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Two Spectrally Different Forms of the Phytochrome Chromophore Extracted from Etiolated Oat Seedlings

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Siegelman, Turner & Hendricks (1966) have described some of the properties of the phytochrome chromophore. Hendricks (1966) has also reported the partial elucidation of the structure of the chromophore. In neither paper was there any mention of the possibility of extracting the chromophore in two spectrally distinguishable forms. We report our findings in this respect.

Materials. Avena sativa (var. Condor) seed (600g. lots) was sown in 24in. × 18in. × 2in. enamelled steel trays. The trays, containing 500g. of horticultural vermiculite and 3l. of tap water at 25°, were fitted with 4in.-deep light-tight metal lids, and kept at 25° in a constant-temperature cabinet. After 6 days, the seedlings were chilled overnight by switching off the cabinet heater. The cabinet temperature fell to 5–7°.

Extraction procedure. The seedlings were harvested in a cold-room (3–4°) by the light of two 40w green fluorescent strip lights each covered by two layers of green gelatin (Withrow & Price, 1957). The seedlings were cropped close to the seed, and macerated in 1·5kg. lots with 1·5l. of 0·1M-potassium phosphate buffer, pH 7·8, in a stainless steel 4l. Waring Blendor. Unless otherwise stated, all buffers used in the extraction procedure were at pH 7·8 and contained 0·35% of 2-mercaptoethanol.

The macerated mixture was filtered on Büchner funnels with Solka Floc cellulose as a filter aid. The filtrate was clarified by centrifugation and concentrated by stirring sufficient dry Sephadex G-50 coarse beads into the extract to form a thick slurry. The slurry was filtered on Büchner funnels and the Sephadex cake rinsed with 0·01M-phosphate buffer. This concentration procedure was repeated twice. The concentrated mixture was centrifuged in the preparative Spinco model L ultracentrifuge with the no. 21 rotor at 15000rev./min. for 30min. This gave 1·7l. of clarified concentrated mixture from 14l. of dilute extract, with approx. 75% recovery of photoreversible material as measured by the method of Hendricks, Butler & Siegelmann (1962) by using the relationship:

$$[\Delta(\Delta E)] = (\Delta E_{725}^{660})^{730\ \text{irrad.}} - (\Delta E_{725}^{660})^{645\ \text{irrad.}}$$

The active concentrated mixture was loaded on to four columns (60cm. × 10cm.) of Sephadex G-100 equilibrated with 0·01M-phosphate, pH 7·8, and eluted with 0·01M phosphate at 150–200ml./hr. The eluate was collected in 30ml. fractions, and small samples were monitored for photoreversibility.

The fractions showing highest photoreversibility were pooled and loaded on to a column (30cm. × 7cm.) of DEAE-cellulose equilibrated to 0·01M-phosphate. The column was washed with 1l. of 0·01M-phosphate and eluted with a linear gradient of 2l. of each of 0·01M- and 0·25M-phosphate buffer at 200–250ml./hr. The eluate was collected in 30ml. fractions, which were monitored for E_{280} and photoreversibility.

Again the most active fractions were pooled, giving 700ml. of photoactive solution for which the $[\Delta(\Delta E)]$ was 0·036/cm.

Hydrolysis and chromophore extraction. The photoactive solution was divided into two equal parts. One half was irradiated with red light and the other half with dark-red light for 20min. Protein was then precipitated from each solution by the addition of 15g. of trichloroacetic acid/100ml. of solution. The precipitates were washed twice with cold methanol, each wash being followed by centrifugation in aluminium-foil-covered buckets, dispersed as far as possible, and then hydrolysed for 3½hr. under reflux in 100ml. of 1% ascorbic acid in methanol (Siegelman *et al.* 1966). The hydrolysis apparatus was covered with aluminium foil. After hydrolysis the supernatant liquors were decanted into separating funnels, diluted with 200ml. of water and extracted with 3 × 100ml. of chloroform. The pooled extracts were each evaporated nearly to dryness on a water bath at 55–60° under pure nitrogen, and the residues were dissolved in 2ml. of chloroform, or 2ml. of 12N-HCl–methanol (1:19, v/v) for the spectral determinations. All these procedures were carried out in a darkened room.

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Concentration of phytochrome solution for visible absorption-spectra measurements. A photoactive solution, prepared in a manner identical with that used for hydrolysis, was equilibrated by passing it down a Sephadex G-50 column (60cm. × 7cm.) equilibrated with 0·01M-phosphate. The equilibrated solution was irradiated with dark-red light for 20min., adsorbed on to a small DEAE-cellulose column (5cm. × 2·5cm.) equilibrated with 0·01M-phosphate, and finally eluted with 0·25M-phosphate buffer. The eluate was collected in 5ml. fractions. The most active fractions (25–30ml.) were pooled.

Photoreversibility measurements. Photoreversibilities were measured on a Unicam SP.500 spectrophotometer at 660mμ and 725mμ in 4cm. cuvettes. The samples were irradiated in the sample compartment of the spectrophotometer for 2min. Irradiation was provided by a microscope lamp with a 48w, 12v tungsten bulb. Red light was obtained by using an interference filter with peak transmission at 645mμ, half-width 30mμ. Dark-red light was obtained by using an Ilford infrared filter no. 207, with maximum transmission at 760mμ and longer wavelengths, and complete cut-off at 720mμ and shorter wavelengths.

