Detection of Phytochrome in Green Plants[1]

H. C. Lane, H. W. Siegelman, W. L. Butler, & E. M. Firer
ARS & AMS, U. S. Department of Agriculture, Plant Industry Station, Beltsville, Maryland

Action-spectra studies of the photocontrol of seed germination, flowering, and stem elongation showed that a reversible photochromic pigment plays a dominant role in the control of plant development (1). The pigment, now called phytochrome, was measured photometrically in dark-grown seedlings and was extracted as a soluble protein (2). Phytochrome has not been detected photometrically in the leaves of green plants even though physiological evidence for its presence is abundant. The present paper reports the presence of phytochrome in partially purified extracts of green plants.

Materials & Methods

Plants selected for extraction of phytochrome represented daylength-responsive plants of several types, and were obtained from the greenhouse and market. The tissue was frozen with dry ice and finely ground in a single-end-runner mill. A weighed amount was allowed to thaw in approximately an equal volume (w/v) of 0.05 M sodium pyrophosphate solution containing 0.001 M disodium ethylene diaminetetraacetate (EDTA) and .01 M 2-mercaptoethanol, pH 9.2. Some plants required more extracting solution to maintain the pH of the homogenate above 7.4. All subsequent steps were carried out at 2°.

The homogenate was filtered through cheesecloth and the filtrate centrifuged for 10 minutes at 16,000 × *g*. The clarified filtrate was made to 50 % saturation with solid ammonium sulfate. The pH was maintained at 7.4 during precipitation by addition of 2 N ammonium hydroxide. The mixture was stirred 10 minutes and the precipitate was collected by centrifugation at 5,000 × *g* for 10 minutes. The precipitate was dissolved in a volume of 0.01 M potassium phosphate buffer, pH 7.5, equal to one-tenth that of the initial homogenate. All buffers used contained 0.001 M EDTA and 0.01 M 2-mercaptoethanol. The solution was centrifuged 1 hour at 140,000 × *g*. Phytochrome could be detected in most extracts at this point.

Elimination of substances of low molecular weight was accomplished by gel filtration on a column of Sephadex G-50 equilibrated and developed with 0.01 M potassium phosphate buffer, pH 7.5. The active fractions from the column were combined and made to 33 % saturation with saturated ammonium sulfate solution adjusted to pH 7.5 with ammonium hydroxide. After stirring and sedimentation, the precipitate was dissolved in the same volume of buffer and centrifuged at 16,000 × *g* for 10 minutes. At this point the pigment was sufficiently concentrated in the extracts of most plants that a reliable assay could be made. Further gel filtration or ammonium sulfate fractionation gave only small increases in phytochrome concentration. In order to increase concentration, an alternate procedure was adopted, which gave higher yields and greater purification of phytochrome from extracts of spinach leaves and squash fruits. In this procedure the volume of the initial extract was reduced to 1/20th of its initial volume by ultrafiltration (4). The concentrated solution was centrifuged 10 minutes at 16,000 × *g*, made to 50 % saturation with solid ammonium sulfate, stirred, and sedimented as before. The precipitate was dissolved in a volume of 0.01 M potassium phosphate buffer, pH 7.5, equal to one-tenth that of the concentrated extract and clarified by centrifugation at 38,000 × *g* for 15 minutes.

The phytochrome in extracts was determined with a dual-wavelength photometer, which measured the optical-density differences between 660 and 730 mμ (Δ OD = $OD_{660} - OD_{730}$). The amount of phytochrome was indicated by the change in the optical-density-difference reading after irradiation of the sample, first with red light and then with far-red light [$P_{total} = \Delta(\Delta \text{ OD}) = \Delta \text{ OD}_r - \Delta \text{ OD}_{fr}$]. Relative concentrations of phytochrome in the extracts are reported as Δ (Δ OD)/5 cm, ‡0.001 Δ OD, and specific activities as Δ (Δ OD)/cm mg protein values.

Protein concentrations were estimated by the biuret method following precipitation with 10 % trichloroacetic acid (3) and washing with methanol.

Results & Discussion

Phytochrome was demonstrated in extracts of most of the plants examined (table I). It was easily detected in extracts from leaves of Hyoscyamus, spinach, and sugarbeet, which are typical long-day plants. The pigment was detected in extracts from some short-day plants such as sorghum and tobacco but was not found in others such as chrysanthemum, Perilla, soybean, and Xanthium (table I). The final extracts of chrysanthemum, Perilla, and soybean contained large amounts of chlorophyll, which could

[1] Received Nov. 27, 1962.

