Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2431–2435, October 1971

Immunocytochemical Localization of Phytochrome

(photomicrographs/peroxidase visualization/oat shoot)

LEE H. PRATT AND RICHARD A. COLEMAN

Department of General Biology, Vanderbilt University, Nashville, Tennessee 37203

Communicated by Sterling B. Hendricks, July 19, 1971

ABSTRACT **An immunocytochemical assay, which localizes phytochrome *in situ*, indicates that this plant chromoprotein is associated with both nuclei and plastids, in addition to the cytoplasm. In an etiolated oat shoot, phytochrome is most abundant just behind the apex of the coleoptile, where it is associated with parenchyma cells; it is apparently absent at the extreme apex of the coleoptile. Further back from the tip, phytochrome is found in the epidermal cells of the coleoptile; it decreases in concentration toward the node, where it is again abundant. Phytochrome is also abundant in the extreme apical cells of young adventitious roots and in association with the procambium of the mesocotyl.**

Phytochrome is a chromoprotein that undergoes photoreversible absorbancy changes upon exposure to light and which serves as the photoreceptor for a large number of photomorphogenic responses in plants (1, 2). Two general hypotheses, each suggesting a different intracellular localization of phytochrome, have been developed in attempts to understand the primary mode of action of phytochrome. One hypothesis argues that phytochrome functions directly via differential gene regulation (1); the other argues that phytochrome regulates membrane permeability (2). Information about the subcellular distribution of phytochrome should assist in determining its primary mode of action.

The subcellular localization of phytochrome has been determined by several methods, but so far no consistent pattern has emerged. Phytochrome has been found associated (*a*) with the mitochondrion (3) and (*b*) with an unidentified particulate fraction (4) by differential centrifugation of aqueous plant homogenates, (*c*) with the nucleus by microspectrophotometry (5), and (*d*) with the plasmalemma by indirect physiological experiments (6). In addition, *in vivo* dual-wavelength spectrophotometry (7, 8) has been used to determine the distribution of phytochrome throughout etiolated seedlings, although the resolution of these measurements was limited by the size (generally 5–10 mm) of the plant segments assayed.

We have adapted an immunocytochemical technique to the problem of phytochrome localization in order to determine its distribution with greater sensitivity and with increased resolution as compared to other methods. Such a technique simultaneously provides information about the subcellular localization of phytochrome and about its distribution throughout an entire seedling.

MATERIALS AND METHODS

Tissue preparation. Oat seeds (*Avena sativa* L., cv. Garry) were germinated and grown in complete darkness at 25°C and 90% relative humidity on pads of moist cellulose packing material (Kimpack #6234, Kimberley-Clark Corp.) in plastic cafeteria trays. Shoots from 3- or 5-day-old seedlings were excised under dim green light, immediately placed in 4% formaldehyde in 0.09 M potassium phosphate, pH 7.8, and fixed overnight in complete darkness at 25°C. With no further precautions regarding lighting, the fixed shoots were dehydrated through an ethanol series, embedded in paraffin, sectioned 10-μm thick, and placed on glass microscope slides; the paraffin was removed with xylene.

Procedure for phytochrome localization. After rehydration of the sections through an ethanol series, phytochrome was localized by an adaptation of a technique described in detail by Sternberger *et al.* (9). The tissue is sequentially incubated, at room temperature, with (*a*) rabbit anti-phytochrome serum, which binds to phytochrome, (*b*) sheep anti-rabbit immunoglobulin G serum, which binds to the antiphytochrome immunoglobulin, and (*c*) immunospecifically purified rabbit anti-peroxidase–peroxidase complex, which binds to the remaining free binding site on the sheep anti-rabbit immunoglobulin G; an enzymatic assay for peroxidase is then performed.

The sections were incubated in serum by covering them with a few drops of the serum and gently agitating the slide

TABLE 1. *Protocol for an immunocytochemical localization of phytochrome*

Treatment	Duration
Prior incubation with undiluted, nonimmune sheep serum	15 min
Incubation with rabbit serum diluted 40-fold, (± anti-phytochrome)	15 min
Running saline wash	15 sec
Standing saline wash	15 min
Prior incubation with undiluted, nonimmune sheep serum	5 min
Incubation with sheep anti-rabbit immunoglobulin G serum, diluted 100-fold	15 min
Running saline wash	15 sec
Standing saline wash	15 min
Prior incubation with undiluted, nonimmune sheep serum	5 min
Incubation with purified rabbit anti-peroxidase-peroxidase complex, diluted 100-fold	15 min
Running saline wash	15 sec
Standing saline wash	30 min
Incubation with 3,3′-diaminobenzidine and H_2O_2	7–10 min