Spectra. The spectra shown in Figs. 1(*a*), 1(*b*) and 1(*c*) were measured in a Perkin–Elmer 137 u.v. spectrophotometer in 1cm. cuvettes; difference spectra were always obtained with the 645mμ-irradiated sample in the reference beam.

Results and discussion. The spectra and difference spectra of the two concentrated extracts in chloroform and in 12N-HCl–methanol (1:19, v/v) are shown in Figs. 1(*a*) and 1(*b*). The spectra of phytochrome in P_R and P_{FR} forms and the difference spectrum of the two forms are shown in Fig. 1(*c*).

Thin-layer chromatography of the extracts in the solvent systems used by Siegelman *et al.* (1966) indicated that the chromophore solutions were homogeneous. The spots were fluorescent under u.v. light. On spraying with zinc acetate–ethanol solution followed by ammoniacal iodine–ethanol the spots fluoresced orange–red.

The results which we have obtained so far are not inconsistent with the suggestion that the phytochrome chromophore is a tetrapyrrole. Although the spectra of the two extracted forms are quite different, they both resemble in some respects other published spectra of biliverdin-type pigments (Gray, Kulczycha & Nicholson, 1961).

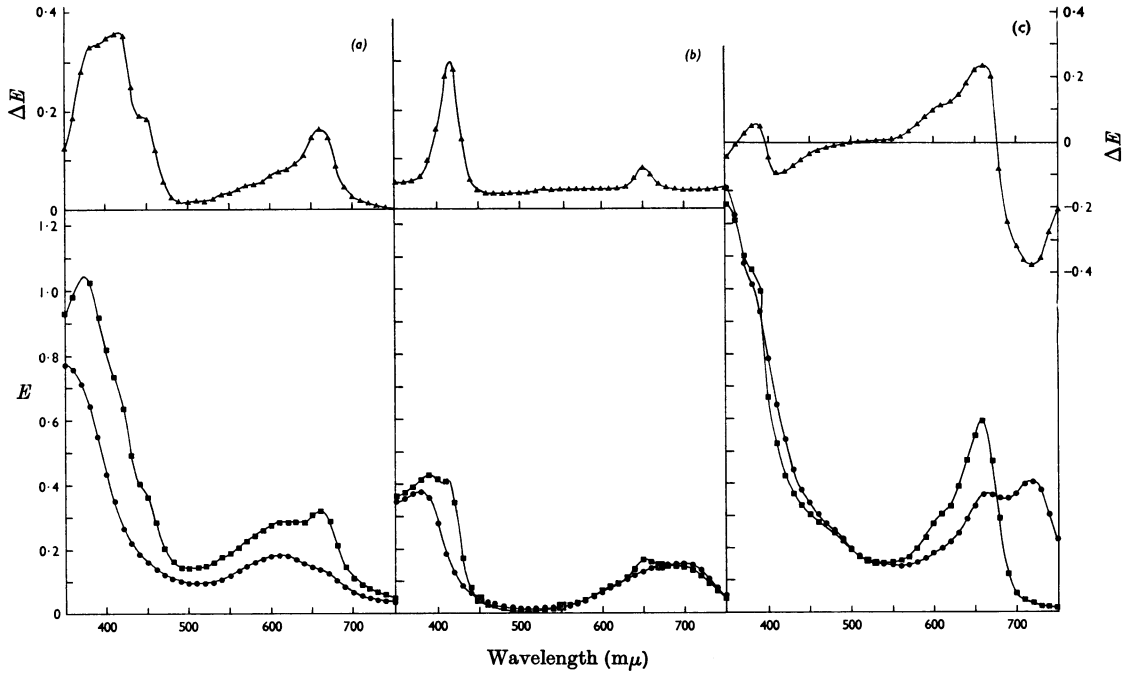

Fig. 1. Visible absorption spectra of (*a*) the P_R and P_{FR} chromophores and their difference spectrum in chloroform, (*b*) the same in 12N-HCl–methanol (1:19, v/v) and (*c*) P_R and P_{FR} native phytochromes and their difference spectrum in 0·25M-phosphate buffer. ●, Absorption spectrum of native P_{FR} or chromophore; ■, absorption spectrum of native P_R or chromophore; ▲, difference spectrum.

Siegelman & Hendricks (1965) have referred to some of the changes that might take place in the chromophore to produce the absorption changes in phytochrome. The striking spectral differences in the chromophores extracted from the two forms of phytochrome indicate that a major configurational change occurs in the chromophore during the $P_R \underset{730}{\overset{645}{\rightleftharpoons}} P_{FR}$ reaction.

The difference spectrum of the two chromophores in chloroform shows that a complex series of shoulders between 530 mμ and 620 mμ is associated with the P_R chromophore. Platt (1952) has shown that this type of spectrum is associated with the ring structure of the porphyrins. Whether this indicates that the phytochrome chromophore in the P_R form is cyclic will require further chemical investigation.

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