Purification and *N*-Terminal Analyses of Algal Biliproteins

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1. R-, B- and C-phycoerythrins and R- and C-phycocyanins were isolated and purified on a preparative scale by calcium phosphate chromatography, ammonium sulphate fractionation and crystallization. 2. The *N*-terminal residues of these biliproteins were analysed. Methionine is the only *N*-terminal residue of all the phycoerythrins, there being about 14 *N*-terminal residues per molecule of R- and B-phycoerythrins (mol.wt. 290000) and about 8 per molecule of C-phycoerythrin (mol.wt. 226000). Threonine (1 residue) is *N*-terminal in C-phycocyanin (mol.wt. 138000), and both threonine (about 1·3 residues) and methionine (5 residues) are *N*-terminal in R-phycocyanin (mol.wt. 273000). 3. Results suggest that the apoproteins of the various phycoerythrins are closely related, whereas C-phycocyanin has quite a different gross structure, and that R-phycocyanin contains two types of sub-unit, one related to C-phycocyanin and the other to the phycoerythrins.

Algal biliproteins comprise the phycoerythrins and phycocyanins. These are respectively red and blue photosynthetically active chromproteins of red and blue–green algae, and they contain covalently bound bile-pigment prosthetic groups. R-phycoerythrin is the dominant pigment of most species of red algae, but in four known species it is replaced by B-phycoerythrin. Most red algae also contain relatively small amounts of R-phycocyanin. Blue–green algae contain C-phycocyanin, C-phycoerythrin or both (cf. Ó hEocha, 1962). These various types of biliproteins are characterized by their visible absorption maxima (see Table 1, column 2).

Most work on the biliproteins has been concerned with their prosthetic groups, and relatively little information regarding the apoproteins is available. This paper reports comparative studies of their *N*-terminal amino acids.

EXPERIMENTAL

The macroscopic red algae *Porphyra laciniata*, *Ceramium rubrum* and *Rhodochorton floridulum* were collected on the sea shore near Galway. The microscopic blue–green algae *Phormidium persicinum* and *Nostoc muscorum* were cultured respectively in enriched artificial sea-water medium (Pintner & Provasoli, 1958) and in the medium of Clendenning, Brown & Eyster (1956), under white fluorescent light. The cells were harvested by centrifugation at 900 *g*.

Calcium phosphate gel was prepared by mixing M-disodium hydrogen phosphate and 1·5 M-calcium chloride solutions in equal proportions. The gel that was precipitated was washed five times with water by centrifugation and decantation and was stored under distilled water. Columns of the gel, mixed with Celite as support, were prepared and used as described by Haxo, Ó hEocha & Norris (1955) except that the use of saline was dispensed with. The columns were washed with water instead, and the biliprotein solutions were dialysed against water before chromatography. Stepwise elution was effected with phosphate buffers, pH 6·5, of graded concentration (5–100 mM). Columns of adsorbent 3·5 cm. × about 35 cm. enabled the isolation and purification of biliproteins in 200 mg. batches.

Biliproteins, at a concentration of 1%, were crystallized by buffering at the isoelectric point (approx. pH 4·5 in all cases) with sodium acetate buffer, followed by slow addition of saturated ammonium sulphate solution to a concentration of 20–25% saturation, or until the biliprotein solutions became slightly turbid. They were then allowed to stand at 3° and examined at intervals under a microscope. If crystallization did not commence, or if much biliprotein remained in solution, more saturated ammonium sulphate solution was added carefully. The crystalline biliproteins were washed with distilled water, in which they were almost completely insoluble, and stored under 20% ammonium sulphate. They could be redissolved in tap water or in carbonate buffer, pH 8·5, which was then quickly neutralized and dialysed out.

Biliprotein concentrations were determined spectrophotometrically by using extinction coefficients at the principal visible absorption maxima (Table 1). These extinction coefficients were determined by measuring the extinctions of sample solutions of the purified biliproteins (obtained as described below) in phosphate buffer, pH 6·5, and then determining the concentrations of the proteins gravimetrically after precipitation, denaturation, washing and drying to constant weight.