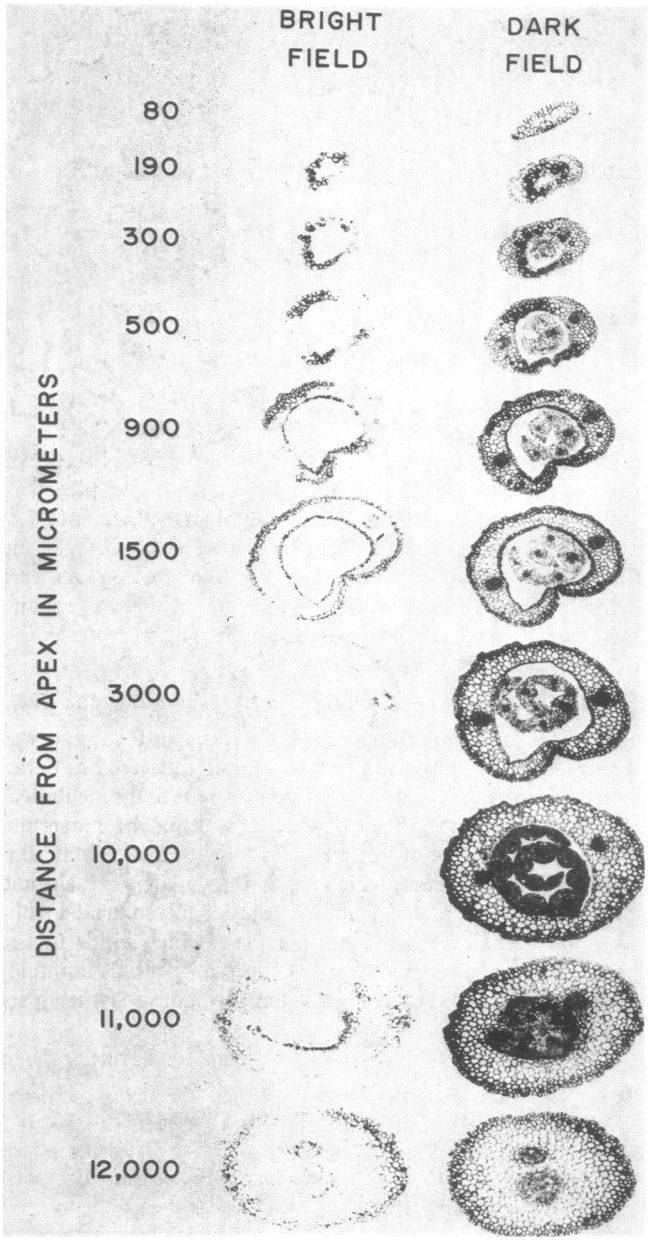

FIG. 1. Distribution of phytochrome in a 3-day-old etiolated oat shoot. Cross sections (10 μm thick), obtained at several distances behind the coleoptile apex, were immunochemically assayed for phytochrome and photographed with both bright-field and dark-field illumination. Magnification = $\times$23.

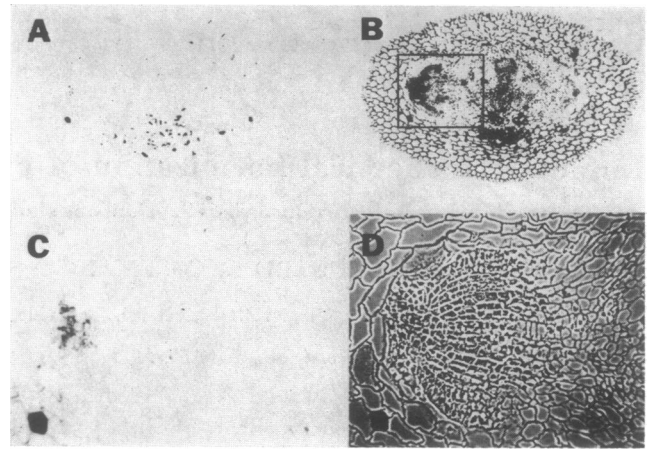

FIG. 2. Phytochrome distribution in a 10 μm thick cross-section obtained from immediately below the node of a 5-day-old etiolated oat shoot that was immunochemically assayed for phytochrome. Photomicrographs were taken with bright-field (*A*, *C*), dark-field (*B*), and phase-contrast illumination (*D*). The outlined area in *B* is presented at a higher magnification in *C* and *D*. Magnification of *A* and *B* = $\times$24; magnification of *C* and *D* = $\times$80.

on a linear shaker. Excess serum was removed by first rinsing the sections with a stream of 0.15 N NaCl and then placing the slide in an unagitated saline bath. In order to minimize nonspecific binding of active serum proteins to the sections, the latter were first incubated with a nonimmune sheep serum (which contained no anti-rabbit immunoglobulins) before incubation with each active serum and, in addition, the active sera were diluted up to 100-fold with the nonimmune sheep serum.

