Spectral Properties of the Phycobilins. II. Phycoerythrobilin*

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The bile pigment phycoerythrobilin, isolated from phycoerythrins by the method of C. Ó hEocha (*Arch. Biochem. Biophys. 73*, 207, 1958), is shown to be a chemically unaltered prosthetic group of these biliproteins. Additional properties and reactions of phycoerythrobilin have been determined and a bilidiene IXα structure, isomeric with mesobiliviolin IXα, is proposed for it. Such a structure has been erroneously associated in the past with a pigment preparation termed mesobilirhodin by W. Siedel (*Z. Physiol. Chem. 237*, 8, 1935), and a pigment isolated by R. Lemberg and G. Bader (*Ann. Chem. 505*, 151, 1933) from R-phycoerythrin. This latter, which was originally regarded as phycoerythrobilin, is shown to have been a urobilinoid artifact of the native prosthetic group. Phycoerythrobilin is isomerized by concentrated hydrochloric acid to a mesobiliviolin and a urobilin which differs from *i*- and *d*-urobilin in that it forms refractory covalent linkages with thiol compounds, including the apoprotein of phycoerythrins. A pigment obtained from *d*-urobilin by isomerization is closely related to phycoerythrobilin, though not identical with it, and the two pigments are considered to be side-chain isomers.

Phycoerythrins are red proteins which occur in most red and some blue-green algae (see Ó hEocha, 1962). Lemberg and Bader (1933) showed that R-phycoerythrin contains a tetrapyrrolic prosthetic group closely related to the meso bile pigments. From hydrolysates of several types of phycoerythrins, Ó hEocha (1958) isolated what he considered to be a native prosthetic group, but it differed both from the pigment isolated by Lemberg and Bader (1933), who used more drastic methods of isolation, and from all bile pigments known at the time. The term phycoerythrobilin was applied by both authors to their pigments. Lemberg and Bader (1933) considered that their pigment was identical with a dehydrogenation product of mesobilirubinogen which they named mesobilierythrin and which was later renamed mesobilirhodin and assigned structure Ia (see Scheme I) by Siedel and Möller (1940). Our results indicate that this structural assignation was incorrect.

The literature dealing with the properties of mesobilirhodin and its relationship to phycoerythrobilin is confused. In order to simplify discussion the following conventions regarding nomenclature will be adopted in this paper (these conventions are amplified in the Discussion):

(1) The pigment isolated from phycoerythrins by Ó hEocha (1958), which we consider the native prosthetic group, will be referred to as phycoerythrobilin, while that described by Lemberg and Bader (1933) will be termed "*phycoerythrobilin.*"

(2) The terms mesobilirhodin and bilirhodinoid will be applied to bile pigments considered by us to contain the conjugated system of structure Ia, and the pigment preparation for which structure Ia was originally proposed by Siedel (1935) will be termed "*mesobilirhodin.*"

Experimental Procedure and Results

The marine red algae *Ceramium rubrum* and *Rhodochorton floridulum* were collected on the shore near Galway. Their phycoerythrins (R- and B-, respectively) were extracted with distilled water and purified by fractional precipitation with ammonium sulfate (Svedberg and Lewis, 1928) and by chromatography on tricalcium phosphate–Supercel columns (Haxo *et al.*, 1955). R-Phycoerythrin had absorption maxima in the visible region of the spectrum at 498, 540, and 568 mμ, and B-phycoerythrin at 546 and 565 mμ. *Phormidium persicinum*, a blue-green alga, was cultured in enriched sea water in white fluorescent light. C-Phycoerythrin (λ_{max} 562 mμ) was extracted from ground cells for 3 hours at 2°, and purified by chromatography on tricalcium phosphate.

HCl (12 N) was Dupont Reagent Grade and 10 N HCl was supplied by May and Baker, Dagenham, England. Figures 1, 3, and 5A were determined with a Beckman DK spectrophotometer; the spectrophotometric titration curve (Fig. 4) was obtained under conditions described elsewhere (Ó hEocha, 1963). Other absorption spectra were determined with a Unicam SP 500 spectrophotometer. Figure 2 was determined with a microfluorospectrophotometer which was calibrated against a neon source and Figure 3A was determined with the instrument of Olsen and Amesz (1960), as described by Ó hEocha and Ó Carra (1961).

Isolation of Phycoerythrobilin.—R- and B-phycoerythrins yielded phycoerythrobilin on hydrolysis with concentrated hydrochloric acid. Following hydrolysis two methods of extraction were used which yielded spectrally identical pigments. The preferred method followed that employed for the release of phycocyanobilin 655 from C-phycocyanin (Ó hEocha, 1963). The dried and powdered phycoerythrin was suspended in 12 N HCl and kept at room temperature in the dark for 30 minutes. The hydrolysate was diluted, neutralized with sodium acetate, and then extracted with diethyl ether. The ethereal solution of phycoerythrobilin was next extracted with 2.8 N HCl and this solution was extracted in turn with chloroform. The final chloroform solution was washed eight times with freshly distilled water in order to ensure complete removal of urobilinoid

