Proc. Nat. Acad. Sci. USA
Vol. 73, No. 1, pp. 138–142, January 1976
Botany

Localization of phytochrome in etioplasts and its regulation *in vitro* of gibberellin levels

(photomorphogenesis/membrane permeability/hormone transport)

A. EVANS AND H. SMITH

University of Nottingham, Faculty of Agricultural Science, Sutton Bonington, Loughborough, LE12 5RD, Great Britain

Communicated by Winslow R. Briggs, September 5, 1975

ABSTRACT **Etioplasts isolated from barley leaves and purified on a Sephadex G-50 (coarse) column were characterized by electron microscopy and nucleic acid analysis. The majority of etioplasts retained an intact outer envelope, and contamination by other fragments was extremely low. The level of gibberellin-like substances extractable from intact etioplast suspensions was enhanced within 5 min of the termination of a saturating red irradiation, and the response was far-red reversible. Ultra-sonication caused a 3-fold increase in extractable activity both in dark control suspension and suspensions treated with red light. It is concluded that phytochrome, as a function of its interconversions, probably causes the transport of gibberellin from inside the etioplast into the surrounding medium. This leads to increased production of active gibberellins, possibly by release of feedback control of late steps of the biosynthetic pathway. Dual wavelength difference spectrophotometry has demonstrated the presence of a proportion of total cellular phytochrome within the etioplast.**

The pigment phytochrome exists in two forms: Pr, with an absorbance maximum at 660 nm; and Pfr, with an absorbance maximum at 730 nm. Photoconversion between the two forms occurs in response to irradiation, and through this property the molecule controls a large number of developmental processes occurring in plants.

Physiological evidence points strongly to an association of phytochrome with membranes. When phytochrome is present in the presumably active, Pfr form, a change in the functional properties of the membrane is thought to occur as an early consequence of Pfr action (1–4). The primary action of phytochrome at the membrane level, and the way in which an initial change in membrane properties leads to the observed developmental effects, are still unknown. Valuable knowledge, however, would be obtained from the study of a cell-free system in which phytochrome controls a specific biochemical process. In this communication we report evidence that some of the cellular phytochrome is located in the etioplasts, where it controls rapidly, and *in vitro*, the efflux of gibberellin-like substances across the etioplast envelope.

METHODS AND MATERIALS

Preparation of Plant Material. Seedlings of *Hordeum vulgare* L. cultivar Julia (Stevens, Shardlow, Derbyshire, England) were grown on moist cotton wool at 24° for 6 days in total darkness. Harvesting, and all subsequent procedures, were carried out under a dim green safe light. Segments of the first leaf were cut 3 cm long, 1 cm from the apex and immediately chilled on ice. All further procedures were carried out at 5°.

Abbreviation: M_r, molecular weight.

Isolation of Etioplasts. Etioplasts were isolated by a modification of the method of Wellburn and Wellburn (5). The laminae were finely chopped with a razor blade and gently homogenized in 25 mM *N*-morpholino-3-propanesulfonic acid (MOPS) containing 3 mM EDTA (disodium salt), 250 mM sucrose, and 0.2% bovine serum albumin, adjusted to a final pH of 7.5. In all experiments involving phytochrome estimation, 2-mercaptoethanol was added to a concentration of 14 mM. A tissue to buffer ratio of 1:2 was used. The homogenate was gently exuded through 12 layers of cheese cloth. The resulting filtrate had a pH of 7.1, which was maintained throughout the isolation procedure. The filtrate was centrifuged at 6000 × *g* for 1 min in a Sorvall model RCB-2 Superspeed centrifuge, and the pellet was washed and recentrifuged. The final crude plastid pellet derived from 80 g fresh weight of leaf tissue was resuspended in 4 ml of buffer and loaded onto a loosely packed Sephadex G-50 (coarse) gel filtration column, 50 cm long and 1.1 cm diameter. Fractions, 0.5 ml in volume, were eluted from the column, and one-drop fractions were taken at intervals for routine measurement on a Unicam SP 1800 ultraviolet spectrophotometer. Fractions composed of intact etioplasts were pooled and used in the subsequent experiments.