Peroxidase was visualized by a final incubation with a few drops of a fresh solution of 5 mg of 3,3′-diaminobenzidine tetrahydrochloride in 10 ml of 0.05 M Tris·HCl, pH 7.6, with 0.01% (v/v) H_2O_2. After dehydration to xylene and mounting of slides in Permount, the orange, insoluble reaction product was clearly visible with the light microscope. The complete protocol is summarized in Table 1.

Reagents. Rabbit anti-phytochrome serum was prepared by challenging a rabbit with about 1 mg of highly purified phytochrome (A_{280nm}/A_{670nm} = 0.90) in complete Freund's adjuvant ($^1/_3$ intraperitoneally, $^1/_3$ intramuscularly, $^1/_3$ subcutaneously), followed by a second, subcutaneous injection of about 0.5 mg of phytochrome (A_{280nm}/A_{670nm} = 0.85) one month later. After an additional week, the rabbit was bled terminally and the serum was separated and stored at -20°C. The anti-phytochrome serum was found to be specific for phytochrome when tested by immunodiffusion against oat extracts of widely different phytochrome purity but equal phytochrome concentration.

Phytochrome used to challenge the rabbits was purified from 4-day-old etiolated oat seedlings, as described by Rice (10) for low molecular weight oat phytochrome, except that 2-mercaptoethanol was not used after the initial brushite column. The purification involved four column chromatographic steps, in the following order: brushite, DEAE-cellulose, CM-Sephadex, and Bio-Gel P-150. The molecular weight of our preparations, as determined with a calibrated P-150 column, was about 60,000. Since recent evidence suggests that phytochrome of this size results from proteolytic degradation of a larger molecular weight molecule (ref. 11 and manuscript submitted to Plant Physiology), we tested our serum by immunodiffusion and found that it crossreacted with high molecular weight preparations of phytochrome (molecular weight about 400,000, as determined with a calibrated Bio-Gel P-300 column) from oats.

Sheep anti-rabbit immunoglobulin G and rabbit anti-peroxidase sera were gifts from Dr. Ludwig Sternberger, Medical Research Laboratory, Edgewood Arsenal, Md. 21010. Horseradish peroxidase, which was used in the preparation of both the anti-peroxidase serum and the peroxidase–antiperoxidase complex, had an RZ of 3.0 (12). Antiperoxidase was immunospecifically purified and complexed with peroxi-

