Spectral Properties of the Phycobilins. I. Phycocyanobilin*

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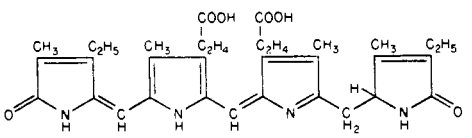
Three different pigments were obtained from C-phycocyanin by closely related methods of isolation. *p*H difference spectra indicate that one of these, phycobilin 630, obtained by direct chloroform extraction after hydrolysis of C-phycocyanin with 12 N HCl for short periods at 25°, is the native prosthetic group (phycocyanobilin). Phycobilin 630 is converted to phycobilin 608 and to phycobilin 655 on standing in 12 N and 9–11 N HCl respectively; 12 N HCl also converts phycobilin 655 to phycobilin 608. Phycobilin 608 has many properties of mesobiliviolin, but on methylation it is converted to phycobilin 655, unlike mesobiliviolin. Phycobilin 655, a dibasic tetrapyrrole, is also formed when an aqueous solution of phycobilin 630 is neutralized with sodium acetate. These findings indicate that the procedure employed for the isolation of the phycobilins must be selected with caution if artifact-pigment formation is to be avoided. Earlier results, indicating interspecific difference in the prosthetic group of C-phycocyanin, must now be attributed rather to variations in the experimental techniques designed to liberate phycocyanobilin.

C-Phycocyanin, a blue-colored, red-fluorescing biliprotein of algae, was reported by Lemberg (1928, 1930) and by Lemberg and Bader (1933) to contain a prosthetic group of the mesobiliviolin type, which was identified as mesobiliviolin IXa (I) (see also Lemberg and Legge, 1949).



(I)

More recently, another pigment was obtained from C-phycocyanin under somewhat milder conditions, and, because it was converted to a mesobiliviolin-type pigment under the conditions employed by Lemberg for hydrolyzing the biliprotein, it was considered to be the prosthetic group (phycocyanobilin) of C-phycocyanin or a very close derivative (Ó hEocha, 1958). Later, when C-phycocyanin from a different algal source was studied under apparently identical conditions, very little pigment was obtained, but on longer hydrolysis a third pigment was recovered which differed from the two already mentioned (Ó hEocha and Lambe, 1961). This pointed to the possibility that C-phycocyanin from different plant sources may contain different prosthetic groups.

A study has been made of the experimental conditions which lead to the recovery of these three pigments from C-phycocyanin. The accumulated spectral evidence indicates that the pigment termed phycocyanobilin in an earlier paper (Ó hEocha, 1958) is the prosthetic group of C-phycocyanin, regardless of its algal source. The discrepancies in the literature are attributed to the fact that slight variations in the methods of hydrolysis and purification lead to the isolation of different pigments from C-phycocyanin.

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EXPERIMENTAL PROCEDURE

The blue-green alga *Microcystis aeruginosa* was collected as a floating thick scum (15 kg) at Lake Como, St. Paul, Minn., in September, 1961, and immediately frozen. On thawing, a viscous algal soup resulted, to which distilled water was added (*ca.* 250 ml per liter of algal material), and extraction of phycocyanin was allowed to proceed for 4 days at 5° in the dark. The algal mass was then removed by centrifugation and the extract clarified by filtration through a bed of Hyflo Super-Cel (Johns Manville). Twenty per cent solid ammonium sulfate (w/v) was added, and the resultant precipitate was removed by centrifugation. The supernatant was discarded and the precipitate taken up in a small volume of water and dialyzed against distilled water, followed by centrifugation at 4°. The supernatant was subjected to fractional precipitation with increasing concentrations of ammonium sulfate. Six pigment fractions were thus obtained. Absorption spectrophotometry indicated that all except the final fraction contained C-phycocyanin (λ_{max} 615 mμ) as the sole biliprotein; the phycocyanin was purified by solution, dialysis, and fractional precipitation of each of the original fractions. In some instances (*e.g.*, the phycocyanin preparation used for determining difference and fluorescence spectra) the C-phycocyanin was further purified by chromatography on tricalcium phosphate Super-Cel columns (Haxo *et al.*, 1955). The C-phycocyanin was quantitated spectrophotometrically, with $E^{1\%}_{1\,cm}$ = 65.0 (Ó Carra, 1962). C-Phycocyanin from *Microcystis* was used throughout, except where indicated otherwise. Allophycocyanin (λ_{max} 650 mμ) was purified by fractional precipitation and chromatography of the final ammonium sulfate fraction. *Nostoc muscorum* was cultured in white fluorescent light in the medium of Clendenning *et al.* (1956) and its C-phycocyanin released from the frozen and thawed cells and purified by ammonium sulfate precipitation, followed by chromatography.

Preparation of the Phycobilins.—(*a*) PHYCOBILIN 655. C-phycocyanin (200 mg dry weight) was hydrolyzed by dissolving in 12 N HCl (50 ml) in the dark for 30 minutes at room temperature (Ó hEocha, 1958). The hydrolysate was added to distilled water (200 ml), treated with a saturated solution of sodium acetate to *p*H 6–7, and centrifuged. The supernatant was extracted with peroxide-free ether (75 ml × 3), giving a deep blue-violet solution (λ_{max} 595 mμ). The ethereal solution was ex-

tracted with 2.8 N HCl (25 ml × 4), and this was in turn extracted with chloroform (25 ml × 4). The acid chloroform solution (λ_{max} 655 mμ) was washed with distilled water (200 ml × 5), and this shifted its absorption maximum to 595 mμ. The hydrolysis and extraction procedure was repeated on the precipitated, unhydrolyzed phycocyanin, and eventually four chloroform extracts were obtained which were united, filtered through chloroform-moistened filter paper, and evaporated to dryness *in vacuo* at < 30°. All these manipulations were conducted in dim light.

(*b*) PHYCOBILIN 608. This was prepared by dissolving phycobilin 655 in 12 N HCl for 21 hours, followed by dilution, neutralization, and extraction as described for phycobilin 655. A pigment having similar properties was prepared from C-phycocyanin by the method of Lemberg and Bader (1933).