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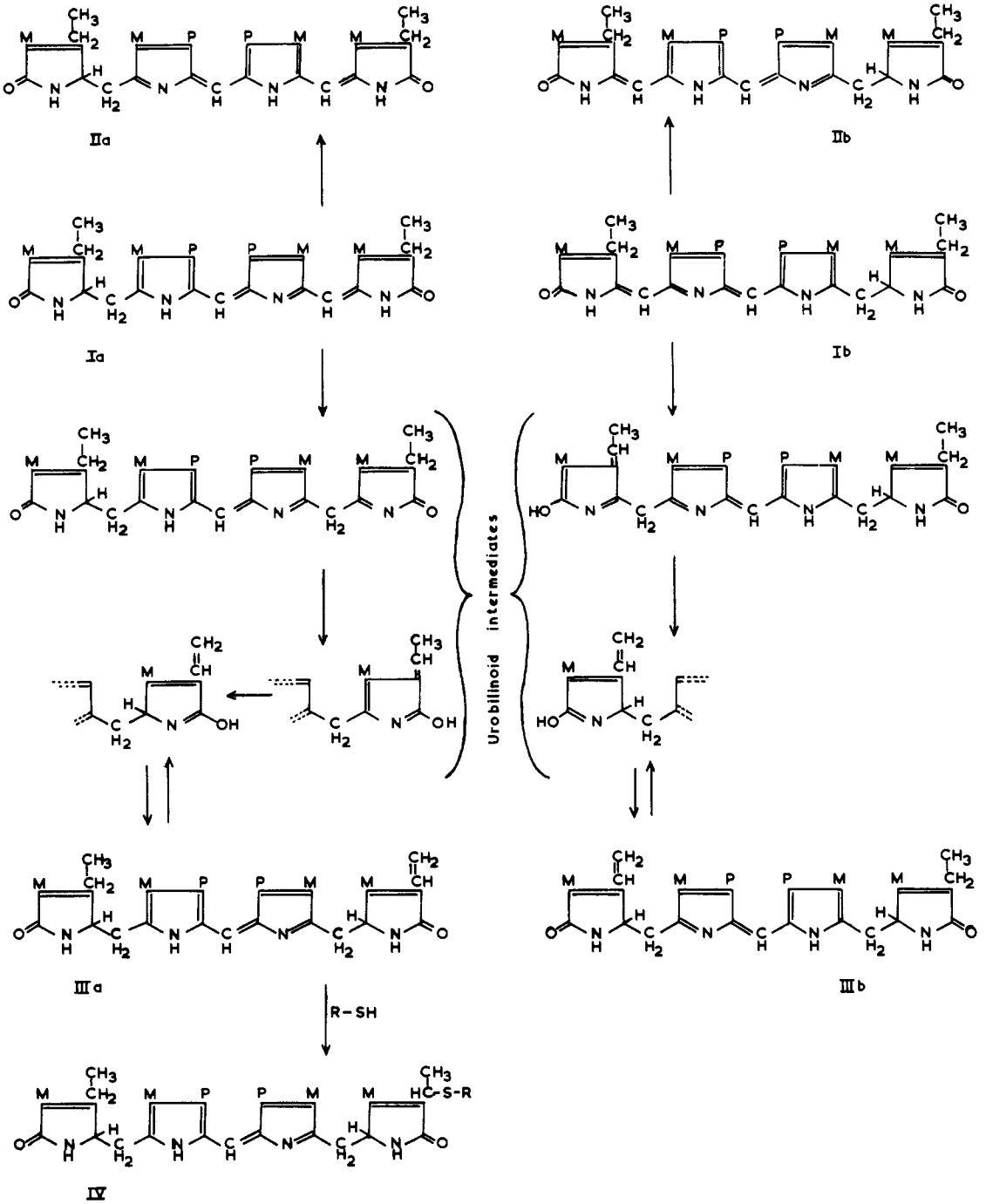


SCHEME I.—M, —CH_3; P, —CH_2—CH_2—COOH.

pigments. The second method was that of Ó hEocha (1958). In a typical experiment dried phycoerythrin (700 mg) was suspended in 12 N HCl (70 ml) and the solution was kept for 30 minutes in the dark at room temperature. It was then poured into distilled water (300 ml) and centrifuged. The supernatant fluid was extracted with chloroform (50 ml × 5) and the pellet treated again with 12 N HCl, diluted, and centrifuged, and the supernatant fluid was extracted with chloroform. The colored chloroform solutions were combined, washed once with distilled water, dried by filtration through chloroform-moistened filter paper, and evaporated to dryness *in vacuo* at 30°. The residue was dissolved in ether and washed with water. The solution was concentrated to 2 ml by evaporation and left overnight at 0°. The ether was poured off leaving ice crystals which were washed with chilled ether. The combined ethereal solutions were evaporated to dryness and the residue was left overnight in a vacuum desiccator. Efforts to crystallize the phycoerythrobilin, as hydrochloride, proved unsuccessful.

Properties and Reactions of Phycoerythrobilin.—The absorption-spectral characteristics of phycoerythrobilin in chloroform are given in Figure 1 and Table I. Phycoerythrobilin had absorption maxima at 307 and 556 mμ in 1 N HCl, and a visible maximum at 507 mμ in 2,6-lutidine. In the latter solvent the pigment emitted orange fluorescence; (λ_{max} 623 mμ; Fig. 2). The absorption and fluorescence properties of phycoerythrobilin in lutidine solutions were quite stable. Chloroform solutions of the zinc complex salt of phycoerythrobilin had absorption maxima at 320, 540 (shoulder), and 583 mμ, and a fluorescence maximum at 592 mμ (Fig. 3). Spectrophotometric titration indicated that