Light Sources and Treatments. Suspensions of isolated etioplasts were rapidly brought to 24° at which time treatment commenced. This temperature was maintained for the duration of the treatments, which were terminated by the addition of ice-cold absolute methanol to a final concentration of 75%. The red light source consisted of four 15 W "warm white" fluorescent tubes filtered through one layer of no. 1 Yellow Cinemoid and one layer of no. 14 Ruby Cinemoid (Rank Strand Electric, Kingsway, London, England). The resulting irradiance was 2.3 μE $m^{-2}s^{-1}$ at the sample surface. The source of far-red light consisted of four 250 W single coil tungsten bulbs filtered through 10 cm of running water, one layer of no. 5A Deep Orange and one layer of no. 20 Deep Blue Primary Cinemoid. Irradiance at the sample surface was 4.6 μE $m^{-2}s^{-1}$.

Extraction and Fractionation of Gibberellin-Like Substances. The methanolic extract was solvent partitioned according to the method of Reid and Crozier (6). Fractionation by thin-layer chromatography was as described by Reid *et al.* (7).

Estimation of Gibberellin-Like Activity. Gibberellin-like activity was estimated by a modification of the method of Jones and Varner (8). α-Amylase activity induced in the barley half seed was measured by estimating the amount of maltose produced in the hydrolysis reaction with a pure amylose substrate (9).

Estimation of Protein Content. Protein was estimated, where possible, by the method of Lowry (10). Bovine serum

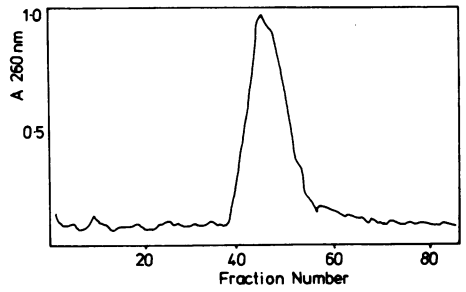

FIG. 1. Separation of crude etioplast preparation by Sephadex G-50 (coarse).

albumin was removed from the medium by repeated centrifugation and washing of the pellet with buffer free of bovine serum albumin. A certain amount of pigment remains in the supernatant at each stage of washing. It seems likely that this is accompanied by a loss of plastid protein, so that whilst this method may be used to estimate the protein content of purified etioplast preparations, it is not possible to make comparisons on a protein basis between stages of the isolation procedure.

Estimation of Phytochrome Content. Total phytochrome was measured at 25° using a Perkin Elmer 156 Dual Wavelength Spectrophotometer. Calcium carbonate was used to increase light scattering over a 1 cm pathlength. Actinic and measuring beams were set at 660 nm and 730 nm. All samples were pre-irradiated with sufficient red light to effect maximum conversion of protochlorophyllide to chlorophyllide prior to measurement. Actinic irradiations were of 30 sec duration. The difference spectrum was constructed at 0° using 800 nm as a reference wavelength.

Estimation of Nucleic Acid Content. Total nucleic acids were extracted and fractionated on polyacrylamide gels according to the method of Jackson and Ingle (11).

Electron Microscopy. The purified etioplast suspension was recentrifuged at 6000 × *g* for 1 min and the resulting pellet fixed in 1% Millonigs buffer (12), washed several times in buffer solution, and dehydrated in a series of graded ethanols. The pellet was embedded in Araldite and sectioned on a Reichert OMU 2 ultramicrotome. The sections were double stained with aqueous uranyl acetate and lead citrate and viewed in a D.E.O.L. JEM 6C electron microscope.

