having been pelleted? If this were the case one would expect the mobilities of $Pfr^{p'le}$ and Pfr^{sup} not to differ from Pr^{sol}. Both Pfr^{sup} and $Pfr^{p'le}$ electrophorese identically with $Pfr^{p'ed}$; that is, with reduced rates compared to Pr^{sol} (Figs. 2–4). This observation indicates that phytochrome not associated with the particulate fraction *in vitro* is modified. Thus, the reduced electrophoretic mobility of $Pfr^{p'ed}$ does not occur as a result of phytochrome having been pelleted. Taken together with the conclusion reached in the preceding paragraph it is clear that there is no evidence to indicate a causal relationship between phytochrome pelletability induced *in vivo* and the phytochrome modification described here.

Does differential sensitivity to different extraction conditions lead to the modification? Since $Pfr^{p'le}$ was extracted and partially purified under precisely the same conditions as for Pr^{sol}, any change in $Pfr^{p'le}$ may not be attributed to different handling conditions. Since $Pfr^{p'le}$ exhibited the same reduced mobility seen for $Pfr^{p'ed}$ (Fig. 3), different extraction conditions are not responsible.

Are the Pr and Pfr forms of phytochrome differentially labile to the extraction conditions? If this were the case, then partially purified phytochrome, added to the grinding medium as Pfr and exposed to the same extraction and isolation conditions as endogenous $Pfr^{p'le}$, would exhibit a reduced mobility. Exogenous Pfr added to extraction medium that was used for homogenization of R-irradiated oat shoots did not show changed mobility compared to exogenous phytochrome added as Pr. Exogenous Pfr migrated more rapidly than $Pfr^{p'le}$ as expected (Fig. 15). Thus, reduced electrophoretic mobility is not due to a simple differential lability of Pfr to extraction conditions.

In view of the more extensive observations presented here, the suggestion of Yu and Carter (17, 18) that phytochrome is altered as a simple consequence of its conversion to the pelletable condition needs to be reevaluated. Since neither Yu and Carter (17, 18) nor Grombein and Rüdiger (3) investigated alternative possibilities for causing the alteration in gel chromatographic behavior properties that they observed, it may not be concluded that there was any causal relationship between phytochrome pelletability and their observations.

In an attempt to explain our observations, we note that our data are consistent with the hypothesis that the modification we have described occurs *in vitro*, when two conditions are met. First, phytochrome must be extracted as Pfr or must be converted to Pfr soon after extraction. $Pfr^{p'le}$ and Pfr^{sup} showed reduced mobility (Figs. 2–5), as did R-irradiated Pr^{sol} (Fig. 13). Second, phytochrome must remain in the Pfr form for at least a brief period (less than 30 min, but otherwise undefined) in order to be modified in such a way as to exhibit reduced electrophoretic mobility. This

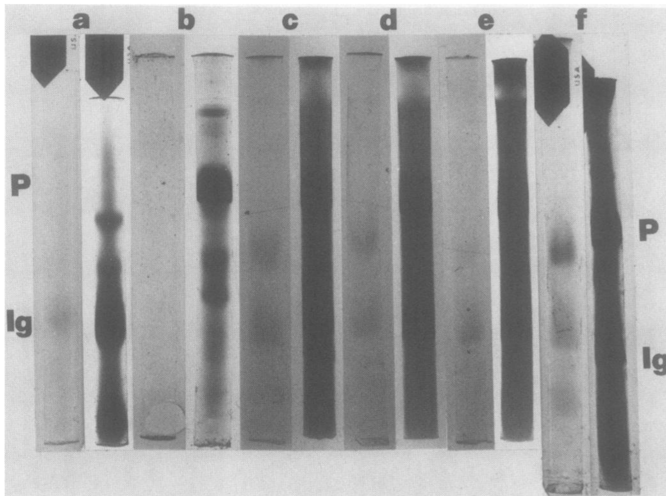

FIG. 16. Photograph of pairs of SDS polyacrylamide gels with identical loads after staining with PAS (left gel of pair) or Coomassie blue (right gel of pair). The positions of phytochrome and immunoglobulin are indicated by P and Ig, respectively. P and Ig at left refer to gels a through e. P and Ig at right refer to the pair of gels labeled f. Commercially obtained immunoglobulin G (100 μg) was electrophoresed in each gel in a. Approximately 80 μg of immunopurified Garry oat phytochrome was applied to each gel in b. Commercially obtained immunoglobulin G (100 μg) was added to immunoprecipitates of conventionally purified barley (40 μg) (c), rye (60 μg) (d), and pea (75 μg) (e) phytochrome. Since between 250 and 280 μg of immunoglobulin G was electrophoresed in gels c-e, the Coomassie blue stain appears as a smear. Absorbance scans of these Coomassie blue-stained gels, however, verified that the expected peaks were present (see Fig. 17). An immunoprecipitate of oat $Pfr^{p'le}$ containing about 75 μg phytochrome in addition to immunoglobulin G was applied to the gel in f. Following staining with PAS the same gel was washed extenisvely and then stained with Coomassie blue.