TABLE I
REPORTED VISIBLE ABSORPTION MAXIMA (IN mμ) OF BILIRHODINOID AND RELATED PIGMENTS

	Pigment[a]	Source of Pigment	Solvent: Chloroform	Solvent: Acid Chloroform	Solvent: HCl (aq), Dilute	Absorption Maxima and Color of Fluorescence of Zinc Complex Salts	Reference
1	Phycoerythrobilin	Acid digestion of phycoerythrins at 15°	505	576	556	(540), 583 (orange)	Present investigation
2	Mesobilirhodin	Alkaline isomerization of *d*-urobilin	500	575	556	540, 581 (orange)	Present investigation
3	Mesobilirhodin	Alkaline isomerization of *d*-urobilin	505, (570–75)	575		(540), 577, (625) (yellow-orange)	D. C. Nicholson (personal commun., 1960)
4	Mesobilirhodin	Oxidation of mesobilirubinogen		(500), 575		(510), (540), 582 (orange)	Present investigation
5	Mesobilirhodin	Oxidation of mesobilirubinogen			560		Watson (1959)
6	*i*-Urobilin	Oxidation of mesobilirubinogen		499	494 (HCl-MeOH)	509.5 (green)	Gray *et al.* (1961b) Lemberg and Legge (1949)
7	Mesobiliviolin	Oxidation of mesobilirubinogen	565	602.5		582.5, 632.5 (red)	Gray *et al.* (1961b)
8	*"Mesobilirhodin"*	Synthesis	(486), 575	497, (557), 605		510, 630 (greenish-brown)	Siedel (1935)
9	Mesobilierythrin (*"Mesobilirhodin"*)	Oxidation of mesobilirubinogen			495	509, (630) (greenish-yellow)	Lemberg and Bader (1933)
10	*"Phycoerythrobilin*	Acid digestion of R-phycoerythrin at 80°			495, (560)	509, (630) (greenish-yellow)	Lemberg and Bader (1933)

[a] See introduction for nomenclature conventions employed.

phycoerythrobilin is monobasic, its conjugate acid having *pK* about 6.4 (Fig. 4).

When dehydrogenated for 40 minutes by the method used by Gray *et al.* (1961a) for the formation of mesobiliverdin (glaucobilin) from mesobilirubinogen, phycoerythrobilin yielded a green pigment which was spectrally identical with mesobiliverdin.

Isomerization of Phycoerythrobilin in Concentrated Hydrochloric Acid.—Phycoerythrobilin in concentrated hydrochloric acid (λ_{max} 560 mμ) was allowed to stand at 15° in an atmosphere of oxygen-free nitrogen or hydrogen. The absorbancy at 560 mμ gradually decreased and a new peak appeared at 498 mμ. The absorbancy at 498 mμ increased over a period of about 14 hours. The product of the reaction was extracted from the diluted reaction mixture with chloroform and its properties indicated that it was a urobilin. Its absorption spectrum (λ_{max} 499 mμ) is shown in Figure 5A; its green-fluorescing zinc complex had λ_{max} 510 mμ in chloroform.

Phycoerythrobilin was heated at 100° in concentrated hydrochloric acid for 15 minutes. The acid was diluted and the pigment was extracted into chloroform. The chloroform was evaporated off *in vacuo* and the residue was dissolved in ether. The ethereal solution was extracted successively with distilled water (fraction I) and 0.2 N hydrochloric acid (fraction II), and the resulting pigment fractions were reextracted into chloroform. Fraction II, as hydrochloride and zinc complex, was spectrally identical with the corresponding derivatives of mesobiliviolin (Gray *et al.*, 1961b; see Table I, line 7). The spectral properties of fraction I were identical with those of the urobilin described.

These reactions of phycoerythrobilin were catalyzed by concentrated acid; urobilin was not formed at a detectable rate in <1 N HCl at 15°. The rates of the 15° and 100° reactions were the same under both oxygen-free nitrogen and atmospheric conditions. Both the violin and urobilin gave a verdinoid pigment when dehydrogenated with ferric chloride for 45 minutes following the method of Gray *et al.* (1961a). This showed that the carbon bridges of phycoerythrobilin were not oxidized to carbonyl groups during the formation of the violin and urobilin. These findings suggest prototropic isomerization as the mechanism of the reactions observed. Such mechanisms have been postulated to explain the conversion of *d*-urobilin to biliviolinoid pigments, and of bilirubin to dihydrobiliverdin under alkaline conditions at elevated temperatures (Gray and Nicholson, 1958; Gray *et al.*, 1961a).

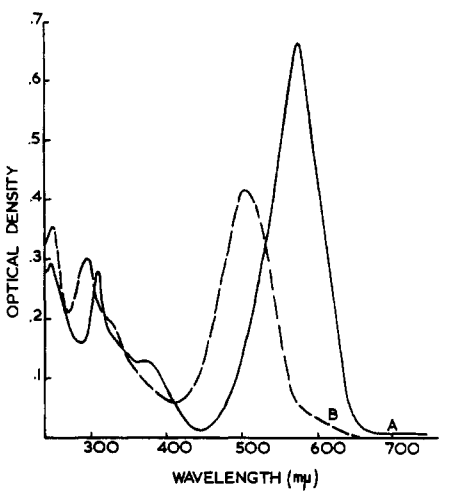


FIG. 1.—Absorption spectra of equal concentrations of phycoerythrobilin in chloroform. A, as hydrochloride; B, as free base.

Extended Hydrolysis of Phycoerythrins.—B-Phyco-

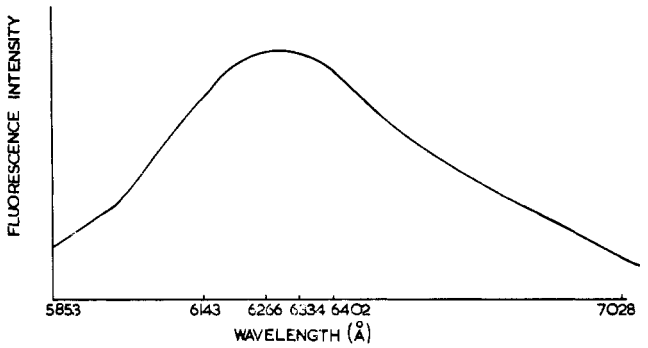


FIG. 2.—Fluorescence spectrum of phycoerythrobilin in 2,6-lutidine. Excitation by mercury high-pressure lamp emitting at 460–547 mμ (peaks at 487 and 546 mμ). Excitation filters: Interference filters 480 and 508 and Corning No. 5030; secondary filter: Corning No. 3486.

