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# The Chromophore of Phytochrome

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*Summary.* The chromophore of phytochrome, the plant photomorphogenic pigment, was cleaved from the associated protein. Chromatographic and spectral properties indicated that it was a bilitriene closely similar to but distinct from the chromophore of C-phycocyanin and allophycocyanin.

The photoreversible changes in absorbance of phytochrome are properties of a specific chromophore attached to a protein. Comparison between action spectra for photomorphogenic responses of higher plants and absorption spectra of the algal chromoproteins C-phycocyanin and allophycocyanin indicated that the several chromophores were probably similar (1–3). The algal chromophores are known to be bile-type pigments (4–6). Purification of phytochrome from plant tissue (7) afforded an opportunity for study of the phytochrome chromophore which is reported here.

## Materials and Methods

Phytochrome (P) from dark-grown oat seedlings was purified by repeated dicalcium phosphate and gel-filtration chromatography as previously described (7, 8). Six kg lots of seedlings gave an average of 30 ml of a purified phytochrome solution having a $\Delta$ ($\Delta$OD) of about 0.8/cm between the 2 forms, $P_R$ and $P_{FR}$ at 660 and 725 m$\mu$ respectively. This amounts to about $1 \times 10^{-7}$ moles of chromophore/ml if $\alpha$ at 660 m$\mu$ for $P_R$ is taken as $1 \times 10^4$, which is the value indicated by the action spectra for photoconversion in vitro if the quantum efficiency is near one (3). Phycocyanin and allophycocyanin from *Plectonema boryanum* were purified by chromatography on dicalcium phosphate essentially by the procedure of Haxo, O'hEocha and Norris (9) or by fractional ammonium sulphate precipitation.

The method of denaturing the proteins was a factor affecting cleavage of the chromophores as will be discussed. The procedure adopted was denaturation by making the protein solutions to 5 % trichloroacetic acid. The precipitated protein was washed with water, followed by repeated washing with $CH_3OH$ at the centrifuge. The $CH_3OH$ washing removed a yellow impurity from phytochrome. Cleavages of the chromophores were effected by refluxing the denatured proteins in $CH_3OH$ for 3 to 4 hours. The solution was filtered, water was added, and the pigments were partitioned into $CHCl_3$. The $CHCl_3$ solution was reduced to a volume of 0.05 to 0.5 ml by heating at 60° in a stream of $N_2$. This final small volume was blue if the cleavage was successful.

Cleavage of the phytochrome chromophore from 5 ml of the purified phytochrome solution gave an average of 0.20 OD/cm in 1 ml of $CH_3OH$-5 % HCl at the absorbancy maximum (690 m$\mu$). This corresponds to about 5 to 10 % yield if $\alpha$ for the chromophore is 10 or $5 \times 10^3$ (3) or about $1 \times 10^{-8}$ moles. The yield was not changed by prolonging boiling of the $CH_3OH$ beyond 4 hours, by repeated treatment, or by refluxing under $N_2$. Assuming the chromophore has a molecular weight near 600, the yield is about 6 $\mu$g of pigment from 5 ml of purified phytochrome solution.

Because of the small amounts of cleavage product obtained from phytochrome, examinations were restricted to measurements of absorbancies (table I) on a Cary 14 recording spectrophotometer, and thin-layer chromatography on silica gel (Adsorbosil 1, Applied Science Laboratories, State College, Pennsylvania). Chromatograms were developed with the several solvent systems shown in the tables II and III. They were examined under visible and ultraviolet (310 to 420 m$\mu$) radiations before and after spraying. Spray reagents used were: (1) a saturated solution of zinc acetate in ethanol to form the zinc salts of the bile pigments which have distinctive fluorescent properties and (2) 0.01 % $I_2$ in ethanol containing 0.2 ml $NH_4OH$/10 ml which serves as an oxidant.

Bile pigments were prepared or obtained for chromatographic comparisons. Urobilins (d- and i-) were obtained from Dr. P. Barrett, National Institutes of Health, Bethesda, Maryland. Glaucobilin (mesobiliverdin) and mesobiliviolin were prepared by ferric chloride oxidation of d-urobilin and i-urobilin, respectively (10). Biliverdin was

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prepared by ferric chloride oxidation of bilirubin (11). Methylation of the pigments was achieved by refluxing with 10 % $BF_3$ in $CH_3OH$ for 2 minutes. Partially esterified materials were obtained by adding 5 ml of methanol and 0.01 ml of 12 N HCl to dry samples of pigments and evaporating the solutions at 60° in a stream of $N_2$.