(*c*) PHYCOBILIN 630. This pigment is phycocyanobilin (Ó hEocha, 1958), and it was prepared as described in the 1958 publication, with use of 12 N HCl; however, the passage of gaseous nitrogen through the hydrolysis mixture was omitted. The diluted hydrolysate was centrifuged and the supernatant extracted directly into chloroform. The drained precipitate of unhydrolyzed phycocyanin was redissolved in acid for 30 minutes, diluted, centrifuged, and again extracted with chloroform. The procedure was repeated a further 2–3 times and all the chloroform extracts were united, washed with 5% HCl and then with water, dried by filtration, and evaporated to dryness *in vacuo* at < 30°.

The Effect of Acid Concentration on the Rate of Pigment Release from C-Phycocyanin.—Samples of C-phycocyanin were dissolved in 10 ml of 9, 10, 11, and 12 N HCl. To 1 ml of each solution was added 3 ml of the appropriate acid, and the absorption spectrum of the solution was determined immediately. After 15 minutes in the dark at 25°, the bulk of the hydrolysate was diluted with 100 ml of distilled water and centrifuged. The supernatant was extracted with chloroform (15 ml followed by 10 ml), and the chloroform solution was washed with 2.8 N HCl and its spectrum was determined. The precipitated, unhydrolyzed phycocyanin was dissolved in acid and the procedure repeated.

Esterification of the Phycobilins.—The phycobilins (2–5 mg) were dissolved in 30 ml of 4% hydrogen chloride in methanol (w/v) and allowed to stand for 6 hours in the dark at room temperature. Chloroform, 100 ml, was added, followed by 100 ml of 1% sodium hydroxide. The chloroform fraction was washed with 7% sodium chloride solution (100 ml × 5) and then with distilled water (200 ml × 2). The chloroform solution was dried by filtration and evaporated to dryness. The residue was extracted with hot petroleum ether (30–60°) (20 ml × 3). The petroleum ether solution was concentrated by evaporation and chromatographed under nitrogen pressure on alumina (Alcoa activated, Grade F20). The alumina columns (22 × 1.2 cm) were prewashed with chloroform and with petroleum ether. The chromatograms were developed with chloroform-diethyl ether (1:2), and the phycobilin esters were eluted as narrow blue zones. The eluates were evaporated to dryness *in vacuo* at < 30°.

Zinc Complex Salts of the Phycobilins.—These were prepared and studied under the conditions described by Gray *et al.* (1961a).

Spectrophotometric Titration Curves.—These were determined by the method of Gray *et al.* (1961b). To 0.5 ml of a stock methanolic solution of the phycobilin was added 4 ml of freshly prepared McIlvaine buffer solution, and the spectral scanning was completed within 5 minutes of the preparation of each solution. After the spectral scanning, the *p*H of each solution was immediately determined. Similar solutions, prepared with 0.01 N HCl, were used at *p*H < 2. No turbidity was noticed in the phycobilin solutions, which were stable at *p*H values less than about 8.5. No variations in titration curves were observed with concentration of pigments.

Reagent-grade chloroform was distilled over potassium carbonate before use. DuPont Reagent hydrochloric acid (12 N) was used. Hydrochloric acid of lower normality was obtained by standing the 12 N acid in a hood for various periods of time; normality was determined by titration against standard alkali. A model G Beckman *p*H meter was used. Absorption spectra in the range 250–850 mμ, which were measured in 4 minutes, were determined with a Beckman DK spectrophotometer; 1-cm cells were used. This instrument records transmittance against wave length, and the curves were replotted manually to read optical density (absorbance) against wave length. Infrared spectra were determined on dry films of the phycobilin esters by means of a modified Perkin-Elmer Model 12-C spectrophotometer, with infrared microscope (Dinsmore, 1959). Fluorescence spectra were determined on microsamples; a recording microfluorospectrophotometer which was calibrated against a neon source was used.

RESULTS

Hydrochloric acid in 12 N concentration was the most satisfactory reagent encountered for the release of protein-free pigment from C-phycocyanin. At lower normalities, hydrochloric acid was progressively less efficient.

Immediately on dissolution in concentrated hydrochloric acid, C-phycocyanin displayed an absorption maximum at 657 mμ with a slight shoulder at 610 mμ, a spectrum similar to that of ethanol-denatured C-phycocyanin at *p*H 2.7. After 30 minutes of hydrolysis the spectrum in concentrated acid of the precipitated C-phycocyanin displayed a peak at *ca.* 610 mμ which was approximately equal in intensity to that at 657 mμ. The ratio of the extinction at the short wave length peak, relative to that at 657 mμ, increased on further hydrolysis. The pigment responsible for the 610 mμ maximum is obviously not extracted by chloroform and probably represents an isomerization product of phycocyanobilin, phycobilin 608, attached to the protein through bonds which are not hydrolyzed by acid.

Sodium methoxide, 0.5 M in methanol, which cleaves the protein-prosthetic group ester linkages of lactoperoxidase at room temperature (D. Holtquist, personal communication), did not yield any phycobilin from C-phycocyanin. A mixture of hydriodic acid and acetic acid was similarly unsuccessful.

Phycobilin 655.—Figure 1 shows the absorption spectral characteristics of acidic and of neutral chloroform solutions of the methyl ester of phycobilin 655. These spectra are identical with those of the unesterified phycobilin. The chromatographically purified methyl ester had $E_{1\,cm}^{1\%}$ = 135 at 595 mμ, and 350 at 365 mμ in chloroform, 350 at 655 mμ, and 320 at 372 mμ in chloroform shaken with 2.8 N HCl.