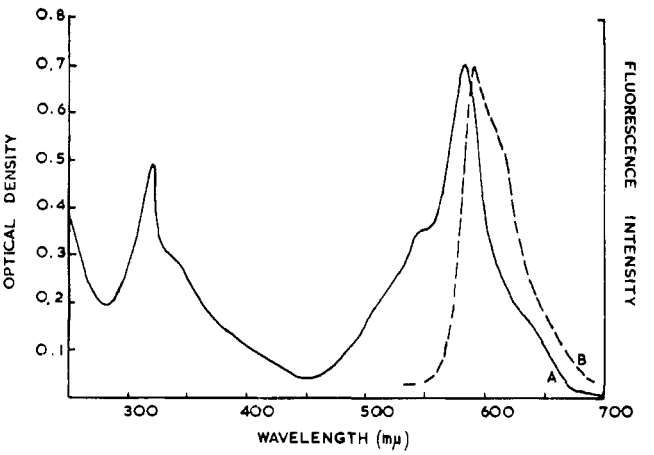


FIG. 3.—Zinc complex salt of phycoerythrobilin, in chloroform. A, absorption spectrum; B, fluorescence spectrum (excitation wavelength, 540 mμ).

erythrin in concentrated hydrochloric acid (λ_{max} 560 mμ) was allowed to stand at room temperature for 4 hours. Its absorption spectrum now showed a sharp decrease in absorbancy at 560 mμ and had a maximum at 498 mμ. No pigment was extracted when the diluted acid solution was shaken with chloroform as described in the preceding section. When the diluted 4-hour hydrolysate was made 5% with respect to trichloroacetic acid, the pigment present was precipitated, indicating its chromopeptide nature. Chromatographic analysis of the acid hydrolysate of this material (5.7 HCl, 105°, 24 hours) revealed the presence of amino acids, confirming this conclusion. The chromopeptide preparation had urobilinoid spectral characteristics both as hydrochloride and zinc-complex salt, and on dehydrogenation with ferric chloride it yielded chromopeptides having violinoid and verdinoid spectral characteristics. The original urobilinoid chromopeptide material was extracted from 2 N HCl by amyl alcohol and on esterification in ethanol-HCl it became soluble in chloroform. The properties of the product resembled those of the "*phycoerythrobilin methyl ester*" of Lemberg and Bader (1933).

Reaction of the Urobilin Isomeride of Phycoerythrobilin with Thiol Compounds.—Phycoerythrobilin (about 0.2 mg) was dissolved in 10 N HCl (3 ml). Crystalline bovine serum albumin (Mann Research Laboratories, New York) (30 mg) was added and the mixture was allowed to stand at 15° for 4 hours, when it was diluted with distilled water (12 ml) and centrifuged. No pigment could be extracted into chloroform from either the weakly colored supernatant fluid or the salmon-pink pellet. The pellet was redissolved in distilled water (5 ml) forming a brownish-red solution. The pigment, which was nondialyzable, was reprecipitated by 30% ammonium sulfate. It had absorption maxima at 278 and 498 mμ in sodium phosphate buffer, *p*H 6.5. This indicated that all the urobilinoid isomerization product of phycoerythrobilin had become attached to bovine serum albumin. In similar experiments neither *i*-urobilin nor *d*-urobilin became attached to bovine serum albumin; these pigments were soluble in chloroform at the end of the experiment.

Phycoerythrobilin was also added to 10 mM solutions of the following amino acids in 10 N HCl and the solutions were allowed to stand for 4 hours at 15°: alanine, leucine, serine, threonine, aspartic acid, lysine, arginine, phenylalanine, and methionine. The rate of isomerization of phycoerythrobilin to urobilin was not altered in the presence of any of these amino acids and, following dilution, the pigments were fully extractable into chloroform, indicating that none of the amino acids had reacted with the urobilin. However, when phycoerythrobilin was added to a 10 mM solution of cysteine in 10 N HCl, its isomerization was accelerated and the resulting pigment (λ_{max} 498 mμ in 10 N HCl; 511 mμ as a green-fluorescing zinc complex in 8 M urea) was not extracted into chloroform from 2 N HCl. This pigment, which was soluble in amyl alcohol, gave a verdinoid pigment on dehydrogenation with ferric chloride and resembled the urobilin-peptide fragments obtainable from B-phycoerythrin on extended hydrolysis (*vide supra*). It is concluded that the urobilin reacted with the thiol group of cysteine, forming a thioether linkage, and that a similar reaction occurred in the presence of bovine serum albumin and of the protein component of phycoerythrins. *d*-Urobilin failed to react with cysteine.

When the isomerization of phycoerythrobilin in 10 N HCl at 15° was carried out in the presence of 1% ethanethiol (ethyl mercaptan), the isomerization was accelerated and proceeded to completion, as happened also in the presence of cysteine or protein. However, the resulting urobilinoid pigment was completely extracted by chloroform from the diluted reaction mixture (λ_{max} 499 mμ in acid chloroform; 510 mμ as green-fluorescing zinc complex in chloroform).

Hydrolysis of Phycoerythrin in the Presence of Ethanethiol (Ethyl Mercaptan).—The purpose of this experiment was to form an ethyl thioether of the urobilin formed by acid isomerization of phycoerythrobilin during its hydrolysis from the protein. Ethanethiol, present in excess, would be expected to compete with –SH groups on the protein for the urobilin, yielding a product which could be extracted from the hydrolysate with chloroform.

HCl (10 N; 10 ml) was shaken with ethanethiol (1 ml) and allowed to equilibrate at 25°. B-Phycoerythrin (20 mg) was added and the solution was allowed to stand at 25° for 10 hours. The hydrolysate was added to distilled water (40 ml) and the pigments were extracted into chloroform. This pigment was identical in properties with that formed on isomerization of phycoerythrobilin in the presence of ethanethiol (*vide supra*).

