*Biochem. J.* (1980) **187**, 311–320
*Printed in Great Britain*



# Structures and Apoprotein Linkages of Phycoerythrobilin and Phycocyanobilin

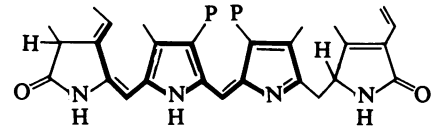
S. Derek KILLILEA,* Padraig O'CARRA† and Richard F. MURPHY‡
*Department of Biochemistry, University College, Galway, Ireland*

(*Received 3 September 1979*)

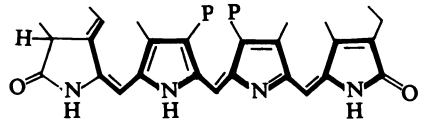
Phycoerythrobilin and phycocyanobilin are covalently attached to the apoproteins of phycoerythrins and phycocyanins. One linkage consists of an ester bond between the hydroxy group of a serine residue and the propionate side chain on one of the inner pyrrole rings (probably ring C). The other linkage is a labile thioether bond between a cysteine residue and the two-carbon side chain on pyrrole ring A. This side chain and both of the $\alpha$-positions of the ring A are in the reduced state. This constitutes an important structural revision, since, in the structures currently accepted for the phycobilins, the two-carbon side chain on ring A is depicted as an ethylidene grouping and this has been regarded not only as a very characteristic feature of the phycobilins, but also as a probable structural feature of the chromophore of phytochrome, largely on the basis of other analogies with the phycobilins. The ethylidene-containing structures apply instead to artefact forms of the pigments released from the apoproteins by treatment with hot methanol. Cleavage of the ring-A linkage involves an elimination reaction releasing the cysteine residue and generating a double bond in the ring-A side chain. During cleavage in methanol the direction of the elimination is towards the ring, generating the ethylidene double bond. Since this is linked to the conjugated system, the methanol-released pigments differ spectrally from the native phycobilins. During acid-catalysed release of the pigments, the elimination apparently goes in the opposite direction, generating a double bond at the outer position of the side chain. Since this double bond is not linked to the conjugated system, the acid-released pigments remain spectrally identical with their protein-bound counterparts.

Phycoerythrobilin and phycocyanobilin, collectively known as phycobilins, are the covalently-bound coloured prosthetic groups of the phycoerythrins and phycocyanins (O'hEocha, 1965, 1966). The structures generally accepted for these phycobilin chromophores are those depicted in Fig. 1: structure I for phycoerythrobilin and structure II for phycocyanobilin (Chapman *et al.*, 1967; Cole *et al.*, 1967; Crespi *et al.*, 1968; Crespi & Katz, 1969; Rüdiger & O'Carra, 1969; Siegelman *et al.*, 1968).

However, these structures apply not to the native phycobilins, but to artefact pigments derived therefrom. As explained in the preceding paper (O'Carra *et al.*, 1980), these artefact pigments are released from the apoproteins by refluxing in hot methanol and were originally referred to simply as 'purple



(I)



P = $-CH_2CH_2CO_2H$

(II)

Fig. 1. *Structures of 'purple pigment' (I) and 'blue pigment' (II)*
Chromophore conjugated systems are emphasized by heavy lines.

* Present address: Department of Biochemistry, North Dakota State University, Fargo, ND 58102, U.S.A.
† To whom reprint requests should be addressed.
‡ Present address: Department of Biochemistry, Queen's University, Belfast, Northern Ireland.



0306-3275/80/050311-10$01.50/1 

pigment' derived from phycoerythrobilin and 'blue pigment' derived from phycocyanobilin. This terminology will be followed here in order to maintain the distinction between these artefacts and the native phycobilins. These methanol-released pigments differ spectrally from the protein-bound phycobilins, whereas pigments released by careful acid treatment of the chromoproteins retain the spectral characteristics of the native pigments [O'Carra & O'hEocha, 1966; the preceding paper (O'Carra *et al.*, 1980)]. Most workers have ignored this distinction, however, and have regarded the methanol-released 'purple pigment' and 'blue pigment' as identical with phycoerythrobilin and phycocyanobilin respectively (Chapman *et al.*, 1967; Cole *et al.*, 1967; Crespi *et al.*, 1968; Crespi & Katz, 1969; Siegelman *et al.*, 1968; Frackowiak & Skowron, 1978; Gossauer & Weller, 1978; Gossauer & Hinze, 1978). The emphasis on the methanol-released artefacts in most previous structural studies must be attributed to the much greater stability of these preparations compared with the acid-released phycobilins.

We attempt to rectify this situation by presenting structural studies on the protein-bound phycobilins and their acid-released counterparts. Owing to the instability of the latter, we were unable to use many of the techniques and procedures that were available for study of the more stable methanol-released derivatives. Thus efforts to crystallize the acid-released phycobilins have been unsuccessful, owing to accumulation of artefact derivatives during purification and particularly during attempts at crystallization. Similar problems have rendered n.m.r. and mass-spectral data impossible to analyse with any confidence. In coming to our conclusions regarding the structures, we have therefore been forced to rely on less direct evidence, based mainly on observations of interconversions or partial similarities with bilins of known structure, together with studies on the mode of linkage of the pigments to the apoproteins.

## Experimental

### *Biliprotein preparations*

R-phycoerythrin (from the red alga *Rhodymenia palmata*), C-phycoerythrin (from the red alga *Phormidium persicinum*) and C-phycocyanin (from the blue–green alga *Nostoc punctiforme*) were isolated and purified as described by O'Carra (1965). Reduction and *S*-aminoethylation of the biliproteins was carried out as described by Raftery & Cole (1966).

