DETECTION, ASSAY, AND PRELIMINARY PURIFICATION OF THE PIGMENT CONTROLLING PHOTORESPONSIVE DEVELOPMENT OF PLANTS

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Responses of plant materials to radiation indicate that flowering and many other aspects of development are controlled by a reversible photoreaction[1,2] involving two forms of a pigment, with action maxima near 660 and 735 mμ. The photoreversible pigment can readily be changed from one to the other form, as indicated by response of the plant to irradiation in the region of the appropriate action maximum. Because the nature of the enzymatic action involved is still unknown, it appeared that direct observation of the pigment in the living material and an assay for its isolation would have to be based on spectrophotometric methods. The pigment should show a change in absorption at 655 and 735 mμ following conversion by radiation.

The location of the pigment and its concentration in specific cells are evidenced by the photoinduced formation of anthocyanin, which depends upon energy transfer from both forms of the excited pigment.[3] The concentration based on a molar absorptivity of 1×10^5 is estimated to be the order of 10^{-6} molar in the most effective cells and probably about 10^{-7} molar in the average tissue.[4] A spectrophotometer suitable for detecting this low concentration of the pigment in tissue must measure absorption of radiation with high sensitivity in dense light-scattering material. Such measurements cannot be made with commercial instruments.

Instrumentation.—The presence of the photoreversible pigment in intact tissue has been demonstrated with a recording, single-beam spectrophotometer. This spectrophotometer[5] employs an end-window multiplier-type phototube placed directly behind the sample to collect a large fraction of the transmitted light. The sample is illuminated by monochromatic light from the exit slit of a double, prism monochromator. Spectral measurements can be made on light-scattering samples having optical densities between 0 and 6, with a sensitivity as high as 0.1 for full scale deflection. The noise level is equivalent to an optical density change of 0.002 for samples having an optical density less than 4.

Since this is a single-beam instrument the recorded curve includes the spectral response of the instrument in addition to the absorption characteristics of the sample. The system response is sufficiently reproducible that valid difference spectra can be obtained by subtracting one recorded curve from another. When the spectral curve is very steep in the region of interest, an electrical compensation can be applied to alter the slope of the curve. This is achieved with a potentiometer geared to the wavelength drum which supplies an additional signal to the Y-axis. The compensation merely alters the system response to make it easier to compute difference spectra.

A more useful instrument for assay of the pigment is one which measures directly the optical density difference between two fixed wavelengths. Such an instrument,

which is a double beam, bichromatic spectrophotometer similar in principle to one developed by Chance,[6] is shown in Figure 1. The close juxtaposition of the end-window, multiplier-type phototube (P) and the sample allows dense light-scattering samples to be used. The two monochromatic beams are obtained with wedge interference filters F_1 and F_2. The spectral purity of the beams is improved with auxili-