Table I

Phytochrome & Protein Contents of Leaf Extracts of Plants

Plant	Phytochrome [△ (△ OD)/5 cm]	Protein (mg/ml)	Specific activity [△ (△ OD)/cm/ mg protein]
Beta vulgaris L. (sugarbeet)	0.010	13.6	0.00014
Brassica oleracea var. acephala DC.	0.010	7.0	0.00028
***Brassica oleracea* var. botrytis L.	0.010	10.0	0.00020
Catalpa bignonioides Walt.	0.000	1.1	0
Chrysanthemum morifolium Ramat.	0.000	10.0	0
***Cucurbita maxima* Duchesne	0.047	9.2	0.0011*
Glycine max (L.) Merr. 'Biloxi'	0.000	15.0	0
Hordeum vulgare L.	0.008	7.0	0.00023
Hyoscyamus niger L. (annual)	0.010	14.0	0.00014
Lactuca sativa L.	0.005	5.0	0.00020
Lycopersicon esculentum Mill. 'Marglobe'	0.012	Not determined	
Melilotus sp.	0.010	11.2	0.00018
Nicotiana tabacum L. 'Maryland Mammoth'	0.010	10.8	0.00019
Nicotiana tabacum L. 'Catterton'	0.010	9.6	0.00021
Perilla frutescens (L.) Britt.	0.000	2.6	0
Saccharum officinarum L.	0.005	5.5	0.00018
Salvia splendens Sello. 'St. Johns Fire'	0.008	10.0	0.00016
Sorghum vulgare Pers. (milo)	0.006	7.6	0.00016
Spinacia oleracea L.	0.060	11.8	0.0012*
Zea mays L. 'U. S. 13'	0.004	12.0	0.00006

* Purified by alternate procedure.
** Curd and fruit extracted.

have interferred with the assay for phytochrome. The low protein values of some extracts suggest that the plants contain endogenous protein precipitants.

The concentration of phytochrome in the extracts was much lower than in extracts of etiolated plants. Other work has shown that phytochrome concentration is markedly reduced in dark-grown seedlings when they are placed in light. (W. L. Butler, H. C. Lane, & H. W. Siegelman. Non-photochemical transformations of phytochrome in vivo. In preparation.)

In all cases with extracts of green plants, the absorption of light by chlorophyll in the final extracts was considerably greater than that by phytochrome. Figure 1 shows the absorption spectrum of a 5-cm path of an extract from spinach and the difference spectrum (at a fivefold increase of photometric sensitivity) of the far-red-irradiated minus the red-irradiated solution. The difference spectrum shows phytochrome of green plants has the same spectral characteristics as that of etiolated plants. As long as the chlorophyll concentration is not too great the overlapping absorption band is of little consequence because of spectrophotometric assay for phytochrome depends on the photoreversible optical-density changes. However, when the chlorophyll concentration is great enough that the intensity of the transmitted measuring beams is comparable to or less than the intensity of chlorophyll fluorescence excited by the measuring beams, the chlorophyll begins to interfere with the measurement of phytochrome.

Reversibility in the extracts was retained for several days at 2° and for several weeks at −15° with slow diminution.

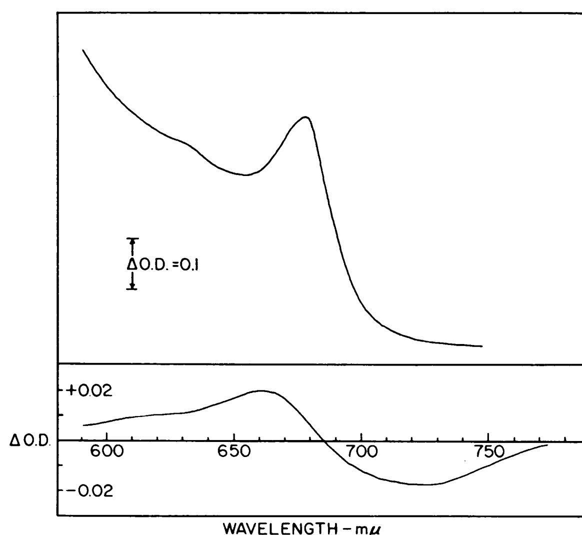

FIG. 1. Absorption and difference spectra of a 5-cm sample of spinach extract. Upper curve is the absorption spectrum; lower curve is the difference spectrum of the far-red-irradiated minus the red-irradiated solution.