### *Bilin preparations*

Acid-released phycoerythrobilin and methanol-released 'purple pigment' were prepared from R-phycoerythrin as described by O'Carra *et al.* (1964) and O'Carra & O'hEocha (1966) respectively. Bilins (1 mg) were esterified (Murray, 1966) by standing them in 1 ml of $BF_3$ in methanol (14% v/v; Sigma, Poole, Dorset, U.K.) for 25 min at 4°C. Ice-cold water (4 ml) was added and the esterified pigment was extracted into chloroform (3 × 2 ml). Non-esterified material was removed by extracting the chloroform extracts with $NaHCO_3$ solution (4%, w/v). Acetylation of acid-released phycoerythrobilin was attempted by standing this bilin (0.1–1 mg) (as the dimethyl ester) in pyridine/acetic anhydride (10:1, v/v at 4°C for 16 h (Falk, 1964) and then extracting the pigment into ether. Comparative t.l.c. of bilin dimethyl ester preparations was carried out on silica-gel G in previously described solvent systems (Rüdiger & O'Carra, 1969; Cole *et al.*, 1967).

### *Treatment with sodium methoxide*

Dry acetone-denatured R-phycoerythrin (10 mg) was suspended in dry methanol (10 ml). A solution (3.3 ml) of 0.2 M-sodium methoxide in methanol was added to the suspension. The cleavage reaction was allowed to proceed at room temperature for 50 min and was then terminated by the addition of 2 M-HCl (2 ml). The suspension was centrifuged and the supernatant was examined spectrally for released pigment. This procedure was found to release phycoerythrobilin (at the same time converting it into a violin). Since sodium methoxide is commonly used to cleave ester bonds and is claimed by Hultquist & Morrison (1963) to be relatively specific for ester bonds, this result seems to confirm the view that the linkage of phycoerythrobilin through ring C is an ester bond (Rüdiger & O'Carra, 1969). The linkage through ring A is more alkali-sensitive than that through ring C (Rüdiger & O'Carra, 1969), and so would not be expected to affect the kinetics of the release by sodium methoxide.

### *Treatment with hot methanol and with proteinases from Streptomyces griseus*

Digestion of biliproteins in hot methanol was carried out as described by O'Carra & O'hEocha (1966) and with mould proteinases as described below in the subsection devoted to the preparation of 'minimal chromopeptide'. The parallel release of the phycobilins and their conversion into 'purple pigment' and 'blue pigment' was monitored by taking samples at intervals during the incubations. Released and protein-bound pigments were separated by acidification of the samples to pH 1.0 followed by extraction with chloroform, into which released pigment passed quantitatively. The aqueous phase, containing the pigment remaining peptide-bound, was made up to 8 M-urea to keep this fraction in solution. Both the aqueous and chloroform phases

were adjusted to the same volume and subjected to careful spectral analysis to identify the nature of the pigment in each phase. For both methanol and proteinase treatment of biliproteins, the spectra indicated that the released pigment in the chloroform phase was entirely converted into the artefact pigment (this was confirmed by t.l.c. analysis; see O'Carra *et al.*, 1980), whereas that remaining peptide-bound in the aqueous phase retained the original phycobilin spectral characteristics, with no trace of any spectral irregularities attributable to the artefact derivatives. The partially digested material from the aqueous phase was also subjected to the chromic acid-degradation procedures of Rüdiger & O'Carra (1969) to identify the ring(s) remaining peptide-bound. The experiments showed that no partial release occurred; all the peptide-bound phycobilin units remained attached via both ring A and ring C. Thus conversion of the phycobilins into the artefact spectral forms and release from the apoproteins by these cleavage methods seem to be simultaneous events.

*Alkali treatment*

Rüdiger & O'Carra (1969) showed that treatment of R-phycoerythrin with hot alkali preferentially cleaves the ring-A–apoprotein linkage of phycoerythrobilin and converts the chromophore into violinoid and verdinoid pigments. A more detailed study of this cleavage has been carried out.

A solution of R-phycoerythrin (25mg) in 1M-KOH (10ml) was kept for up to 2h at 25°C. This procedure released small amounts of a violinoid pigment. Spectral examination of the residual peptide-bound pigment showed that much violinoid pigment in addition to phycoerythrobilin remained attached to the peptide chain. Samples of the reaction mixture were removed at intervals and pigment-bound peptide material was precipitated with trichloroacetic acid. Released pigment was removed by washing the precipitate with acetone. The residual chromopeptide was then subjected to the chromic acid-degradation procedures of Rüdiger & O'Carra (1969) to determine the ring(s) remaining peptide-bound. The results, which are discussed in detail elsewhere (Killilea, 1972), revealed that most of the phycoerythrobilin residues were attached through both ring A and ring C. However, a small amount of the phycoerythrobilin units and all of the violinoid pigment were attached only through ring C. From these results it was concluded that cleavage of the ring-A–apoprotein linkage of phycoerythrobilin must take place before the chromophore can be converted into the violinoid pigment. Thus alkaline-catalysed cleavage of the ring-A–apoprotein linkage probably proceeds by an elimination reaction, with the generation of a double bond in the two-carbon side chain, and this double bond then isomerizes into ring A to become part of the conjugated system, yielding the violin. These results, together with those described in the above subsection, therefore, strongly indicate that the ring-A–apoprotein linkage of the phycobilins involves the two-carbon side chain of this ring, since cleavage of the linkage is associated with the generation of a double bond in this two-carbon side chain.

*Preparation and purification of phycobilin-containing 'minimal chromopeptides'*

Phycobilin-containing 'minimal chromopeptides' were prepared from the biliproteins by digestion with proteolytic enzymes. However, the crude proteinase preparations known as Pronase and Nagarse have been reported to release the chromophores from the biliproteins (Murphy, 1968; Siegelman *et al.*, 1967). Therefore, in addition to proteinases from *Streptomyces griseus* (Pronase B from Calbiochem, Bishop's Stortford, Herts., U.K., and 'protease type IV' from Sigma), a range of other commercially available proteinase preparations were also tested for their ability to cleave the chromophore–apoprotein linkages. R-Phycoerythrin (25mg) that had been reduced and aminoethylated was incubated at 37°C for 24h under $N_2$ with proteinase preparation (5–25mg) in 0.1M-Tris/HCl buffer, pH7.5, except in the cases of pepsin and papain where 0.1M-KCl/HCl buffer, pH2.0, and 0.1M-sodium acetate buffer, pH4.5, were used respectively. The incubation mixtures were then acidified to pH1.0, and any released pigment was extracted into chloroform.