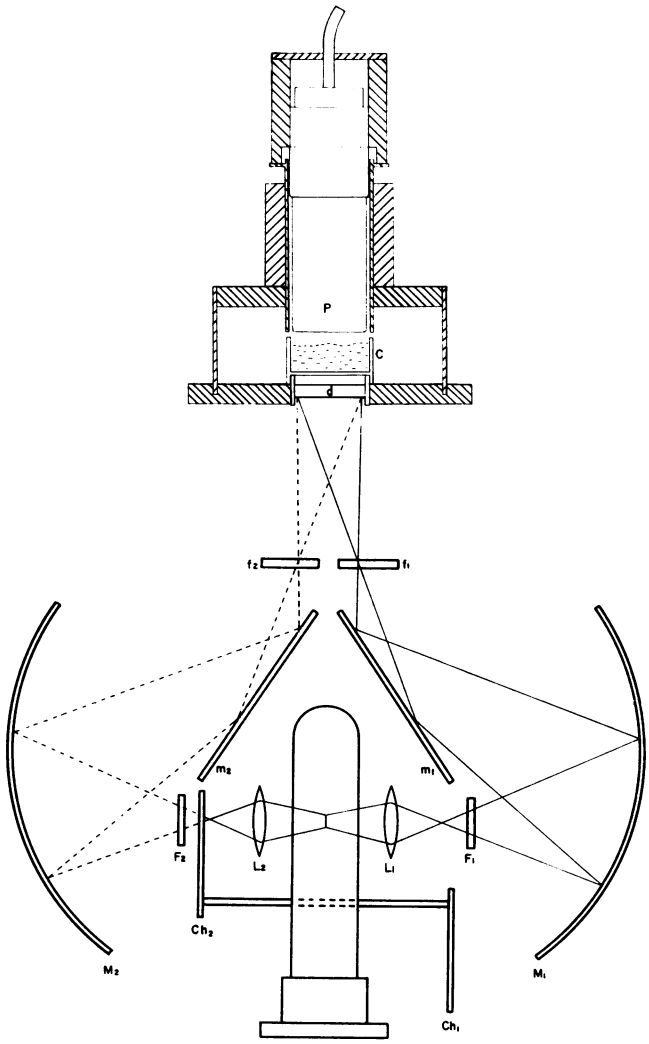

FIG. 1.—A differential spectrophotometer suitable for assay of the reversible pigment in living tissue or solution.

ary filters f_1 and f_2. The rotating chopper blades Ch_1 and Ch_2 cause the sample to be illuminated alternately with the two beams through the diffusing disk d. The phototube is synchronized with the chopper blades so that the measuring circuit records the optical-density difference ($O.D._{\lambda 1} - O.D._{\lambda 2}$). This instrument can be operated at an optical-density sensitivity as high as 0.05 for full-scale deflection, with a noise level of 0.0005.

The radiation source to change the state of the pigment was a focused beam from a 75 watt internal-reflection projection lamp with appropriate filters. The "red" source was a band in the region of 600 to 700 mμ and the "far-red" source in the region >700.

Detection of the Pigment in Living Plants.—The first tissue selected for examination was cotyledons of turnip seedlings (*Brassica rapa* var. white globe purple top) grown in the dark in the presence of chloramphenicol. This selection was based on the marked capacity of such tissue to form anthocyanin in light[7] and on the low content of protochlorophyll and chlorophyll which interfere with observations in the region of 670 mμ. The cotyledons were loosely pressed into the sample holder to a depth of about 1.5 cm and irradiated for about a minute with red radiation. This radiation converted any protochlorophyll present to chlorophyll and the pigment to the form with its absorption maximum near 735 mμ (later referred to as P_{735}). The curve of the optical densities of this tissue versus wavelength was measured in the 570 to 850 mμ region. The pigment was then converted to the form with the absorption maximum near 655 mμ (later referred to as P_{555}) by irradiation with far-red radiation for about a minute, and the optical density curve of the tissue was recorded again in the 570 and 850 mμ region.

The pigment was clearly evident by the optical density changes. Far-red radiation caused the optical density to decrease in the far-red region of the spectrum with a maximal change at 735 mμ and to increase in the red region with a maximal change at 655 mμ. Red radiation had the opposite effect.

A preliminary survey of etiolated parts of several seedling plants was then made and the shoots of 3-day-old maize seedlings were found to be particularly responsive. The shoots were cut and loosely pressed into a sample holder. A record from the spectrophotometer for a 1.5 cm thick sample of this material after irradiation with red and far-red radiation is shown in Figure 2. The difference spectrum is also plotted. It is evident that P_{735} has an absorption maximum at 735 mμ and P_{655} has an absorption maximum at 655 mμ. The reversibility between P_{655} and P_{735} by the action of red and far-red light can be repeatedly demonstrated. These results are in complete agreement with numerous action spectra[8] as also is the absorption in the region of 600 to 650 mμ.

The chlorophyll absorption near 672 mμ in Figure 2 is the result of the conversion of protochlorophyll by the initial irradiation with the red source. For a short period after the initial conversion, absorption in the red region of the spectrum changes because of a shift of the newly formed chlorophyll with an absorption maximum at about 680 mμ to a form with an absorption maximum at about 670 mμ.[9] Subsequent shifts of the chlorophyll peaks are sufficiently slow as not to interfere with the assay for the photoreversible pigment. No resynthesis of protochlorophyll is evident in maize seedlings for about 2 hours after the initial irradiation. Thereafter, the assay for the pigment is complicated by the snythesis of protochlorophyll and its conversion to chlorophyll by red irradiation.

A sample of maize shoots examined on the differential spectrophotometer gave a $\Delta(\Delta O.D.) = (O.D._{655} - O.D._{735})$ far-red irradiated $- (O.D._{655} - O.D._{735})$ red irradiated $= 0.2$. The concentration of the pigment and the sensitivity of the instrument were such that the distribution of the pigment along a single shoot could be determined by limiting the sample to a 5 mm segment. Values of $\Delta(\Delta O.D.)$

of 0.01 were observed with the coleoptile and 0.005 with the mesocotyl. Leaves of the maize seedlings were not responsive.