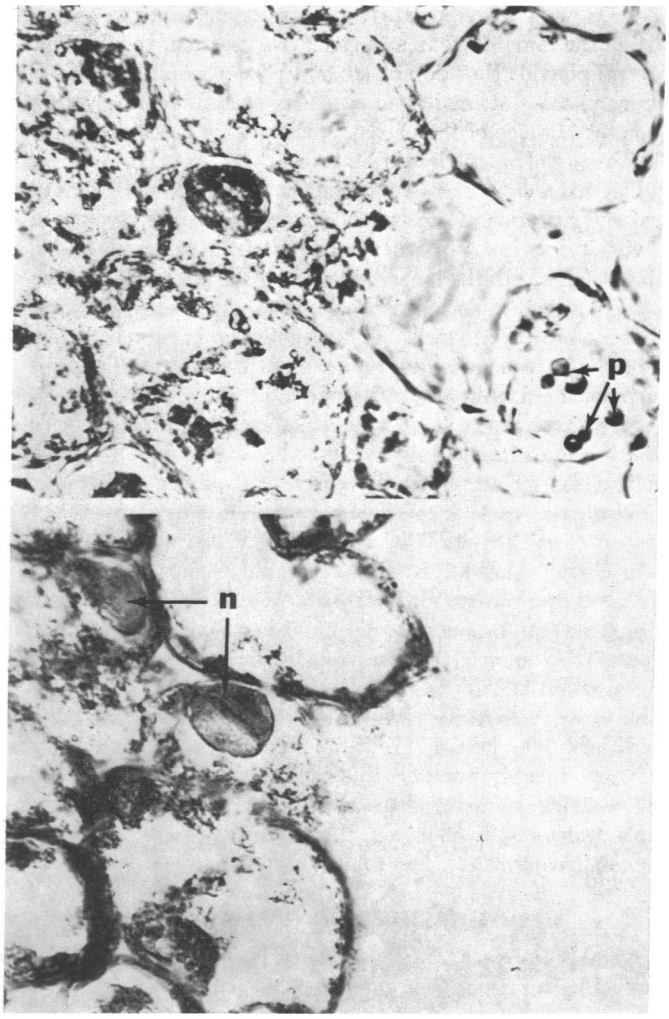

FIG. 3. Photomicrographs, taken with both phase-contrast (*upper*) and bright-field (*lower*) illumination, of coleoptile parenchyma cells in a cross section (10 μm thick) obtained from about 0.3 mm behind the tip of a 3-day-old etiolated oat shoot that was immunochemically assayed for phytochrome. Plastids are designated by *p*, nuclei by *n*. Magnification = $\times$860.

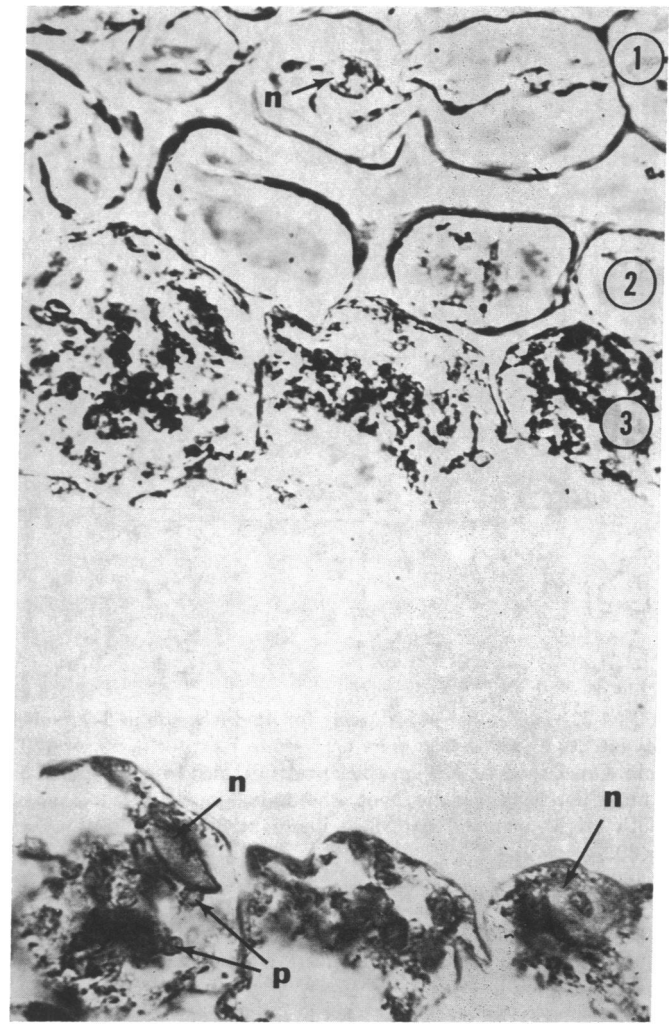

FIG. 4. Photomicrographs, taken with both phase-contrast (*upper*) and bright-field (*lower*) illumination, of a cross section (10 μm thick) obtained from about 0.3 mm behind the apex of a 3-day-old etiolated oat shoot that was immunochemically assayed for phytochrome. Cell layer (**1**) is primary leaf epidermis, layer (**2**) is coleoptile inner epidermis, and layer (**3**) is coleoptile parenchyma. Plastids are designated by *p*, nuclei by *n*. Magnification = $\times$780.

dase as described by Sternberger *et al.* (9). Nonimmune sheep and rabbit sera were obtained from Miles Laboratories.

Photomicrography. All sections were photographed with a Zeiss microscope under bright-field and either dark-field (Figs. 1, 2, 5) or phase-contrast (Figs. 3–5) illumination. Photographs taken with dark-field illumination are printed as negative images for clarity. Dark-field and phase-contrast photographs are presented in order to show the general structure of the sections. Dark areas in the bright-field micrographs indicate the presence of phytochrome activity, which is also visible as comparatively dark areas under dark-field illumination, since the insoluble, oxidized form of diaminobenzidine scatters light strongly.