In addition to the proteinase preparations from *Streptomyces griseus*, the following preparations released the chromophore from R-phycoerythrin as 'purple pigment' with a variable proportion of urobilinoid isomerization product: pepsin (Sigma), crude cathepsin preparations from ox liver (extracts from an acetone-dried powder supplied by Sigma), snake-venom proteinase preparations from the cobra *Naja nivea* and the puff adder, *Bitis arientans* (Koch–Light, Colnbrook, Bucks., U.K.) and a fungal proteinase preparation from *Aspergillus oryzae* (Sigma). A yield of 10% free pigment from R-phycoerythrin but only about 1% from C-phycocyanin was obtained with the 'type IV protease' from *S. griseus* and lower yields were obtained with other proteinases. The mammalian pancreatic and intestinal proteinases and peptidases [either as pure or crude preparations (Sigma)], aminopeptidase M (Carl Roth, Karlsruhe, Germany) and papain (Sigma) were found to be free of chromophore-releasing activity.

In the preparation of 'minimal chromopeptides', the aim was to 'trim' away as much as possible of the polypeptide chain while not cleaving the native linkages of the chromophore to amino acid residues.

Clearly the proteinase preparations containing chromophore-releasing activity are unsuitable. Although in all cases the cleavage was slow compared with the proteinase activity, it is also possible that these preparations might catalyse undetected alterations in the chromophore–apoprotein linkages before the cleavage reaction and thus spurious results could be obtained. For preparing the 'trimmed' chromopeptides, therefore, only mammalian pancreatic and intestinal protease preparations were used.

Also, as discussed previously (O'Carra *et al.*, 1980), reduced and *S*-aminoethylated biliproteins were used in these digestions to prevent covalent modification of the chromophores with free thiol groups on the apoproteins. To prevent such covalent modification with any thiol groups on the digestive enzymes, 2-mercaptoethanol was added to the digests to compete with such thiol groups.

Phycoerythrobilin-containing and phycocyanobilin-containing 'minimal chromopeptides' were prepared with C-phycoerythrin and C-phycocyanin respectively by the following procedure. Reduced and *S*-aminoethylated biliprotein (200mg) was dissolved in 0.2M-Tris/HCl buffer (5ml), pH8.0, containing 8M-urea and then diluted with 0.2M-Tris/HCl buffer (15ml), pH8.0. Trypsin (10mg), chymotrypsin (20mg) and 2-mercaptoethanol (0.2ml) were added and digestion was carried out for 4h at 25°C under $N_2$. Extra chymotrypsin (20mg) was added, and digestion was continued for a further 20h. The digest was then acidified to pH2.0 and centrifuged. The supernatant was filtered through a column (3cm × 15cm) of talc (Dus *et al.*, 1962), which bound the chromopeptides. The column was washed with water (100ml) to remove colourless peptide material. The chromopeptides were then eluted off as a sharp band with ethanol/water/$NH_3$ (sp.gr. 0.88) (49:49:2, by vol.). The eluate was immediately acidified with a few drops of (50%, v/v) acetic acid and evaporated to dryness at 40°C under reduced pressure. The chromopeptide preparation was then dissolved in 0.1M-phosphate buffer (15ml), pH7.5. Carboxypeptidase A (2.5mg) and aminopeptidase M (2mg) were added, and digestion was carried out at 37°C under $N_2$ for 36h. Trichloroacetic acid (5% w/v) was added and insoluble material was removed by centrifugation. The phycobilin-containing 'minimal chromopeptides' in the supernatant were purified by adsorption chromatography on columns (2cm × 10cm) of talc. The columns were washed with equal volumes (50ml) of 10mM-, 1mM- and 0.1mM-HCl and finally with water to remove colourless peptides and amino acids (Dus *et al.*, 1962). The chromopeptides were eluted off as a sharp band with ethanol/water/$NH_3$ (sp.gr. 0.88) (49:49:2, by vol.) and after acidification were evaporated to dryness as described above. The chromopeptides were then subjected to gel filtration in the dark on columns (1.37cm × 40cm) of Sephadex G-50 equilibrated with phenol/acetic acid/water (1:1:1, by vol.; Synge & Youngson, 1961). The phycoerythrobilin-containing 'minimal chromopeptides' were eluted as a single symmetrical peak, whereas the phycocyanobilin-containing 'minimal chromopeptides' were partially resolved into two fractions, which were separately pooled for amino acid analysis. Extraction of the eluted chromopeptide fractions with peroxide-free ether removed most of the phenol and some of the acetic acid. Most of the chromopeptide material remained in the aqueous phase. The small amount that was extracted into the organic phase was salvaged by re-extraction of this phase with water. The aqueous phases were combined and evaporated to dryness. To test for the presence of non-chromopeptide material the 'minimal chromopeptide' preparations were subjected to descending paper chromotography on Whatman no. 1 paper in butanol/acetic acid/water (4:5:1, by vol.). In this solvent system the chromopeptides travelled near the solvent front ($R_F$ 0.8–0.9). No ninhydrin-positive material could be detected in any preparation of 'minimal chromopeptide'.

Quantitative amino acid analyses of the 'minimal chromopeptides' by ion-exchange chromatography (Moore *et al.*, 1958) was carried out on a Spinco amino acid analyser after hydrolysis at 110°C in 6M-HCl for 20h. Before hydrolysis the 'minimal chromopeptides' were subjected to performate oxidation by standing in 1ml of 88% (v/v) formic acid/30% (v/v) $H_2O_2$ (10:1, v/v) for 3h at 18°C. The performic and formic acids were removed by evaporation under a stream of $N_2$ at 60°C.

As Tables 2 and 3 show, all the performate-oxidized chromopeptides were found to contain cysteic acid. In a previous communication we reported qualitative amino acid analyses of phycoerythrins (O'Carra & Killilea, 1970) in which this amino acid was not detected. These analyses, however, were carried out on chromopeptide preparations that had not been subjected to performate oxidation before hydrolysis. The yield of cysteine under these conditions is low (Tristram & Smith, 1963).

