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The Discovery of Phytochrome

The discovery of the photoreversible control of plant growth and development and the subsequent isolation of the photoreceptor pigment, phytochrome, constitutes one of the most fascinating chapters in plant physiology. Primarily, the concerted efforts of a group of investigators at the plant industry Station, U.S. Department of Agriculture, Beltsville, Maryland, working over approximately a twenty-five year period have provided most of the current information. This work was led by two experimentalist, H. A. Bothwick and S. B. Hendricks. Borthwicsk is a plant morphologist by training and a keen student of plant behaviour. Hendricks is a physical chemist and a perceptive analyst of experimental findings. Successful pursuit of the overall problem required collaboration with plant physiologists, electronic engineers, biophysicists, and biochemists. Few pursuits in plant physiology have successfully utilized such a diversity of disciplines.

A fundamental advance in plant photobiology was the discovery that growth responses elicited by red light are reversible by far-red light. It was previously known that the germination of lettuce seed, which requires light, is promoted by red light and suppressed by far-red light. Detailed action spectra for light-sensitive lettuce seed germination shows maximum promotion near 660 nm and maximum suppression near 730 nm. The important idea derived from the action spectra studies was that the seeds are potentiated for germination by red light and the potentiation is reversed by far-red light. If this were true, seed repeatedly exposed to red and far-red light would show a response dependent on the spectral region of the final light exposure. Simple experiments confirmed the idea of a photoreversible photoresponse. The action spectra and energy requirements for seed germination and for control of flowering in cocklebur were found to be similar.

The simplest interpretation of the photoresponse was that a single photoreceptor pigment could exist in two photoconvertible forms:

$$Pr \underset{\text{far red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{fr}$$

The pigment P_r was found to have an action maximum near 660 nm, and P_{fr}, near 730 nm. Absorption of light by either form converted it to the other form. Implicit from the action spectra studies is that a pigment must be acting as a photoreceptor and that its absorption maxima must be close to the action maxima. It could thus be predicted that the pigment would be blue in color, and approximate absorption wavelength maxima could be specified. The low energy requirement suggested that the photo action bring about a change in molecular configuration rather than a transfer of energy to another system.

The Detection of Phytochrome

A number of years passed between the description of the properties of phytochrome deduced from the action spectra studies and the physical measurement of the pigment. The existence of the yet unseen pigment was regarded with considerable scepticism.

A principal problem in measuring phytochrome *in vivo* was the apparent low concentration of the pigment in plant tissues. The molar extinction coefficients of P_r and P_{fr} were determined and found to be quite high. Nonetheless, a pigment with the required properties was not apparent even in albino seedlings which showed typical phytochrome-controlled growth responses. Since the catalytic or regulating function of phytochrome is still unknown, an assay was conceivable based on the spectral properties defined by the action spectra. Direct physical measurement of phytochrome required the development of spectrophotometric equipment which could measure small absorbance changes in optically dense samples. The overriding absorbance of chlorophyll has obscured the measurement phytochrome in green tissues. The proper selection of plant material used for measurement is paramount since phytochrome has not been detected in many phytochrome-containing tissues. The plant response is frequently a more sensitive assay for phytochrome than current methods of physical measurement.

The first tissue in which phytochrome was physically detected was the cotyledons of dark-grown turnip seedlings grown in the presence of chloramphenicol. This tissue was selected because of its low protochlorophyll content and its capacity to show responses to light. The cotyledons were placed in a cuvette and irradiated with red light. The radiation transformed the P_r to P_{fr} and protochlorophyll to chlorophyll. The absorbance of the tissue was measured from 570 to 850 nm. The tissue was then irradiated with far-red light and the absorbance again measured from 570 to 850 nm. The presence of the pigment was clearly evident from the absorbance changes. Far-red radiation caused an absorbance decrease in the far-red region of the

spectrum with maximal change at 735 nm and an absorbance increase in the red region with maximal change at 655 nm. Red radiation had the opposite effect.