OBSERVATIONS

Although we will be referring to the presence or absence of phytochrome throughout the paper, it should be understood that we are in fact visualizing peroxidase activity which is, in turn, associated only with phytochrome that maintains its antigenic activity under the conditions of the immunocytochemical assay. Controls were run in parallel with all sections presented, but no photomicrographs of them are shown because they are invisible under bright-field illumination; this result indicates the absence of both nonspecific binding of active serum-proteins to the sections and endogenous peroxidase activity.

The distribution of phytochrome in several 2 cm long, 3-day-old etiolated oat shoots was examined; 10 of the 30 cross sections assayed from a typical oat shoot are presented in Fig. 1. Phytochrome was not found within 100 μm of the coleoptile apex, but was abundant in parenchyma (i.e., nonepidermal and nonvascular) cells of the coleoptile 100–500 μm behind the apex. 0.5–1.5 mm behind the apex, the pattern of phytochrome distribution shifted from parenchyma cells to both the inner and outer epidermal layers of the coleoptile. Further back from the coleoptile tip, phytochrome content rapidly decreased toward the node and was not detectable in the region from 5 to 10 mm behind the apex, although it was

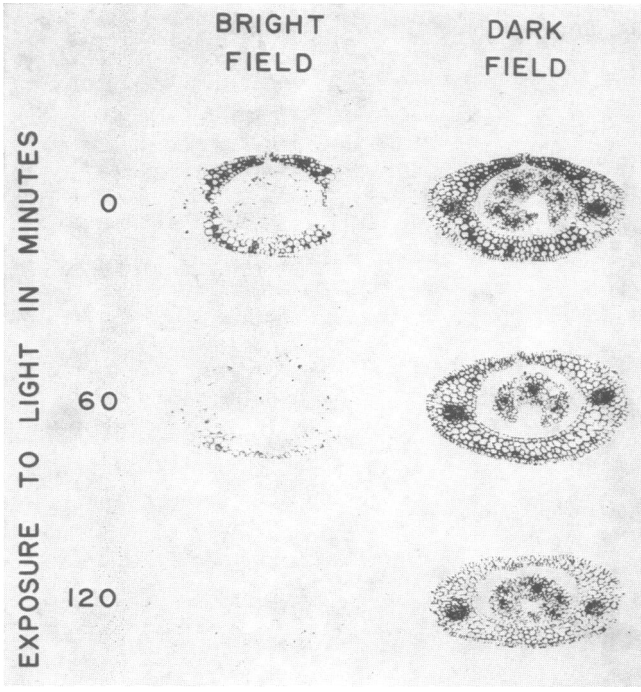

FIG. 5. Immunochemical assay for phytochrome in 3-day-old etiolated oat shoots that were exposed to light for 0, 60, or 120 min. Cross sections (10 μm thick) were obtained from about 0.5 mm behind the tip of the shoot. Photomicrographs were taken by both bright-field and dark-field illumination. Magnification = $\times$30.

found in the vascular tissue of the coleoptile in the 3-mm section. Phytochrome was again abundant at the node (11-mm section) and in the differentiating mesocotyl immediately behind the node (12-mm section), where it was found at the periphery of the stem and also in association with the procambial strands. Only small amounts, if any , of phytochrome were found in the primary leaf. Although not shown, cross sections immediately adjacent to those presented in Fig. 1 were assayed for total protein by a Ninhydrin–Schiff assay (13). We were unable to detect any correlation between total protein and phytochrome concentration.

Phytochrome distribution in 5-day-old etiolated shoots (about 8 cm long) was examined and found to be indistinguishable from that observed in 3-day-old shoots, although the intensity of the phytochrome assay was markedly reduced. Immediately behind the node of these 5-day-old plants, however, phytochrome was consistently found in the extreme apical cells of young adventitious roots, as well as with the procambial strands of the mesocotyl (Fig. 2). (Preliminary observations on older root apices also show a high concentration of phytochrome in the root apex, more specifically within the root cap.)

Cross sections obtained from about 0.3 mm behind the coleoptile apex of a 3-day-old etiolated seedling illustrate the general features of the intracellular distribution of phytochrome (Figs. 3 and 4). In all cells examined in which there is a detectable quantity of phytochrome, it is found associated with both nuclei and plastids, in addition to the cytoplasm.