RESULTS

Characterization of Etioplasts. Separation of etioplasts by the method described here results in a high yield of intact etioplasts with minimal contamination by other fragments. The crude plastid pellet obtained by differential centrifugation is contaminated by mitochondria, nuclei, and broken plastid fragments. When the resuspended pellet is layered onto the column, broken fragments remain at the top whilst etioplasts and mitochondria are rapidly separated. Unlike that of Wellburn and Wellburn (5), the elution profile in Fig. 1 shows only one peak of absorption at 260 nm before the stationary phase of the column packs and further elution is prevented. Fractions 40 to 55 were pooled, examined by electron microscopy, and found to be predominantly composed of intact etioplasts (Fig. 2). It is thought the packing of the column soon after the elution of this peak prevents the elution of those fractions contaminated with mitochondria and broken plastids.

Gel scans of total nucleic acid composition of (A) the filtered homogenate, (B) the second crude plastid pellet, and

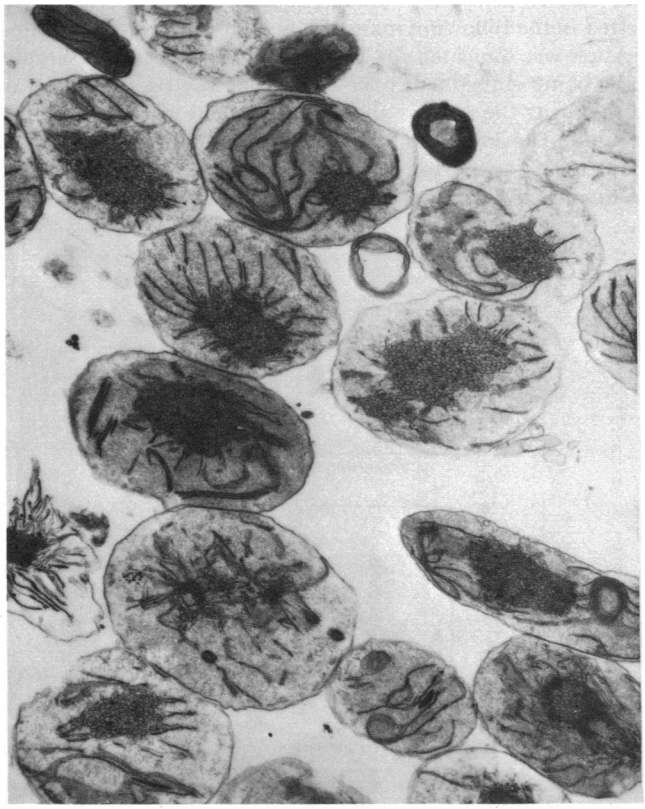

FIG. 2. Electron micrograph of an etioplast suspension purified on a Sephadex G-50 column (× 5800).

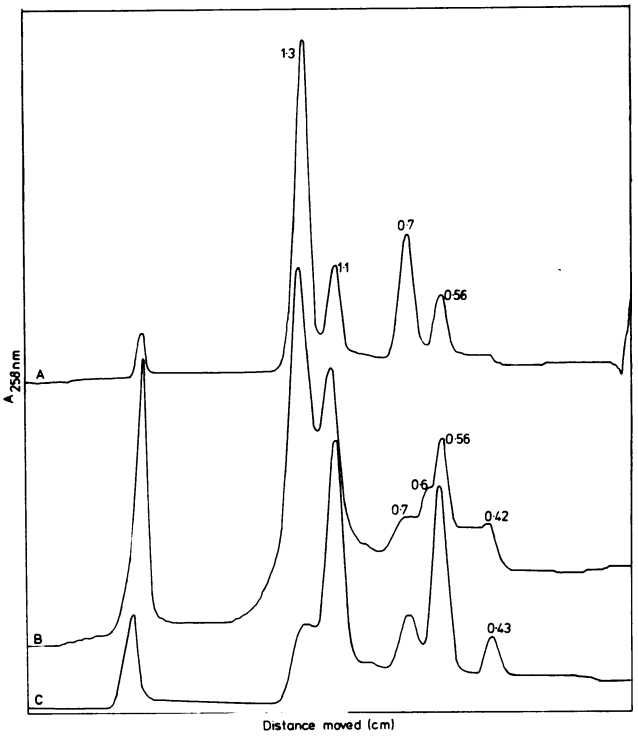

FIG. 3. Polyacrylamide gel electrophoresis of nucleic acids extracted from (A) filtered homogenate, (B) washed, crude plastid pellet, (C) purified etioplast suspension. The numbers refer to the estimated molecular weights of the respective RNA molecules ($\times 10^{-6}$).