It was immediately evident from a preliminary survey of several tissues that the reversible pigment occurs at the maximum observed levels in shoots of several grasses irrespective of anthocyanin formation. Seemingly, the better guide is the responsiveness to etiolation of the shoots of gramineous plants which is displayed by the lengthening of internodes beneath the soil. Maize was selected as a suitable

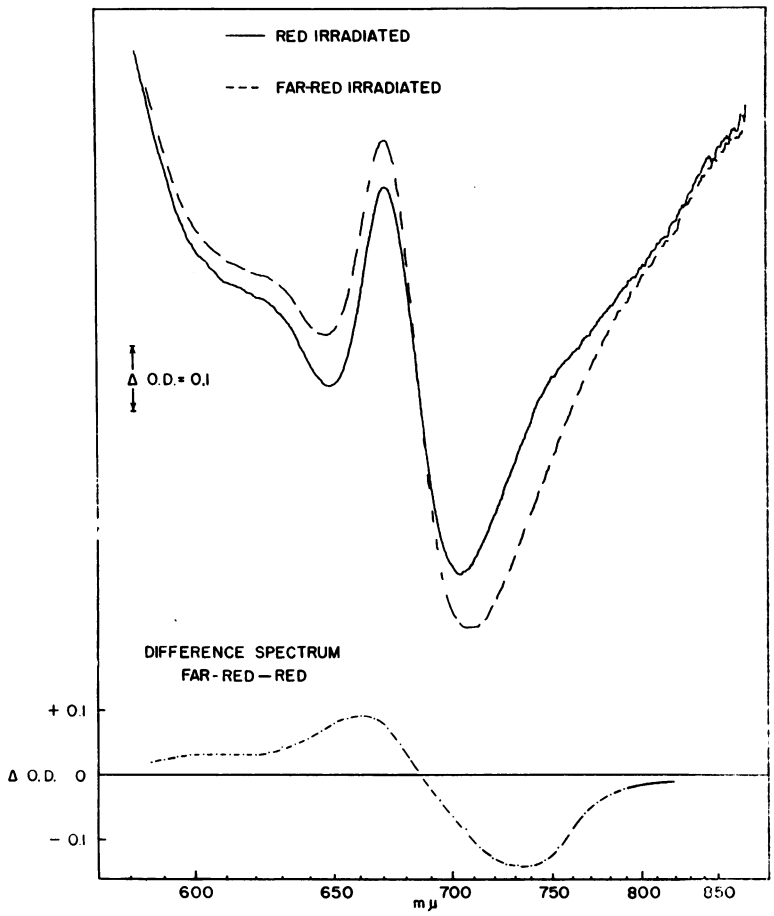

FIG. 2.—Recorded optical density curves from maize shoots in the 580 to 850 mμ region after red and far-red irradiations. The difference spectrum is shown.

source for the pigment because the seed are readily available, the shoots are large, and the pigment response is as great as with any tissue surveyed.

Separation of the Pigment.—Initial stages of pigment purification were effected by straightforward methods of protein chemistry using the Δ(ΔO.D.) values for assay.

A 450 gram lot of 3-day-old seedling maize shoots grown in darkness at 27° of variety US-13 was run in a blendor for 2 min with 450 ml of 0.1 *M* $Na_4P_2O_7$ buffer (pH 8.4) containing 0.01 *M* ascorbate and 0.01 *M* cystiene. The blended material

was passed through cheesecloth and the filtrate was then centrifuged at 40,000 × *g* for 20 min. The supernatant was centrifuged at 140,000 × *g* for 120 min and the resulting supernatant was brought to 0.33 saturation with $(NH_4)_2SO_4$. The precipitate was stirred for 15 min. It was then removed and redissolved in 0.25 of the original volume of solution. All operations were performed at 2°. Results from one of five runs are shown in Table 1. Protein values in this table were determined by the biuret method. The precision of the Δ(ΔO.D.) values was <0.0005/cm.

The pigment was retained upon dialysis at 2° against buffer at pH 8.4. The photoreversibility was retained for a period of at least two weeks upon holding solutions at −15°. It was lost upon heating to 50°.