*Preparation of haematinic acid-containing peptides from phycoerythrobilin-containing 'minimal chromopeptides'*

Purified phycoerythrobilin-containing 'minimal chromopeptide' material (from 100mg of R-phycoerythrin) was subjected to chromic acid degradation at 20°C essentially as described by Rüdiger & O'Carra (1969). Released imides were removed

from the peptide-bound imides in the aqueous phase by ether extraction. Sufficient solid ascorbic acid was added to reduce the excess dichromate and the pH was adjusted to 8.5 with 4M-NaOH. The imide-linked peptides were dinitrophenylated by the procedure of Matheson (1963). After the removal of excess dinitrophenol (Mills, 1952) the ether-soluble dinitrophenyl-peptide fraction was subjected to paper chromatography by the method of Blackburn & Lowther (1951). Eight dinitrophenyl derivatives, resolved as yellow bands, were eluted off the paper in 1% $NaHCO_3$ (10ml) and, after acidification, taken into ether. Each dinitrophenyl derivative was analysed for bound imide(s) by the method of Rüdiger & O'Carra (1969). Four of the derivatives were found to contain haematinic acid, and these were hydrolysed to their constituent amino acids in 6M-HCl at 110°C for 20h. The hydrolysates were dinitrophenylated (Matheson, 1963) and the component amino acids were analysed by t.l.c. as described by Brenner *et al.* (1961). Two of these haematinic acid-containing fractions were found to contain serine, glycine and alanine. The two other fractions contained amino acids additional to those listed above. These results provide further evidence that the ring-C–apoprotein linkage of phycoerythrobilin is to a serine residue. The other expected imide, methylethylidenesuccinimide, was not detected in any of the bands, and it was assumed that this imide was lost during the procedure owing to the instability of the linkage through this ring (Rüdiger & O'Carra, 1969).

## Results and Discussion

### *Phycoerythrobilin*

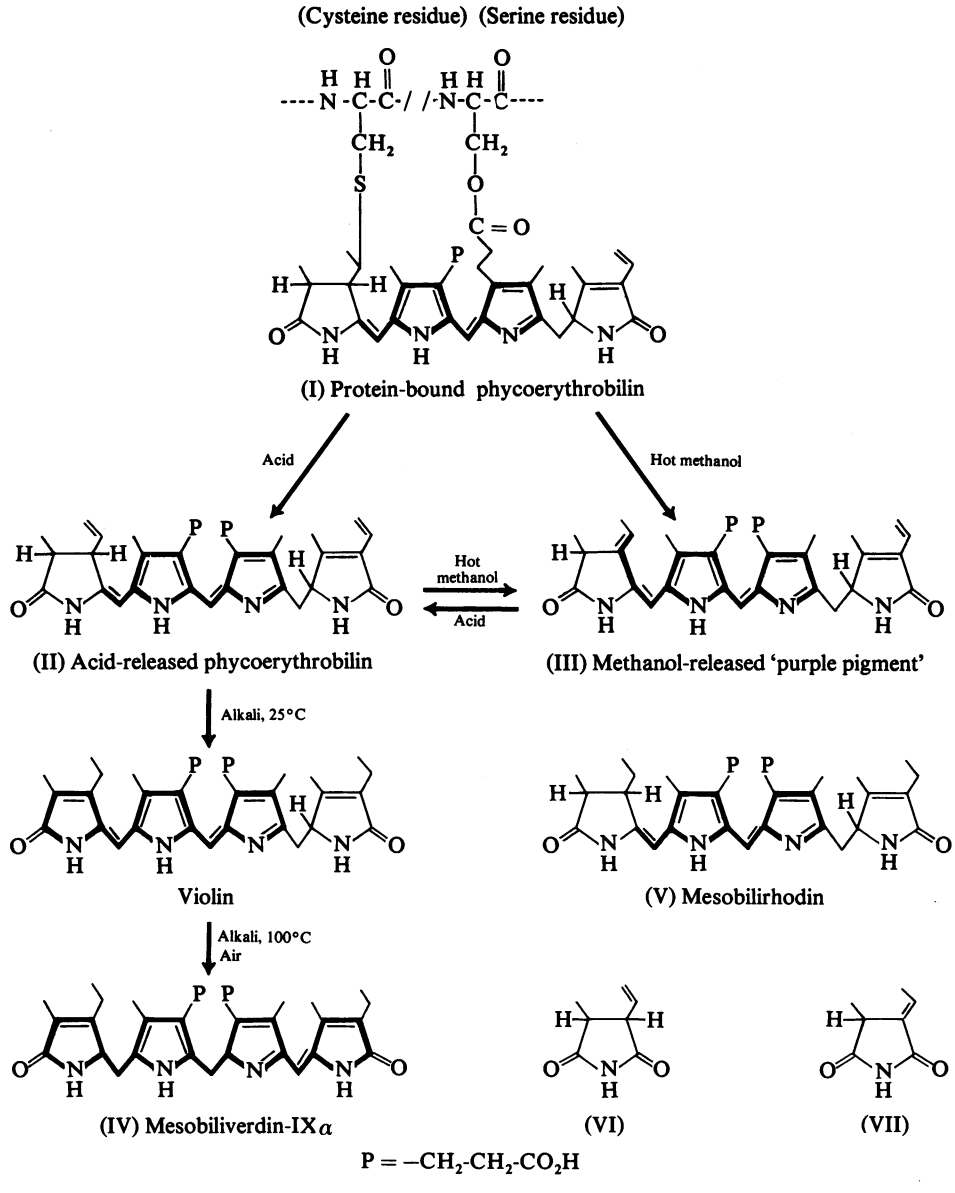
We propose structures I and II (Scheme 1) for protein-bound and acid-released phycoerythrobilin respectively. The evidence for the various features of these structures is as follows.

*Carbon skeleton and empirical formula.* When released from the apoprotein, phycoerythrobilin readily isomerizes in alkaline solution to a product that has been identified unambiguously by O'Carra & Colleran (1970) as mesobiliverdin-IX$\alpha$ (structure IV, Scheme 1). Since the conditions of release and conversion seem unlikely to cause any alteration in the carbon skeleton, this indicates a IX$\alpha$-skeleton structure for the phycoerythrobilin and an empirical formula identical with that of mesobiliverdin.