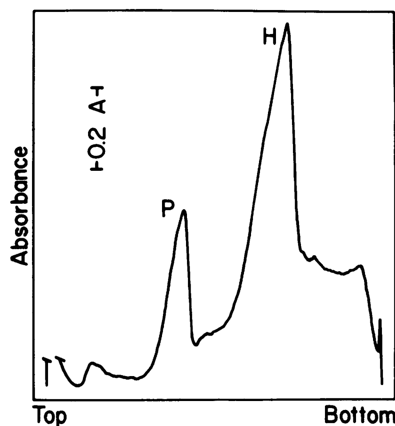

FIG. 17. Absorbance scan of the Coomassie blue-stained gel presented in Figure 16e. The gel was scanned with a Shimadzu MPS-50L spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan) at 466 nm. The two major bands are undegraded phytochrome (P) and immunoglobulin heavy chain (H).

requirement would explain why $Pr^{p'le}$, $Pr^{p'ed}$, and FR-irradiated $Pfr^{p'le}$ did not exhibit reduced mobility (Figs. 3, 8, 9 and 14). The necessity for phytochrome to be in the Pfr form in order to be modified is not unreasonable since Hunt and Pratt (5) have reported that Pfr as compared to Pr has one more each of histidine and cysteine that react immediately with modifying reagents.

The question of why $Pr^{p'ed}$ electrophoreses more rapidly than Pr^{sol} is raised here (Figs. 3 and 8) but no attempt is made to answer

Table I. *Relative Electrophoretic Mobilities of Different Phytochrome Preparations*

The relative electrophoretic mobilities of the preparations that head columns (labeled with ^{35}S) with respect to those that begin rows (labeled with ^{3}H) are given. + Indicates more rapid mobility, = indicates an indistinguishable mobility, and − indicates a slower mobility. A numerical superscript indicates the number of times that specific comparison was made with different phytochrome preparations. Replication of a comparison with the same phytochrome preparations was not counted although it was typically done. Note that the total number of times two phytochrome preparations were compared independently is the sum of two specific comparisons (*e.g.*, $Pfr^{p'le}$ and Pfr^{sol} were compared twice, once with $Pfr^{p'le}$ heading a column, and once with Pfr^{sol} heading a column).

	Pr^{sol}	Pfr^{sol}	$Pfr^{p'le}$	$Pfr^{p'ed}$	Pfr^{sup}	$Pr^{p'le}$	$Pr^{p'ed}$	$Pr^{sol'}$
Pr^{sol}	$=^{a}$	$-^{2}$	$-^{4}$	$-^{2,b}$	$-^{2}$	$=^{3}$	$+^{2}$	$=^{2}$
Pfr^{sol}	+	NT[c]	$-^{d}$	−	$-^{2,a}$	NT	NT	NT
$Pfr^{p'le}$	$+^{4}$	+	NT	$=^{3,a}$	NT	NT	NT	NT
$Pfr^{p'ed}$	$+^{5,b}$	$+^{2}$	$=^{a}$	$=^{a}$	$=^{2,a}$	NT	+	+
Pfr^{sup}	$+^{3}$	$+^{2,a}$	NT	$=^{a}$	$=^{a}$	NT	NT	NT
$Pr^{p'le}$	$=^{3}$	$-^{2,a}$	$-^{2,a}$	$-^{a}$	NT	NT	$+^{a}$	NT
$Pr^{p'ed}$	$-^{3}$	$-^{a}$	NT	−	NT	NT	NT	NT
$Pr^{sol'}$	=	NT	NT	−	NT	NT	NT	NT

[a] These comparisons have not been presented elsewhere in this paper.
[b] Comparison presented in an earlier report (1).
[c] NT two preparations not coelectrophoresed.
[d] Where no numerical superscript, an entry of 1 is implied.

it. It might be that a proposed subtle alteration of phytochrome occurs *in vivo* and causes it to become pelletable *in vitro*. This subtle alteration, which is itself not observed, might then lead *in vitro* to either a reduced mobility ($Pfr^{p'le}$, $Pfr^{p'ed}$, Pfr^{sol}, Pfr^{sup}) or to an enhanced mobility ($Pr^{p'ed}$) depending upon the precise state and form in which phytochrome is extracted and partially purified.