Dinitrophenyl-biliproteins

A modification of Sanger's (1945) method was found most satisfactory for dinitrophenylating the biliproteins. A

0·5–1% solution of the biliprotein was made 5% (w/v) with respect to sodium hydrogen carbonate. Twice the volume of 5% (w/v) fluorodinitrobenzene in ethanol was added slowly with shaking and the mixture was shaken at room temperature for 4 hr. The precipitate of DNP-biliprotein was then centrifuged down and washed in succession with water (in which the DNP-biliproteins were completely insoluble), acetone, twice with 0·1 N-hydrochloric acid, thrice with ethanol and thrice with ether. The product was air-dried at 30°, powdered finely and dried *in vacuo*. Weighed samples (8–10 mg.) of the DNP-biliprotein were hydrolysed in 5·7 N-hydrochloric acid (1 ml.) at 105° in sealed glass tubes. Preliminary experiments, in which the time of hydrolysis was varied between 6 and 24 hr., indicated that the highest yeilds of DNP-*N*-terminal residues were obtained from the DNP-phycoerythrins after 10 hr., and from the DNP-phycocyanins after 12 hr. hydrolysis. The DNP-*N*-terminal residues were extracted and identified by paper chromatography as described by Fraenkel-Conrat, Harris & Levy (1955) with Whatman no. 1 paper and the following solvent systems: 2-methylbutan-2-ol saturated with 3% (w/v) ammonia; M-phosphate buffer, pH 6·5; toluene–pyridine–2-chloroethanol–ammonia ('toluene' solvent; Biserte & Osteux, 1951); 2-methylbutan-2-ol–phthalate buffer, pH 6 (Blackburn & Lowther, 1951). Standards of authentic DNP-amino acids were run in all cases. These were prepared by the procedure of Sanger (1945). Methionine sulphone was prepared by oxidation of methionine with performic acid. The elution and quantitative determination of the DNP-amino acids was carried out in duplicate as described by Fraenkel-Conrat *et al.* (1955), the weight of biliprotein used in the calculations being taken as equivalent to 80% of the weight of the DNP-biliprotein sample used. The results were corrected for destruction of DNP-*N*-terminal residues during hydrolysis and chromatography by applying correction factors calculated from the percentage recovery of samples of the relevant authentic DNP-amino acids added to samples of the DNP-biliproteins and passed through the entire hydrolysis and isolation procedure.

Isolation of biliproteins

Spectral analysis of aqueous extracts of *Porphyra laciniata* revealed that R-phycocyanin represented 60% of the biliprotein content of this alga in March and April and this material was found to be an excellent source of R-phycocyanin. This biliprotein is generally only a minor, and sometimes absent, component of the biliprotein complement of red algal species. It does not seem to have been previously reported as the dominant biliprotein in any species.

R-phycocyanin and R-phycoerythrin from *Porphyra* were isolated and purified as follows. The washed algal fronds were minced and extracted in water (approx. 1·5 l./kg. of fresh alga) for 14 days at room temperature in closed vessels. A few drops of toluene were added daily to inhibit bacterial action. The deep-purple extract was filtered through cheesecloth, centrifuged at 1000 *g* for 15 min. and then filtered through hard-packed Celite in a Buchner funnel under suction. The biliproteins were isolated from the resulting clear orange-fluorescing solution by chromatography on calcium phosphate–Celite. The columns were loaded with the solution until the coloured band had spread one-third of the way down. Almost all the R-phycocyanin could then be eluted cleanly as a blue band when the columns were washed with 3 column-vol. of distilled water. The red R-phycoerythrin spread slowly down the columns during this washing, and was gathered and eluted with 10 mM-phosphate buffer. A relatively small amount of phycoerythrin and phycocyanin which remained adsorbed was discarded.

The chromatographically isolated R-phycoerythrin and R-phycocyanin both precipitated within the range 10–25% (w/v) of ammonium sulphate. Solid ammonium sulphate was added to each chromatographic eluate to 10% (w/v) concentration and the slight sediment after centrifugation was discarded. The supernatant was then brought to 25% (w/v) of ammonium sulphate, centrifuged, and the supernatant discarded. The precipitated biliprotein was redissolved in distilled water to a concentration of 0·5–1%, and the ammonium sulphate fractionation was repeated. Each biliprotein was again redissolved in water to a concentration of 0·5%, dialysed against distilled water for 24 hr., and rechromatographed on calcium phosphate–Celite columns. The R-phycocyanin was generally found to behave differently in this second chromatographic step from the first one. Apart from a minor band eluted with water, the R-phycocyanin was now more strongly adsorbed, being eluted with 40 mM-phosphate. This change of chromatographic properties is discussed below.

The eluted biliproteins were reprecipitated with ammonium sulphate, then redissolved in water to a concentration of 1%, and crystallized from ammonium sulphate. The R-phycoerythrin crystallized very readily within a few hours as broad red needles, and was always crystallized twice. The R-phycocyanin crystallized as violet–blue rhombohedric plates, but its crystallization was more difficult, generally taking about a week to accomplish. It was crystallized once in routine preparations.