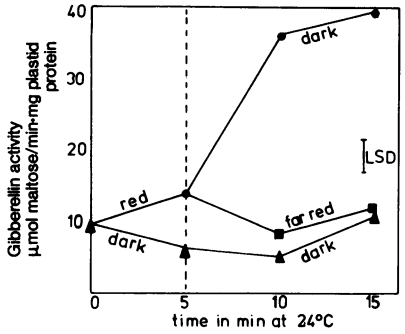

FIG. 4. The effect of red and far-red light on the level of gibberellin-like activity extractable from suspensions of intact etioplasts. LSD, least significant difference when $P = 0.05$.

(C) the purified etioplast suspension, can be seen in Fig. 3. The presence of various breakdown products makes quantification of the different components difficult; however, it is evident that the final separation step greatly enhances the proportion of plastid RNA as compared with previous stages. The small amount of cytoplasmic RNA, shown most accurately by the 1.3×10^6 M_r (molecular weight) fraction, still present in the purified etioplast preparation may be due to the adsorption of cytoplasmic ribosomes to the exterior of the etioplast envelope. The relatively large 0.7×10^6 M_r peak is mainly due to breakdown of the 1.1×10^6 M_r chloroplast component. It is notable, however, that the extent of breakdown observed after column separation (C) is much less than in the crude plastid pellet (B). This might be taken as an indication of the intactness of the etioplasts obtained after purification.

Light-Mediated Changes in Gibberellin-Like Substances. Fig. 4 demonstrates the effect of red and far-red light on the level of gibberellin-like substances extractable from suspensions of intact etioplast. Etioplasts were subjected to a saturating red irradiation of 5 min duration and then replaced in darkness. The level of gibberellin-like activity extractable into 75% methanol is substantially increased within 5 min of the termination of irradiation, whereas the level of activity extractable from suspensions held in darkness is not significantly changed. There is no increase in the level of activity in samples irradiated with far-red light immediately after red light.

Thin-layer chromatography of extracts (Fig. 5) indicates a major zone of activity in the dark controls at R_F 0.3 to R_F 0.6. After 5 min of red light followed by 5 min of darkness (Fig. 5f), the level of activity in this zone is significantly enhanced but migrates into two distinct peaks. There is an accompanying increase in activity at zones 0.2 and 0.9. This pattern is maintained after 10 min of darkness with some further increase in these zones. Far-red irradiation after red light treatment prevents the increase in activity seen in samples held in darkness after red irradiation. The major zone of activity in red/far-red treated samples was distinguishable between 0.4 and 0.6 R_F after 5 min of far-red light. No distinct pattern was distinguishable after 10 min of far-red light. Results of a generally similar nature were recently reported for wheat plastid preparations (13). Unfortunately, the significance of this report cannot be assessed due to the complete absence of any characterization of the plastid preparations used.

The rapidity of this response suggests that red light stimulates the production of the freely extractable hormone from a pre-existing form. As it is known that more gibberellin-like activity can be found in extracts of isolated chloroplasts whose membranes have been ruptured by ultrasonication than in extracts of intact chloroplasts (14), it is possible that substances with gibberellin-like activity are confined within the etioplast and are not freely extractable into 75% methanol from suspensions of intact etioplasts. This hypothesis was tested in the following manner: one half of an etioplast preparation was sonicated for 1 min in 20-sec bursts, the sample tube being embedded in ice to prevent any rise in temperature during the process; the remaining half was retained untreated. The intact and sonicated samples were brought to 24° and extracted for acid ethyl acetate-soluble gibberellin as described. From Fig. 6 it can be seen that three times the