*Arrangement of double bonds.* The spectral properties of bilins have been found in the past to be an accurate index of the length of the systems of conjugated double bonds (Gray *et al.*, 1961). The position of the longest-wavelength absorption maximum and the p$K$ of the pyrroline group in the conjugated system relate consistently in an inverse manner with the length of the conjugated system. This is illustrated by the properties of the bilins listed in Table 1. On this basis the spectral properties of phycoerythrobilin indicate that it contains a conjugated system of six double bonds, one less than that of 'purple pigment' and the same number as that of mesobilirhodin. Indeed, as we have stated previously (O'Carra & Killilea, 1970), the very characteristic spectral properties of phycoerythrobilin coincide so closely with those of mesobilirhodin that the two pigments must contain identical conjugated systems. Since structure V (Scheme 1) has been reliably established for mesobilirhodin by the studies of O'Carra & Killilea (1970), Rüdiger *et al.* (1970) and Chapman *et al.* (1972), the conjugated system shown in structures I and II (Scheme 1) can be assigned to phycoerythrobilin. The remaining features of the structure are assigned on the basis of the ready conversion of the chromophore into 'purple

Table 1. *Spectral absorption maxima of bilins and derivatives*
The major maxima are given in italics and minor maxima or absorption shoulders are in parentheses.

| | λ (nm) | | | p$K$ of pyrroline group | Reference |
|---|---|---|---|---|---|
| | | Hydrochloride | | | |
| Bilin | Free base in chloroform | In acid chloroform | In 0.1M-HCl in methanol | Zinc complex in chloroform/pyridine (4:1, v/v) | |
| *i*-Urobilin | *454* | *499* | *494* | *510* | 7.4 | Gray *et al.* (1961); O'Carra & Killilea (1970) |
| Mesobilirhodin | 302, *504* | 312, *576* | 307, *555* | 319, (543), *583* | 6.2 | O'Carra & Killilea (1970) |
| Acid-released phycoerythrobilin | 304, *505* | 312, *576* | 307, *556* | 319, (540), *583* | 6.4 | O'Carra *et al.* (1964); O'Carra (1962) |
| Methanol-released 'purple pigment' | 320, *532* | 329, *602* | 328, *590* | 326, 340, 560, *605* | 6.6 | O'Carra & O'hEocha (1966) |
| Mesobiliviolin | *325*, 568 | 327, *602* | *326*, 580 | *337*, (583), 630 | 4.0 | O'Carra & Killilea (1970); Gray *et al.* (1961) |




Scheme 1. *Proposed structures for protein-bound phycoerythrobilin and its protein-free derivatives*
Chromophore conjugated systems are emphasized by heavy lines.

pigment', for which structure III is already well established. The interconversions of the two pigment preparations (O'Carra & O'hEocha, 1966; Murphy, 1968) are readily explained as prototropic shifts of the double bond in the side chain of ring A in and out of the conjugated system (Scheme 1).

As reported previously by Rüdiger & O'Carra (1969), chromic acid degradation of phycoerythrobilin and 'purple pigment' yield cyclic imide products. Identical imide products are to be expected from rings B, C and D, but ring A of structure II proposed for phycoerythrobilin might be expected to yield methylvinylsuccinimide (structure VI, Scheme 1) rather than the observed methylethylidenesuccinimide (structure VII). This imide is, of course, formed directly from 'purple pigment'. Efforts to synthesize methylvinylsuccinimide or close derivatives, however, have so far been foiled by

spontaneous conversion into methylethylidenesuccinimide (W. Rüdiger, personal communication), and it seems likely that the production of methylethylidenesuccinimide from phycoerythrobilin arises by a similar isomerization when ring A is oxidized to the imide. A shift in the double bond of the vinyl group on ring A of phycoerythrobilin to form an ethylidene group is proposed in Scheme 1 as the mechanism of formation of the 'purple pigment' derivative.

A number of lines of evidence, outlined below, indicate that the protein-bound form of phycoerythrobilin lacks the side-chain double bond in ring A, since this group seems to be involved in a thioether linkage to a cysteinyl residue (Scheme 1). This would explain the much greater stability of protein-bound phycoerythrobilin compared with the acid-released pigment.

Rüdiger (1971) has suggested that the release of the chromophore in acid involves hydrolysis of the ring-A linkage, producing a hydroxyethyl side chain. Our efforts to detect such a hydroxy group by acetylation experiments, however, have yielded negative results. Moreover, in our experience, the introduction of a side-chain hydroxy group into bilin structures leads to substantial decrease in the chromatographic mobility when the dimethyl ester derivatives are subjected to t.l.c. on silica gel in a number of hydrocarbon-based solvent systems [e.g. benzene/light petroleum (b.p. 100–120°C)/methanol, 9:5:1, by vol.]. Thus we would expect the dimethyl ester derivative of acid-released phycoerythrobilin to move more slowly than the dimethyl ester derivative of 'purple pigment' if the hydrated structure were correct. Instead the dimethyl esters of the two pigments have very similar chromatographic mobilities in the above-mentioned chromatographic systems, and this is consistent with the isomeric relationship proposed here.

*Attachment to the apoprotein.* The degradative studies of Rüdiger & O'Carra (1969) on protein-bound phycoerythrobilin showed that the pigment is attached to the apoprotein through two rings, ring A and one of the two inner rings, B or C. Rüdiger (1968, 1970) has presented evidence favouring ring C rather than ring B, and to simplify discussion it is assumed that the protein-linked inner ring is ring C as depicted in structure I of Scheme 1.