The rate of release of pigment from B-phycoerythrin was determined under the above conditions. (The isomerization of phycoerythrobilin and the reaction of the product with ethanethiol were assumed to be independent of the hydrolysis and not to affect the rate of release of pigment.) Aliquots (1 ml) of the hydrolysate were withdrawn at intervals over a 20-hour period. Each aliquot was added to distilled water (5 ml) and extracted with chloroform (1.5 ml × 3). The chloro-

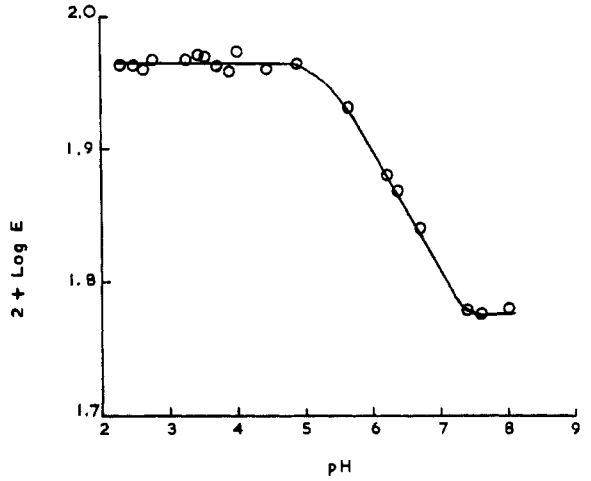


FIG. 4.—Spectrophotometric titration curve of phycoerythrobilin in McIlvaine buffers.

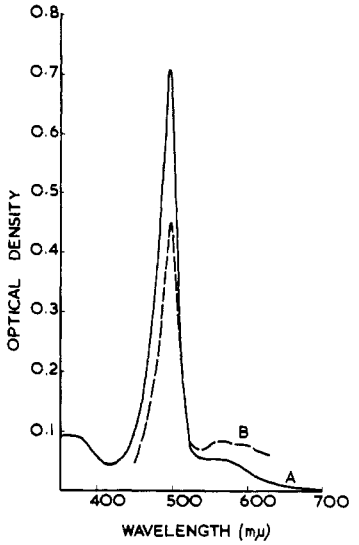


FIG. 5.—Absorption spectrum in chloroform of, A, the urobilin isomeride of phycoerythrobilin and, B, the urobilin isomeride of the mesobilirhodin from *d*-urobilin.

form extracts were diluted to 5 ml and the absorbancy (A) was determined at 499 mμ. During the first 45 minutes of hydrolysis, some of the extracted pigment was unisomerized phycoerythrobilin, and the absorbancies at 525 and 576 mμ were also determined for these samples. From a study of the absorbancies of pure solutions of phycoerythrobilin and the urobilin ethyl thioether, the following equation was applied to the extracts obtained during the first 45 minutes:

$$A_{499} \text{ (calcd) of phycoerythrobilin as its urobilin isomeride} = 1.2\,(A_{576} - A_{525})$$

The value obtained was added to A_{499} in the case of these aliquots.

The A_{499} values in the complete series of experiments were plotted against time (Fig. 6). The yield of pigment reached a maximum value in 6–7 hours. At this stage the hydrolysate contained only urobilinoid pigment, about 75% of which was soluble in chloroform. This yield was not increased on prolongation of the hydrolysis. It is calculated from Figure 6 that, of the phycobilin which became chloroform-soluble after hydrolysis with 10 N HCl at 25°, 50% was released in about 40 minutes.

Determination of Free ε-Amino Groups of Lysine in Phycoerythrins.—R- and B-phycoerythrins were dinitrophenylated and their DNP derivatives (5 mg) hydrolyzed for 24 hours following the method of Sanger (1945). DNP methionine from the N-terminal end of the proteins (Ó Carra and Ó hEocha, 1962), and its breakdown products, were extracted into ether. In each case the aqueous layer was evaporated to dryness *in vacuo* over sodium hydroxide flakes, and the residue was taken up in distilled water (1 ml). This solution (0.05 ml) was subjected to two-dimensional paper chromatography (Levy and Chung, 1953). The only colored spot observed corresponded in its chromatographic behavior with authentic DNP-lysine. When the chromatogram was treated with the ninhydrin reagent, the presence of sixteen free amino acids was revealed, but lysine was not among them. This indicates that no detectable proportion of the lysine ε-amino groups in R-phycoerythrin are involved in covalent linkages. (When untreated R-phycoerythrin was hydrolyzed and the hydrolysate was similarly characterized, lysine was detected.)

The Preparation of Bilirhodinoid Pigments.—(A) ISOMERIZATION OF *d*-UROBILIN.—The method of Gray and Nicholson (1958; and personal communication) was followed. To crystalline *d*-urobilin (2.5 mg) in methanol (2.5 ml) was added 2 N sodium hydroxide (0.5 ml), and the mixture was refluxed at 100° for 15 minutes in an atmosphere of hydrogen. A color change from yellow to red was noted. The cooled solution was diluted with water (15 ml) and treated with saturated sodium acetate solution (1.25 ml) and glacial acetic acid (2.5 ml). It was then extracted with ether (100 ml) which was extracted in turn with water (20 ml × 8), and the aqueous solution was then extracted with small quantities of chloroform. The mesobilirhodin hydrochloride, which was formed on shaking the chloroform solutions with 3 N HCl, displayed an absorption maximum at 575 mμ, and in this and other spectral properties it resembled phycoerythrobilin (Table I, lines 1 and 2). The close relationship between these two pigments was further demonstrated by studying the effect of concentrated hydrochloric acid on the mesobilirhodin from *d*-urobilin. Its solution in 10 N HCl was placed in the dark at 15° for 24 hours and, following dilution with three volumes of distilled water, the acid solution was extracted with chloroform. The absorption spectra of the resulting pigment (λ_{max} 499 in chloroform; Fig. 5,B) and of its green-fluorescing zinc salt (λ_{max} 510 mμ in chloroform) corresponded with those of a urobilin. A similar product was obtained when the mesobilirhodin in 10 N HCl was heated for 15 minutes at 100° in an atmosphere of nitrogen or of hydrogen.

