THE AMINO ACID CONSTITUENTS OF THE PHYCOBILIN CHROMOPROTEINS OF THE RED ALGA PORPHYRA[1]

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The phycobilin chromoproteins, phycocyanin and phycoerythrin of red algae, because of their stability and water-solubility, have been studied physico-chemically by many investigators. They have been shown to have definite molecular weight, characteristic isoelectric points, mobility, diffusion and adsorption properties (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932; Tiselius, 1930, 1937; Swingle and Tiselius, 1951). Their behavior as accessory pigments in photosynthesis is also well established (Haxo and Blinks, 1950; French and Young, 1952; Blinks, 1954a, 1954b; Yocum and Blinks, 1954). A comparative study of the chromatographically separated phycobilins of red and blue-green algae has recently revealed the occurrence of individual phycobilin pigments other than the classical varieties (Haxo, O'hEocha and Norris, 1955; Tiselius *et al.*, 1956). The former authors also established the presence of allophycocyanin as a natural, (although minor) component of the chromoproteins of several of the red and blue-green algae. The water-soluble chromoproteins of *Porphyra naiadum,* after separation by column chromatography and electrophoresis, have been shown to contain an appreciable quantity of allophycocyanin as well as phycoerythrin and phycocyanin (Airth, 1955; Blinks and Airth, 1957). A highly ionized lavender fraction was also found to be present. This behaved as a homogeneous entity and moved as an anion in the electrophoretic cell even at a pH of 5.0 where phycocyanin and phycoerythrin are nearly isoelectric.

Quantitative analyses of crystallized chromoproteins by Kylin (1910), Kitasato (1925) and Fujiwara (1955) show very little difference in elementary composition. Wassink and Ragetli (1952) reported on the amino acid composition of phycocyanin isolated from a species of the blue-green alga *Oscillatoria.* These authors detected sixteen ninhydrin-reactive spots of which thirteen were identified.

The present paper is concerned with the amino acid composition of several chromatographically pure phycobilin chromoproteins isolated from *Porphyra naiadum, Porphyra perforata* and *Porphyra Nereocystis,* all primitive red algae.

Material and Method

The algae were freshly collected from the shores of the Monterey Peninsula, California. *P. Nereocystis* was found growing epiphytically upon the stripes of the large kelp *Nereocystis Luetkeana. P. naiadum* was collected from the leaves of the

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flowering plant, *Phyllospadix* growing at mean low tide. The species *P. perforata* was collected from the rocks high up in the intertidal zone. Each species occupied, therefore, a different ecological habitat.

Extraction of pigments

The algae were returned to the laboratory in sea water, then washed twice with distilled water before extraction. In the case of *P. naiadum* the washed tissue was covered with distilled water and allowed to stand in the refrigerator for 24 hours at 5° C. (see Blinks and Airth, 1957). With *P. Nereocystis* and *P. perforata* it was found necessary to macerate the algal tissue in a Waring Blendor for two minutes and to allow the macerated material to stand in the refrigerator for 48 hours at 5° C. to obtain maximum extraction. The material was then filtered through cheesecloth and the filtrate passed through Whatman No. 1 filter paper pulp to remove chloroplasts and other fine organic debris. In each case the clear filtrate, exhibiting intense fluorescence, was precipitated with ammonium sulphate. Although the extracted chromoproteins of *P. Nereocystis* and *P. perforata* were precipitated with ammonium sulphate at 50% saturation, with *P. naiadum* precipitation was only complete at 90% saturation. After the purple-red precipitate was allowed to settle out overnight in the refrigerator it was removed by centrifugation, redissolved in distilled water and dialyzed against running tap water for 24 hours at 10° C., followed by 0.1 *M* acetate buffer at pH 5.0 for a further 24 hours. The non-dialyzable pigment solution was finally concentrated by pervaporation.