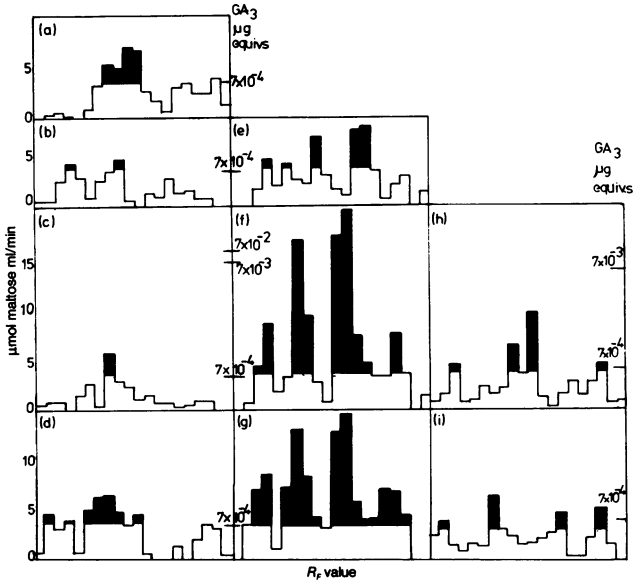

FIG. 5. Thin-layer chromatography of acidic ethyl acetate fractions of extracts from intact etioplast suspensions. (a) Zero time in dark, (b) 5 min in dark, (c) 10 min in dark, (d) 15 min in dark, (e) 5 min in red light, (f) 5 min in red light and 5 min in dark, (g) 5 min in red light and 10 min in dark, (h) 5 min in red light and 5 min in far-red light, (i) 5 min in red light and 10 min in far-red light. GA, gibberellic acid. ■, Significant promotion at 5% level of probability.

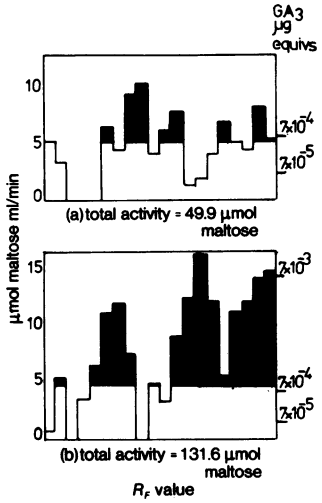

FIG. 6. Thin-layer chromatography of acid ethyl acetate fractions of extracts from (a) intact etioplast preparation, (b) ultrasonicated preparation, at time zero in dark. ■, Significant promotion at 5% level of probability.

Table 1. Comparison of gibberellin-like activity extractable from intact etioplasts and etioplast preparations ultrasonicated immediately prior to extraction

	Gibberellin-like activity (μmol of maltose/min·mg of protein)				
	Dark	5 min red light	5 min red light + 5 min dark		
Etioplasts	T	T	T	P	S
Intact	4.0	10.8	22.0	3.0	24.1
Sonicated	17.5	17.9	24.9	—	—

Extracts were made either of the total suspension (T), or of pellet (P) and supernatant (S) fractions after centrifugation.

activity extractable from intact etioplast suspensions may be extracted from suspensions after membrane rupture by ultrasonication. There is a marked increase in the content of nonpolar, i.e., rapidly migrating, substances extractable from ultrasonicated preparations. The intact etioplast envelope, therefore, prevents the expression of activity of gibberellin-like substances present within the etioplasts held in darkness.

Table 1 shows a comparison of the level of gibberellin-like substances extractable from intact suspensions of etioplasts and etioplast suspensions that have been sonicated immediately prior to extraction. The level of activity extractable from intact suspensions increases rapidly after red irradiation, the greatest activity being observed after 5 min of red light plus 5 min of darkness. Differential centrifugation of this suspension at 6000 × *g* for 1 min to pellet the etioplasts reveals that the bulk of activity is retained in the supernatant. The level of activity extractable from irradiated preparations sonicated just before extraction remains constant over the period of red irradiation and exhibits a slight increase after a 5-min dark period after irradiation. It appears from these data that the initial increase in gibberellin level observed in the response to red light is due entirely to release of activity from the etioplast, but there are rapidly initiated reactions that involve a detectable stimulation of gibberellin production over that already present in the etioplast.