The results of a spectrophotometric titration of phycobilin 655 are presented in Figure 2. The titration curves indicate that phycobilin 655 is dibasic; the diprotonated form dissociates with a *pK* of about 3.5 and the monoprotonated form with a *pK* of about 5.7. In the methanol-buffer system used, the ultraviolet maximum of phycobilin 655 was at 363 mμ re-

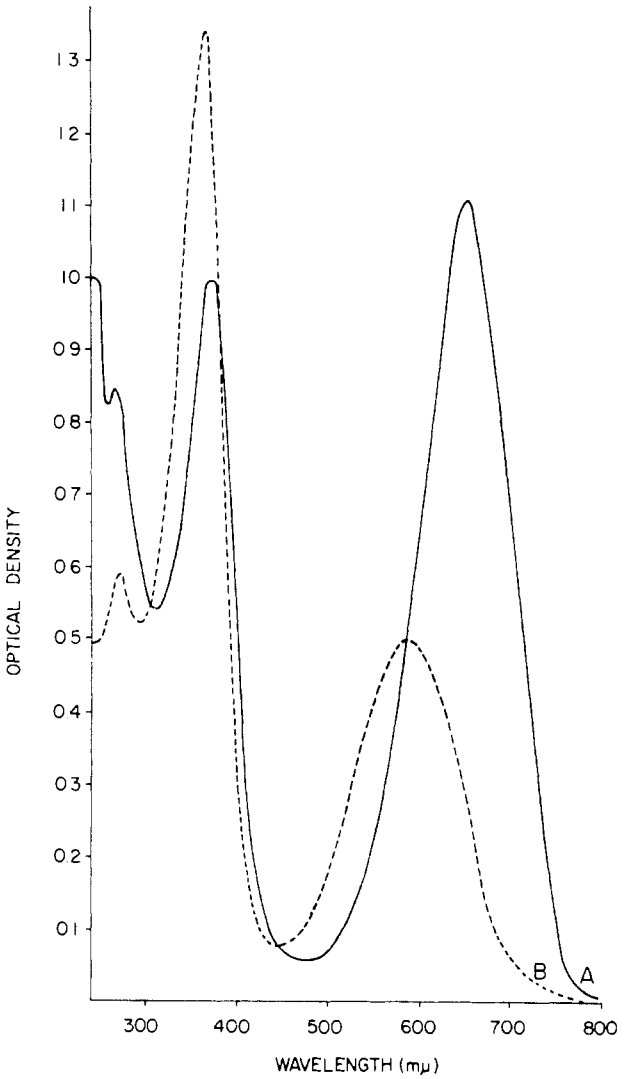


FIG. 1.—Absorption spectra of equal concentrations of phycobilin 655 methyl ester in chloroform. *A*, as hydrochloride; *B*, as free base.

gardless of *p*H, while the red maximum of the diprotonated form was at 665 mμ, that of the monoprotonated form at 656 mμ, and that of the free base at 605 mμ.

The zinc complex salt of phycobilin 655 has absorption maxima at 658 and 378 mμ, and, after oxidation with iodine, at 627, 584, and 515 mμ (Fig. 3). Its light-absorption characteristics are intermediate between those of the zinc complexes of verdins and of violins (Gray *et al.*, 1961a,b). When irradiated, the phycobilin 655 zinc salt displays a red fluorescence (Fig. 4*C*), while the iodine-oxidized salt emits orange fluorescence.

A solution of phycobilin 655 in 2,6-lutidine emits a weak, dull-red fluorescence under ultraviolet irradiation. Preparations of phycobilin 655 which had been stored in the dark in a vacuum desiccator for some weeks had a single-banded fluorescence spectrum (Fig. 4*B*), but fresh samples displayed broad emission between 650 and 700 mμ. The absorption spectra of the stored and fresh samples of phycobilin 655 were identical. The fluorescence of phycobilin 655 in other bases (*e.g.*, pyridine, ammonia) was less intense than in lutidine. Its absorption spectrum in 2,6-lutidine had maxima at 585 mμ and 370 mμ.

Phycobilin 655 is unstable in 12 N HCl at room temperature, being converted to phycobilin 608 on standing for 7 to 21 hours.

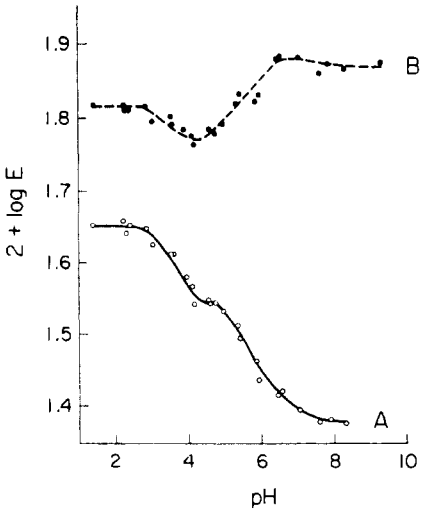


FIG. 2.—Spectrophotometric titration curves of phycobilin 655 in McIlvaine buffers. *A*, maximal absorbance at 665–605 mμ; *B*, absorbance at 363 mμ.

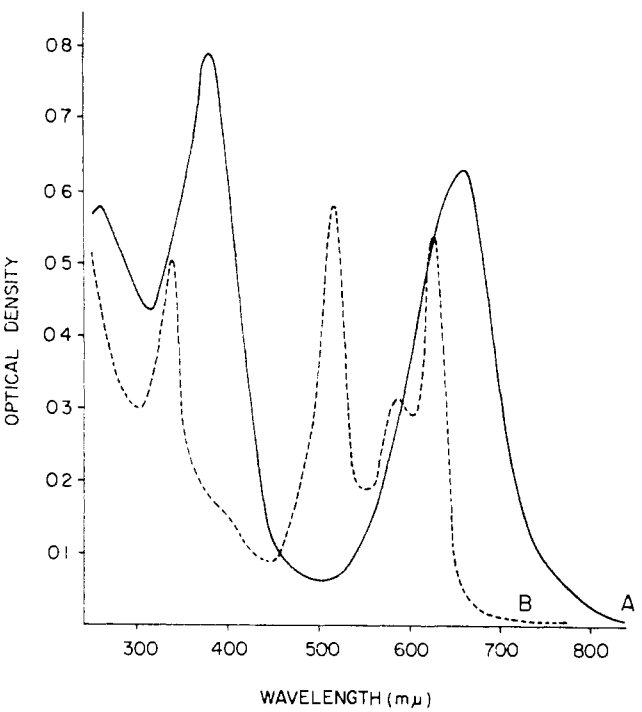


FIG. 3.—Absorption spectra of the zinc complex of phycobilin 655. *A*, in ethanol; *B*, after addition of iodine.