The presence of traces of SO_4^{2-} ions promoted the adsorption of R-phycocyanin on the calcium phosphate columns. For example, ammonium or sodium sulphate added to a concentration of 50 mM to the algal extracts before the first chromatographic step caused most of the R-phycocyanin to be eluted after the R-phycoerythrin rather than before it. This effect also resulted from ammonium sulphate fractionation of the algal extracts and was difficult to eliminate by dialysis, suggesting that SO_4^{2-} ions become adsorbed on the R-phycocyanin. When R-phycocyanin was eluted off the columns after R-phycoerythrin it was always heavily contaminated with residual phycoerythrin, and was very difficult to purify in good yield. Therefore, when R-phycocyanin is being isolated it is best to postpone ammonium sulphate fractionation until after the two biliproteins have been separated by chromatography. This effect of SO_4^{2-} ions also explains the change of chromatographic properties of R-phycocyanin between the first and second chromatographic steps.

B-phycoerythrin from *Rhodochorton floridulum* and R-phycoerythrin from *Ceramium rubrum* were isolated and purified in essentially the same way as described above for the R-phycoerythrin from *Porphyra*. These two algae contained only a relatively small proportion of phycocyanin, as compared with *Porphyra*, and when this was not being isolated it was found convenient to interpose a preliminary ammonium sulphate-fractionation step before the first chromatographic step. The R-phycoerythrin from *Ceramium* crystallized as hexagonal prisms, as reported by

Kylin (1910) and Lemberg (1928), and the B-phycoerythrin crystallized as sheaves of fine needles.

C-phycoerythrin was isolated from *Phormidium pericinum* by extraction of the ground cells for 3 hr. at 2°, and purified by chromatography twice on calcium phosphate. Efforts to crystallize this biliprotein were unsuccessful.

C-phycocyanin was isolated from *Nostoc muscorum*. The harvested cells were ground with sand at 2° for 30 min. and extracted in distilled water at 2° for 3 hr. The temperature was kept below 3° throughout the following purification, which was carried out as rapidly as possible. The extract was filtered through Celite and fractionated twice with ammonium sulphate. The fraction precipitating between 15 and 30% (w/v) ammonium sulphate was collected. The precipitate was redissolved in water, dialysed against frequently changed distilled water for 10 hr. and chromatographed on calcium phosphate–Celite. The eluted C-phycocyanin was dialysed and rechromatographed as before. It was crystallized readily from ammonium sulphate as blue–green hexagonal plates.

N-*Terminal amino acid residues*

The 2-methylbutan-2-ol–ammonia and phosphate buffer solvents were used two-dimensionally to identify and isolate the DNP-N-terminal residues from all the biliproteins, and the other solvent systems were used to verify the identifications in phycoerythrins. Some initial difficulty was encountered in identifying the DNP derivative of the N-terminal methionine from biliproteins, as the R_F and shape of the spots in the phosphate buffer solvent system were generally slightly distorted relative to those of the standard DNP-methionine. The identity was proved by mixing samples of the DNP-N-terminal residue with authentic DNP-methionine and subjecting the mixtures to prolonged chromatography in all solvent systems. No tendency towards resolution into two spots was observed. The barium hydroxide procedure of Mills (1950) was used to regenerate the free amino acid from the DNP-N-terminal residue of the phycoerythrins. The products were subjected to chromatography in butan-1-ol–acetic acid–water (4:1:5, by vol., top phase) with standards and treated with ninhydrin. Two spots were revealed, one corresponding to methionine and the other to methionine sulphone. The latter is formed by oxidation of methionine during the regeneration procedure. N-Terminal analyses were also carried out on R-phycocyanin and phycoerythrins oxidized with performic acid (Sanger & Thompson, 1953). In all cases the DNP-methionine was replaced by a DNP-amino acid having R_F values identical with those of authentic DNP-methionine sulphone.

The percentage destruction of the DNP-N-terminal residues during hydrolysis of the DNP-biliproteins was in all cases found to be unusually high (50–60%), giving high correction factors for the quantitative estimations.

RESULTS AND DISCUSSION

Results are summarized in Table 1.

The R-phycoerythrin from *Ceramium rubrum* is of the classical type, having three absorption peaks in the visible region (Lemberg, 1928; Svedberg & Eriksson, 1932), and it is distinguished here as R-phycoerythrin I. The R-phycoerythrin from *Porphyra lacinata* was found to be similar to the two-peaked variant R-phycoerythrin from *Porphyra perforata* reported by Haxo *et al.* (1955) and it is distinguished here as R-phycoerythrin II. The other biliprotein preparations had the typical absorption spectra associated with their types (Table 1, column 2).