## Results and Discussion

Phytochrome denatured with trichloroacetic acid was previously observed to remain blue colored, although the photoreversibility was destroyed (12). When it was denatured with $CH_3OH$ or by boiling in water the denatured protein was only very lightly colored and no chromophore was obtained upon boiling with $CH_3OH$. Attempts to cleave the chromophore from trichloroacetic acid-denatured protein by hydrolysis with alkali or 12 N HCl (13, 14), which gives products with algal biliproteins, were ineffective with phytochrome. A poor yield of the phytochrome chromophore was obtained by refluxing the trichloroacetic acid-denatured protein in $CHCl_3$ containing a few drops of concentrated HCl. Refluxing the trichloroacetic acid-denatured phytochrome protein in 1 % ascorbic acid in $CH_3OH$ was satisfactory, but not more so than $CH_3OH$ alone. Fujita and Hattori (15) refluxed $CH_3OH$-washed cells of *Tolypothrix tenuis* and *Anabaena cylindrica* in 1 % ascorbic acid in $CH_3OH$ and obtained a blue pigment. They recognized the product as a bilitriene-type of bile pigment, but because of the poor yield and mild conditions of cleavage suggested that it was not the chromophore of phycocyanin but that it was a precursor of the pigment. The yield of chromophore obtained by us from allophycocyanin and phycocyanin, never exceeded 20 % based on absorbancies and the yield from phytochrome did not exceed 10 %. The prior purification of these materials indicates that the cleaved product is a constituent of the chromoprotein and is the chromophore. The low yields rather seem to involve some feature of the denaturation process that is not now understood. The yield was not improved, but often greatly reduced, by many variations of procedure and use of other cleaving agents.

Table I. *Wavelength Maxima of the Phytochrome, Allophycocyanin, and Phycocyanin Chromophores and Authentic Bile Pigments*

All spectra measured in 5 % HCl-MeOH.

| Compound | Wavelength maxima (mμ) |
|---|---|
| Phytochrome chromophore | 380 , 690 |
| Phycocyanin chromophore | 375 , 685 |
| Allophycocyanin chromophore | 375 , 685 |
| Biliverdin | 372 , 680 |
| Glaucobilin | 358 , 682 |
| Mesobiliviolin | 327*, 565* |

* Gray et al. (24).

Availability of authentic bile pigments and the chromophores of phytochrome, allophycocyanin, phycocyanin permitted spectral and chromatographic intercomparisons. The absorbance maxima (table I) of the algal biliprotein and phytochrome chromophores in 5 % HCl-$CH_3OH$ were similar to, but not identical with, the maxima of the bilitrienes, biliverdin and glaucobilin but differed markedly from the maxima of the bilidiene, mesobiliviolin.

The $R_F$ values of the dimethyl esters of allophycocyanin and phycocyanin chromophores were identical in several solvents on thin-layer chromatograms (table II) and were distinctly different from $R_F$ values of biliverdin, glaucobilin, and mesobiliviolin dimethyl esters. Phytochrome dimethyl ester $R_F$ values differed from all of these. Three spots were present on the chromatograms of the partially esterified methyl esters of each material (table III). The $R_F$ values of the phytochrome esters were only slightly different in the particular solvents from those of phycocyanin and allophycocyanin which were equal. The lutidine-$H_2O$-$NH_4OH$ solvent is known to separate porphyrin pigments according to the number of carboxyl groups (16–18). The 3 spots were in accord with presence of 2 carboxyl groups as the free acids, mono-, and di-methyl esters.

Fluorescence under ultraviolet radiation and changes in color and fluorescence after spraying the chromatograms gave further information. The phytochrome, phycocyanin and allophycocyanin chro-

Table II. *Thin-layer Chromatography of the Dimethyl Esters of the Phytochrome, Allophycocyanin and C-Phycocyanin Chromophores and Authentic Bile Pigments*

| Compound | $R_F$ values in various solvents | | |
|---|---|---|---|
| | $C_6H_6$ / $C_2H_5OH$ 10 / 0.25 | $CCl_4$ / $CH_3COOH$ 10 / 2.5 | $CCl_4$ / $CH_3COOCH_3$ 10 / 4 |
| Phytochrome chromophore | 0.61 | 0.42 | 0.72 |
| Allophycocyanin chromophore | 0 52 | 0.35 | 0.67 |
| Phycocyanin chromophore | 0.52 | 0.35 | 0.67 |
| Biliverdin | 0.37 | 0.54 | 0.50 |
| Glaucobilin | 0.48 | 0.49 | 0 65 |
| Mesobiliviolin | 0.24 | ... | ... |