Phycobilin 608.—The visible absorption maxima of phycobilin 608 (Figure 5) and of its zinc complex salt are close to those of mesobiliviolin (Table I). The maximum at 332 mμ is also close to that of mesobiliviolin (327 mμ, according to Gray *et al.*, 1961b); phycobilin 608 is the only one of the pigments we have isolated from C-phycocyanin which displays this ultraviolet peak. Unlike mesobiliviolin, phycobilin 608 has a maximum at *ca.* 360 mμ, the position and relative extinction of which were constant in all preparations of the pigment. However, the possibility cannot be ruled out that this peak is caused by contamination with incompletely isomerized phycobilin 655.

Methylation of phycobilin 608 yielded a pigment with the spectral characteristics of phycobilin 655,

TABLE I
ABSORPTION MAXIMA (mμ) OF BILE PIGMENTS AND PHYCOBILINS

Pigment	In Neutral Chloroform			In Acid Chloroform		
	λ_1	λ_2	E_2/E_1[a]	λ_1	λ_2	E_2/E_1[a]
Biliverdin	640–645	377.5	3.10[b]	665	379	1.2
Phycobilin 655	595	367	2.10	655	367	0.85
Esterified product from phycobilin 608	597	367	2.25	655	367	0.95
Phycobilin 630	612	360	1.95	630	365	0.75
Phycobilin 608	565 (360)	332	1.65	608 (365)	332	0.82
Mesobiliviolin	565	327	1.54[b]	602.5	327.5	0.93[b]

[a] Ratio of absorption at the two maxima. [b] Data of Gray *et al.* (1961a,b).

regardless of whether methanolic hydrochloric acid or the BF_3-methanol reagent was used to effect the reaction. The spectrum of phycobilin 608 was unaltered on standing for a number of hours in methanol or in 4% aqueous hydrochloric acid; immediately after solution in 4% HCl–methanol its visible spectrum consisted of two peaks (λ_{max} 607 and 660 mμ), and after 10 minutes it displayed only one peak (λ_{max} 667 mμ) and corresponded closely to the spectrum of phycobilin 655 in the same solvent. At the end of the esterification period (6 hours) the ester was purified by chromatography and found to be spectrally identical (ultraviolet, visible, and infrared) with phycobilin 655 methyl ester (see Table I). A notable feature of the esterification was the disappearance of the mesobiliviolinoid ultraviolet maximum at 332 mμ.

Phycobilin 630.—This pigment is characterized by absorption maxima at 630 and 365 mμ in acid chloroform (Fig. 6). The positions and relative intensity of the peaks were not altered by repeated washing with 2.8 N HCl. Previously (Ó hEocha, 1958), no shift was observed in the position of the red absorption maximum of this pigment, termed phycocyanobilin, on washing with water, but owing to loss of pigment this was not investigated fully. With larger quantities of pigment, it has now been observed that the absorption maximum is shifted reversibly to 612 mμ on washing with freshly distilled water (× 3) (Fig. 6). A solution of freshly prepared phycobilin 630 in 2,6-lutidine (λ_{max} 583 mμ) was found to be nonfluorescent under ultraviolet irradiation, differing thereby from phycobilin 655.

Spectrophotometric titration of the red maximum of phycobilin 630 indicated that it is a monobasic pigment having a *pK* of about 4.8.

Phycobilin 630 was esterified with 4% HCl–MeOH for 6 hours; the recovered ester corresponded in its spectral properties with phycobilin 630. However, after chromatographic purification on alumina, its spectrum (ultraviolet, visible, and infrared) corresponded to that of phycobilin 655 methyl ester. Because of its lability, it did not prove possible to purify phycobilin 630, and the crude pigment from chloroform extracts of the C-phycocyanin hydrolysate was used in the study of this pigment.

Of particular interest was the effect of various concentrations of hydrochloric acid on phycobilin 630. When this pigment was dissolved in 10.9 N HCl for several hours in the dark (times varying between 7

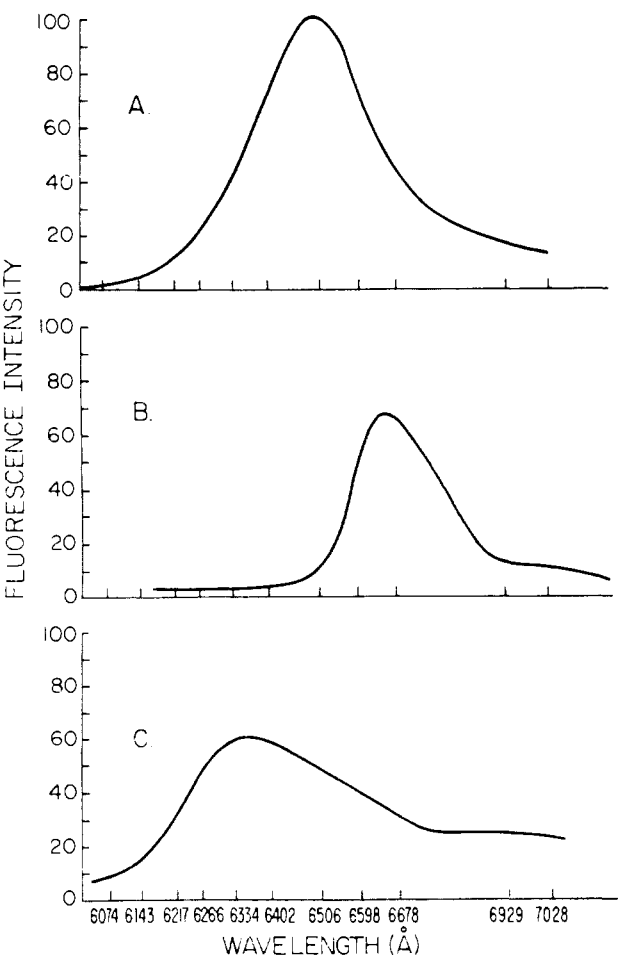


FIG. 4.—Fluorescence spectra. *A*, C-phycocyanin in phosphate buffer (*p*H 6.8). *B*, an aged preparation of phycobilin 655 in 2,6-lutidine. *C*, zinc complex of phycobilin 655 in ethanol.