Fractionation

For the separation of the individual phycobilins the concentrated solution was subjected to column chromatography as employed by Airth (1955). Columns were prepared using one part tricalcium phosphate (dry weight) to five parts of washed Celite filter air. Gentle suction was used in forming the column which was washed with NaCl (1%) followed by 0.1 *M* acetate buffer at pH 5.0. The pigment extract was introduced onto the column and the individual phycobilins eluted with the appropriate buffer solutions, details of which are shown in Table I. Each fraction was further chromatographed on individual columns to ensure complete separation and elution.

The phycobilins from *P. naiadum* were also separated using a Tiselius electrophoresis apparatus. At pH 5.0 with acetate buffer, the individual chromoproteins were found to be homogeneous.

Absorption spectra were determined for each pigment over the range 250–700 mμ using a Beckman Model DU spectrophotometer.

The fractions, phycocyanin, phycoerythrin, allophycocyanin and the "highly ionized fraction" from *P. naiadum* were concentrated by pervaporation and a quantitative analysis of the amino acids released on acid hydrolysis undertaken. Because of the small amount of *P. Nereocystis* available at the time of the experiments, only phycocyanin and phycoerythrin were investigated from this alga.

Acid hydrolysis

The protein fractions were hydrolyzed using a mixture of equal volumes of concentrated hydrochloric acid and glacial acetic acid containing 4 per cent of stannous

TABLE I

Buffer solutions used in the chromatographic separation of the various phycobilins

Species	Phycobilin	Buffer	Absorption data
P. naiadum	Phycocyanin	1 *M* acetate pH 5	λ max. 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 545 mμ "Shoulder" 560–565 mμ
	Highly ionized fraction	2 *M* acetate pH 5	λ max. 565 and 615 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ
P. perforata	Phycocyanin	1 *M* acetate pH 5	λ max. 557 and 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 495 and 565 mμ "Shoulder" 540–550 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ
P. Nereocystis	Phycocyanin	1 *M* acetate pH 5	λ max. 557 and 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 495 and 565 mμ "Shoulder" 540–550 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ

chloride dihydrate (Fowden, 1954). Protein concentrations were adjusted to about 10 mg. per ml. and the hydrolysis performed in sealed tubes heated at 105° C. for 24 hours. After completion of hydrolysis the mixtures were evaporated to dryness *in vacuo* to remove the volatile acids, and the residues redissolved in 5 ml. distilled water. The amino acids were absorbed on the cation exchange resin Zeo-Karb 225, eluted with 1 N NH_4OH and finally dried in a vacuum desiccator over $CaCl_2$ and NaOH. Before chromatographing, the amino acids were taken up in 0.2 ml. isopropanol (10%) to which was added a little HCl.

Chromatographic procedure

Whatman No. 1 chromatography paper was used throughout. Spots were applied to the paper with calibrated micropipettes, the size of the spot being kept as small as possible (about 5 to 8 mm. diameter).

For two-dimensional chromatography, n-butanol : acetic acid : water (4 : 1 : 5) was used for the first direction and phenol-water (80 : 20) containing 0.04% 8-hydroxyquinoline in an atmosphere of NH_3 (1%) for the second direction. The butanol solvent was run for 28 hours at 20° C. and the solvent allowed to drip on the paper. The phenol solvent was run for 24 hours at a temperature of 20° C. The butanol was removed from the paper by drying the sheets in a current of warm air for two hours. The phenol was removed by the ether-wash technique of Fowden (1951). The amino acids phenylalanine, leucine and isoleucine were resolved