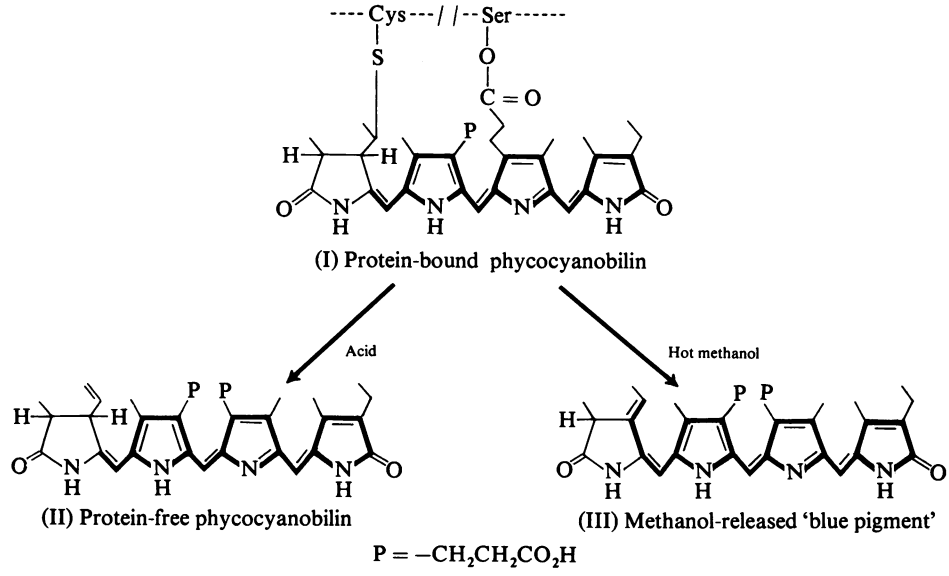
Studies on the kinetics of cleavage of the linkages suggest that the inner ring is linked through an ester bond involving its propionic acid side chain, whereas ring A is linked through a more labile linkage (O'Carra *et al.*, 1964; Rüdiger & O'Carra, 1969). We find that this ring-C linkage is readily cleaved by treatment at room temperature with sodium methoxide, a reagent generally considered to be relatively specific for ester bonds (Hultquist & Morrison, 1963). Studies described below, aimed at identifying the amino acid residues attached to the chromophore, indicate that ring C is linked to a serine residue. It is therefore proposed that protein-bound phycoerythrobilin is linked by an ester bond between the propionic acid side chain of ring C and the side-chain hydroxy group of the serine residue (structure I, Scheme 1).

The linkage of ring A was originally considered by Rüdiger & O'Carra (1969) to consist of a bond between the ring nitrogen group and a carboxy group on the protein. On the basis of this suggestion and preliminary studies of the chromophore-linked amino acid residues, we proposed that the ring-A linkage involved a glutamic acid residue (Killilea & O'Carra, 1968). As indicated in the Experimental section, however, the procedures used in these early studies were unsuitable for the detection of cysteine and, as a result, a cysteine residue close to the chromophore was overlooked. Current evidence now indicates that a cysteine residue, rather than the glutamic acid residue, is linked via its thiol group to ring A, as indicated in structure I (Scheme 1).

The conclusion, that the site of the linkage to ring A is the two-carbon side chain, is based largely on studies on the cleavage of this linkage by hot methanol, by Pronase and by treatment with alkali. In each case cleavage is associated with an alteration of the chromophore involving the generation of a double bond in the two-carbon side chain of ring A. Cleavage by hot methanol or Pronase generates the ethylidene double bond in 'purple pigment'. The formation of the double bond can be monitored spectrally during the cleavage process, whereas the cleavage itself can be directly monitored by measuring the proportion of ring A directly releasable as imide by chromic acid degradation. The two events run exactly in parallel, suggesting that cleavage and double-bond formation at the two-carbon side chain are closely associated processes. The close association is readily explained as an elimination reaction, as indicated in Scheme 1. An elimination reaction in the opposite direction under acid conditions readily explains the generation of acid-released phycoerythrobilin retaining the same conjugated system, and hence the same spectral properties, as the protein-bound chromophore. As mentioned above, the protein-bound phycoerythrobilin is much more stable than the released pigment, even when the phycoerythrins are in the denatured state, and this disparity in stability is probably attributable to the generation of the labile centre of unsaturation during release of the pigment.

*Phycocyanobilin*

Structure I (Scheme 2) is proposed for protein-bound phycocyanobilin. On most points the evidence and arguments for this structure closely resemble those presented above for phycoerythro-




Scheme 2. *Proposed structures for protein-bound phycocyanobilin and its protein-free derivatives.* Chromophore conjugated systems are emphasized by heavy lines.

bilin. Thus the isomerization of released phycocyanobilin to mesobiliverdin-IX$\alpha$ (O'Carra & Colleran, 1970) establishes the carbon skeleton and empirical formula, and the interconversions of acid-released phycocyanobilin and methanol-released 'blue pigment' (O'Carra & O'hEocha, 1966) indicated a relationship analogous to that established above between phycoerythrobilin and 'purple pigment'. Since the structure of 'blue pigment' is well-established as structure III (Cole *et al.*, 1967; Crespi *et al.*, 1968; Rüdiger & O'Carra, 1969), this analogy indicates structure II for acid-released phycocyanobilin. The proposed apoprotein linkages are identical with those proposed for phycoerythrobilin, almost identical results being obtained in experiments relevant to the apoprotein linkages when these are conducted on either phycoerythrins or phycocyanins (Rüdiger & O'Carra, 1969).

*Amino acids involved in the chromophore apoprotein linkages*

The identification of the amino acids to which rings A and C are linked was achieved as follows. C-phycoerythrin and C-phycocyanin were digested with a mixture of proteinases and peptidases in an effort to trim away the peptide material attached to the chromophore until only the directly-attached amino acid residues remained. Bacterial and mould proteinases were not used, since such preparations were found to cause alterations and partial cleavage of the chromophore–apoprotein linkages. No such problems were encountered with the mixture of mammalian proteinases finally used, but, as outlined in the experimental section, special precautions were required to avoid formation of artefact linkages between the chromophores and the apoproteins. The chromopeptide preparations obtained after exhaustive digestion were purified and are referred to as 'minimal chromopeptides'. In no case could they be trimmed further to leave only the two directly attached amino acid residues. This resistance to complete hydrolysis by the peptidases may be attributable to a masking effect by the bulky aromatic chromophores. Such steric hindrance has been encountered in similar studies on a haem–peptide from 'RHP' cytochrome (Dus *et al.*, 1962).