A solution of the pigment gave the response shown in Figure 3 on the differential spectrophotometer. The reversibility of the pigment is also illustrated. In fact,

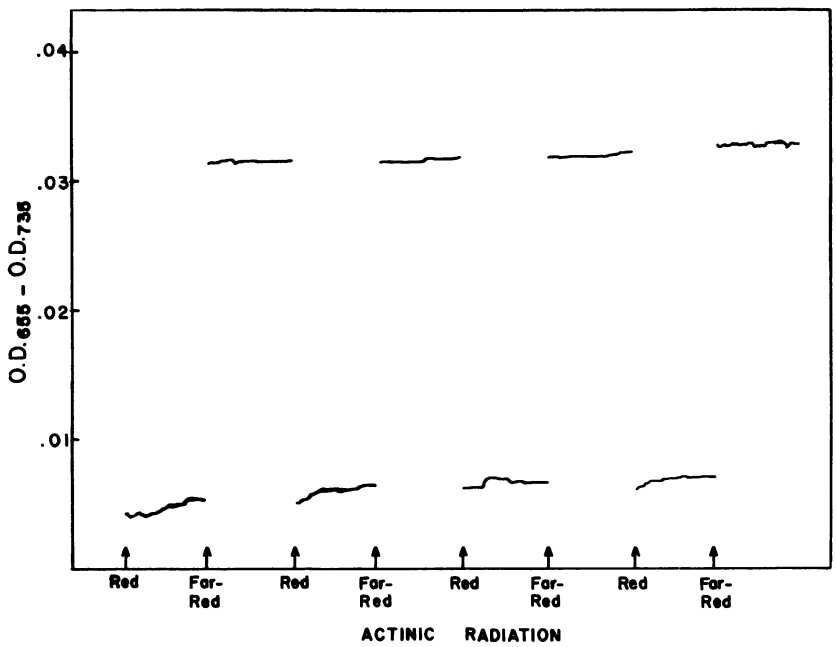

FIG. 3.—Photoreversible response to red and far-red radiation of a solution obtained from maize shoots.

TABLE 1

PURIFICATION FROM 450 GM. OF DARK-GROWN SHOOTS OF SEEDLING MAIZE FOR THE PIGMENT CONTROLLING PHOTORESPONSIVE DEVELOPMENT OF PLANTS

Fraction	Volume ml	Protein gm	Δ(ΔOD) /cm	Purification factor
40,000 × *g* supernatant	590	6.25	0.004	1.0*
140,000 × *g* supernatant	550	3.85	0.004	1.6
$(NH_4)_2(SO_4)$ 0.0 to 0.33 saturation	135	3.02	0.012	6.0

* Subject to some error from change in optical path-length by scattering.

it is necessary to lower the intensities of the analyzing light beams by about 100-fold, relative to the arrangement used for higher optical densities of unseparated plant material to prevent the beams from driving the reaction. This stage of purification brings the assay to the threshold of usual laboratory procedures. The presence of

the pigment in a clear solution of the 0.33 $(NH_4)_2SO_4$ saturated precipitate has been detected by means of $\Delta(\Delta O.D.)$ values obtained from the four appropriate measurements on conventional spectrophotometers.

Discussion.—Although many aspects of the nature of the pigment effective for control by light of plant development were found during the last seven years, attempts to separate it in several laboratories were unsuccessful and were usually left undescribed. This work supplies three needed elements for further progress: A source of the pigment, a method of assay, and a system for separation. There would seem to be no essential barrier to finding the nature of the enzymatic action of the pigment, P_{735}, which constitutes the limiting pacemaker[10] or "bottleneck" of control evident in plant development and to elaborating physiological and biochemical aspects of its action.

Summary.—The photoreversible pigment controlling many aspects of plant development was observed in living tissue by direct spectrophotometry. The pigment was separated from the tissue by usual methods of protein chemistry using differential spectrophotometry for assay.

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*MOLECULAR INTERACTION OF ISOALLOXAZINE DERIVATIVES. II**

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Understanding of the mode of action of the flavoproteins might be aided greatly by systematic study of the interaction of *iso*alloxazine derivatives with nonprotein compounds of known structure and state. The present paper describes spectrophotometric and potentiometric experiments which extend observations reported previously.[1]

Experimental.—Materials: 3-Methyllumiflavin (3,6,7,9-tetramethyl*iso*alloxazine) was synthesized by condensation of methylalloxan[2] with 2-amino-4,5-dimethyl-*N*-methylaniline, prepared by a modification of the method of Kuhn and Reinemund.[3] Melting point: 290°–291°C, with decomposition. Analysis: calculated, C 62.19, H 5.22, N 20.74; found, C 62.20, H 5.11, N 21.54. All other chemicals