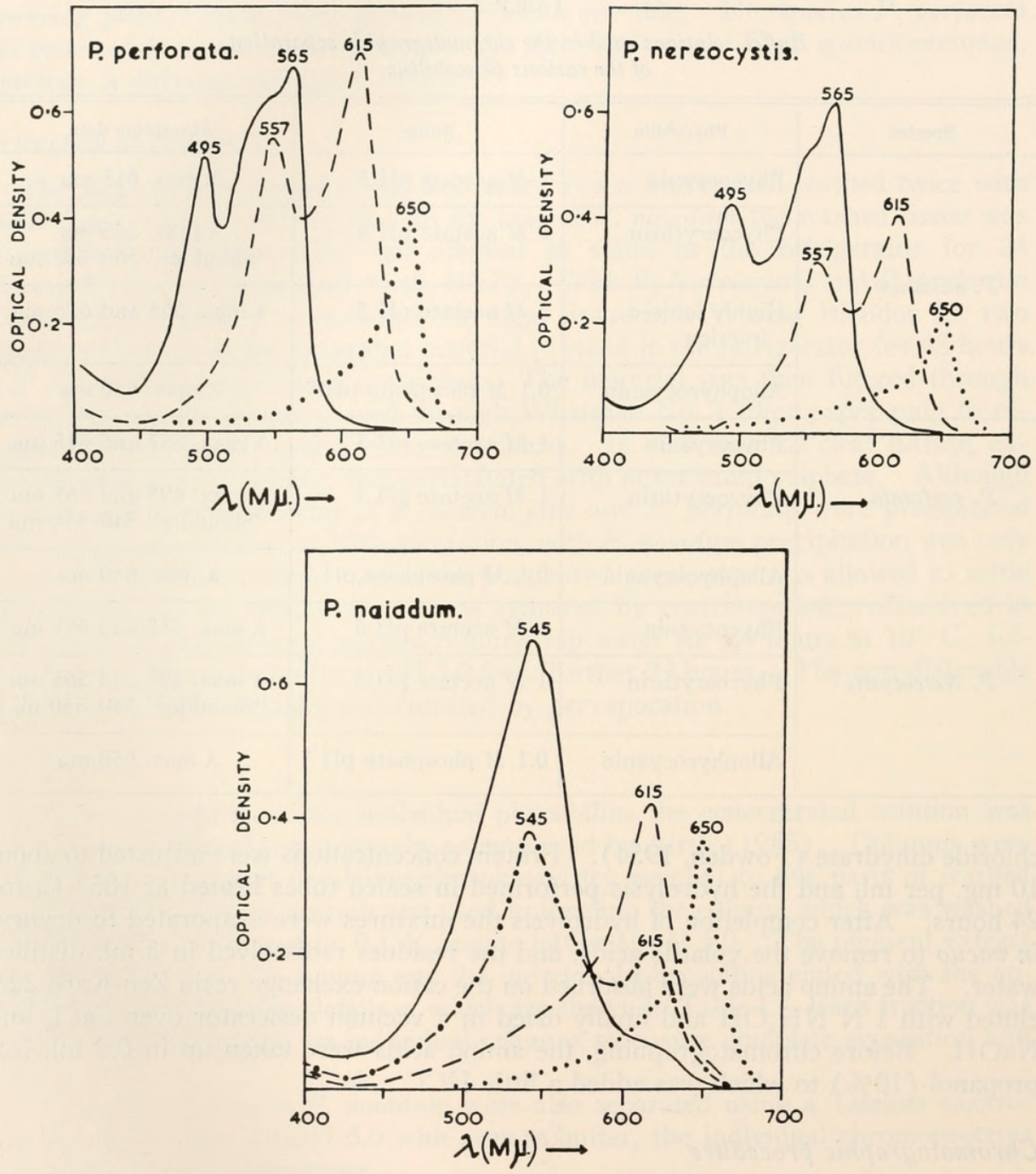

FIGURE 1. Absorption spectra of isolated phycobilins from three species of *Porphyra*. ———— phycoerythrin; – – – – – – phycocyanin; . . . . . allophycocyanin; –·–·–·–·– highly ionized fraction.

by one-dimensional chromatography using continued development for four days in water-saturated tertiary amyl alcohol in an atmosphere of 1% diethylamine. The solvent was removed from the paper by drying in a current of warm air for two hours.