(B) DEHYDROGENATION OF MESOBILIRUBINOGEN.—A number of attempts were made to prepare a mesobilirhodin by oxidation of mesobilirubinogen (Siedel, 1935). A violinoid pigment was the main product on each occasion. Only once did we obtain (by chromatography) a pigment having properties similar to phycoerythrobilin and this pigment was undoubtedly contaminated with some urobilin (see Table I, line 4).

DISCUSSION

Phycoerythrobilin differs considerably in spectral properties from native phycoerythrins. However, the close spectral correspondence of phycoerythrobilin and its derivatives with those of *denatured* phycoerythrins in the ultraviolet and at wavelengths >530 mμ (Table II) indicates that the native prosthetic group has the same structure as the isolated pigment. In all *native* phycoerythrins, the spectrum of phycoerythrobilin is altered by noncovalent interactions with the proteins (Ó hEocha and Ó Carra, 1961). C-Phycoerythrin

TABLE II
ABSORPTION MAXIMA (Mμ) OF DENATURED PHYCOERYTHRINS AND THEIR PHYCOBILINS

	λ_{max} in Aqueous Acid[a]				λ_{max} of Zinc Salt[b]			
Phycoerythrobilin	307	(375)		556	320		(540)	583
Phycourobilin			495			509		
C-Phycoerythrin	307	(375)		556	320		(542)	586
R-Phycoerythrin	307	(370)	498	556	320	512	(542)	586

[a] 1 N HCl (phycobilins) and *p*H 1 (phycoerythrins). [b] In $CHCl_3$ (phycobilins) and 8 M urea plus 50 mM zinc acetate (phycoerythrins).

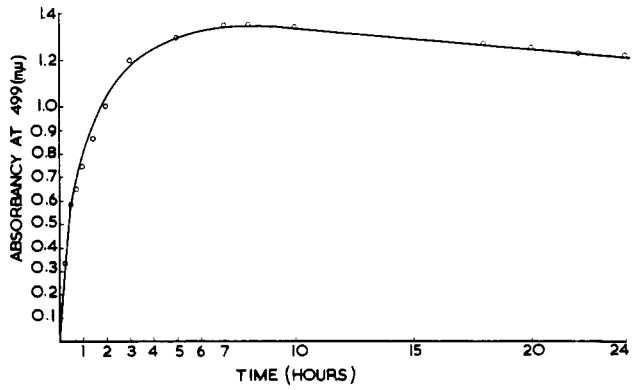


FIG. 6.—The rate of release of phycobilin from B-phycoerythrin at 25° in 10 N HCl saturated with ethanethiol. Points represent the corrected absorbancies of chloroform extracts from the diluted hydrolysate (see text).

contains phycoerythrobilin as its only colored prosthetic group, but R-phycoerythrin contains, in addition, a urobilinoid pigment (phycourobilin) which accounts for the maxima in the region 490–520 mμ (Table II).

The general properties of phycoerythrobilin indicate that it is a bile pigment. Its absorption spectra (Fig. 1) lack a Soret band (typical of porphyrins) and it can be readily converted to typical bile pigments, e.g., a bilene (urobilin) on isomerization and a bilitriene (verdinoid bile pigment) on dehydrogenation. This latter reaction confirms that none of the carbon bridges which join its pyrrole rings is a carbonyl group.

The long-wavelength-absorption maxima of phycoerythrobilin and its derivatives (Table I, line 1) are intermediate in position between those of the corresponding derivatives of the bilitrienes, whose chromophores contain four pyrrole rings in conjugation, and of the bilenes, (e.g. urobilins) which contain only two rings in conjugation. This classifies phycoerythrobilin with the violinoid bile pigments as a bilidiene containing three rings in its conjugated system. However, the wavelength of the absorption maxima of phycoerythrobilin and its derivatives is 30–50 mμ shorter than the wavelengths of the maxima of mesobiliviolin and its corresponding derivatives (Table I, line 7). This suggests that the conjugated system of the violins is more extensive than that of phycoerythrobilin. Confirmation for this comes from spectrophotometric titration of phycoerythrobilin (Fig. 4), which shows that the pigment contains one pyrrolenine nitrogen (−N =) with a *pK* value of about 6.4, which falls between mesobiliviolin (*pK* 4.0) and the urobilins (*pK* 7.2–7.6) (Gray *et al.*, 1961b). According to those authors, the basicity of a bile pigment pyrrolenine nitrogen is inversely related to the extent of the conjugated double-bond system in which it is located.

