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CLEAVAGE OF PHYCOCYANOBILIN FROM C-PHYCOCYANIN

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SUMMARY

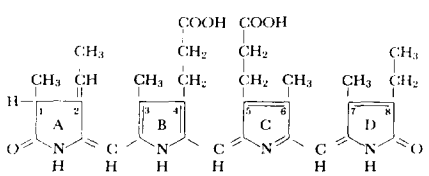
The bile pigment chromophore of C-phycocyanin, phycocyanobilin, was cleaved from the protein by several procedures. Comparison of the crystalline phycocyanobilin dimethyl ester by chemical, spectral, and chromatographic properties was made. The pigment was found to be identical by all procedures. Highest yields of phycocyanobilin were obtained by the methanol cleavage. The conversion of phycocyanobilin to mesobiliverdin by KOH was confirmed.

INTRODUCTION

The plant biliproteins are a unique group of chromoproteins having important roles as photoreceptors in photosynthesis and in regulation of plant growth and development. The availability and abundance of the algal biliproteins can provide useful systems for studies in energy transfer mechanisms and for physicochemical examination of chromophore–protein interactions. The nature of the binding of the bile chromophore to the protein is not yet known, but it is apparently different from the known types of heme binding to proteins. The capacity of algae to synthesize large amounts of bile pigments should be of value in biosynthetic studies on the mechanism of conversion of porphyrin to bile pigment.

Complete identification of the algal bile pigments is essential in any further studies. LEMBERG[1–3] initially characterized the chromophore of phycocyanin, phycocyanobilin, as a bile pigment which was cleaved with conc. HCl at 80°. Ó'hEOCHA[4,5] found that cleavage could be attained with conc. HCl at 25°. FUJITA AND HATTORI[6] and later Ó'CARRA AND Ó'hEOCHA[7] isolated a pigment directly from cells of blue-green algae with warm methanol. The bile pigments isolated by the above workers were reputedly different on the basis primarily of spectral absorbance properties, and were considered variously as artifacts of extraction, native chromophore or biosynthetic intermediates.

A modification of the FUJITA–HATTORI cleavage procedure was devised[8] to split the chromophore from several purified biliproteins. The structure of phycocyanobilin was recently established as[9]:



In the present report, careful and systematic examinations of phycocyanobilin obtained by several cleavage methods were compared. The phycocyanobilin obtained by all procedures was identical in all physical and chemical properties examined. Large-scale methods of protein purification were devised to provide sufficient materials for these studies.

MATERIALS AND METHODS

The biliprotein C-phycocyanin was prepared from the Cyanophyte *Phormidium luridum*. The alga was grown in the D medium of KRATZ AND MYERS[10] in a mass-culture apparatus[11]. Thin-layer chromatography was performed on layers of silica gel (Adsorbosil 5) and magnesium silicate (Adsorbosil M-1) obtained from Applied Science Laboratories, State College, Pa. Solutions of 14% BF_3 in methanol were obtained from Applied Science Laboratories.

C-Phycocyanin was extracted from 250-g lots of harvested cells by repeated freezing and thawing in 0.1 M potassium phosphate buffer first at pH 7, then at pH 6. The extracts were centrifuged (16000 × *g*, 20 min) and the supernatants combined. The phycocyanin was precipitated by the addition of solid $(NH_4)_2SO_4$ to 55% satn. and allowed to stand at 4° until the protein had completely settled out.