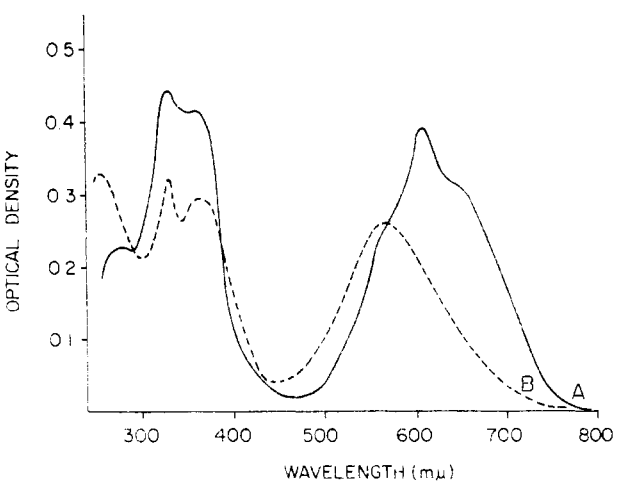
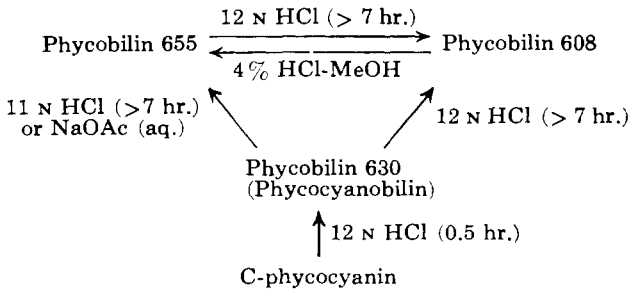


FIG. 5.—Absorption spectra of equal concentrations of phycobilin 608 in chloroform. *A*, as hydrochloride; *B*, as free base.

and 21 hours gave similar results), then diluted with 3–4 volumes of distilled water and extracted into chloroform, the resulting pigment displayed absorption maxima at 655 and 370 mμ. Similar results were obtained when 9.6 N HCl was used, indicating that phycobilin 630 is converted to phycobilin 655 on standing in 9.6–11 N HCl. On the other hand, when phycobilin 630 was dissolved in 12 N HCl under the same conditions, it was transformed to phycobilin 608, as indicated by the absorption properties of the resulting pigment. Phycobilin 630 is stable in 2.8 N HCl for at least 24 hours.

The findings concerning the interconversion of the phycobilins isolated from C-phycocyanin may be summarized as follows:



When C-phycocyanin, rather than phycobilin 630, was treated with acid similar results were obtained. Hydrolysis of the biliprotein for 20 hours at room temperature with 10.9 N HCl followed by dilution and direct extraction into chloroform yielded phycobilin 655. This is the pigment we reported from the C-phycocyanin of *Nostoc muscorum* (Ó hEocha and Lambe, 1961). However, when C-phycocyanin from *Nostoc* was hydrolyzed with 12 N HCl at room temperature, phycobilin 630 was obtained after 30 minutes and phycobilin 608 after 20 hours. British commercial hydrochloric acid (about 10 N) was used by Ó hEocha and Lambe (1961), and the results obtained are evidently accounted for in terms of this concentration, rather than by any difference in the nature of the prosthetic groups of C-phycocyanins from different algae.

Difference Spectra.—The fluorescence of C-phycocyanin (Fig. 4A) is rapidly quenched by protein-denaturing agents such as acid, urea, and ethanol. An approximately 1000-fold decrease in fluorescence intensity was observed when a solution of C-phycocyanin at *p*H 8 was made 50% with respect to ethanol. Simultaneously, the red absorption maximum of the biliprotein was shifted from 615 mμ to 600 mμ, with a sharp fall in optical density (Ó hEocha and Lambe, 1961). The higher optical density of native phycocyanin is apparent from the difference spectrum (Fig. 7). It is also apparent that there is a concomitant increase in extinction at 350 mμ when C-phycocyanin is denatured. A similar spectrum was obtained with thermally denatured C-phycocyanin, prepared by heating at 50° for 60 minutes. When denatured at *p*H 2.7, the C-phycocyanin difference spectrum displayed an additional peak at 665 mμ, which is attributable to the protonated form of the prosthetic group (Fig. 8). If the solutions of ethanol-denatured phycocyanin were dialyzed against 1% sodium chloride immediately after preparation, denaturation at both *p*H values was found to be reversible to a large degree; this indicates that the prosthetic group had not undergone any irreversible chemical change, such as isomerization to a mesobiliverdin (Ó hEocha and Lambe, 1961).

*p*H difference spectrophotometry was employed in an effort to determine which of the three isolated

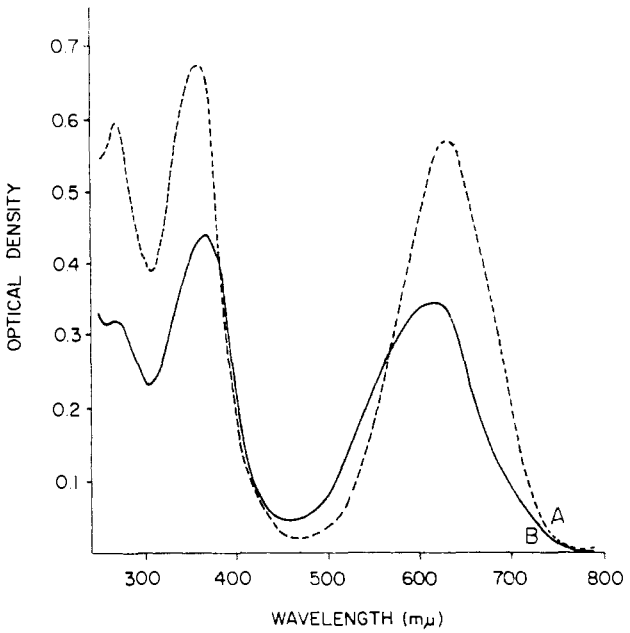


FIG. 6.—Absorption spectra of equal concentrations of phycobilin 630 (phycocyanobilin) in chloroform. *A*, as hydrochloride; *B*, as free base.