Detection of Etioplast Phytochrome. Since it is evident that isolated etioplasts respond specifically to red and far-red irradiation, the possible presence of phytochrome in the etioplast was investigated optically in fractions eluted from the Sephadex column. Phytochrome was detectable in those fractions eluted from the column that have been shown to contain etioplasts (Fig. 7). The pigment was not detectable at any other point along the elution profile. At pH 7.1 in the absence of Mg^{++} ions the binding of phytochrome to membranes isolated from unirradiated tissue is extremely low (15). It would, therefore, be expected that any phytochrome not specifically associated with the etioplast would be retained in the supernatant during the initial stages of the separation procedure, and it can be concluded from these data that a proportion of total cellular phytochrome is either bound to the etioplast or present within the etioplast.

Additional evidence for etioplast phytochrome is given by the difference spectrum of the etioplast suspension (Fig. 8). The difference spectrum is clearly that of phytochrome, although there is some distortion due possibly to partial degradation of the high levels of protochlorophyllide present in these samples.

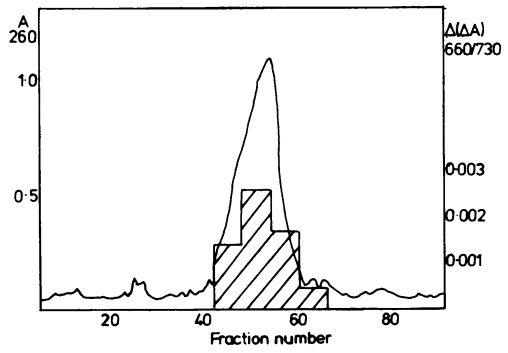

FIG. 7. Distribution of etioplasts and phytochrome on a Sephadex G-50 column. ▨, Absorbance at 260 nm; (—)Δ(ΔA) at 660 and 730 nm.

DISCUSSION

Significant levels of gibberellin-like activity have been found associated with chloroplast preparations from the leaves of several higher plants (14). The level of extractable activity may be increased by ultrasonication of the preparations, and this result has been interpreted as an indication of gibberellins being confined within the plastid and released on membrane rupture (14). It is evident from these data that a similar situation exists in etioplasts isolated from dark-grown barley leaves. Low levels of gibberellin-like substances are extractable from intact etioplast suspensions held in darkness. Much higher levels of activity are extractable after either red light irradiation or membrane rupture by ultrasonication. These results are most easily interpretable as being due to the transport of pre-existing gibberellin from the etioplast into the ambient medium brought about by the photoconversion of phytochrome from the Pr to the Pfr form. It seems unlikely that phytochrome actually transports the gibberellin across the etioplast membrane because of the 5-min lag usually seen before the onset of a detectable increase in gibberellin level. It is more likely that phytochrome changes the permeability of the membrane with respect to gibberellins. The far-red reversibility of this response is consistent with this concept. The amount of gibberellin-like substances extractable at the onset of far-red irradiation is, in most experiments, not significantly greater than at time zero; a change in the permeability of the membrane at this point due to the photoconversion of Pfr to Pr would prevent any further release of gibberellin from the etioplast.

It is apparent from comparisons between chromatograms of dark sonicated extracts and extracts of samples irradiated with red light that the total gibberellin extractable after Pfr activity cannot be entirely accounted for by a simple release mechanism. This is clearly seen in Table 1, where the level

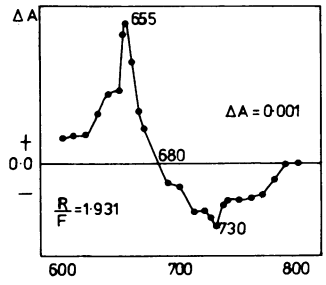

FIG. 8. Difference spectrum of etioplast phytochrome. Δ*A*, absorbance of far-red irradiated sample minus absorbance of red irradiated sample; R/F, ratio of Δ*A* at 655 nm to Δ*A* at 730 nm.