Chromatography of the phycocyanin

All chromatographic operations were performed at 4°. The bulk of the supernatant solution from the precipitated protein was removed. Then 3 to 4 l diatomaceous earth (Celite 545-Johns Manville Co.) were added to the precipitated protein. The thick blue slurry was poured into a 15 cm × 35 cm column and allowed to settle. The residual supernatant was allowed to elute from the column, and the column was washed with 55% satd. $(NH_4)_2SO_4$ solution until the eluant was colorless. The column was eluted with a linear gradient of 4 l of 55% satd. $(NH_4)_2SO_4$ solution and 4.4 l of 15% satd. $(NH_4)_2SO_4$ solution. The flow rate was maintained at 20 ml/min with a peristaltic pump and 300-ml fractions were collected. Allophycocyanin was eluted first as a pale blue-green fraction followed by the deep blue C-phycocyanin. C-Phycocyanin remaining on the column at the completion of the gradient was eluted with 5% satd. $(NH_4)_2SO_4$ solution. The allophycocyanin fractions ($A_{653nm}/A_{615nm} > 1.5$) were pooled, and the C-phycocyanin fractions which contained no spectroscopically detectable allophycocyanin impurity were pooled. All intermediate fractions were discarded. The pooled C-phycocyanin fractions were denatured by making to 1% with trichloroacetic acid. The denaturation was aided by the presence of $(NH_4)_2SO_4$ in the solution. Acetone denaturation was achieved by adding 0.5 vol. of acetone to the C-phycocyanin. The acetone denaturation required removal of most of the $(NH_4)_2SO_4$ by precipitation of the C-phycocyanin with $(NH_4)_2SO_4$ and redissolving the protein in water. After denaturation, the precipitated protein was collected by centrifugation and washed twice with water, then thrice with methanol.

Hydrolysis of the chromophore

The HCl cleavage of phycocyanobilin was performed essentially by the method of Ó'hEOCHA[4]. The denatured protein was not dried prior to cleavage. The washed, denatured phycocyanin was dispersed in conc. HCl and held at 25° for 30 min. The

conc. HCl–C-phycocyanin mixture was diluted to 3 M HCl with water. A blue residue (Residue A) precipitated out, and was removed by filtration and retained. The free chromophore was transferred to chloroform, which was washed with water, dried by passage through a chloroform-soaked filter paper and evaporated to dryness. The chromophore was redissolved in a few ml of methanol.

The methanol cleavage of phycocyanobilin was performed by the method of SIEGELMAN, TURNER AND HENDRICKS[8]. The methanol-washed denatured C-phycocyanin was not dried prior to cleavage. It was dispersed in methanol and refluxed for 16 h. Bumping was avoided by constant stirring with a magnetic stirrer hot plate. The blue solution was filtered from the green residue (Residue B) and evaporated to a minimal volume. Residue B was retained. The cleavage procedure of FUJITA AND HATTORI[6] was tested with approx. 200 g of *P. luridum* cells extracted with cold acetone to remove the chlorophyll and carotenoids. The resultant blue cellular residue was refluxed in methanol for 16 h with the aid of a magnetic stirrer hot plate. No ascorbate was included in the reflux mixture. The blue methanol solution was filtered and reduced to a minimal volume.

Esterification and chromatography

The diesters of phycocyanobilin are more stable and easier to purify and crystallize than the diacid. Esterification to the dimethyl ester was performed by refluxing the phycocyanobilin for 3 min in 7 % BF_3 in methanol. A few ml of chloroform were added to the mixture, and the diester was transferred to the chloroform by the addition of water. The chloroform solution was thoroughly washed with water to deprotonate the ester (the color of the chloroform solution changes from blue-green to deep blue). The chloroform solution was dried by filtration through chloroform-moistened filter paper. Other esterification procedures such as use of BF_3–methanol at 5°, 5 % HCl–methanol, 5 % H_2SO_4–methanol, diazomethane, or methane disulfonic acid–methanol did not eliminate the small amount of degradation or isomerization products formed. Other diesters such as ethyl, butyl, bromoethyl, benzyl and nitrobenzyl were also prepared, but offered no advantage over the dimethyl ester.

The dimethyl ester was purified by thin-layer chromatography on 1-mm layers of silica gel with carbon tetrachloride–methyl acetate (2:1, v/v). Other solvent systems used primarily for analytical work were benzene–ethanol (9:1, v/v) and ethylene dichloride–ethyl acetate (8:3, v/v). The free acid was chromatographed on silica gel impregnated with 0.3 M oxalic acid[12] with the solvent system benzene (or toluene)–isopropanol (55:45, v/v); or magnesium silicate layers with methyl ethyl ketone–acetic acid (95:5, v/v); or silica gel layers with methyl ethyl ketone–water–acetic acid (100:10:1, v/v).