Amino acid analyses of typical 'minimal chromopeptide' preparations are shown in Tables 2 and 3. Of the amino acids remaining in significant molar ratio with respect to the chromophore, alanine and glycine contain no side-chain functional group and so seem unlikely to be involved in the direct linkage to the chromophore [*N*- and *C*-terminal analyses have already eliminated the possibility of a linkage through an *N*-terminal amino or a *C*-terminal carboxy group (O'Carra, 1965; Raftery & O'hEocha, 1965)]. The amino acids with functional side-chain groups that appear in significant amounts are serine, cysteine, glutamic acid and aspartic acid. The linkage of a serine residue to an inner ring (probably ring C, see above) was indicated when phycoerythrobilin-containing 'minimal chromopeptides' were subjected to further degradative studies using

Table 2. *Quantitative amino acid analyses of phycoerythrobilin-containing 'minimal chromopeptides'*
Peptide preparations I–IV were oxidized with performate before hydrolysis. Values for serine and threonine were corrected for destruction during hydrolysis assuming a 10 and 5% loss respectively (Moore & Stein, 1963).

| | | Content (mol/mol of cysteic acid) | | | |
|---|---|---|---|---|---|
| Amino acid | Preparation ... | I | II | III | IV |
| Cysteic acid | | 1.00 | 1.00 | 1.00 | 1.00 |
| Glutamic acid | | 0.50 | 0.68 | 2.10 | 0.63 |
| Aspartic acid | | 0.28 | 0.42 | 0.70 | 0.37 |
| Serine | | 0.50 | 0.69 | 0.74 | 0.56 |
| Threonine | | 0.09 | 0.12 | 0.30 | 0.27 |
| Glycine | | 1.18 | 1.50 | 3.70 | 1.30 |
| Alanine | | 0.36 | 0.62 | 1.26 | 0.91 |
| Leucine | | 0.24 | 0.28 | 0.15 | 0.09 |
| Isoleucine | | 0.30 | 0.25 | 0.26 | 0.09 |

Table 3. *Quantitative amino acid analyses of phycocyanobilin-containing 'minimal chromopeptides'*
Peptides were oxidized with performate before hydrolysis. Chromophore content is based on an $\varepsilon$ of 24000 mol · litre$^{-1}$ · cm$^{-1}$ at 660nm for phycocyanobilin (O'Carra, 1962). Fractions I and II correspond to the first and second phycocyanobilin-containing chromopeptide fractions eluted off the Sephadex G-50 columns (see the Experimental section). Values for serine and threonine were corrected for destruction during hydrolysis assuming 10 and 5% loss respectively (Moore & Stein, 1963).

| | Content (mol/mol of chromophore) | |
|---|---|---|
| Amino acid | I | II |
| Cysteic acid | 0.95 | 1.03 |
| Glutamic acid | 0.74 | 1.02 |
| Aspartic acid | 0.98 | 1.15 |
| Serine | 1.07 | 1.09 |
| Threonine | 0.54 | 0.74 |
| Glycine | 1.20 | 1.33 |
| Alanine | 0.63 | 0.69 |
| Proline | 0.33 | 0.48 |
| Valine | 0.36 | 0.46 |
| Leucine | 0.20 | 0.20 |
| Isoleucine | 0.60 | 0.50 |
| Lysine | 0.05 | 0.20 |
| Arginine | 0.10 | 0.14 |

chromic acid to cleave the bridges between the pyrrole rings. The resulting material was fractionated and a peptide-bound imide was isolated that, on hydrolysis, yielded haematinic acid (the imide derivative from the inner pyrrole rings), together with serine, glycine and alanine. Similar attempts to isolate and characterize the ring-A-containing fragment were unsuccessful. This is attributed to the lability of the ring-A linkage.

Of the remaining amino acid residues revealed in the 'minimal chromopeptides', cysteine, linked through its thiol group as shown in Schemes 2 and 3, seems to accord best with the evidence indicating a linkage through the two-carbon side chain of ring A, which may be cleaved by an elimination reaction. Further evidence for the involvement of the thiol group of cysteine in the linkage was the apparent inaccessibility of the sulphur group to *S*-aminoethylation. Although the biliproteins were subjected to prior *S*-aminoethylation, the cysteine derivative in the chromopeptides remained non-derivatized and was released as cysteic acid after performate oxidation and hydrolysis. Such procedures do not convert *S*-aminoethylcysteine into cysteic acid (S. D. Killilea & P. J. MacGillivray, unpublished work). Other workers have adduced complementary evidence in favour of a linkage of ring A through a cysteine residue (Crespi & Smith, 1970; Byfield & Zuber, 1972; Köst-Reyes *et al.*, 1975; Brown & Troxler, 1978; Williams & Glazer, 1978; Bryant *et al.*, 1978; Glazer *et al.*, 1979).

On the basis of experiments with 'trimmed' chromopeptides, Brooks & Chapman (1972) proposed a mode of linkage of phycoerythrobilin involving no linkage through ring A, whereas Crespi & Smith (1970) and Byfield & Zuber (1972) suggested that phycocyanobilin is linked only through ring A and not through ring C. These proposals conflict with the clear-cut evidence adduced from chromic acid-degradation studies (see above; Rüdiger & O'Carra, 1969; Köst *et al.*, 1975). A possible explanation for these anomalous proposals may reside in the fact that these workers used either pepsin or Pronase in the preparation of their chromopeptides. As indicated above and in the Experimental section, these proteinase preparations cause cleavage and possibly other modifications of the chromophore–apoprotein linkages. Brown & Troxler (1978) used chromic acid to degrade subunits of phycocyanin that had been reduced and carboxyamidated. They suggest that ring I of each phycocyanobilin residue is linked to the apoprotein, one to a cysteine residue and the other to a different amino acid, because more cysteine residues appeared to be susceptible to carbamoylation than would be possible if each chromophore were attached by a thioether. The possible release of the chromophore by iodoacetamide was discounted, because no free bilin could be extracted into chloroform and the spectral properties of the biliprotein appeared normal when the excess reagent was removed. The anomalous result, however, might also be explained if iodoacetamide were to convert the thioether into a sulphonium derivative, which, by an elimination reaction, could give rise to additional

carbamoylated cysteine residues with the formation of non-derivatized side group of ring I. Free pigment would not be released, however, because of the ester linkage to ring III. Glazer and associates (Williams & Glazer, 1978; Bryant *et al.*, 1978) could not find evidence to support the presence of the second linkage through ring C in peptides produced when biliprotein subunits were treated with CNBr in 70% (v/v) formic acid to cleave peptide bonds at methionine residues. Although the ester linkage may also have been broken by this treatment, they consider that a definitive answer to the question of the nature of bilin attachment is not yet available (Bryant *et al.*, 1978). Also, because of the lack of homology between two bilin-binding sites in the $\beta$-subunit of C-phycocyanin, they mention the possibility that bilin attachment at different points in the polypeptide chains involves different modes of linkage. The experimental evidence presented here indicates that chromophores are attached to biliproteins through thioether and ester linkages.