of gibberellin activity measurable in preparations of etioplasts sonicated after light treatment increases after 5 min of red light followed by 5 min of darkness. Release from the etioplast envelope is, therefore, selective and a component of gibberellin production must be involved in the response to red light. Recent studies of gibberellin metabolism have suggested that the chloroplast may be an important site of gibberellin synthesis and may have a regulatory function in the compartmentation of early intermediates of the gibberellin interconversion pathways (16, 17). It is conceivable that intermediates of such a pathway exist in equilibrium within the etioplast. Release of end products of such a pathway in response to Pfr action would result in the disturbance of the internal equilibrium and a stimulation of earlier steps of the pathway towards a re-establishment of the equilibrium. Physiologically active gibberellins released from the etioplast may be involved in a series of reactions culminating in the final display of photomorphogenic phenomena observed in the process of de-etiolation of cereal leaves.

Phytochrome has been shown, both by red/far-red control of gibberellin levels and by direct difference spectroscopy, to be associated with the etioplast. Taken with the demonstrable affinity of phytochrome for membranes *in vitro* (18) and its reported association with other subcellular membrane structures such as the plasmalemma (4), nucleus (19), rough endoplasmic reticulum (20), and mitochondria (21), this evidence supports the hypothesis that phytochrome is located in intimate association with specific critical membranes over which it exerts a regulatory function (1, 22).

It appears likely that release of gibberellin from the etioplast is an active transport process. The question as to whether phytochrome regulates the passage of gibberellin in a specific manner involving gibberellin "transport factors" or in a nonspecific manner such as by the regulation of ATP levels in the etioplast is open to speculation. Whilst the first alternative represents a mechanism specific to gibberellin control, the second is generally applicable to phytochrome phenomena. The cell-free system described here provides an excellent opportunity for investigating these possibilities and should prove of value in achieving a greater understanding of the role and action of phytochrome.

We thank Miss J. Elliott, Dr. M. Jackson, and Mrs. A. Tomlinson for technical assistance, and the Ministry of Agriculture, Food and Fisheries for supporting this work.

1. Hendricks, S. B. & Borthwick, H. A. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 2125–2130.
2. Satter, R. L., Marinoff, P. & Galston, A. W. (1970) *Am. J. Bot.* **57**, 912–926.
3. Tanada, F. (1968) *Proc. Nat. Acad. Sci. USA* **59**, 376–380.
4. Haupt, W. (1970) *Physiol. Veg.* **8**, 551–563.
5. Wellburn, A. R. & Wellburn, F. A. M. (1971) *J. Exp. Bot.* **23**, 972–979.
6. Reid, D. M. & Crozier, A. (1970) *Planta* **94**, 95–100.
7. Reid, D. M., Tuing, M. S., Durley, R. C. & Railton, I. D. (1972) *Planta* **108**, 67–75.
8. Jones, R. L. & Varner, J. (1967) *Planta* **72**, 155–161.
9. Bernfield, B. (1955) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press Inc., New York), Vol. I, pp. 149–150.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–273.
11. Jackson, M. & Ingle, J. (1973) *Plant Physiol.* **51**, 412–414.
12. Millonig, G. (1961) *J. Appl. Phys.* **32**, 1637.
13. Cooke, R. J. & Saunders, P. F. (1975) *Planta* **123**, 299–302.
14. Stoddart, J. L. (1968) *Planta* **81**, 106–112.
15. Marme, D., Boisard, J. & Briggs, W. R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3861–3865.
16. Railton, I. D. & Reid, D. M. (1974) *Plant Sci. Lett.* **2**, 157–163.
17. Railton, I. D. & Reid, D. M. (1974) *Plant Sci. Lett.* **3**, 303–308.
18. Roux, S. J. & Yguerabide, J. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 762–764.
19. Galston, A. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 454–460.
20. Williamson, F. A. & Morre, D. J. (1974) *Plant Physiol. Ann. Supp.* **46**.
21. Manabe, K. & Furuya, M. (1974) *Plant Physiol.* **53**, 343–347.
22. Smith, H. (1970) *Nature* **227**, 655–668.