Thin-layer chromatography separated the esterified phycocyanobilin into a prominent leading blue zone and a lesser trailing violet zone. Other very minor isomerization and oxidation products were ignored. The prominent blue zone (phycocyanobilin dimethyl ester) was scraped from the plates and eluted in ethanol. The diester was crystallized as long fibrous crystals from chloroform–ethanol and recrystallized from chloroform–methanol.

Absorption spectra

Acidified chloroform solutions, prepared by shaking chloroform with HCl solutions, were not satisfactory for spectral examination of the bile pigments. Proto-

nation was incomplete because of the low and indeterminate acidity of such solutions. Visible–ultraviolet absorption spectra were recorded in 5 % HCl in methanol[13] with a Cary-14 recording spectrophotometer. Infrared absorption spectra were measured in KBr discs in a Perkin–Elmer Infracord spectrophotometer. Absorption spectra of the zinc complex of phycocyanobilin were measured in ethanol saturated with zinc acetate.

RESULTS

Methanol cleavage

Phycocyanobilin dimethyl ester prepared by methanol cleavage of both acetone- and trichloroacetic acid-denatured C-phycocyanin was found to be identical on the basis of spectral properties (visible–ultraviolet and infrared) and non-separation in mixed chromatograms in the three solvent systems. It was obtained as long fibrous crystals, and occasionally prisms, with the absorption spectral properties given in Table I.

TABLE I

ABSORPTION SPECTRA MAXIMA OF PHYCOCYANOBILIN DIMETHYL ESTER AND FREE ACID IN VARIOUS SOLVENTS

Solvent	λ (*nm*)			
	Phycocyanobilin dimethyl ester		*Phycocyanobilin as free acid*	
	Band 1	*Band 2*	*Band 1*	*Band 2*
Chloroform	598	366	590	367
Methanol	618	363	614	361
5 % HCl–methanol	689	374	680	372
Zinc complex (in ethanol)	664	375	647	374

HCl cleavage

Phycoyanobilin was prepared from C-phycocyanin by cleavage in conc. HCl according to the procedure of Ó'hEocha[4], converted to the dimethyl ester, purified by thin-layer chromatography and crystallized. The dimethyl ester of this HCl-cleaved phycocyanobilin was found to be identical with the phycocyanobilin dimethyl ester prepared by methanol cleavage of trichloroacetic acid-denatured C-phycocyanin. The absorption spectra (visible–ultraviolet, infrared) were the same and there was no indication of heterogeneity on mixed chromatography in the three solvent systems. The blue residue form the HCl cleavage (Residue A) was refluxed in methanol for 16 h. It yielded about 3 times as much phycocyanobilin as the original HCl cleavage. The residue from the methanol cleavage (Residue B) yielded only a small amount of phycocyanobilin on further methanol refluxing or digestion in HCl.

Methanol cleavage of cells

Phycocyanobilin was released from acetone- and methanol-extracted cells of *Phormidium luridum* by refluxing the cellular residue in methanol for 16 h. It was

converted to the dimethyl ester, purified by thin-layer chromatography and crystallized. Comparisons of the visible–ultraviolet and infrared absorption spectra of phycocyanobilin dimethyl ester cleaved from the whole cells were made with those of phycocyanobilin dimethyl ester cleaved by the above two methods and all were found to be identical. Mixed chromatography by the three solvent systems confirmed the identity as phycocyanobilin dimethyl ester.

Quantitative results

The low yield of phycocyanobilin from the HCl cleavage has been a limiting factor in earlier work on the pigment. Methanol cleavage provided a milder cleavage with much better yields. The yields of phycocyanobilin from C-phycocyanin by different methods are compared below. The results are empirical since they are based on total absorbance units of C-phycocyanin before denaturation and of the obtained phycocyanobilin in neutral chloroform. The recovery is expressed as a percentage of the original C-phycocyanin absorbance: methanol hydrolysis, 10.3 %; HCl hydrolysis, 2.5 %; methanol hydrolysis of Residue A, 8.5 %.