With the assurance that the specific characteristics of phytochrome derived from action spectra were confirmed by photoreversible absorbance changes at the proper wavelengths, a rapid method was devised for detection. A dual wavelength difference photometer was devised by Birth (1960) which measured directly the absorbance difference (ΔA) between 660 and 730 nm, $\Delta A = A_{660} - A_{730}$ the relative amount of phytochrome in a sample was calculated from the difference between the ΔA readings after irradiation of the sample with actinic sources of red and far-red light: $(\Delta A) = \Delta A f_{r\ irrad} - \Delta A_{r\ irrad}$. The assay based on absorbance changes at 660 and 730 nm measures the sum of the photoreversible P_r and P_{fr} absorbances. P_r and $P_f r$ can be determined separately by measuring the ΔA between 660 and 800 nm and between 730 and 800 nm, respectively. In some cases the 730 v/s 800 nm measurement may be the most reliable assay.

The availability of a simple, sensitive, rapid, and specific physical assay permitted the examination of a large number of plant materials which might serve as useful sources for extraction and purification of phytochrome. A survey indicated that phytochrome could be detected in several dark-grown seedlings and the cauliflower head. Hillman (1964) found a high content of phytochrome in several non-green plant tissues. The distribution of phytochrome in several dark-grown seedlings was examined in vivo. The concentration of phytochrome in monocotyledon seedlings was highest at the apex of the coleoptile and at the junction of the mesocotyl and node. In legume seedlings the maximum concentration of phytochrome occurred in the growing tip and hypocotyls arch.

Dark-grown seedlings, which can be grown reproducibly in relatively large amounts, are an enriched source of the pigment. They have a higher content of phytochrome than light-exposed seedlings and a minimum of chlorophyll contamination. Phytochrome has now been detected in all parts of higher plants including roots, hypocotyls, cotyledons, inflorescences, and developing fruits, and in both primary and secondary growth.

Isolation and Purification

The isolation and purification of phytochrome was dependent on the photometric assay and the augmented content of phytochrome in dark-grown seedlings. Methods for extracting proteins were used in the isolation. Consideration of some properties of phytochrome suggested that it was a protein with a chromophoric group. The very low radiant energy needed to bring about a physiological response suggested a need for amplification analogous to the functioning

of an enzyme. Substances present in trace amounts are usually physiologically active by virtue of binding to a protein. Initial attempts to isolate phytochrome as a protein were successful, but subsequent purification required considerable time. Attempts to isolate the phytochrome chromophore directly from plant tissue by methods used in pigment chemistry have not yet been successful.

The purification of pytochrome has been satisfactorily achieved to date only from dark-grown monocotyledonous seedlings. Dark-grown five-day-old seedlings of *Avena sativa* (oats) provided a useful source of easily extractable phytochrome. The amount of phytochrome extracted is largely dependent on the final pH after the initial extraction. At pH values above 7.3, the supernatant solution after filtration and centrifugation contained the maximal extractable pigment. At pH 6.2 or below, the phytochrome was only in the sediment. The intercellular location of phytochrome has not been systematically pursued.

Satisfactory methods for the purification of many plant proteins have only recently become available. There are several problems peculiar to the purification of plant proteins. The growing plant cell is highly vacuolated and has a low content of protein. Protein precipitants, acidic substances, and phenolase substrates are frequently found in considerable concentration in plant vacuoles. These substances must be rapidly and effectively counteracted or the protein will be denatured or rendered insoluble. Some of these can be inhibited by the use of reduced sulphydryl compounds such as 2-mercaptoethanol. Sufficient amounts of the reduced sulphydryl compound should be used to ensure that a detectable amount remains after the initial extraction. Polyvinyl pyrrolidone may prove useful in the isolation of some plant proteins. Low temperature during protein isolation reduces protein denaturation and possible proteolysis.

The low proportion of proteins extracted from plants often requires concentration before purification methods can be applied. Concentration by sultrafiltration is simple and convenient.

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