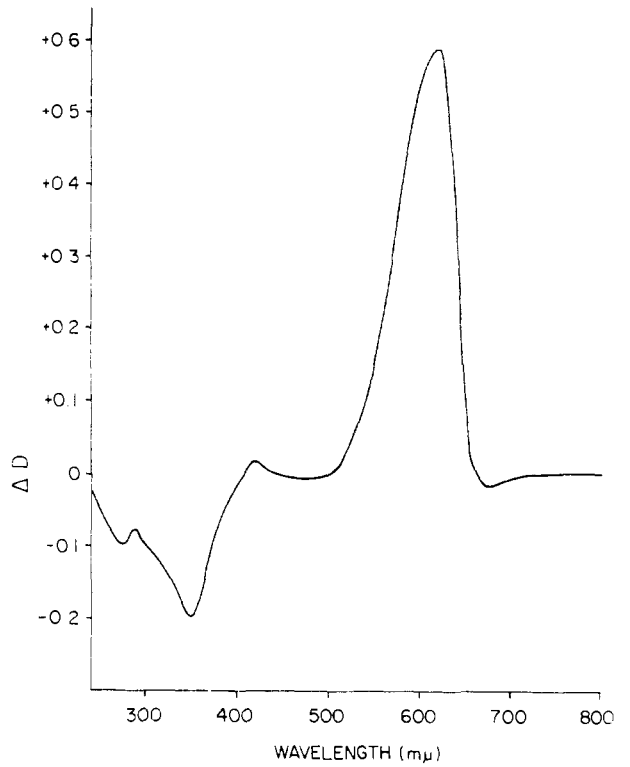


FIG. 7.—The difference spectrum of native (*p*H 6.8) and ethanol-denatured (*p*H 8.0) C-phycocyanin (0.11 mg/ml).

phycobilins represents the native prosthetic group of C-phycocyanin. The difference spectrum of C-phycocyanin denatured with ethanol at *p*H 2.7 and at *p*H 8.0 showed a peak at 658 mμ (Fig. 9, *A*). A corresponding peak was evident in the difference spectrum of the acidic and basic forms of phycobilin 630, but in the same solvent system phycobilin 655 displayed a maximum at 675 mμ (Fig. 9, *B* and *C*). The ultraviolet maxima were similar in all three cases. The difference spectrum of phycobilin 608 was radically different from that of C-phycocyanin under these condi-

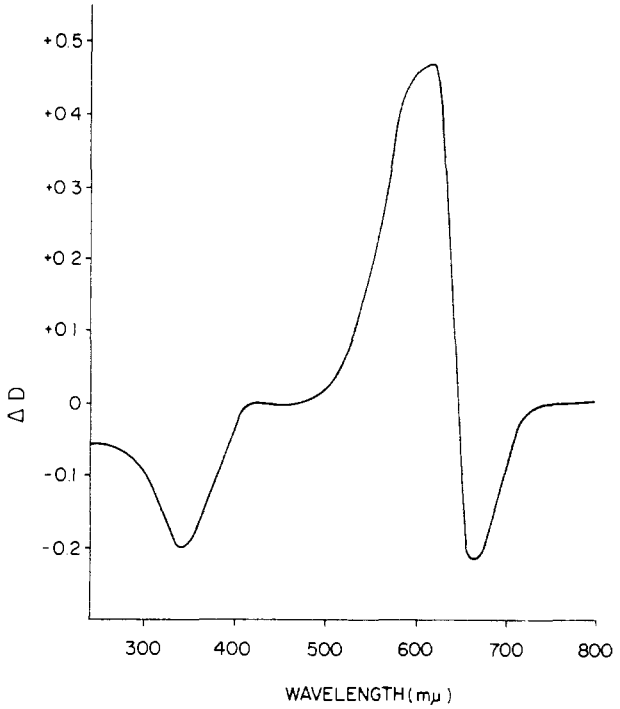


FIG. 8.—The difference spectrum of native (*p*H 6.8) and ethanol-denatured (*p*H 2.7) C-phycocyanin (0.11 mg/ml).

tions in that it displayed major peaks at 595 and 328 mμ. These *p*H difference spectra provide clear evidence that phycobilin 630 is the prosthetic group of C-phycocyanin.

Earlier studies indicated that the prosthetic groups of C-phycocyanin and allophycocyanin (λ_{max} 650 mμ) are very closely related, if not identical (Ó hEocha, 1958). As further confirmation of this, the spectra of denatured allophycocyanin in dilute hydrochloric acid (λ_{max} 655 mμ) and 50% ethanol (λ_{max} 595–600 mμ) were found to correspond to the maxima of C-phycocyanin in these solvents (Lemberg, 1930; Ó hEocha and Lambe, 1961). However, the nature of the protein environment surrounding the phycocyanobilin in the two chromoproteins must differ if the difference in their absorption spectra in the native state is to be accounted for.

DISCUSSION

(*a*) *Identification of the Native Prosthetic Group of C-Phycocyanin.*—It has been pointed out by Rabinowitch (1951) that the spectral properties of mesobiliviolin are difficult to correlate with those of C-phycocyanin. *p*H difference spectrophotometry clearly rules out the mesobiliviolinoid phycobilin 608 as the prosthetic group of C-phycocyanin. This technique (Fig. 9) points to phycobilin 630 as the native pigment, phycocyanobilin. This conclusion is also supported by the fact that phycobilin 655 and phycobilin 608 are both formed from phycobilin 630. Efforts to reverse these reactions have failed. Furthermore, the absorption maximum of the free base of phycobilin 630 (612 mμ in chloroform) is very close to that of native C-phycocyanin (615 mμ). In view of these findings, phycobilin 630 will henceforth be referred to as phycocyanobilin.