Roux *et al.* (14) reported detecting carbohydate on phytochrome by PAS staining of SDS polyacrylamide gels in 80 μg of a 60% pure sample, or on about 50 μg of phytochrome. We were unable to obtain a positive reaction with up to 80 μg of phytochrome. The procedures used here detected sugar on 100 μg immunoglobulin G, which contains 2.9% carbohydrate (6), following electrophoresis (Fig. 16a). Thus, assuming any sugar residues on phytochrome react equally as well as those on immunoglobulin G, our procedure would have detected a carbohydrate content of 4%, which is the value reported by Roux *et al.*

It is not possible to evaluate the data of Roux *et al.* (14) critically. They did not present the results of any control, such as attempted PAS staining of a known nonglycoprotein, designed to demonstrate that the apparent stain for carbohydrate was not an artifact. This point is critical since it has been documented that residual SDS associated with protein bands in acrylamide gels will give positive activity by PAS staining (2). In fact, we did observe an apparently positive reaction associated with the phytochrome band in our earliest experiments. After taking appropriate precautions to eliminate artifactual staining, however, we were no longer able to obtain a positive reaction with phytochrome even though authentic glycoproteins still stained appropriately. The positive result obtained by Roux *et al.* is therefore suspect, especially since Roux (personal communication cited in Ref. 11) and W. O. Smith, Jr., (personal communication) have also reported that they have recently failed in attempts to repeat the observation. In addition, other analyses reported by Roux *et al.* (14) were performed on phytochrome samples for which no rigorous documentation of purity was presented leaving open the possibility that a contaminant could have resulted in the detection of the amino sugars and carbohydrate that they reported. Roux *et al.* evaluated the purity of their phytochrome preparations only by Purity Index (A_{278}/A_{667}) values. Since they have presented no other evidence con-

cerning purity and since their phytochrome preparations, which for these analyses were proteolytically degraded, are different from those studied by others, it is not possible to evaluate properly the significance of their Purity Index values.

Because of our inability to repeat one of the observations leading to the conclusion that phytochrome is a glycoprotein, and because of other uncertainties in the data used to support this conclusion as discussed above, we conclude that the question of whether phytochrome is a glycoprotein must be reevaluated. We also conclude that, since no sugar was detected in phytochrome preparations that electrophorese with reduced mobility in SDS polyacrylamide gels, the reduced mobility may not be attributed to a change in the carbohydrate composition of phytochrome that is detectable by this staining procedure.

LITERATURE CITED

1. Boeshore ML, LH Pratt 1980 Phytochrome modification and light-enhanced, *in vivo*-induced phytochrome pelletability. Plant Physiol 66: 500–504
2. Glossmann H, DM Neville 1971 Glycoproteins of cell surfaces: comparative study of three different cell surfaces of the rat. J Biol Chem 246: 6339–6346
3. Grombein S, W Rüdiger 1976 On the molecular weight of phytochrome: a new high molecular phytochrome species in oat seedlings. Z Physiol Chem 357: 1015–1018
4. Hunt RE, LH Pratt 1979 Phytochrome immunoaffinity purification. Plant Physiol 64: 332–336
5. Hunt RE, LH Pratt 1981 Physicochemical differences between the red- and the far-red-absorbing forms of phytochrome. Biochemistry, 20: 941–945
6. Kabat EA 1976 Structural Concepts in Immunology and Immunochemistry, Ed 2. Holt, Rinehart, and Winston, New York, p. 227
7. Levine L, H Van Vunakis 1967 Micro complement fixation. Methods Enzymol 11: 928–936
8. Marmé D 1977 Phytochrome: membranes as possible sites of primary action. Annu Rev Plant Physiol 28: 173–198
9. Pratt LH 1973 Comparative immunochemistry of phytochrome. Plant Physiol 51: 203–209
10. Pratt LH 1978 Molecular properties of phytochrome. Photochem Photobiol 27: 81–105
11. Pratt LH 1979 Phytochrome: function and properties. *In* KC Smith, ed, Photochemical and Photobiological Reviews, Vol. 4. Plenum Press, New York, pp 59–124
12. Pratt LH, D Marmé 1976 Red light-enhanced phytochrome pelletability: reexamination and further characterization. Plant Physiol 58: 686–692
13. Quail PH, WR Briggs 1978 Irradiation-enhanced phytochrome pelletability: requirement for phosphorylative energy *in vivo*. Plant Physiol 62: 773–778
14. Roux SJ, SG Lisansky, BM Stoker 1975 Purification and partial carbohydrate analysis of phytochrome from *Avena sativa*. Physiol Plant 35: 85–90
15. Satter RL, AW Galston 1976 The physiological functions of phytochrome. *In* TW Goodwin, ed, Chemistry and Biochemistry of Plant Pigments, Ed 2 Vol 1. Academic Press, London, pp 681–735
16. Weber K, M Osborn 1969 The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244: 4406–4412
17. Yu R, J Carter 1976 Chromatographic behaviour of phytochrome following its *in vivo* phototransformation. Plant Cell Physiol 17: 1321–1328
18. Yu R, J Carter 1976 Gel filtration of soluble and solubilized phytochrome. Ann Bot 40: 647–649
19. Zacharius RM, TE Zell, JH Morrison, JJ Woodlock 1969 Glycoprotein staining following electrophoresis on acrylamide gels. Anal Biochem 30: 148–152