The amino acids were located by dipping the chromatograms in 0.2% isatin in acetone and heating at 100° C. for three minutes to reveal proline and then dipping them into 0.2% ninhydrin in acetone and heating at 100° C. for 15 minutes to detect the other amino acids (Jepson and Smith, 1953; Smith, 1953).

TABLE II

Amino acid composition of phycobilin chromoproteins isolated from three species of Porphyra
(Percentage by weight)

Amino acid	*P. naiadum*				*P. perforata*			*P. Nereocystis*	
	PC	PE	APC	"I"	PC	PE	APC	PC	PE
Aspartic	12.9	15.3	12.8	12.9	10.7	12.5	9.4	12.6	11.3
Glutamic	12.3	13.9	16.3	17.5	12.9	10.1	11.2	12.5	8.7
Serine	3.1	0.2	3.5	3.1	2.4	2.5	3.5	8.9	8.0
Glycine	5.4	5.7	7.2	5.2	6.8	9.1	9.2	11.9	13.5
Threonine	5.8	1.6	8.6	3.7	6.1	3.6	4.5	5.7	2.8
Alanine	12.3	18.3	10.9	9.5	12.6	20.3	12.5	12.3	11.7
Histidine	1.0	2.1	+	0.8	1.4	2.1	1.0	1.3	1.9
Lysine	2.6	1.4	5.1	2.3	3.1	4.9	3.6	3.4	6.5
Arginine	2.0	1.1	5.8	1.4	2.0	4.6	4.2	2.9	7.1
Proline	5.8	4.1	6.2	4.9	5.1	2.9	5.2	3.8	4.6
Valine+methionine	10.6	7.4	11.7	6.0	10.5	9.1	6.9	6.3	3.1
Phenylalanine	6.5	10.7	3.1	6.3	2.0	4.2	7.4	3.4	5.6
Leucine	11.2	10.1	5.1	10.5	12.2	8.9	11.1	7.8	6.8
Isoleucine	9.1	6.3	3.9	8.8	6.8	4.4	7.8	3.1	5.2
Tyrosine	−	2.0	+	5.0	4.4	1.9	+	4.1	3.7
Cystine	−	+	−	+	−	+	−	−	+

PC = phycocyanin; PE = phycoerythrin; APC = allophycocyanin; "I" = highly ionized fraction. + = present but too small to determine. − = not detected.

Quantitative estimation

The chromatograms, developed as described above, were utilized for quantitative estimation by a densitometric method. The maximum spot color density (*i.e.*, average blank reading minus the minimum reading for the given spot) multiplied by the spot area is a constant under the same conditions (Block, 1950). A densitometer suitable for the analysis of the chromatograms was constructed; this consisted of a photoelectric cell, a constant voltage light source and a galvanometer (Weston Model 440 No. 10623). Rapidity of operation was improved by fixing the photoelectric cell on a movable arm which held it over a circular light source of diameter 0.5 cm. Before use the light source was adjusted to produce a suitable standard transmission. For the paper blanks percentage transmission readings of 90–100 were obtained while the amino acid spots varied between 5 and 80 per cent transmission for the concentrations employed. The area of the amino acid spot was determined by tracing the spot on uniform paper and weighing the cut-out spot with a torsion balance. Standard chromatograms of known amino acid composition were developed at the same time as the hydrolysates. For the standard amino acids linear relationships were obtained over the range of 1–25 μgm. amino acid with an error of ± 12%.

All analyses were carried out in duplicate for each alga.

RESULTS

The three species of *Porphyra* investigated exhibit a graded increase in the ratio of phycocyanin to phycoerythrin. The deep water form, *P. Nereocystis*, has the

least phycocyanin. *P. naiadum* possesses comparatively large amounts of both phycocyanin and phycoerythrin. *P. perforata* has proportionately the most phycocyanin and the least phycoerythrin. Allophycocyanin is present in all three species of *Porphyra,* but is most abundant in *P. naiadum.* The absorption spectra for the various isolated and chromatographically purified phycobilins are shown in Figure 1.