(*b*) *Pigment-Protein Interaction in C-Phycocyanin.*—The results reported indicate the need for rigorous control of the experimental conditions employed in the study of the pigment moiety of C-phycocyanin. At

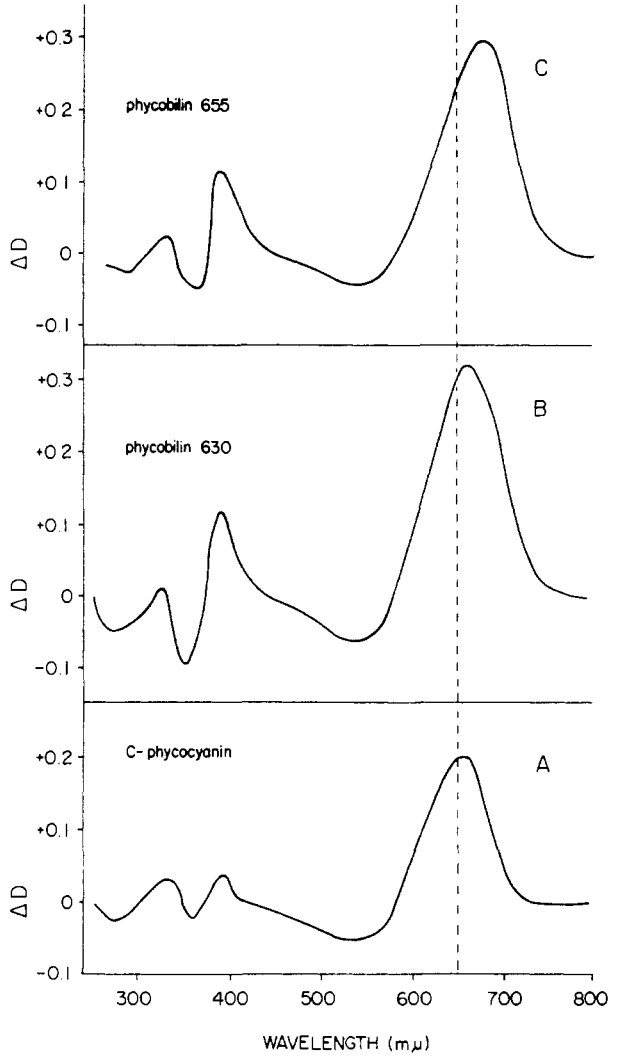


FIG. 9.—Difference spectra in ethanol-McIlvaine buffer (*p*H 2.7 and 8.0): *A*, C-phycocyanin (0.11 mg/ml); *B*, phycobilin 630 (phycocyanobilin); *C*, phycobilin 655.

room temperature, an optimal rate of pigment release occurred when the biliprotein was hydrolyzed with 12 N HCl. The use of hydrochloric acid of lower normality undoubtedly accounts for the failure of Ó hEocha and Lambe (1961) and of Garnier (1961) to obtain appreciable quantities of pigment from C-phycocyanin after hydrolysis at room temperature for 30 minutes. Phycobilin 608, an acid isomerization product of phycocyanobilin, appears to form unhydrolyzable bonds with the protein of C-phycocyanin, and this must explain the low yield of pigment (2%) obtained by Lemberg and Bader (1933) from C-phycocyanin. Clendenning (1954) reported 4% pigment in C-phycocyanin, a value not far from that of 5.1%, recently deduced by Brody and Brody (1961) from fluorescence polarization studies.

Lemberg (see Lemberg and Legge, 1949) surmised that the covalent protein-phycobilin linkage in C-phycocyanin may be a peptide bond between the propionic acid side-chains of the prosthetic group and amino groups on the protein. However, proteolytic enzymes do not release phycocyanobilin (Clendenning, 1954). The peptide bonds of proteins which are least stable in hydrochloric acid are those involving an amino group of a β-hydroxyacid, *i.e.*, serine or threonine (Desnuelle and Casal, 1948). Their selective cleavage is favored at high acid concentrations and at low tem-

peratures of hydrolysis. Thus 20–30% of these bonds present in a protein were split by 10 N HCl after 1 hour at 30°, under which conditions very few other peptide bonds were hydrolyzed. Ten normal HCl was the highest concentration used in these experiments, which were interpreted as involving an N → O peptidyl shift and subsequent rupture of an ester bond.

During hydrolysis of C-phycocyanin some isomerization of phycocyanobilin occurs, some of the isomerization products become irreversibly bound to the protein, and some loss of unhydrolyzed protein-pigment complex occurs during the batch procedure employed in liberating the prosthetic group. Consequently it is difficult to obtain an accurate estimate of the rate of release of pigment from the protein. Of the phycobilin which became chloroform soluble after hydrolysis with 12 N HCl at 25°, 50% was released in about 45 minutes, and this value might indicate that an ester, rather than a peptide bond, joins the phycocyanobilin to the protein. However, the failure of sodium methoxide in methanol to release any pigment from C-phycocyanin at room temperature is difficult to reconcile with this, because this reagent has proved very successful in cleaving the ester linkage between the prosthetic group of lactoperoxidase and its apoprotein (D. Holtquist, personal communication).

The effect of ethanol and urea in denaturing proteins is due to their breaking hydrophobic interactions present in proteins in their native configurations (Whitney and Tanford, 1962). The immediate denaturing effect of these reagents on C-phycocyanin, changing its absorption spectral properties to those of phycocyanobilin (*cf.* Fig. 9, *A,B*), indicates that the phycocyanobilin residues are enclosed in hydrophobic regions of the protein, as is true of the prosthetic group of myoglobin (Kendrew *et al.*, 1961). Some support for this view comes from the fact that phycocyanobilin in neutral chloroform solution has an absorption at 612 mμ (Fig. 5) close to that of C-phycocyanin (615 mμ). However, the interaction of the chromophore and native protein leads to two phenomena which are not duplicated in any solvent system we have used for phycocyanobilin, namely, red fluorescence and an E_{365}/E_{615} = *ca.* 0.15. The value for this ratio given by phycocyanobilin in chloroform is 1.95 (Table I).

