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Some Properties of Phytochrome Isolated from Dark-grown Oat Seedlings (Avena sativa L.)[1]

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Abstract. Phytochrome was partially purified from etiolated seedlings of *Avena sativa* L. Several properties of the red-absorbing (P_R) and far-red absorbing (P_{FR}) forms of the pigment were compared. The 2 forms could not be shown to differ with respect to their sedimentation velocity in sucrose density gradients, elution volume from Sephadex G-200 columns, binding properties on calcium phosphate, or electrophoretic mobility. P_{FR}, however, was more labile than P_R during precipitation with 50 % ammonium sulfate. Sephadex G-200 elution diagrams obtained with fresh phytochrome preparations revealed 2 components of different molecular weights, 1 roughly 180,000, and 1 roughly 80,000. Native phytochrome had an absorption spectrum *in vivo* showing an absorption maximum for P_R of 667 nm. Both the large and small forms of phytochrome mentioned above can be maintained with an absorption maximum for P_R of 667 nm. However, allowing them to remain for several hours as P_{FR}, even at 4°, shifted this peak to 660 nm. The protein conformational change during phytochrome transformation may be quite small, though the various comparative techniques used do not strictly rule out a fairly large one. The need for maintaining the pigment as P_R during all steps of purification, but particularly during ammonium sulfate precipitation is underscored.

Since the initial spectrophotometric detection of the plant pigment phytochrome (2), considerable progress has been made both in purifying it and in studying its properties *in vitro*. Siegelman and Firer (7) developed techniques for the isolation and partial purification of phytochrome from oat seedlings, and Mumford and Jenner (6) published techniques for obtaining material of high purity from the same plant source. Currently Correll *et al.* (4) are reporting preparations of high purity obtained from rye seedlings. Butler *et al.* (3), meanwhile, showed that the far-red-absorbing form of phytochrome (P_{FR}) was far more susceptible to denaturation by urea or damage by sulfhydryl-reacting agents than was the red-absorbing form (P_R). They showed further that pronase and trypsin would attack phytochrome readily only during P_R to P_{FR} transformation. They suggested that the light-induced interconversions of P_R and P_{FR} involved not just changes within the chromophoric group, a bilitriene (8), but changes in protein conformation as well.

The present paper describes experiments comparing some of the physical properties of P_R and P_{FR}, and presents evidence for both spectral and molecular weight changes which may occur during the purification procedure.

Materials and Methods

Phytochrome was extracted and partially purified from oat seedlings by a modification of the methods described by Siegelman and Firer (7). Oat seeds (*Avena sativa* L., cv USDA CI-2020) were sown on moist cellulose packing material (Kimpak 6223, Kimberly-Clark) and grown for 5 days at 25° in plastic boxes. On the day they were harvested, the seedlings were cooled to 4° in the dark before they received any white light. All further steps were carried out at that temperature. Lots of tissue weighing approximately 500 g were harvested and ground in a chilled mortar with 0.05 M tris-HCl buffer (1.3 ml buffer per g tissue), pH 8.1, containing 0.7 % (v/v) 2-mercaptoethanol. After thorough grinding, the material was filtered through cheesecloth and the filtrate centrifuged for 45 minutes at 14,000 × *g*. The supernatant was passed through a Sephadex G-50 column equilibrated with 0.001 M phosphate buffer, pH 7.8, containing 0.5 % mercaptoethanol, adsorbed on calcium phosphate (brushite) equilibrated with the same solution, washed with a liter of buffer without the mercaptoethanol, and then eluted with a convex buffer gradient running from 0.001 to 0.4 M. Active fractions were pooled, and the phytochrome was precipitated by the gradual addition of an equal volume of saturated ammonium sulfate solution (pH 7.8). Following centrifugation, the pellet was redissolved in 25 to 50 ml of 0.001 M phosphate buffer, pH 7.8, and the resulting solution passed through a Sephadex G-200 column. The pooled active fractions from

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this last step were used for most of the experiments described below, although the phytochrome was frequently concentrated by a second ammonium sulfate precipitation followed by redissolving in a small volume of buffer.

The ultrafiltration step of Siegelman and Firer (7) was omitted, and great care was taken to maintain the phytochrome as P_R, particularly during the ammonium sulfate precipitation. Under these conditions, active preparations showing from 30- to 50-fold purification were obtained, with a final yield of 30 to 50 % of the starting material. Phytochrome was assayed using a Ratiospect R-2 difference spectrophotometer (Agricultural Specialty Company, Incorporated), and activity is expressed as the sum of the optical density differences inducible at 660 and 730 nm by red and far-red light [$\triangle(\triangle OD)$].

Results and Discussion

Butler *et al.* (3) had shown that P_{FR} was more labile in the presence of proteolytic enzymes, urea, and sulfhydryl reagents than P_R. Since there was frequently a substantial decrease in yield of phytochrome during the ammonium sulfate step, we felt that this loss might be reduced by doing the ammonium sulfate precipitation with material first transformed to P_R. A comparison of the loss from ammonium sulfate fractionation performed with P_R and P_{FR} showed that in the latter case, 25 to 30 % of the phytochrome was lost, while in the former, only 10 to 15 % disappeared. Clearly P_{FR} is more labile in the presence of high concentrations of ammonium sulfate than P_R, as one might expect from the results of Butler *et al.*; and all subsequent preparations were handled as P_R at this step. Furthermore, at no time during the procedure was the pigment allowed to remain as P_{FR} for more than 2 hours.

Since the accumulated evidence suggested that phytochrome transformation might involve a protein conformational change, P_R and P_{FR} were compared physically to see whether evidence other than relative lability could be obtained for such a change. Preliminary experiments using ultracentrifugation on sucrose density gradients suggested that P_R sedimented somewhat more rapidly than P_{FR}. As will be shown below, however, it soon became clear that these early preparations consisted of phytochrome components of substantially different molecular weights (see figs 2, 3). During the relatively long ultracentrifuge run, the larger material may well have broken down more rapidly to the small when the pigment was spun as P_{FR} than as P_R (there was no loss of total reversibility). A later experiment, using only the small molecular weight component is shown in figure 1. In all 3 tubes, 2 ml of phytochrome solution containing 0.38 $\triangle(\triangle OD)$ were layered on the sucrose gradient (8–20 %). The material was spun for 40 hours in an International

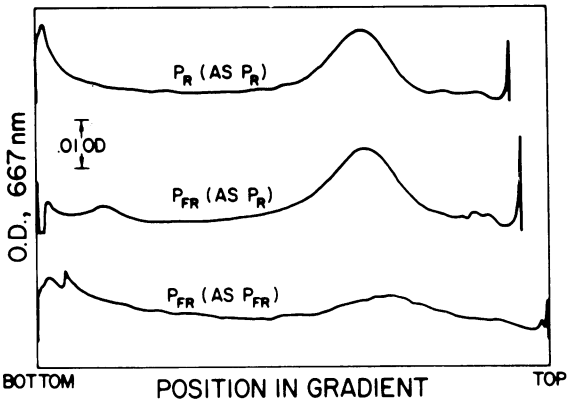

FIG. 1. Sedimentation of P_R and P_{FR} on sucrose gradients (8–20 %). Form of phytochrome sedimented shown above each curve. Form in which phytochrome was monitored following centrifugation shown in parentheses.

B-60 ultracentrifuge with the SB-110 head, at 4°. Phytochrome was monitored in the tubes by passing the gradient solution through a flow cell of the Gilford Model 2000 multiple sample absorbance recorder and monitoring optical density at 667 nm. The sample spun as P_{FR} (at 4°, reversion of P_{FR} to P_R was negligible) was first converted to P_R before monitoring. Figure 1, top 2 curves, shows that under the conditions used, there was no detectable difference between the sedimentation rates of P_R and P_{FR}. The lowest tracing was obtained from a sample in which the phytochrome was deliberately kept as P_{FR} during the spectrophotometric analysis of the gradient. The reduced optical density at 667 nm under these conditions shows that the light-absorbing material detected was indeed phytochrome, as subsequently verified by direct spectrophotometric analysis in the Ratiospect.

During purification, analysis of the elution pattern of phytochrome from the Sephadex G-200 column revealed 2 forms of substantially different molecular weights. Figure 2 shows these 2 components clearly. An analytical Sephadex G-200

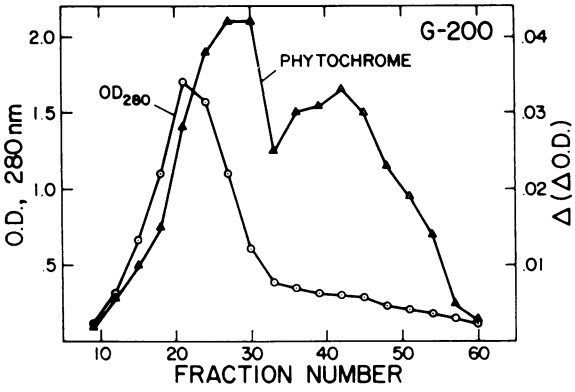

FIG. 2. Elution pattern for fresh phytochrome sample from a Sephadex G-200 column.

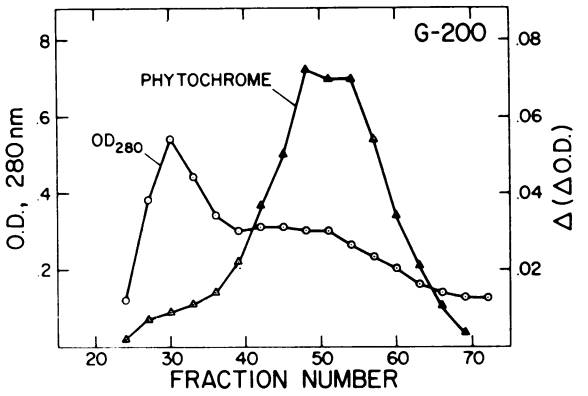

FIG. 3. Elution pattern for aged phytochrome sample from a Sephadex G-200 column.

experiment, using cytochrome *c* (Calbiochem, mol wt 12,500), catalase (Calbiochem, mol wt 225,000), and blue dextran (Pharmacia, mol wt 2,000,000) as markers, yielded approximate molecular weights for the large and small forms of phytochrome of 80,000 and 180,000 respectively. These molecular weight values should not be regarded as very precise since chromatographic behavior on Sephadex was the only criterion applied; furthermore, the smaller molecular weight component may itself be polydisperse, judging from the relatively broad elution bands in figures 2, 3, and 4. Mumford and Jenner (6) have reported a value of 60,000 for highly purified oat phytochrome. Figure 2 was obtained with a fresh preparation, placed on the Sephadex G-200 column immediately after the ammonium sulfate step. When preparations were allowed to stand for a day after this step and before the Sephadex chromatography, the large

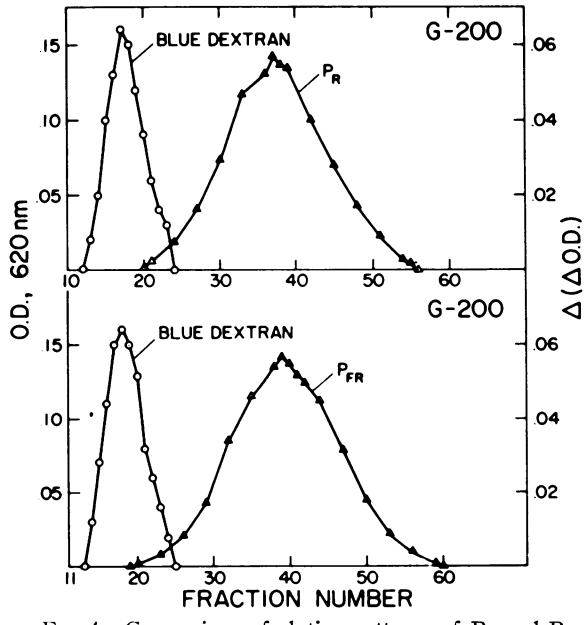

FIG. 4. Comparison of elution patterns of P_R and P_{FR} from matched Sephadex G-200 columns.

molecular weight component disappeared, as shown in figure 3. The conditions which determine whether one obtains the large or small molecular weight material are currently under investigation. However, since roughly equal starting volumes or concentrations of phytochrome yield either a single or double peak on Sephadex G-200 chromatography, depending upon freshness of sample, the large molecular weight form cannot be considered as simply an aggregation resulting from high protein concentration in the starting material. Parameters such as pH, salt concentration, and spectral status of the pigment—whether present as P_R or P_{FR}—are currently under investigation. In all experiments reported below, only fractions containing the small molecular weight form were used for the P_R-P_{FR} comparisons.

Figure 4 shows that P_R and P_{FR} have identical elution volumes on analytical Sephadex G-200 columns, as one might have expected from their sedi-

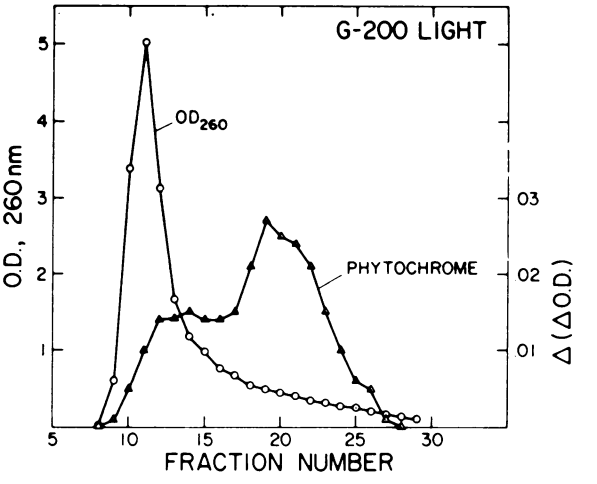

FIG. 5. Elution pattern for phytochrome from Sephadex G-200 column, column continuously illuminated during run.

mentation behavior in the ultracentrifuge. The results shown were obtained by running P_R and P_{RF} simultaneously through 2 precisely matched columns, with blue dextran added as a large molecular weight marker. The ratio of phytochrome elution volume to blue dextran elution volume was the same in both cases. Figures 5 and 6 illustrate an experiment in which samples containing both large and small molecular weight forms were run through the same analytical Sephadex columns, 1 sample illuminated with white light from a 25 w fluorescent bulb placed 6 inches from the column, and the other kept as P_R and run in the dark. The object of the experiment was to determine whether continuous cycling of the pigment from one form to the other, through the various intermediates known to occur on both the P_R to P_{FR} pathway and the reverse pathway (1, 5)

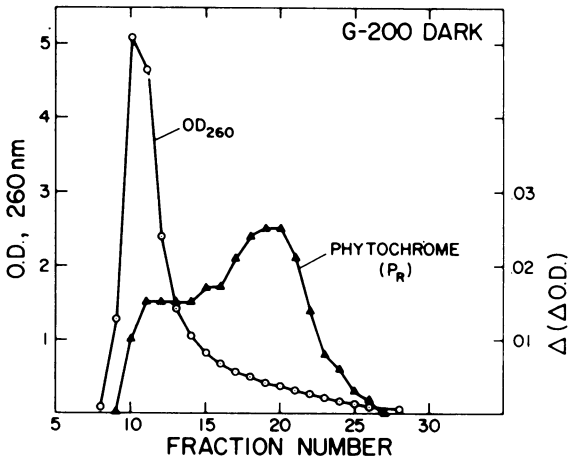

FIG. 6. Elution pattern for phytochrome from Sephadex G-200 column, column run in darkness, phytochrome applied as P_R. Sample size, concentration, and column dimensions identical with those for figure 5.

would in any way alter the chromatographic behavior of the pigment (a long-lived intermediate might have a very open conformation). The 2 figures show clearly that during the 4 hours of the run, cycling the pigment did not alter the elution pattern of either the large or small phytochrome, nor did it cause any measurable change in the detectable amount of the 2 forms.

Figure 7 shows that the binding capacity of phytochrome to calcium phosphate is the same whether the pigment is placed on the column, washed, and eluted as P_R or as P_{FR}. Finally, figure 8 shows that P_R and P_{FR} have the same electrophoretic

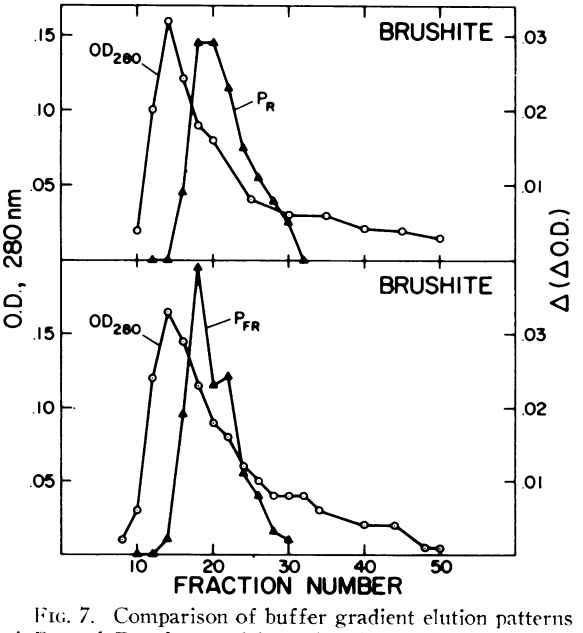

FIG. 7. Comparison of buffer gradient elution patterns of P_R and P_{FR} from calcium phosphate (brushite).

mobility on polyacrylamide gels at 2 different pH's (the Buchler Polyacrylamide Apparatus was used, with gels prepared according to their brochure; the samples were applied in 0.2 M sucrose instead of in a sample gel).

In the comparison of P_R with P_{FR}, above, the only difference found was in relative lability in the

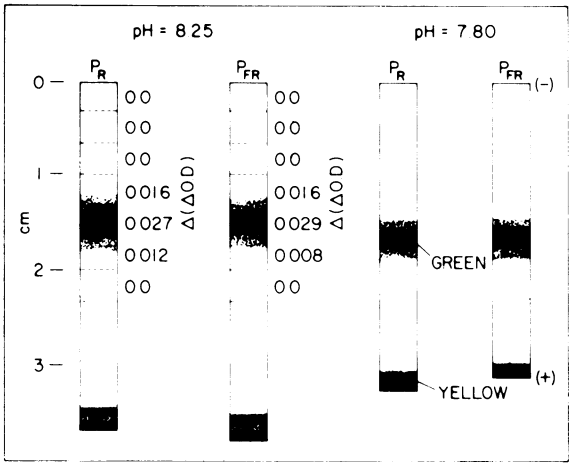

FIG. 8. Comparison of electrophoretic mobility of P_R and P_{FR} on polyacrylamide gels made at 2 different pH's.

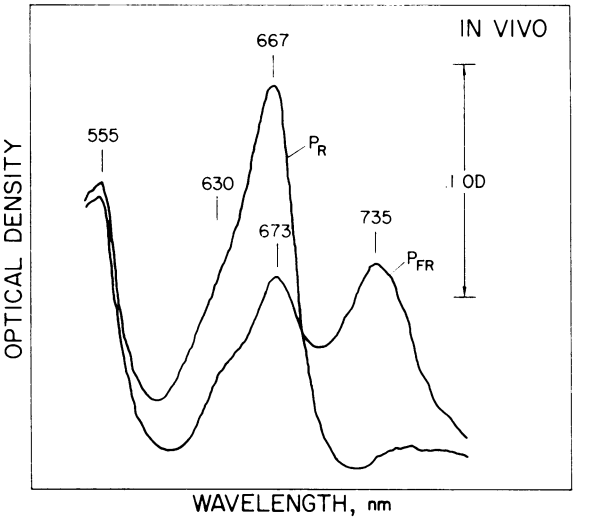

FIG. 9. Absorption spectra of roughly 0.5 g oat coleoptile tips following saturating far red (P_R curve) or red irradiation (P_{FR} curve).

presence of 50 % ammonium sulfate. The 2 forms of the pigment did not differ in their sedimentation velocity, electrophoretic mobility, capacity for binding on calcium phosphate, or elution pattern from Sephadex G-200. Thus the protein conformational change suggested by the results of Butler *et al.* (3) may be small, though the present results certainly do not rigorously exclude a significant one.

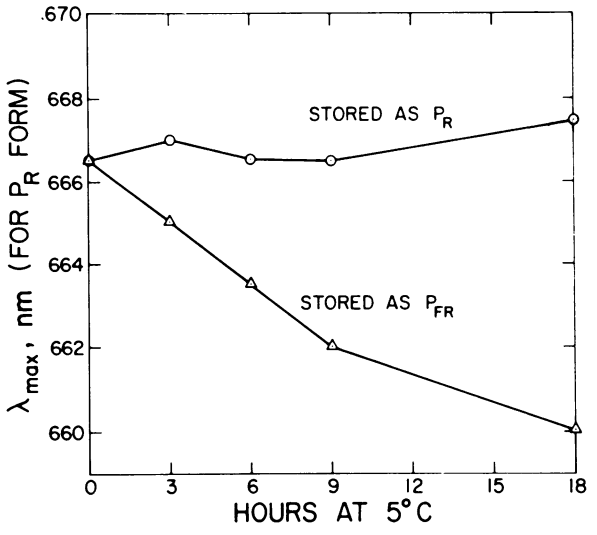

FIG. 10. Change in the absorption maximum for P_R for phytochrome stored either as P_R (top curve) or P_{FR} (lower curve).

Both the large and small components of phytochrome, obtained from the Sephadex G-200 column, can be shown to have, for the P_R form, absorption maxima near 667 nm. Figure 9 shows the absorption spectrum of oat phytochrome *in vivo* (obtained using a Biospect Model 61 spectrophotometer, single beam, adapted for tissue samples, Agricultural Specialty Company, Incorporated). The sample consisted of approximately 0.5 g of oat coleoptile tips 0.2 cm in length, free of primary leaf tissue. The phytochrome *in vivo* clearly shows an absorption maximum of P_R of 667 nm. Thus the extracted material, with its 667 nm absorbancy, evidently approximates the native condition for the chromophore. Allowing a purified sample to remain overnight as P_{FR}, even in the dark and at 4°, however, results in a shift of this absorption maximum from 667 to 660 nm, as shown in figure 10. Thus rapid procedure, and maintenance of P_R during as much of the isolation procedure as possible are required if what one desires in material spectrally similar to the native material.

Acknowledgments

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