Fig. 3, which shows only coleoptile parenchyma cells, demonstrates the general observation that all cells of the same type within a given cross section contain the same quantity of phytochrome if they contain any at all—that is, there are no cells with intermediate concentrations of phytochrome within the same tissue in any given cross section. In addition, several plastids that do not contain phytochrome are visible under phase-contrast in one parenchyma cell, demonstrating that the absence of the chromoprotein is not associated with the absence of organelles within the cell.

Fig. 4 is a cross section through the epidermis of a primary leaf and a protion of the surrounding coleoptile. Phytochrome is visible only in the parenchyma cells of the coleoptile and is absent from both the inner epidermis of the coleoptile and the leaf epidermis. A nucleus is visible in the phase-contrast view that is not associated with phytochrome, again demonstrating that the absence of a detectable quantity of phytochrome is not associated with the absence of organelles.

Since exposure to light is known to result in a decrease *in vivo* in phytochrome activity, as assayed spectrophotometrically (14), we examined the effect of exposure to light on phytochrome activity by our immunochemical assay. A tray of 3-day-old etiolated seedlings was placed 15 cm from four closely spaced Sylvania Gro-lux fluoresecent light bulbs at 22°C and representative shoots were removed at various times for both immunochemical and *in vivo*, dual-wavelength spectrophotometric (14) assays. After 60 min of exposure to light, about 50% of the initial spectrophotometrically assayable activity remained, while after 120 min only about 25% remained. The loss of activity in the immunochemical assay (Fig. 5) roughly parallels this observed decrease in spectrophotometrically assayable activity. In addition, we were unable to detect any change in the subcellular pattern of localization from that observed in fully etiolated tissue.

DISCUSSION AND SUMMARY

Several reasons may be given to justify our assertion that we are visualizing phytochrome with our immunocytochemical assay. First, by use of the protocol given in Table 1, immunochemical activity is wholly dependent upon the use of a specific anti-phytochrome serum. Second, endogenous peroxidase activity is not observed under the conditions of our assay. Third, the distribution of activity is in good agreement with the distribution of photoreversible activity observed in oat seedlings by Briggs and Siegelman (7). Fourth, the intensity of the immunocytochemical assay (Fig. 5) decreases in a roughly parallel fashion with the loss of spectral photoreversibility after exposure of the seedlings to light.

Assuming that we are, in fact, visualizing phytochrome *in situ*, several generalizations may be made regarding the distribution of the chromoprotein in etiolated oat shoots. First, the distribution of phytochrome is highly specific, with respect both to organs and to tissues. Near the tip of the shoot, for example, phytochrome is found only in the parenchyma cells of the coleoptile (Fig. 1, 190- and 300-μm sections), while farther back from the tip it is found only in the epidermal cells and vascular strands of the same organ (Fig. 1, 1.5- and 3-mm sections). In the region of the node, phytochrome is found only in the periphery of the shoot and in association with the differentiating vascular strands and the apices of adventitious root primordia (Fig. 1, 11- and 12-mm sections; Fig. 2). Also, in a given section, it is clear that phytochrome is not found in all cells of the same tissue (Fig. 3). Near the apex of the coleoptile, for example, phytochrome is found primarily in those regions of the coleoptile away from the two polar vascular strands (Fig. 1, 500-μm section) and is

not observed in the parenchyma cells surrounding the vascular bundles.

Second, phytochrome content is not positively correlated with total protein distribution. For example, phytochrome is not observed in the basal region of the primary leaf where the total protein concentration [Ninhydrin–Schiff assay (13)] is very high (Fig. 1, 10-mm section), but is abundant in the coleoptile where the total protein concentration is relatively low (Fig. 1, 190-μm section). Third, the absence of phytochrome is not related to the absence of organelles within a cell, since both plastids and nuclei may be found that are not associated with phytochrome (e.g., Figs. 3 and 4). Fourth, if a cell contains phytochrome, it contains as much as any other cell that also has phytochrome in the same tissue and in the same cross section.

Fifth, within an etiolated cell that contains phytochrome, the chromoprotein is always associated with both plastids and nuclei (if present in the field of view) as well as the cytoplasm. In addition, this general intracellular pattern of phytochrome distribution did not change after exposure to light, during which time some 90% of the spectrophotometrically detectable activity was lost. Unfortunately, the absence of any specific association of phytochrome, as observed with the light microscope, with a particular organelle or region of the cytoplasm does not allow one to use these observations to exclude either the gene-regulation or membrane-permeability hypotheses relating to the primary mode of phytochrome action.

This research was supported by National Science Foundation Grant GB-17057.

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