The zinc complex salt of phycocyanobilin is red-fluorescent, but its absorption maximum in the red (630 mμ in chloroform solution; Ó hEocha, 1958), differs from that of C-phycocyanin. It also possesses a high extinction in the near ultraviolet. Recently, Hattori and Fujita (1959) reported an ash-free preparation of C-phycocyanin, which argues against the occurrence of a tetrapyrrolic metal complex in the biliprotein. A further argument is the similarity of the *p*H difference spectra of denatured C-phycocyanin and of phycocyanobilin (Fig. 9); the phycobilin would not be expected to retain a complexed metal after being dissolved in 12 N, followed by 3 N HCl, during its isolation.

(*c*) *Phycocyanobilin and Derivatives.*—The formation of different products as a result of the action at room temperature of 11 N and of 12 N HCl on phycocyanobilin is difficult to interpret, but it undoubtedly explains the findings of Ó hEocha (1958) and Ó hEocha and Lambe (1961), who hydrolyzed C-phycocyanin with concentrated hydrochloric acid of different normalities. Since phycobilin 655 is converted to phycobilin 608 by 12 N HCl, it might appear to be an intermediate between phycocyanobilin and phycobilin 608. However, a time course study of the reaction (Ó hEocha, 1958, Fig. 3) did not indicate the formation of phycobilin 655 at any intermediate stage.

Protoporphyrin, which contains two vinyl side-chains, is hydrated on standing at 20° in hydrochloric acid (Falk *et al.*, 1956). The degree of hydration (and possibly also hydrochlorination) was found to increase with increasing normality of acid; 10 N HCl, the highest normality used in these experiments, led to the formation of 80% hydration product, probably hematoporphyrin (2 —CH=CH_2 → 2 —CHOH—CH_3). Hematoporphyrin was not dehydrated on standing in 8.2 N HCl, the highest normality used in this particular experiment.

If phycocyanobilin were to contain vinyl side-chain(s), the work of Falk *et al.* (1956) suggests that hydration or hydrochlorination of these groups would take place on standing of the pigment in hydrochloric acid. However, these studies do not suggest any explanation for the fact that different products are obtained in 12 N and 10 N HCl. Saturation of vinyl groups would be expected to lead to a bathochromic shift in absorption maximum, such as occurs in 12 N HCl (630 mμ to 608 mμ, determined in acid chloroform). In the porphyrin series, the long wave length maximum of protoporphyrin lies about 5 mμ farther toward the infrared than that of hematoporphyrin (Fischer and Orth, 1937).

The pigment obtained by Lemberg and Bader (1933) after hydrolysis of C-phycocyanin at 80° analyzed for $C_{34}H_{44}O_8N_4$, as against $C_{33}H_{40}O_6N_4$, the empirical formula for mesobiliviolin. These authors argued that noncrystalline bile pigments usually give too high oxygen values. However, the experimental value for oxygen would agree with that expected for a hydrated biliviolin, containing two hydroxyethyl side-chains. The suggestion was made by Fischer and Orth (1937) that phycocyanobilin may contain vinyl rather than ethyl groups as postulated by Lemberg. This suggestion was rejected by Lemberg and Legge (1949) on the grounds that mesobiliverdin was isolated from C-phycocyanin under conditions (boiling 10% methanolic alkali) which do not convert biliverdin to mesobiliverdin. However, Gray *et al.* (1961a) have demonstrated that sodium hydroxide and sodium methoxide catalyze a prototropic isomerization of bilirubin to dihydrobiliverdin, thereby converting a vinyl to an ethyl substituent. The same reagents also isomerize *d*-urobilin to a mesobiliviolinoid pigment, a reaction which may also convert a vinyl to an ethyl substituent (Gray and Nicholson, 1958).

The possibility exists therefore that phycocyanobilin contains vinyl side-chain(s) which can be hydrated in acid, forming a hydrated biliviolin, and reduced by isomerization in alkali, forming a verdin.

Since the carboxyl group of mesobiliviolin IXa (I) is separated by two methylene groups from the conjugated double-bond system of the tetrapyrrole, esterification would not be expected to cause any appreciable change in the visible and ultraviolet spectra of this pigment. Such has been the general experience in the natural bile pigment series. Lemberg and Bader (1933) reported that the mesobiliviolin they obtained from C-phycocyanin formed a dimethyl ester whose absorption spectral properties were identical with those of the parent compound. However, they reported that the ester was more labile than the free acid, being converted to a green compound, even under nitrogen.

Phycobilin 608, the isomerization product of phycobilin 655 in 12 N HCl, has absorption spectral properties somewhat similar to those of mesobiliviolin (Table I). However, esterification of phycobilin 608 produced a

pronounced spectral change, the product having the spectral properties of phycobilin 655; phycobilin 608 apparently contains an esterifiable group intimately conjugated with the tetrapyrrolic chromophore.

Since phycobilin 655 is formed from phycocyanobilin on neutralization of its aqueous solution, a close structural relationship must exist between these two pigments. Spectrophotometric titration establishes phycobilin 655 as a dibasic tetrapyrrole (Fig. 2), while a similar titration indicates that phycocyanobilin is monobasic. The basicity of tetrapyrroles depends on the number of pyrroline nitrogens (—N=) in the molecule, and it may be that one of the basic electron pairs of phycocyanobilin is unavailable for protonation but is readily made available on neutralization in aqueous solution. This does not occur in chloroform solution, since, in this solvent, the basic forms of phycocyanobilin (Fig. 6) and of phycobilin 655 (Fig. 1) have different spectral properties.

The dibasic nature of phycobilin 655 distinguishes it from the monobasic verdins and mesobiliviolin IXa studied by Gray *et al.* (1961b). In this property it resembles the porphyrins (Phillips, 1958). However, most of the properties of phycobilin 655 and phycocyanobilin are closer to those of the bile pigments than to the porphyrins. Their light absorption and extinction properties are intermediate between those of the verdins and of mesobiliviolin (Table I), but no fully acceptable structure can yet be proposed for these phycobilins.

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