## References


Blackburn, S. & Lowther, A. G. (1951) *Biochem. J.* **48**, 126–128

Brenner, M., Niederwieser, A. & Pataki, G. (1961) *Experientia* **17**, 145–153

Brooks, C. & Chapman, D. J. (1972) *Phytochemistry* **11**, 2663–2670

Brown, A. S. & Troxler, R. F. (1978) *FEBS Lett.* **82**, 206–210

Bryant, D. A., Hixson, C. S. & Glazer, A. N. (1978) *J. Biol. Chem.* **253**, 220–225

Byfield, P. G. H. & Zuber, H. (1972) *FEBS Lett.* **28**, 36–40

Chapman, D. J., Cole, W. T. & Siegelman, H. W. (1967) *J. Chem. Soc.* **89**, 5976–5977

Chapman, D. J., Budzikiewicz, H. & Siegelman, H. W. (1972) *Experientia* **28**, 876–878

Cole, W. J., Chapman, D. J. & Siegelman, H. W. (1967) *J. Am. Chem. Soc.* **89**, 3643–3645

Crespi, H. L. & Katz, J. J. (1969) *Phytochemistry* **8**, 759–761

Crespi, H. L. & Smith, U. H. (1970) *Phytochemistry* **9**, 205–212

Crespi, H. L., Smith, U. H. & Katz, J. J. (1968) *Biochemistry* **7**, 2232–2242

Dus, K., Bartsch, R. G. & Kamen, M. D. (1962) *J. Biol. Chem.* **237**, 3083–3093

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, p. 145, Elsevier Publishing Co., Amsterdam

Frackowiak, D. & Skowron, A. (1978) *Photosynthetica* **12**, 76–80

Glazer, A. N., Hixson, C. S. & De Lange, R. J. (1979) *Anal. Biochem.* **92**, 489–496

Gossauer, A. & Hinze, R.-P. (1978) *J. Org. Chem.* **43**, 283–285

Gossauer, A. & Weller, J.-P. (1978) *J. Am. Chem. Soc.* **100**, 5928–5933

Gray, C. H., Kulczycka, A. & Nicholson, D. C. (1961) *J. Chem. Soc.* 2268–2276

Hultquist, D. E. & Morrison, M. (1963) *J. Biol. Chem.* **238**, 2843–2846

Killilea, S. D. (1972) Doctoral Thesis, National University of Ireland, Galway

Killilea, S. D. & O'Carra, P. (1968) *Biochem. J.* **110**, 14P

Köst, H. P., Rüdiger, W. & Chapman, D. J. (1975) *Justus Liebigs Ann. Chem.* 1582–1593

Köst-Reyes, E., Köst, H. P. & Rüdiger, W. (1975) *Justus Liebigs Ann. Chem.* 1594–1600

Matheson, N. A. (1963) *Biochem. J.* **88**, 152–155

Mills, G. L. (1952) *Biochem. J.* **50**, 707–712

Moore, S., Spackman, D. H. & Stein, W. H. (1958) *Anal. Chem.* **30**, 1185–1190

Moore, S. & Stein, W. H. (1963) *Methods Enzymol.* **6**, 819–831

Murphy, R. F. (1968) Doctoral Thesis, National University of Ireland, Galway

Murray, M. F. (1966) M.Sc. Thesis, National University of Ireland, Galway

O'Carra, P. (1962) Doctoral Thesis, National University of Ireland, Galway

O'Carra, P. (1965) *Biochem. J.* **94**, 171–174

O'Carra, P. & Colleran, E. (1970) *J. Chromatogr.* **50**, 458–468

O'Carra, P. & Killilea, S. D. (1970) *Tetrahedron Lett.* 4211–4214

O'Carra, P. & O'hEocha, C. (1966) *Phytochemistry* **5**, 993–997

O'Carra, P. & O'hEocha, C. & Carroll, D. M. (1964) *Biochemistry* **3**, 1343–1350

O'Carra, P., Murphy, R. F. & Killilea, S. D. (1980) *Biochem. J.* **187**, 303–309

O'hEocha, C. (1965) *Annu. Rev. Plant Physiol.* **16**, 415–434

O'hEocha, C. (1966) in *Biochemistry of the Chloroplasts* (Goodwin, T. W., ed.), vol. 1, pp. 407–421, Academic Press, New York

Raftery, M. A. & Cole, R. D. (1966) *J. Biol. Chem.* **241**, 3457–3461

Raftery, M. A. & O'hEocha, C. (1965) *Biochem. J.* **94**, 166–170

Rüdiger, W. (1968) in *Porphyrins and Related Compounds* (Goodwin, T. W., ed.), pp. 121–130, Academic Press, New York

Rüdiger, W. (1970) *Angew. Chem. Int. Ed. Engl.* **9**, 473–480

Rüdiger, W. (1971) *Fortschr. Chem. Org. Naturst.* **29**, 61–139

Rüdiger, W., Köst, H. P., Budzikiewicz, H. & Kramer, V. (1970) *Justus Liebigs Ann. Chem.* **738**, 197–201

Rüdiger, W. & O'Carra, P. (1969) *Eur. J. Biochem.* **7**, 509–516

Siegelman, H. W., Chapman, D. J. & Cole, W. J. (1967) *Arch. Biochem. Biophys.* **122**, 261

Siegelman, H. W., Chapman, D. J. & Cole, W. J. (1968) in *Porphyrins and Related Compounds* (Goodwin, T. W., ed.), pp. 107–120, Academic Press, New York

Synge, R. L. M. & Youngson, M. A. (1961) *Biochem. J.* **98**, 31P

Tristram, G. R. & Smith, R. H. (1963) *Adv. Protein Chem.* **18**, 227–250

Williams, V. P. & Glazer, A. N. (1978) *J. Biol. Chem.* **253**, 202–211