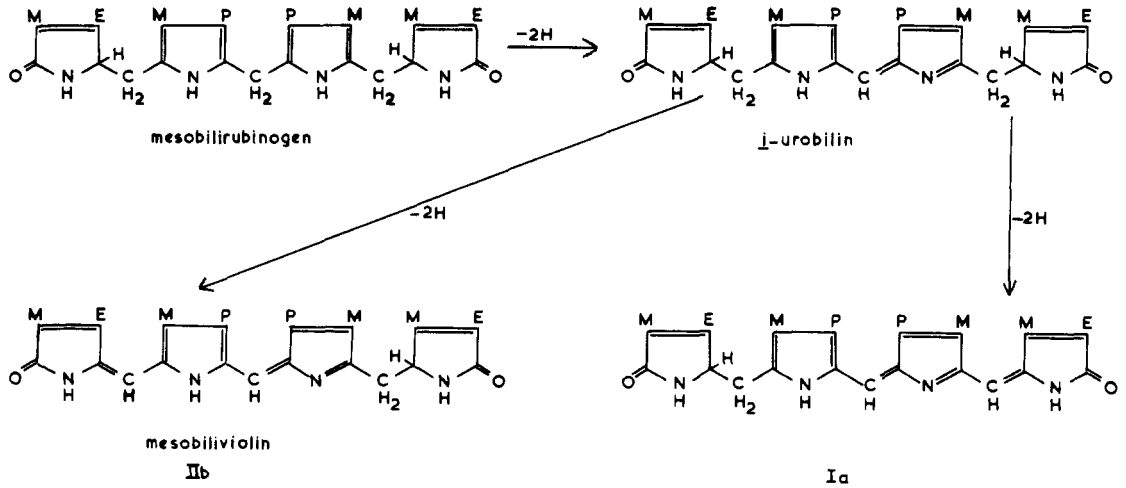
Phycoerythrobilin yields ethylmethylmaleimide on oxidative degradation and this indicates that it is a meso bile pigment (having two ethyl side chains) (W. J. Cole and C. Ó hEocha, unpublished). It is thought to have a IXα order of side chains (Lemberg and Bader, 1933).

Phycoerythrobilin in concentrated hydrochloric acid solution at 15° isomerizes to a urobilin, while at 100° in the same solvent it forms, in addition, an isomeride seemingly identical with mesobiliviolin. Structure Ia and its side-chain isomer Ib (Scheme I) appear to be the only bilidiene IXα structures, containing three rings in conjugation, which are isomeric with mesobiliviolin (IIa and IIb).[1] The properties and reactions of phycoerythrobilin can be satisfactorily interpreted on the basis of either structure Ia or Ib. The chromophore of these structures differs from that of mesobiliviolin (IIa and IIb) in the relative arrangement of the ring types (pyrrole and pyrrolenine). The chromophores of all four structures contain eight double bonds; but while all those in the mesobiliviolin chromophore are linearly conjugated, that in the β,β position of the pyrrolenine rings of Ia and Ib is cross-conjugated. This is consistent with the apparently less-extensive chromophore of phycoerythrobilin as compared with mesobiliviolin.

On the basis of structure Ia, prototropic isomerizations of phycoerythrobilin to a mesobiliviolin (IIa) and a monovinyl urobilin (IIIa) are envisaged as shown in Scheme I. The isomerisation of Ib to a mesobiliviolin (IIb) and a monovinyl urobilin (IIIb) is similar but in the case of urobilin formation not identical, owing to the different position, relative to the chromophore, of the ethyl group involved.

The mesobilirhodin formed on isomerization of *d*-urobilin in methanolic sodium hydroxide (Gray and Nicholson, 1958) closely resembles phycoerythrobilin in spectral properties, but is not identical with it (Table I, lines 1 and 2). It isomerizes to a urobilin in concentrated hydrochloric acid at 15°, but does so more rapidly than phycoerythrobilin. In concentrated hydrochloric acid at 100°, mesobilirhodin and phycoerythrobilin yield different ratios of urobilin and violin. The mesobilirhodin may be the side-chain isomer of phycoerythrobilin, i.e., Ia or Ib, depending on which of these is phycoerythrobilin. The formation of mesobilirhodin from *d*-urobilin can be represented as a reversal of one of the isomerizations Ia → IIIa or Ib → IIIb. This reaction of *d*-urobilin lends support to the assignment of either Ia or Ib to mesobilirhodin, as the structure of *d*-urobilin is most likely to be IIIa, IIIb, or a structure similar to one of the intermediate urobilins shown in Scheme I (Siedel, 1956; Gray and Nicholson, 1958). Gray and Nicholson (1958) have

[1] Structures IIa and IIb are side-chain isomers within the IXα series differing only in the relative positions of the ethyl and methyl side chains on the terminal rings. Most workers have assumed structure IIb for their preparations of mesobiliviolin IXα, whereas that obtained, for example, by dehydrogenation of mesobilirubinogen (the commonest method of preparation) must yield a mixture of the isomers IIa and IIb (see Lemberg and Legge, 1949, pp. 125–26). Throughout this paper IIa and IIb are both referred to as mesobiliviolin; where necessary the isomers are distinguished by reference to the numbered structures.



SCHEME II.—After Siedel and Möller, 1940. M, $-CH_3$; E, $-CH_2-CH_3$; P, $-CH_2-CH_2-COOH$.

formulated a somewhat similar scheme to account for the formation of mesobilirhodin from *d*-urobilin.

In aqueous acid, the urobilin isomeride of phycoerythrobilin attaches to bovine serum albumin and to the apoproteins of phycoerythrins. The artifact linkage formed between the proteins and the urobilin is of a refractory covalent nature, since further hydrolysis of the protein-urobilin products in concentrated HCl yields urobilin-peptides rather than free urobilin. Of the types of functional groups present in bovine serum albumin and phycoerythrins, only the thiol group of cysteine reacts with the urobilin isomeride of phycoerythrobilin. Ethanethiol also reacts with the urobilin and, unlike the other adducts, the product is hydrophobic. Since phycoerythrobilin itself does not react with these thiol compounds, it seems that the reactive group on the pigment arises during the isomerization; the attachment probably takes place by the addition of a thiol group across the vinyl side chain of the urobilin isomeride (e.g., IIIa), with formation of a thioether bond (IV).

