C-Phycocyanin and Allophycocyanin in Two Species of Blue-Green Algae

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1. The biliproteins C-phycocyanin and allophycocyanin were purified from the blue-green alga *Anabaena variabilis* by ammonium sulphate fractionation and gel filtration. 2. An assay procedure that enabled the proportion of the two pigments, present as a mixture, to be determined was devised by using the data provided by spectrophotometric analysis of the purified biliproteins. 3. The degree of association and relative proportions of the two pigments were analysed by the application of this procedure to the components separated by thin-layer gel filtration. 4. The C-phycocyanin/allophycocyanin ratio remained essentially constant in algal extracts prepared at various stages throughout the growth cycle or after growth under conditions of reduced illumination. 5. The behaviour of the C-phycocyanin aggregate species from *Anacystis nidulans* suggested that they were of appreciably lower molecular weight than those observed in extracts of *Anabaena variabilis*.

In the blue-green algae a significant proportion of the total protein is contributed by intensely absorbing blue pigments to which a function as accessory pigments in photosynthesis has been attributed. Two of these linear tetrapyrrole conjugates, generally termed biliproteins, commonly found in the Cyanophyceae are C-phycocyanin and allophycocyanin. The former pigment is known to exist as a reversibly associating system built on the various aggregate states of a monomer, which has a molecular weight of about 30 000 (Berns, Scott & O'Reilly, 1964). Recently attention has been directed to a study of the physical properties of C-phycocyanin extracted from several blue-green algae and in particular to its state of aggregation in various ionic environments (Hattori, Crespi & Katz, 1965; Scott & Berns, 1965; Berns & Morgenstern, 1966). From electron-micrographic evidence Berns & Edwards (1965) suggest that in *Plectonema calothricoides* C-phycocyanin exists, when in the hexameric configuration, as round structures that could facilitate the formation of chlorophyll-phycocyanin complexes.

Allophycocyanin was originally presumed to be a degradation product of other biliprotein material (Lemberg & Bader, 1933), but reports of its wide distribution and its successful extraction from fresh material (Haxo, Ó hEocha & Norris, 1955) support its position as a minor chromoprotein of both Rhodophyceae and Cyanophyceae.

Previous methods for separating C-phycocyanin and allophycocyanin have been based on adsorption chromatography or on ammonium sulphate precipitation. Neither method lends itself to a rapid

assessment of the amount of chromoprotein present. Scott & Berns (1965) have further suggested that adsorption chromatography may lead to an irreversible alteration in the degree of association of C-phycocyanin. Gel filtration has been widely successful in protein purification and has the advantage of not being dependent on the net charge of the protein concerned. It therefore seemed of particular relevance to the separation of C-phycocyanin and allophycocyanin, where a technique that did not interfere with the ionic character of the protein aggregates was demanded. Nolan & Ó hEocha (1967) have used column gel filtration to examine the biliproteins from several Rhodophyceae and cryptomonad algae.

The present paper describes a convenient and rapid assay employing thin-layer gel filtration that was used to determine the proportion of the two biliproteins and their state of aggregation in *Anabaena variabilis* and *Anacystis nidulans*.

MATERIALS AND METHODS

Organisms. The blue-green algae used (*Anabaena variabilis* Kützinger and *Anacystis nidulans*) were maintained as described by Carr & Hallaway (1965).

Media and growth conditions. Organisms were grown on medium C (Kratz & Myers, 1954), supplemented with 0.05% (w/v) NaHCO₃ and gassed with air + CO₂ (95:5, v/v). With *Anabaena variabilis* a magnetic stirrer was employed to prevent settling, but this was not necessary with *Anacystis nidulans*. Growth was followed turbidimetrically, and a calibration curve used to relate EEL colorimeter reading (no filter) to the dry wt. (mg./ml.). Cultures for experimental purposes were grown at 32–34° either in Roux

bottles (11.) or in Pyrex flat-bottomed flasks (51.). Illumination, except where stated, was provided by a bank of four 60 w tungsten bulbs 6 in. distant from the centre of the growth vessel.

Preparation of cell-free extracts. Algal cells were harvested at room temperature by centrifugation at 1500g for 10 min. The pellet was washed in distilled water and once in 25 mm-sodium phosphate buffer, pH 6.8, before suspension in the same buffer to a final concentration of 20–40 mg. dry wt./ml. Disruption of the cells was carried out by extrusion through a French Pressure Cell at 10000–15000 lb./in.², or by treatment for four periods of 45 sec. in an MSE 60 w ultrasonic disintegrator. In each case precautions were taken to ensure thorough cooling of the samples. Cell debris was removed by centrifugation at 150000g for 45 min. at 0°. (A preliminary low-speed centrifugation to remove whole cells was not employed, as leaching of the phycocyanin from damaged cells into the supernatant would lead to incorrectly high values for the biliprotein/dry wt. ratio.)

Electrophoresis. Starch-block electrophoresis was carried out with the Phorograph-original (type 64) Electrophoresis Unit. A block holder with additional buffer compartments was constructed from Perspex, providing strips of support media 6 cm. × 0.5 cm. × 30 cm.; 10 mm-sodium phosphate buffer, pH 6.7, was used to equilibrate the starch grains and also in the electrode compartments. The sample was introduced on a filter-paper strip inserted in a razor slit cut in the starch block. A potential gradient of 20–30 v/cm. (30–20 mA) was applied for 5 hr.