Examination of the absorption spectra of the purified biliprotein preparations showed that they were free from contamination with other biliproteins or other coloured impurities. Subjection of samples to further repeated crystallization and/or chromatography on calcium phosphate and on Sephadex G-75 did not result in any changes in the extinction coefficients (Table 1, column 3) or in the ratio of visible to ultraviolet (280 mμ) extinction, suggesting the absence of colourless impurities. Except with C-phycocyanin the results of the N-terminal analyses were quite clean and no N-terminal residues other than those listed in

Table 1. N-*Terminal residues and extinction coefficients of biliproteins*

R-phycoerythrin I and R-phycoerythrin II (column 1) are from *Ceramium* and *Porphyra* respectively. Absorption shoulders are in parentheses; the most intense absorption maximum is italicized (column 2) and $E^{1\%}_{1\,\text{cm.}}$ (column 3) is measured at the italicized wavelength. Numbers in parentheses in column 4 are the corrected quantitative estimations of the N-terminal residues. Molecular weights used in the calculations (column 5) are taken from Eriksson-Quensel (1938), Airth & Blinks (1957) and Hattori & Fujita (1959).

Biliprotein	Absorption maxima (mμ)					$E^{1\%}_{1\,\text{cm.}}$	N-Terminal residue (and no./mol.)	Mol.wt.
R-phycoerythrin I	498,	540,		*568*		80·2	Methionine (14)	291000
R-phycoerythrin II	498,			*564*		81·5	Methionine (13)	290000
B-phycoerythrin	(498),	*546*,		(565)		82·3	Methionine (14)	290000
C-phycoerythrin				*563*		125	Methionine (8)	226000
C-phycocyanin					*615*	65	Threonine (1)	138000
R-phycocyanin			553		*615*	66	Methionine (5)	273000
							Threonine (1·3)	

column 4 of Table 1 were detected, indicating the absence of protein impurities.

In early experiments, C-phycocyanin was extracted from *Nostoc* after repeated freezing and thawing of the cells, and was purified at room temperature by ammonium sulphate precipitation and chromatography on calcium phosphate. *N*-Terminal analyses of C-phycocyanin prepared thus were not very reproducible and revealed the presence of many *N*-terminal amino acids, among which leucine or isoleucine and threonine generally predominated. However, when C-phycocyanin was extracted and purified quickly and at low temperature, as described in the Experimental section, the only significant DNP-amino acid on the chromatograms was identified as DNP-threonine. A trace of what was tentatively identified as DNP-leucine or DNP-isoleucine was also present. These results may possibly be due to degradation of the C-phycocyanin by proteolytic enzyme(s) from the alga during the less rigorous isolation procedure. Proteolytic enzymes have been demonstrated in extracts from a number of blue–green algae (Allen, 1952) and proteolytic activity has been detected in extracts from *Nostoc muscorum* (D. Mitchell, personal communications).

Owing to the high correction factors necessary, the quantitative estimations of the *N*-terminal residues of the biliproteins must be tentative (cf. Fraenkel-Conrat *et al.* 1955). The quantitative value for R-phycoerythrin I agrees fairly well with the quantitative estimation of the *C*-terminal residues (12 alanine residues) of the same biliprotein obtained by Raftery & Ó hEocha (1965) and indicates that R-phycoerythrin I contains 12–14 probably similar peptide chains. R-phycoerythrin II and B-phycoerythrin seem to have gross structures very similar to that of R-phycoerythrin I, but that of C-phycoerythrin appears to be somewhat different. C-phycoerythrin also differs from the other phycoerythrins in molecular weight and in being much more sensitive to denaturing agents. However, the occurrence of methionine as the only *N*-terminal residue of all the types of phycoerythrin suggests that the apoproteins as well as the chromophore systems (Ó hEocha, 1958) are closely related, since methionine is a very minor component of all biliproteins and it is improbable that its occurrence in such a characteristic position as *N*-terminal in different biliproteins could be fortuitous.

The results for C-phycocyanin indicate that the molecule (mol.wt. 138000) consists of one peptide chain with *N*-terminal threonine. Its gross structure must therefore be different from those of the phycoerythrins. The prosthetic group of C-phycocyanin is a blue pigment, phycocyanobilin (Ó hEocha, 1963), and the prosthetic group common to all the phycoerythrins is a red pigment, phycoerythrobilin (Ó Carra, Ó hEocha & Carroll, 1964). R-phycocyanin contains both phycocyanobilin and phycoerythrobilin (Ó hEocha, 1960) and the *N*-terminal analysis results indicate that it is composed of two types of peptide chains, one type having *N*-terminal threonine, as has C-phycocyanin, and the other type having *N*-terminal methionine, as have the phycoerythrins. This correspondence of *N*-terminal and prosthetic groups suggests that one type of sub-unit in R-phycocyanin is related to C-phycocyanin and the other to the phycoerythrins. It has been shown conclusively that R-phycocyanin is a discrete molecular species and not a mixture of biliproteins (P. Ó Carra & C. Ó hEocha, unpublished work).

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