The amino acid composition of these chromoproteins is given in Table II. The distribution of the amino acids is expressed as a percentage of the total weight of the amino acids in the protein. It is seen that, in the phycobilins isolated, the same

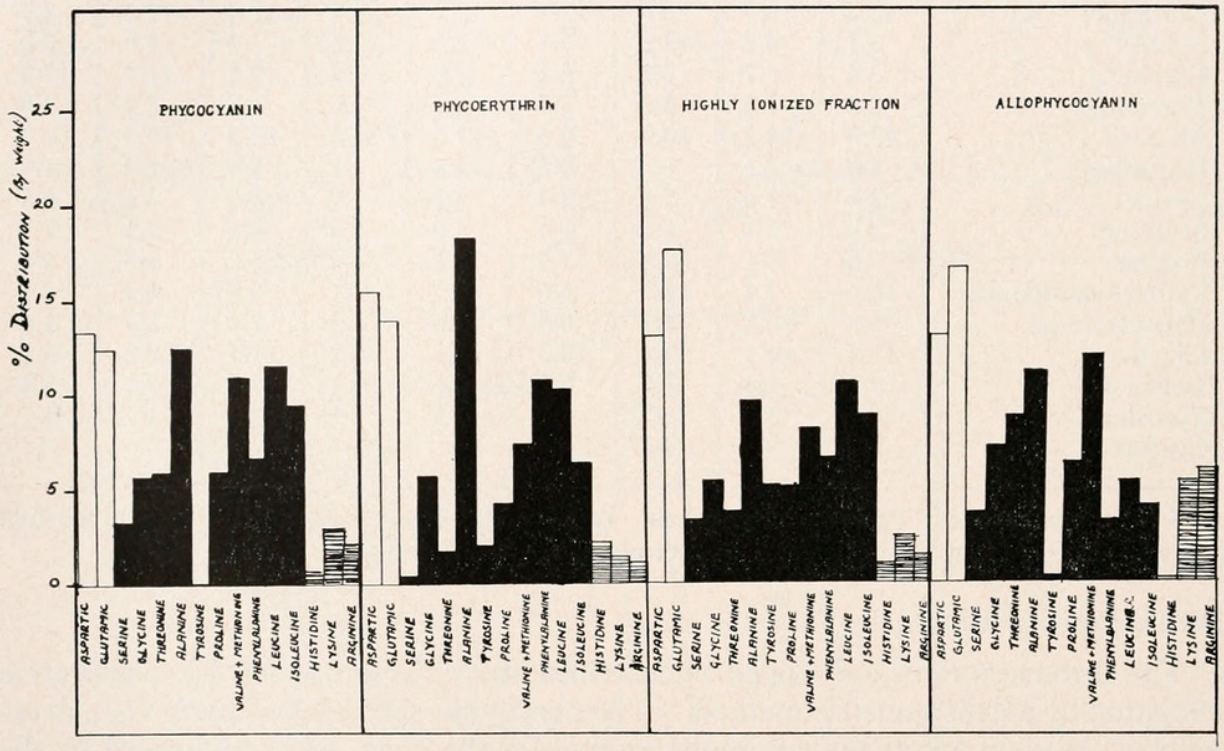

FIGURE 2. The major amino acid components of four chromoprotein fractions of *Porphyra naiadum.* Acidic amino acids are shown in white, basic, cross-hatched, and neutral, in black. The amounts are indicated as percentage of the total.

amino acids are present. Although tyrosine was not detected in the phycocyanin from *P. naiadum* it may well be due to the fact that this amino acid exists in concentrations considerably lower than that of any other amino acid present. The quantitative distribution of each amino acid varies for each protein analyzed. In all cases the dicarboxylic amino acids predominate and are comparable. Of the neutral amino acids, alanine and the two leucines are highest, although in the phycoerythrin isolated from *P. Nereocystis* glycine is present in relatively high concentration. It is interesting to note that alanine is present in greater amount than any other amino acid in the phycoerythrins isolated from *P. naiadum* and *P. perforata.* The basic amino acids of all the phycocyanins are low compared with the other phycobilin proteins. A histogram of the distribution in *P. naiadum* is shown in Figure 2.

