Phytochemistry, 1966, Vol. 5, pp. 993 to 997. Pergamon Press Ltd. Printed in England

BILINS RELEASED FROM ALGAE AND BILIPROTEINS BY METHANOLIC EXTRACTION*

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(*Received* 4 *January* 1966)

Abstract—The purple and blue pigments obtained from *Tolypothrix tenuis*[1] are prepared from a number of Cyanophyta and Rhodophyta by a modified procedure which permits isolation in much larger yield. These pigments are established as artifacts (formed by the action of hot methanol in releasing and modifying phycobilin prosthetic groups) rather than precursors of phycoerythrobilin and phycocyanobilin as originally assumed. Further properties of these pigments are described and they are shown convertible to phycoerythrobilin and phycocyanobilin respectively on solution in concentrated hydrochloric acid. The separation of bilin from apoprotein in hot methanol is shown to occur without methylation of the propionic acid side-chains in the former moiety.

INTRODUCTION

FUJITA and Hattori[1,2] reported the isolation of two different free bilins from the blue–green alga *Tolypothrix tenuis*; a purple one when the alga was cultured under conditions favouring the synthesis of phycoerythrin, and a blue one when the conditions favoured the synthesis of phycocyanin. The pigments were extracted from the alga by treatment with methanol–1% ascorbic acid for 20–30 min at 60°, and were referred to as the *purple pigment* and *blue pigment* respectively. The authors proposed that these pigments are precursors of phycoerythrobilin and phycocyanobilin, or are involved in some way in their biosynthesis. However, the present results, which arose out of successful attempts to isolate similar pigments from red algae, demonstrate that the postulated precursors are artifacts formed from phycoerythrin and phycocyanin, the covalently-bound prosthetic groups of which were extracted from algae by the modified method but again with molecular change.

RESULTS

The spectral properties of the purple and blue pigments and their derivatives, obtained in the present investigation, are summarized in Table 1. These properties are essentially in agreement with those published by Fujita and Hattori.[1,2] All pigment preparations identified as the purple or blue pigments in the investigation had spectral properties identical with those described in Table 1.

The blue pigment was isolated from the phycocyanin-rich Cyanophyta *Nostoc muscorum* and *Anabaena cylindrica*. The purple pigment was isolated from the phycoerythrin-rich Cyanophyte *Phormidium persicium* and from the following Rhodophyta: *Ceramium rubrum*, *Cystoclonium purpureum*, *Polysiphonia elongata* and *Rhodomela* sp. These Rhodophyta

* Supported by the Air Force Office of Scientific Research, O.A.R., through the European Office, Aerospace Research, United States Air Force (AF EOAR 63-18) and by the Charles F. Kettering Foundation.

[1] Y. FUJITA and A. HATTORI, *J. Gen. Appl. Microbiol.* (*Tokyo*) **9**, 253 (1963).

[2] Y. FUJITA and A. HATTORI, *J. Biochem.* (*Tokyo*) **51**, 89 (1962).

also yielded variable amounts of the blue pigment, formed probably from phycocyanin, and this proved very difficult to separate from the purple pigment.

The extraction method of Fujita and Hattori[1,2] was first used, but a modified procedure gave greatly improved yields of the same pigments. The principal modifications were pre-extraction of the algae with methanol at room temperature to remove the bulk of the chlorophylls and carotenoids, and considerable extension of the time of the subsequent extraction with hot methanol.

C-phycocyanin and R-phycoerythrin also yielded the blue pigment and purple pigment respectively at a slow rate over 2–3 days when subjected to the methanol–ascorbic acid extraction procedure. Isolated, purified, phycoerythrobilin was completely converted to the purple pigment when subjected to the extraction conditions for 48 hr.

Spectrophotometric titration showed that the purple pigment contains a pyrrolenine (—N=) group with a pK' of about 6·6, and the blue pigment a similar group with a pK' of

TABLE 1. SPECTRAL PROPERTIES OF THE PURPLE AND BLUE PIGMENTS AND THEIR DERIVATIVES

	λ_{max} nm*					
	Free base	Hydrochloride			Zn complex	Fluorescence of zinc complex
Compound	5% Pyridine in $CHCl_3$	Acid-$CHCl_3$	0·1 N HCl	5% HCl in MeOH	$CHCl_3$	
Purple pigment:						
This investigation:	320, 532	329, 602	328, 590	589	326, 340, 560, 605	Red
Fujita and Hattori[1]:		593		588	562, 607	
Blue pigment:						
This investigation:	373, 590	660–680†	374, 684	375, 686	378, 665	None
Fujita and Hattori[2]:		680		375, 685	374, 661	

* Main peak indicated by underlining.
† Variable, depending on concentration of acid with which chloroform solution is equilibrated.

about 5·7. Both pigments had the same R_f value as did free mesobiliviolin (0·78) on paper chromatography in lutidine–water–ammonia, while the R_f value of mesobiliviolin di-methyl ester was 1·00. In this solvent system the R_f values of porphyrins and bilins are inversely proportional to the number of free carboxyl groups per molecule: the chromatographic behaviour of each of the isolated pigments is compatible with the presence of two carboxyl groups per molecule.[3–5] The presence of such groups is also suggested by the ready extractions of the pigments from chloroform into 1% sodium bicarbonate solution.