Table III. *Thin-layer Chromatography of the Products of Partial Esterification of Phytochrome, Allophycocyanin and C-phycocyanin Chromophores*

| Chromophore | $R_F$ value in: 2,6-lutidine 8 / $H_2O$ 2 / $NH_4OH$ vapor | $CH_3COC_2H_5$ 10 / $H_2O$ 1 |
|---|---|---|
| Phytochrome | 0.03, 0.25, 0.98 | 0.22, 0.50, 0.90 |
| Phycocyanin | 0.03, 0.23, 0.98 | 0.24, 0.52, 0.90 |
| Allophycocyanin | 0.03, 0.23, 0.98 | 0.24, 0.52, 0.90 |

mophores, biliverdin, and glaucobilin, all were blue and did not fluoresce under uv-light. Mesobiliviolin was red colored and was reddish-purple fluorescent under uv-light. After spraying with zinc acetate solution, mesobiliviolin remained red fluorescent and the other compounds still did not fluoresce. The chromatograms sprayed with zinc acetate were oversprayed with the alkaline $I_2$ solution. The mesobiliviolin changed to an orange color and was greenish fluorescent, and the other pigments became bright-red fluorescent. The fluorescence of the untreated mesobiliviolin was probably due to heavy metal contamination of the thin layer bed.

The allophycocyanin and phycocyanin chromophores are identical on the basis of our findings. This is in agreement with the earlier conclusions of O'hEocha (14, 19) who considered that the chromophoric groups were very closely related, if not identical. He further considered the phycocyanin chromophore, phycocyanobilin, prepared by acid cleavage of the purified biliprotein was intermediate in properties between a verdin and a violin (14). The phytochrome chromophore is similar in structure to the phycocyanin chromophore and is a diacid but must differ in some detail. Complete physical and chemical examination of the readily obtainable

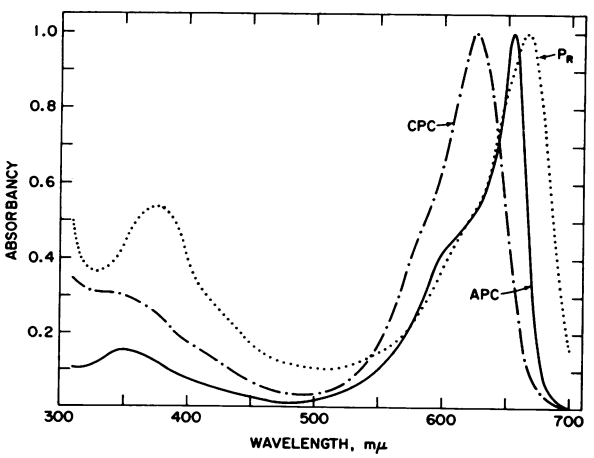


FIG. 1. Absorption spectra of allophycocyanin (APC) and C-phycocyanin (CPC) from *Plectonema boryanum* and the red-absorbing form of phytochrome from *Avena* ($P_R$).

algal chromoprotein chromophores are required to fully establish the exact structures.

While the chromophores of phytochrome, phycocyanin, and allophycocyanin are apparently very similar, the proteins might not be related and surely differ in the number of chromophores per molecule. The numbers are probably 1 for phytochrome (7) 12 for allophycocyanin (20) and about 22 for C-phycocyanin (20). The algal biliproteins are accessory pigments in photosynthesis and have been found only in certain algae. Phytochrome has some other, but still unknown biochemical function such as enzymatic or hormonal action through which its morphogenic function is attained. Physiological evidence indicates that it is present in *Spermatophyta, Bryophyta, Pteridophyta,* and the order *Conjugales* in the division *Chlorophyta* (21). In vertebrates, bilirubin is known to associate with protein (22), but no active function has been considered as derived from the association.

Phytochrome also differs from C-phycocyanin and allophycocyanin in that it has 2 photoconvertible forms $P_R$ and $P_{FR}$. Attempts to observe such interconversion in phycocyanin have been unsuccessful. Results of flash photolysis of phytochrome further shows that at least a total of 6 transitory forms exist between $P_R$ and $P_{FR}$ (23). These all involve structural modification of the chromophore, either within itself or because of proximity to the associated protein. Spectral changes have not been observed following irradiation of the isolated phytochrome chromophore.

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