Alkaline cleavage

LEMBERG AND BADER[3] clearly showed that refluxing phycocyanin with 10 % KOH in methanol for 15 min yielded mesobiliverdin. We have confirmed this observation. Trichloroacetic acid-denatured C-phycocyanin was refluxed for 15 min in 1 M KOH–methanol, and the resulting green solution was acidified to pH 4 with acetic acid. The pigment was transferred to chloroform, then methanol, methylated and purified by thin-layer chromatography. Mesobiliverdin dimethyl ester was obtained crystalline. Identity with authentic mesobiliverdin dimethyl ester prepared from bilirubin[14] was established by visible–ultraviolet and infrared absorption spectra and co-chromatography. Mesobiliverdin was also prepared directly from phycocyanobilin dimethyl ester and from acetone–methanol-extracted whole cells by similar alkaline methanol treatment.

Violinoid artifact

A trailing violet (violinoid) zone (λ_{max} 652, 351 nm, 5 % HCl–methanol) was found on chromatography of the crude esterified products of the original methanol cleavage. It was considered to be an artifact of the preparative procedure.

DISCUSSION

Phycocyanobilin obtained from denatured C-phycocyanin was cleaved by a variety of methods and found to be identical on the basis of absorption spectra (visible–ultraviolet and infrared) and chromatographic properties. It was prepared as the crystalline dimethyl ester from all procedures.

The problems with phycocyanobilin encountered by previous investigators can be traced to a multiplicity of causes. Large amounts of purified C-phycocyanin were not readily available. The yield of phycocyanobilin from the HCl cleavage was low and the diacid is not stable. The improved yield from the methanol cleavage and the stability of the diester have been important factors in the current work. The incom-

plete protonation in acid chloroform gave variable spectral behavior. This was avoided by measuring the visible–ultraviolet spectra in 5 % HCl in methanol[13].

Ó'hEocha[5] clearly showed that HCl cleavage was drastic and that the product was dependent upon time, temperature and acid normality. Consequently he showed that Lemberg's 'phycocyanobilin' was a result of the high temperature employed, and proposed that phycocyanobilin 630 was the native chromophore. His interpretation is confirmed by our observations. Phycocyanobilin prepared by the various procedures is identical to an acid-cleaved phycocyanobilin, which in all probability is identical to phycocyanobilin 630 of Ó'hEocha.

Methanol treatment of acetone-denatured protein is a very mild procedure and the cleaved phycocyanobilin can be considered as the native chromophore. There was no evidence (*cf.* absorption spectra of ester and acid) that esterification caused any chemical alteration of the pigment except methylation of the two carboxyl groups. Crespi *et al.*[15] refluxed the undenatured protein in 90 % methanol to cleave phycocyanobilin, and they proposed a structure for phycocyanobilin slightly different from that proposed by Cole, Chapman and Siegelman[9], who used the methanol procedure outlined here. There is no evidence to indicate that these two methods would provide different cleavage products. The different suggested structures are probably due to individual interpretations of the data and to variations in the choice of diacid and diester as the starting form of phycocyanobilin.

The violinoid observed on the thin-layer chromatograms was considered to be an artifact of the preparative procedure for several reasons. The artifact was always present only as a minor zone on the initial chromatogram. Acetone denaturation did not yield the violinoid zone but instead a minor verdinoid zone (λ_{max} 684, 360 nm, 5 % HCl–methanol). Repetition of the esterification procedure with purified phycocyanobilin dimethyl ester yielded small amounts of the violinoid. Purification of the phycocyanobilin diacid by chromatography prior to esterification lowered the yield of the violinoid to trace amounts, much less than could account for the quantities of violinoid ester. Absorption spectral data indicate that this violinoid is not the same as Lemberg's violinoid or the phycocyanobilin 608 of Ó'hEocha.

Methanol hydrolysis has provided a very mild method for the cleavage of the chromophore. The recovery of the chromophore, even by prolonged methanol hydrolysis is never complete. This would suggest that some of the chromophores, at least, are bound or shielded in a different manner. The conversion of phycocyanobilin to mesobiliverdin provided evidence that phycocyanobilin must be a IXα-substituted form, or readily converted to such.

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