Conversion of the Purple and Blue Pigments to Phycoerythrobilin and Phycocyanobilin

When dissolved in 10 N HCl at room temperature the purple and blue pigments were converted within 10 min to pigments very similar to phycoerythrobilin and phycocyanobilin respectively. These products, which had solubility properties identical with those of phyco-

[3] R. E. H. NICHOLAS and C. RIMINGTON, *Scand. J. Clin. Lab. Invest.* **1**, 12 (1949).
[4] R. TIXIER, *Mem. Mus. Nat. Hist. Nat.* **5**, Ser. A, 41 (1952).
[5] C. Ó hEOCHA, *Arch. Biochem. Biophys.* **73**, 207 (1958).

erythrobilin and phycocyanobilin, were purified in the same way as the phycobilins.[6,7] Their spectral properties are compared with those of the phycobilins in Table 2 and are seen to be almost identical with them. The pigment obtained by acid treatment of the purple pigment further resembled phycoerythrobilin[7] in being converted slowly to a urobilin on longer standing in 10 N HCl. This urobilin condensed with cysteine to form an adduct which was insoluble in chloroform, although the reaction was not quantitive, as in the case of the urobilin formed from phycoerythrobilin.

TABLE 2. BILINS OBTAINED FROM PURPLE AND BLUE PIGMENTS BY TREATMENT WITH 10 N HCl, COMPARED WITH PHYCOERYTHROBILIN AND PHYCOCYANOBILIN

	λ_{max} nm*				
	Free base	Hydrochloride		Zn Complex	Fluorescence of zinc complex
Compound	Neutral $CHCl_3$	Acid $CHCl_3$	Dil. HCl	$CHCl_3$	
Bilin from purple pigment	304, 506	311, 574	306, 555	321, (540), 584	Orange
Phycoerythrobilin†	304, 505	312, 576	307, 556	320, (540), 583	Orange
Bilin from blue pigment	— —	360, 635	— 656	375, 630	Very weak red
Phycocyanobilin‡	— —	357, 630	— 655–660§	378, 630	Weak red

* Main peak indicated by underlining
† From Ref. 7. ‡ From Ref. 5. § λ_{max} of C-phycocyanin in 0·1 N HCl.

DISCUSSION

The following three points indicate that the purple and blue pigments are artifacts and not *in vivo* pigments as Fujita and Hattori[1,2] deduced.

1. The pigments are released only on prolonged treatment with methanol at 60°. Cold methanol or ethanol does not extract them, nor does hot acetone. Only traces are released by hot ethanol.

2. The yield of purple or blue pigment is proportional to the time of treatment and roughly to the amount of phycoerythrin or phycocyanin respectively present in the algal sample. The colour of the algae, due to the biliproteins, decreases noticeably as the purple or blue pigment is extracted. For example, samples of *Phormidium persicinum* or *Ceramium rubrum* after extraction of most of the carotenoids and chlorophylls with acetone and methanol at room temperature, were a bright red phycoerythrin-colour. (No detectable purple pigment was extracted in these preliminary washings.) When these samples were then treated with methanol-ascorbic acid at 60° for 8 hr a high yield of purple bilin was obtained and the red colour of the samples was considerably reduced. Re-extraction, as before, for a further 10 hr gave a further, lower yield of purple pigment and the algal samples were then a light grey-brown colour. Further extraction gave very low and progressively decreasing yields of purple pigment. Similar results were observed in the extraction of the blue pigment from *Nostoc*.

3. Isolated phycoerythrobilin is completely converted to the purple pigment by the extraction conditions. Similar treatment of R-phycoerythrin and C-phycocyanin yields purple and blue pigments respectively but at a much slower rate than from the isolated

[6] C. Ó hEOCHA, *Biochemistry* **2**, 373 (1963).
[7] P. Ó CARRA, C. Ó hEOCHA and D. M. CARROLL, *Biochemistry* **3**, 1343 (1964).

phycoerythrobilin or the algal samples. The probable reason for this is the formation of dense insoluble grains of biliprotein in which the phycobilin prosthetic groups remain inaccessible to the hot methanol.