The vinyl side chains of protoporphyrin react with the thiol groups of cysteine and of bovine serum albumin, forming thioethers (Sano and Granick, 1961; Popper and Tuppy, 1963). The addition occurs much less readily with protoporphyrin than protoporphyrinogen, in which the vinyl groups are confined to single pyrrole rings—as is also true of the vinyl group of IIIa. Our failure to react *d*-urobilin with thiol groups indicates that it may be represented by structures similar to those of the intermediate urobilinoids in Scheme I (see also Siedel, 1956).

The second type of native prosthetic group in R-phycoerythrin is urobilinoid (Ó hEocha and Ó Carra, 1961) and is not released by acid hydrolysis. Preliminary results (C. Ó hEocha, P. Ó Carra, and T. Brennan, unpublished) indicate that this phycourobilin (see Table II) is similar in structure to IV (R = phycoerythrin apoprotein).

In view of the experimental conditions necessary for their cleavage (>10 N HCl at room temperature), the covalent bonds which joint phycoerythrobilin to the protein might be expected to involve carboxyl groups of phycoerythrobilin and either amino groups (Lemberg, 1930) or hydroxyl groups of the protein. A peptide linkage between phycoerythrobilin and protein is unlikely in view of the finding that the NH_2-terminal groups (Ó Carra and Ó hEocha, 1962) and apparently all the ϵ-NH_2 groups of the lysine residues of phycoerythrins are free to react with fluorodinitrobenzene. Hydrolysis of B-phycoerythrin in the presence of ethanethiol indicates that in 10 N HCl at 25°, 50% of the native covalent bonds between phycoerythrobilin and protein are hydrolyzed in 40 minutes (Fig. 6). This rate seems too rapid for a peptide bond (see Desnuelle and Casal, 1948). However, no positive evidence was obtained as to the nature of the bonding involved. Sodium methoxide (0.5 M), which cleaves ester bonds of lactoperoxidase at room temperature (Hultquist and Morrison, 1963), did not release any phycobilin from R-phycoerythrin.

The Terms Phycoerythrobilin and Mesobilirhodin.—There is much confusion in the literature concerning the terms mesobilirhodin and phycoerythrobilin (see introductory paragraphs for nomenclature conventions employed). The earlier work in this field is reviewed by Lemberg and Legge (1949) and Rabinowitch (1956).

Structure Ia has previously been associated with "*phycoerythrobilin*" and mesobilierythrin of Lemberg and Bader (1933) and "*mesobilirhodin*" of Siedel (1935). The last names are synonyms for a pigment preparation obtained, in addition to mesobiliviolin, on dehydrogenation of mesobilirubinogen with ferric chloride. All these pigment preparations had similar spectral properties (e.g., λ_{max} at 495 mμ as hydrochloride), but they differ from those of the phycoerythrobilin and the mesobilirhodin described and discussed previously (see Table I). Examination of the spectral properties reported for the earlier preparations indicates that they consisted of urobilinoid pigments with variable, and generally slight, admixture of mesobiliviolin (compare with the properties of authentic *i*-urobilin and mesobiliviolin, Table I, lines 6 and 7). This conclusion is in agreement with the original observation of Lemberg and Bader (1933), which we have confirmed, that "*phycoerythrobilin*" and mesobilierythrin were converted to mesobiliviolin on dehydrogenation with ferric chloride. This reaction would be expected from urobilins but not from a compound with structure Ia, which is isomeric with mesobiliviolin and should yield mesobiliverdin directly on dehydrogenation.

Under the conditions used by Lemberg and Bader (1933) for preparation of "*phycoerythrobilin*" (digestion of phycoerythrin in concentrated HCl at 80°) we have found that phycoerythrobilin is converted to a urobilinoid pigment which becomes reattached to protein fragments through artifact bonds, yielding urobilinoid products of the general structure IV (in which R represents a peptide chain). Such products resemble the "*phycoerythrobilin*" preparation of Lemberg and Bader (1933), which probably contained a peptide component (Lemberg and Legge, 1949).

The formation of a mixture of mesobiliviolin and a

pigment of structure Ia might be expected on dehydrogenation of mesobilirubinogen, as proposed by Siedel (1935) (see Scheme II). However, our findings indicate that, if formed, Ia would be isomerized to mesobiliviolin and/or urobilinoid pigment under the experimental conditions employed, i.e., ferric chloride in acid at elevated temperatures. Similar considerations apply to Siedel's (1935) attempted synthesis of Ia, since concentrated acid at elevated temperature was used. On one occasion, after complex fractionation of the products of ferric chloride dehydrogenation of mesobilirubinogen, we succeeded in isolating a trace of pigment closely resembling mesobilirhodin and phycoerythrobilin (Table I, line 4). (It clearly remained contaminated with some urobilin, which accounts for the absorption in the 500–510 mμ region.) Watson *et al.* (1960) and C. H. Gray and D. C. Nicholson (personal communication) have also noticed only traces of this bilirhodinoid pigment among the products of this dehydrogenation. The chief products of the reaction were mesobiliviolin and urobilinoid pigments, which separated into a number of mutually contaminated fractions when chromatographed on talc (the method used by Siedel, 1935). Since the spectral properties of Siedel's (1935) preparation resemble those of a mixture of mesobiliviolin and urobilin, the conclusion that his preparation was such a mixture, and not a compound of structure Ia, seems warranted.

We consider therefore that the original association of structure Ia with "*mesobilirhodin*" of Siedel and Möller (1940) and "*phycoerythrobilin*" of Lemberg and Bader (1933) arose through a series of misinterpretations, and that both of these pigment preparations were predominantly urobilinoid in character.

The term phycoerythrobilin is preferably confined to the native red prosthetic group of phycoerythrins, as originally suggested by Ó hEocha (1958). Structures Ia and Ib are consistent with the properties of this pigment and of the red isomer of *d*-urobilin, and we suggest that the term mesobilirhodin be confined to the latter compound or adapted as a general term for pigments having similar properties.

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