Adsorption chromatography. Hydrated tricalcium phosphate was prepared from a calcium complex of sucrose by the addition of H₃PO₄ by following the method of Swingle & Tiselius (1951). A column of internal diam. 2.3 cm. was packed with a slurry (2:1, v/v) of cellulose (100 g./l.) and tricalcium phosphate (30 g./l.) in distilled water. The total volume of the settled slurry was 270 ml. Then 5 ml. of a cell-free extract containing 40 mg. of protein in 2.5 mm-sodium phosphate buffer, pH 6.8, in 0.5% NaCl was layered on to the top of the packed column. Immediately after the sample had entered the column, elution was continued with 0.5% NaCl. The biliproteins were subsequently eluted with stepwise increments in the molarity of sodium phosphate buffer, pH 6.8: 100 ml. of 2.5 mM, 5 mM, 10 mM, 20 mM and 40 mM and finally 300 ml. of 250 mM. All the buffers were prepared in 0.5% NaCl.

Gel filtration. Columns were prepared with Sephadex G-200 (140–400 mesh) packed to a bed volume of 200 or 50 ml., and eluted with 25 mm-sodium phosphate buffer, pH 6.8. Thin-layer gel filtration was carried out on 20 cm. × 20 cm. glass plates coated with a suspension of Sephadex G-200 (Superfine particle size 10–40 μ, lot no. 5761) to a depth of 0.9 mm. The gel at a concentration of 4.3 g./100 ml. was applied by means of Shandon adjustable spreader. The thin-layer plates were accommodated in a Perspex tank designed to allow three plates to be run simultaneously in an atmosphere saturated with water vapour. Sodium phosphate buffer, pH 6.8 (25 mM), was used for the preliminary swelling and for the subsequent development of the thin-layer gel. Inclination at an angle of 18° (hydrostatic head 5.5 cm.) allowed development of the plates overnight (14 hr.). Faster development could be obtained with an angle of inclination of 21°. The subsequent procedure employed for their treatment was based on that of Morris (1964). It was found that removal of the paper 'print' from

the gel surface before drying decreased the tendency of the proteins to migrate during this process and did not significantly alter the sensitivity. The dried filter papers were immersed in a protein stain of bromocresol green (0.2%) in ethanol-acetic acid (19:1, v/v) for 5 min. and then washed with four changes of aq. 5% (v/v) acetic acid. The papers were then exposed, after drying, to an atmosphere saturated with ammonia vapour. Analysis of the zones separated by the technique was also carried out by scraping off the Sephadex gel and resuspending it in buffer; after the suspension had settled for 1 hr. in graduated tubes, the supernatant liquid was removed (by filtration) and analysed spectrophotometrically. Quantitative values for the amount of buffer containing the proteins were derived by correcting the settled gel volume for the content of included buffer and adding this to the supernatant volume. The correction was obtained by utilizing a factor representing the fraction of the gel contributed by the void volume plus the proportion of the internal volume available to the protein. The latter was estimated by using the K_d values given by Morris (1964) for the appropriate molecular weight of the protein species under investigation.

Analytrol scanning. The stained filter-paper 'prints' were cut to size and the qualitative distribution of the protein material was measured with a recording densitometer (Beckman-Spinco Analytrol).

Determination of chlorophyll. Chlorophyll *a* was measured by extracting a known volume of disrupted cell suspension with acetone-water (4:1, v/v) at 0° in the dark for 30 min. The E_{663} of the protein-free supernatant liquid was measured and the concentration of chlorophyll *a* calculated (MacKinney, 1941); chlorophyll *b* is not present in the Cyanophyceae (Bogorad, 1962).

Determination of dry weight. The dry weight of the disrupted suspension was determined by heating 0.2–1.0 ml. of extract to constant weight at 65°. The contribution of the buffer salts to the total mass was subtracted.

Procedure for the purification of C-phycocyanin and allophycocyanin. The total phycocyanin present in the cell-free extract was precipitated by 50%-saturated ammonium sulphate. After removal of cell debris by repeated high-speed centrifugation, the resuspended pellet was subjected to further ammonium sulphate fractionation procedures at 35% and 50% saturation. Under the former conditions the resulting pellet was enriched with respect to C-phycocyanin, and by treating the supernatant to produce 50% saturation a fraction containing allophycocyanin was isolated. This process was repeated three times.

The partially purified extracts were dialysed against 25 mm-sodium phosphate buffer, pH 6.8, and subsequently subjected to fractionation at 40%, 45% and 50% saturation with ammonium sulphate. In each case the precipitated fraction was resuspended in 1.5 ml. of 25 mm-sodium phosphate buffer, pH 6.8, and further purified by gel filtration with a Sephadex G-200 column (bed volume 50 ml.). The eluted fractions that had E_{654}/E_{620} ratios greater than 1.57 (allophycocyanin) and less than 0.25 (C-phycocyanin) were retained and, after treatment with ammonium sulphate to 50% saturation, the biliproteins allowed to crystallize out at 4°.

Chemicals. The proteins used were obtained from the following sources: cytochrome *c* [Seravac Laboratories (Pty) