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*Short Communication*

# Immunological Determination of the Relationship between Large and Small Sizes of Phytochrome

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SUSAN C. CUNDIFF AND LEE H. PRATT
*Department of General Biology, Vanderbilt University, Nashville, Tennessee 37235*

Molecular weight determinations of phytochrome, the photoreceptor for a wide variety of photomorphogenic responses in plants (6), suggest the existence of at least two sizes of the chromoprotein, a larger molecule with a molecular weight of at least 240,000 (large phytochrome) and a smaller molecule of approximately 60,000 mol wt (small phytochrome) (1, 3–5, 11). Recent evidence indicates that small phytochrome is an artifact resulting from the proteolysis of large phytochrome (5) and is not a subunit of an aggregating system as had been earlier reported (3, 4).

By using specific antisera prepared against the two sizes of the chromoprotein, we have both tested the hypothesis that the smaller size results from proteolysis of the larger and investigated the relationship between the two sizes. Our rationale for comparing large and small phytochrome with the two kinds of antisera is based on the assumption that if large phytochrome contains unique primary structure not present in small phytochrome, then antilarge phytochrome serum might contain immunoglobulins specific for these unique segments of large phytochrome and thus provide a means for their assay. Antismall phytochrome serum should not contain immunoglobulins against these unique determinants and would therefore serve as a control.

## MATERIALS AND METHODS

Crude preparations of large phytochrome from etiolated Garry oat (*Avena sativa* L., cv. Garry) seedlings were prepared by chromatography with brushite as described by Gardner *et al.* for their "partially purified" preparations (5). The brushite pool was fractionated with a low concentration of ammonium sulfate (20 g/100 ml) to selectively precipitate large phytochrome (3). Our assumption that these crude phytochrome preparations consist of large phytochrome is based upon the observations that chromatography of such material through Bio-Gel P-300 yields primarily large phytochrome (11), and that its electrophoretic mobility is identical to that of purified large phytochrome (see below). Preparations of purified large and small phytochrome from etiolated Garry oat seedlings were prepared by three chromatographic procedures (brushite, diethylaminoethyl cellulose, Bio-Gel P-300) as described elsewhere (11).

One unit of phytochrome is defined as that quantity which, in a volume of 1 ml, yields $A_{667\ nm}^{1\ cm} = 1$ following saturating far red irradiation. The absorbance ratios ($A_{667\ nm}/A_{280\ nm}$) of our preparations, used as an estimate of purity, were about 0.05 for the crude preparations and about 0.17 for the purified preparations. All experiments were performed under green safelights with phytochrome in the red-absorbing form in 60 mM potassium phosphate buffer, pH 7.8.

Antiserum against small phytochrome is the same as that characterized elsewhere (11). Antiserum against large phytochrome was prepared by injecting a rabbit with an immunospecific precipitate prepared by precipitating large phytochrome (absorbance ratio = 0.13) with a specific antiserum previously prepared against small phytochrome (12). The precipitate, containing 1 unit of phytochrome, was washed thoroughly with 0.15 M NaCl to remove extraneous protein, suspended in complete Freund's adjuvant, and injected into a rabbit (one-half intraperitoneally, one-half intramuscularly). The rabbit was boosted after 1 month by a subcutaneous injection of a similarly prepared precipitate of 0.5 units of large phytochrome and antismall phytochrome immunoglobulins. After an additional week, the rabbit was bled terminally, and the separated serum was stored at −20 C.

Immunoelectrophoresis was performed at a pH of 8.6 in a diethyl barbiturate buffer made 1.0% (w/v) in agarose as described elsewhere (11). Antigen wells required a volume of 3 μl and antiserum troughs were 2 mm wide except for the plates shown in Figure 2, a and b, which have 1.5-μl wells and 1-mm troughs. After washing with 0.15 M NaCl, precipitin bands were stained with coomassie blue. Phytochrome migration was toward the anode.

## RESULTS AND DISCUSSION

Immunoelectrophoretic assays of purified small and large phytochrome (Fig. 1, a and b) yield single precipitin bands against both sera indicating that the sera are specific for phytochrome. Additional evidence that the sera are specific is presented in Figure 2a, since the crude preparation assayed in this experiment contains a wide range of impurities and yet gives only a single precipitin band. Further, it is apparent that the electrophoretic migration rate of large phytochrome is greater under these conditions. Finally, when a mixture of purified small and large phytochrome are assayed together, a spur develops only against the antilarge phytochrome serum suggesting that there are determinants on large phytochrome which are absent from small phytochrome (Fig. 1c).

In order to test the possibility that large phytochrome contains unique primary structure not found in small phytochrome, a freshly isolated crude preparation of large oat phytochrome at a concentration of about 1 unit/ml was allowed to incubate in the dark for 96 hr at 4 C during which time an endogenous protease presumably could degrade the chromoprotein (5, 10). We observed that the initial precipitin band resulting from the presence of large phytochrome is slowly replaced during incubation by at least three new precipitin bands (Fig. 2). One of these new bands is electrophoretically similar to small phytochrome and is formed by both sera, whereas the others are distinct from both molecular weight forms of phytochrome and are observed only with antilarge phytochrome serum (arrows).

We obtained qualitatively identical results starting with

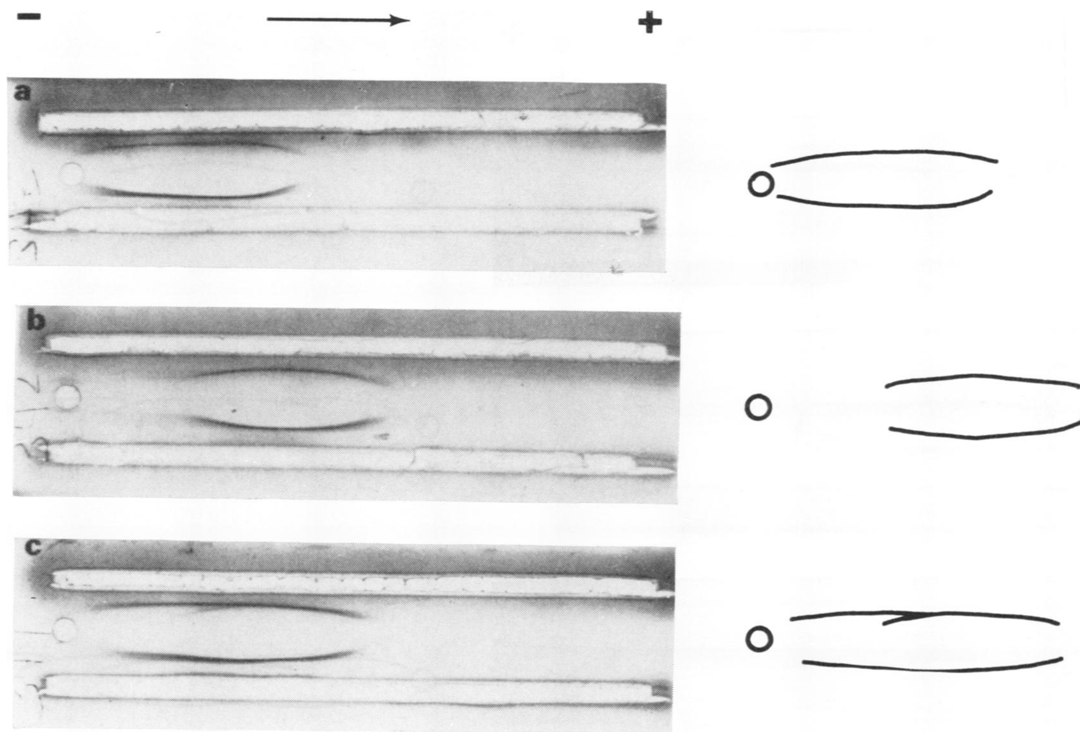


FIG. 1. Immunoelectrophoretic patterns of purified small (a), large (b), and a mixture of small and large (c) phytochrome. Phytochrome, at a concentration of approximately 1 unit/ml, was placed in each well and electrophoresed toward the anode for 2 hr at a potential of 15 v/cm, with the anode to the right. In each pattern, the upper trough was filled with undiluted antilarge phytochrome serum, the lower with undiluted antismall phytochrome serum.

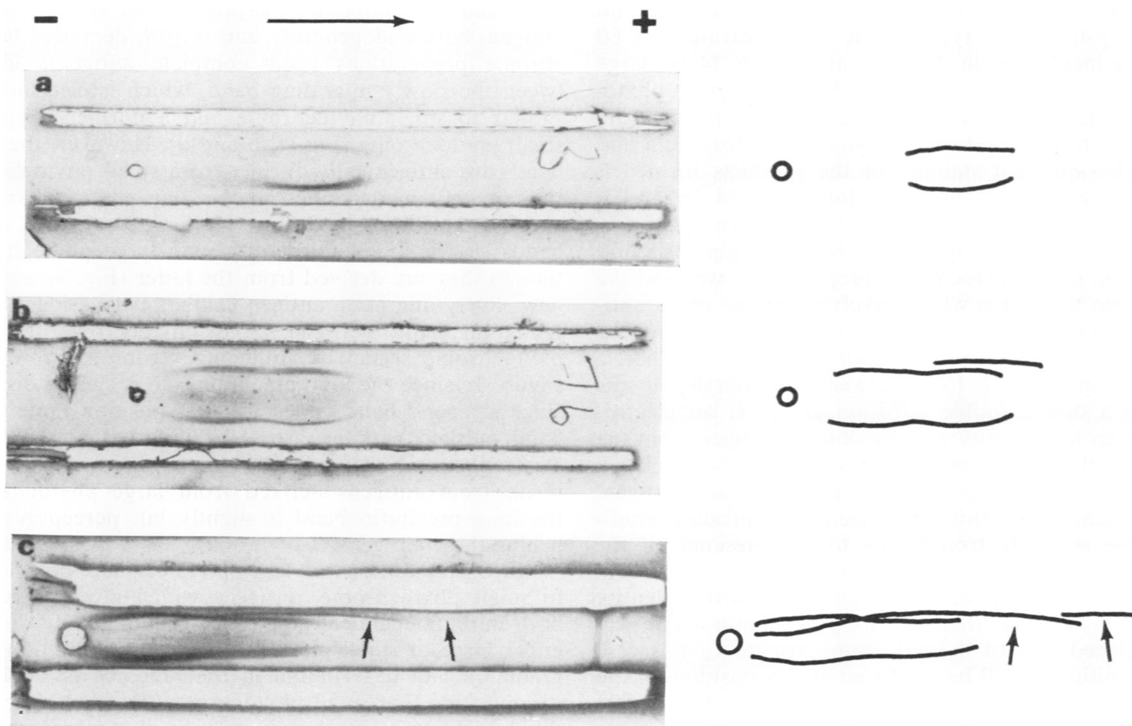


FIG. 2. Immunoelectrophoretic patterns of crude phytochrome at a concentration of approximately 1 unit/ml, at 0 hr (a), 42 hr (b), and 96 hr (c) after redissolving the ammonium sulfate precipitate from the brushite column and beginning of incubation at 4 C. Electrophoresis was performed as described for Figure 1. The upper troughs were filled with undiluted antilarge phytochrome serum, the lower with undiluted antismall phytochrome serum.

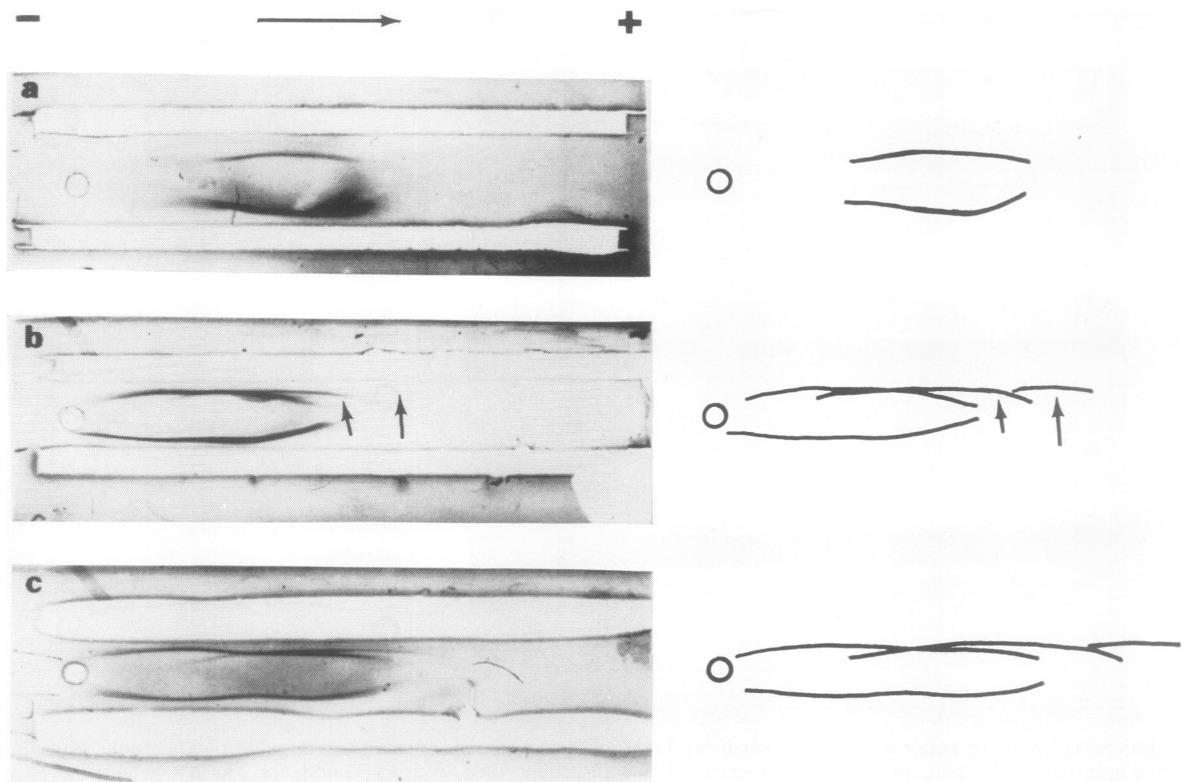


FIG. 3. Immunoelectrophoretic patterns of trypsin degraded purified large phytochrome at a concentration of approximately 1 unit/ml 24 hr (b) and 6 days (c) after the addition of trypsin to a final concentration of 60 $\mu$g/ml with incubation at 4 C. Pattern (a) is a control showing the pattern obtained with an identical phytochrome sample incubated for 24 hr in the absence of trypsin. Electrophoresis was performed as described for Figure 1. The upper troughs were filled with undiluted anti-large phytochrome serum, the lower with undiluted antismall phytochrome serum.

purified large phytochrome at a concentration of 1.5 unit/ml and adding trypsin (Sigma type XI) at a concentration of 60 $\mu$g/ml. During incubation in darkness at 4 C for 24 hr, large phytochrome disappeared and was replaced by small phytochrome and at least two other fragments electrophoretically distinct from both large and small phytochrome (Fig. 3, a and b, arrows). The apparent stability of the products formed is evident when the patterns obtained following 24 hr and 6 days of enzyme digestion are compared (Fig. 3, b and c).

In order to examine the products of both endogenously and trypsin degraded large phytochrome preparations, we used the common antigen technique which involves the use of an antigen trough in place of one of the serum troughs (9). As the antigen from the trough and the antiserum diffuse together, a straight precipitin line will form between and parallel to the troughs as in a direct double diffusion assay. If an electrophoresed antigen has identity with the known antigen from the antigen trough, then the long precipitin line will be displaced toward the antiserum trough by the electrophoresed antigen. This occurs because of a shift in antigen concentration gradient toward the antibody trough due to the presence of the electrophoresed antigen. No "spurs" or secondary bands will be formed. If an electrophoresed antigen has partial identity with the known antigen, then the long precipitin band will again be displaced toward the antiserum trough. In this case a second precipitin arc will be produced at this position on the agarose plate with the possibility of "spurs" or incomplete fusion of the two bands. Finally, if there is complete nonidentity between the electrophoresed antigen and the known antigen, then there will be no displacement of the precipitin line by the electrophoresed antigen.

As evidenced by the displacement of the long precipitin band and its complete incorporation of the electrophoresed antigen, both endogenously and trypsin degraded large phytochrome preparations show complete antigenic identity between the slowly migrating band, which is electrophoretically similar to small phytochrome and a purified preparation of small phytochrome (Fig. 4, b and d). However, the other new bands are antigenically distinct from small phytochrome since they are not incorporated in the long precipitin band. When compared with purified large phytochrome, none of the new bands shows complete identity with large phytochrome even though they are derived from the latter (Fig. 4, a and c). The new slowly migrating antigen exhibits partial identity with the precipitin band between large phytochrome and antilarge phytochrome serum as anticipated from the observations in Figure 1c since the long precipitin line is clearly displaced and since a second band appears at this position (note the second band marked with an arrow behind that obtained with the electrophoresed antigen). The other new bands also seem to result from antigens derived from large phytochrome since the long precipitin band is slightly but perceptibly displaced behind these antigens.

Since preparations of large phytochrome yield, in addition to small phytochrome, antigens which give precipitin bands both immunochemically and electrophoretically distinct from either large or small phytochrome upon both endogenous degradation and degradation in the presence of added trypsin, we conclude that small phytochrome does not arise by simple disaggregation or hydrolysis of large phytochrome into identical subunits. Our observations are in agreement with the conclusions of Gardner *et al.* (5) and, in addition, lead to the conclusion that large phytochrome contains at least two antigenically identifiable moieties in addition to the 60,000 mol wt

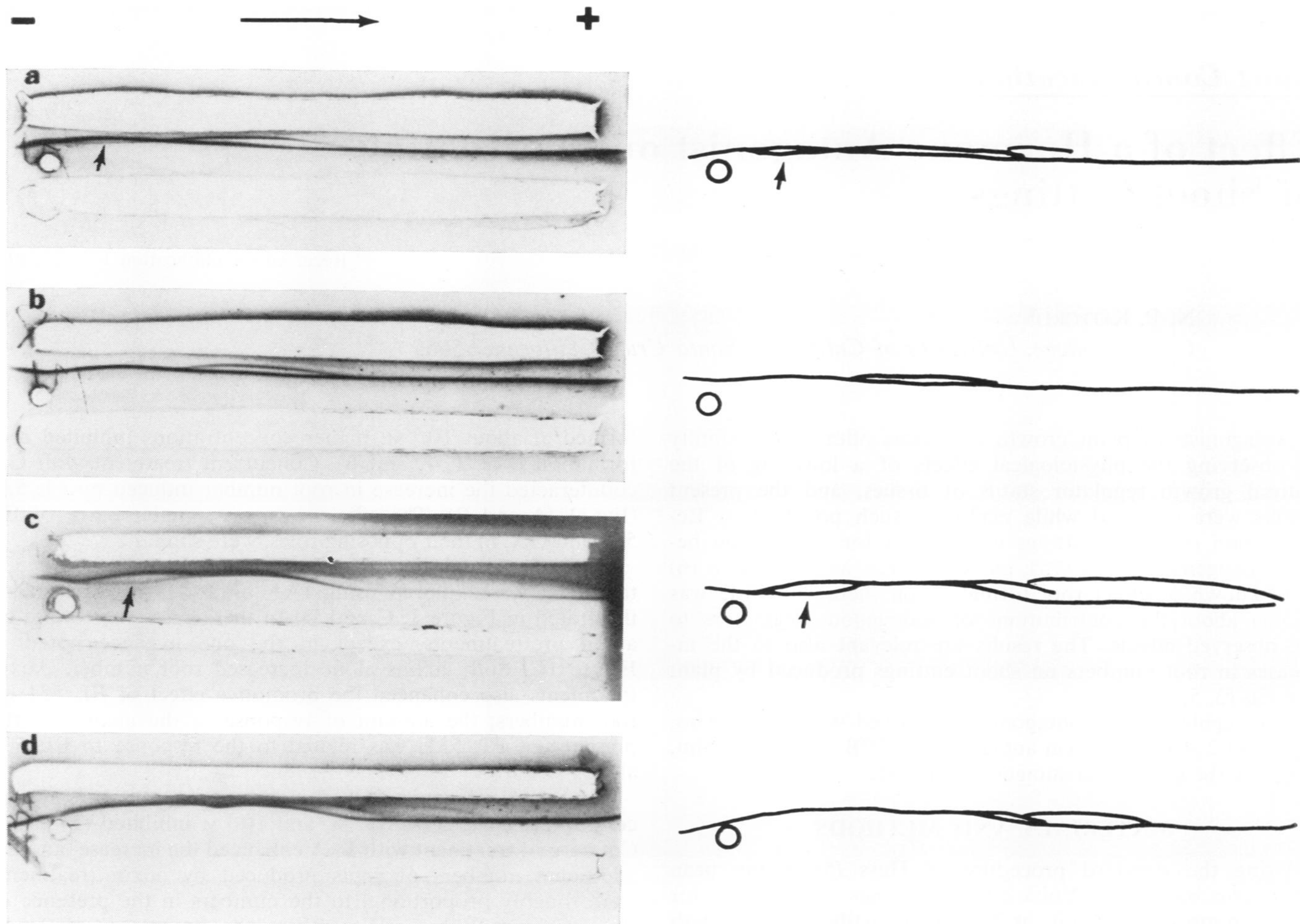


FIG. 4. Immunoelectrophoretic analysis of the degradation products of large phytochrome using the common antigen technique. The 96-hr endogenously degraded sample (a, b) and 24-hr trypsin degraded sample (c, d) were electrophoresed as described for Figure 1 and diffused against undiluted antilarge phytochrome serum in the upper trough. Purified large phytochrome (a, c) or purified small phytochrome (b, d) was placed n the lower trough at a concentration of 0.2 units/ml.

chromophore-containing moiety which has been characterized in several laboratories (7, 8, 13).

Recent preliminary observations resulting from immunoelectrophoretic analysis of eluant fractions from Bio-Gel P-200 chromatography of endogenously degraded large phytochrome indicate that one of the antigens derived from large phytochrome but not recognized by antismall phytochrome serum elutes before, and is therefore larger than, small phytochrome. This antigen possesses no spectral photoreversibility and hence cannot be detected by the usual phytochrome assay (2) even though it apparently represents a large fraction of the original molecule. We are continuing to characterize the products of phytochrome degradation, especially those which are distinct from small phytochrome, using our antisera as an assay system.

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