DISCUSSION

The absorption characteristics of the isolated chromoproteins described in this work agree closely with those published by previous workers (Blinks, 1954a, 1954b; Airth, 1955; Haxo, O'hEocha and Norris, 1955). Typical R-phycoerythrins of the

higher red algae (Florideae) display absorption peaks at 495, 545, and 565 mμ. Of the lower red algae (Bangiales) here investigated, *P. perforata* and *P. Nereocystis* lack a pronounced second peak at 545 mμ. In *P. naiadum,* the phycoerythrin is characterized by a single peak at 545 mμ and a small shoulder at 560 mμ. This phycobilin has been designated B-phycoerythrin (Blinks, 1954b; Airth and Blinks, 1956), and is closest to the C-phycoerythrin of the Cyanophyta which has a single peak at 550 mμ. The phycocyanins of *P. perforata* and *P. Nereocystis* are similar in possessing the characteristic principal maximum at 615 mμ and a small one at 557 mμ. *P. naiadum,* however, contains a phycocyanin which has but a single absorption peak at 615 mμ. This is similar to the C-phycocyanin of blue-green algae. Allophycocyanin with an absorption maximum at 650 mμ is present in all three species of *Porphyra,* thereby substantiating the findings of Haxo, O'hEocha and Norris (1955) that it is a natural, although minor, component of the chromoproteins of marine algae. The highly ionized chromoprotein found only in *P. naiadum* has two absorption maxima, a major one at 545 mμ and a minor one at 615 mμ (probably due to the presence of phycocyanin).

The comparison of the above phycobilin proteins was undertaken to establish whether similar types of protein were present in the different species. The basis of comparison employed here, namely amino acid composition, although useful is subject to certain limitations. The physico-chemical properties of proteins depend not only upon their amino acid composition, but upon the arrangement of the amino acid residues within the protein and the nature of the helical configuration of the molecule. It is therefore realized that an amino acid analysis alone cannot account for all the biological or physico-chemical characters of the proteins.

The data presented show that the amino acid compositions of the various phycobilins differ significantly. Although *P. perforata* and *P. Nereocystis* possess phycobilins of similar absorption spectra, the amino acid composition of the proteins varies. The amino acid analysis of the phycocyanin from *P. naiadum* differs widely from the analysis published by Wassink and Regetli (1952) for the C-phycocyanin of *Oscillatoria* which has similar absorption characteristics. Of particular note is the presence of arginine which was absent from *Oscillatoria* phycocyanin. *P. naiadum* possesses phycobilin chromoproteins which differ from the other species of *Porphyra,* both in amino acid composition and absorption spectra. This is of particular interest since Professor G. J. Hollenberg (University of Redlands) has noted several morphological peculiarities which will probably remove *P. naiadum* from its present genus. The amino acid analysis of the highly ionized fraction present in this species offers little explanation for the high mobility of the molecule when subject to electrophoresis at pH 5.0. The degree of ionization is too great to be accounted for by the carboxyl groups of the amino acids. However, this fraction exhibited high absorption in the U. V. range of 265–280 mμ which suggests that the pigment may be attached to a nucleoprotein, in which case nucleic acid could be responsible for the high mobility.

Summary

1. The phycobilin chromoproteins of three species of *Porphyra* have been separated by column chromatography and their individual absorption spectra recorded. These "chromatographically purified" chromoproteins were subjected to acid hydrolysis and their constituent amino acids resolved by paper chromatography and determined quantitatively by a densitometric method.

2. The quantitative amino acid composition of each chromoprotein differed. The dicarboxylic amino acids alanine, glycine and the two leucines were most abundant. Alanine was found to be present in high concentration in the phycoerythrin of *P. naiadum* and *P. perforata.*

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