Summary

Green tissue from 20 different kinds of plants was examined for phytochrome content. The soluble proteins were extracted in mildly alkaline buffer and precipitated from the solutions with ammonium sul-

fate. Chlorophyll concentrations were reduced by centrifugation. Phytochrome was detected in extracts from most of the plants examined. The spectral properties of phytochrome obtained from green plants were similar to those of the pigment extracted from etiolated tissue.

Literature Cited

1. BORTHWICK, H. A. & S. B. HENDRICKS. 1960. Photoperiodism in plants. Science 132: 1223–28.
2. BUTLER, W. L., K. H. NORRIS, H. W. SIEGELMAN, & S. B. HENDRICKS. 1959. Detection, assay, & preliminary purification of the pigment controlling photoresponsive development of plants. Proc. Natl. Acad. Science 45: 1703–08.
3. GORNALL, A. G., C. J. BARDAWILL, & M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751–66.
4. SIEGELMAN, H. W. & E. M. FIRER. 1962. A convenient ultrafilter for concentrating protein solutions. Anal. Biochem. 3: 435–37.

Inhibition of Metabolism in Avena Coleoptile Tissue by Fluoride [1, 2, 3]

Lawrence Ordin & Betty P. Skoe

Department of Biochemistry & Air Pollution Research Center, University of California, Riverside

Fluoride continues to be a major problem of air pollution. One of the aspects that is most insidious is the effect on growth or on respiration without visible tissue injury (2). Over a decade ago Bonner and Wildman (3) found that fluoride-induced inhibition of phosphatase in Avena coleoptiles was about the same as the inhibition of growth by fluoride. Respiration, on the other hand, was much less affected. Bonner and Thimann (4) concluded that inhibition of enolase was not involved in the inhibition of growth of excised plant sections.

Fluoride inhibition of growth of yeast cells has been linked to interference with cell wall polysaccharide synthesis, particularly by inhibiting the conversion of hexose-6-P to hexose-1-P (6). Christiansen and Thimann (5) found a small inhibition in net synthesis of cellulose and hemicellulose in the presence of IAA and fluoride in pea stem segments. Since partial growth occurred during these observations it is not clear whether the effect was a consequence of inhibited growth or not. Earlier reports (12, 13) have described the effect of some growth inhibitors on cell wall polysaccharides in the absence of growth hormones.

Because of the possibility that inhibition of metabolism of specific cell wall constituents might be the cause of the inhibition of growth, an investigation of the influence of fluoride on incorporation of C^{14} from glucose-U-C^{14} into the cell wall was undertaken. The present report shows that, even in the absence of IAA-induced elongation, growth inhibiting concentrations of fluoride markedly affect metabolism of certain components of the cell wall.

Materials & Methods

Oat seedlings (*Avena sativa*, L. var. Siegeshafer) were grown and harvested as described earlier (12). Coleoptiles were defoliated and 3 mm apical sections were discarded. The next two successive 5 mm sections were used in C^{14} experiments. For growth experiments, similar 5 mm decapitated sections with primary leaves in situ were used.

In each replicate, sections were pretreated in 20 ml of NaF or Na_2SO_4 solution for 6 hours, unless noted otherwise. The number of sections per ml of pretreatment solution varied from 5 for C^{14} experiments to 1 for growth experiments. Preliminary experiments indicated that the use of 5 sections per ml during pretreatment did not affect subsequent growth results when 20 sections were incubated in 20 ml of growth solution.

Since the amount of external fluoride carried over from a pretreatment solution was found to be insufficient to affect the growth results, the sections were blotted without rinsing before transfer to fluoride-free or sulfate-free solutions containing either glucose-C^{14} or IAA. All pretreatments and sub-

[1] Received Nov. 27, 1962.

[2] Part of this work was reported at the Annual Meeting of the American Society of Plant Physiologists at Corvallis, Oregon on August 30, 1962.

[3] This investigation was supported in part by a research grant (AP 00213) from the Division of Air Pollution, Bureau of State Services, United States Public Health Service.