These findings establish that the purple and blue pigments are derived from the prosthetic groups of phycoerythrin and phycocyanin. The mechanism of the formation and release of the pigments remains obscure. Methanol rather than ascorbic acid appears to be the active agent, since the pigments are still formed, although in lower yield, when ascorbic acid is omitted from the extracting methanol, but not when 1% ascorbic acid in acetone is used instead. The phycobilins are thought to be attached to the apoproteins by ester bonds involving the propionic acid side-chains.[6,7] However, since the carboxyl groups of the purple and blue pigments are not esterified, the release of the pigments cannot involve methanolysis of such bonds. (Esterified bilins, such as mesobiliviolin dimethyl ester, were not hydrolysed when subjected to the procedures used in the purification of the purple and blue pigments.) The structural relationship of the artifact pigments to the phycobilins, and the mechanism of regeneration of the latter in 10 N HCl remain equally obscure.

The procedure involved in the release and isolation of the purple and blue pigments from the biliproteins seems milder than the methods necessary to obtain free phycoerythrobilin and phycocyanobilin (involving hydrolysis of the biliproteins with conc. HCl). However, that the latter compounds are the native prosthetic groups has been established by spectral comparison of the isolated pigments with the denatured biliproteins under similar conditions.[6,7] Using the methanol extraction procedure, relatively large yields of apparently reconvertible derivatives of phycobilins can be isolated direct from the algal material, and the method may be of value in identifying phycobilin prosthetic groups when only small samples of material are available. It circumvents the necessity of isolating and purifying the biliproteins and subjecting them to acid hydrolysis which is complicated by side reactions and gives low yields of free phycobilins.[5–7]

EXPERIMENTAL

Extraction and Isolation of the Purple and Blue Pigments

The following modified procedure was found to release the phycobilin prosthetic groups in good yield as the purple and blue pigments, and to facilitate purification of the latter. The algal sample was first extracted (× 3 for 15 min) with methanol at room temperature to remove water and the bulk of carotenoids, chlorophylls, etc. The sample was then immersed in methanol–1% ascorbic acid and incubated at 60° for 8 hr. The supernatant was decanted and filtered. If the alga remained red or blue it was re-extracted for a further 8 hr period. The filtered extracts were evaporated to dryness at about 40° on a rotary evaporator and redissolved in a small quantity of methanol. Four vol. of peroxide-free ether were added, and the resulting solution was extracted into 0·1 N HCl (0·25 vol. × 3), leaving some residual non-bilins in the ether layer. The combined acid extracts were washed with ether (0·25 vol. × 2) and then neutralized by addition of solid sodium acetate. The pigment was then extracted into ether (0·25 vol. × 3) and the combined ether extracts were washed with 1% sodium acetate (1 vol.) and distilled water (1 vol.). The pigment was then re-extracted into 0·1 N HCl (0·5 vol.) and from there into chloroform (0·25 vol. × 2). The chloroform was evaporated under a stream of nitrogen. When not used immediately, the pigments were stored dry at −20°.

The macroscopic Rhodophyta were gathered on the sea-shore near Galway. The Cyano-

phyta *Phormidium persicinum*, *Nostoc muscorum* and *Anabaena cylindrica* were cultured in white fluorescent light.[8] Phycoerythrobilin and the biliproteins, R-phycoerythrin and C-phycocyanin, were prepared as described elsewhere.[7,8] Mesobiliviolin di-methyl ester was prepared by dehydrogenation of mesobilirubinogen with $FeCl_3$–HCl in methanol.[9] The product of this reaction was shown to be a dimethyl ester rather than the free acid by its solubility and chromatographic properties; free mesobiliviolin was obtained from it by hydrolysis in 10 N HCl for 15 min at 80°, followed by fourfold dilution with water and extraction of the pigment into chloroform.

Zinc complexes of bilins in chloroform were formed by addition (one drop per ml) of 2% zinc acetate in ethanol containing 5% pyridine. The pyridine aided formation of the complexes by neutralizing any acid present and did not alter the spectra of the complex salts.

Spectrophotometric titrations were carried out in aqueous solution. The pigment was dissolved in 0·1 N HCl–0·01 M citric acid (pH 1·4) and titrated to pH 9 by addition first of Na_2HPO_4 (0·5 M) to a concentration of 0·05 M, and then 2 N NaOH from a micro-pipette.

The pH and absorbancy at the λ_{max} of the hydrochloride were measured at intervals, and the absorbancies were corrected for the volume change. The pH was returned to 1·4 and the absorbancy, after correction, was checked to see that no appreciable destruction of pigment had taken place during the titration. A correction was also applied for the contribution of the progressively increasing amount of free base to the extinction at the titration wavelength. The absorption spectra of the same concentration of the pigment at pH 1·4 and pH 9·0 were used as standard spectra of the hydrochloride and free base, respectively. The corrected extinction values were plotted against wavelength.

Paper chromatography of bilins in 2,6-lutidine–water–ammonia was carried out as described by Nicholas and Rimington.[3]

[8] P. Ó Carra, *Biochem. J.* **94**, 171 (1965).
[9] C. H. Gray, A. Kulczycka and D. C. Nicholson, *J. Chem. Soc.* 2276 (1961).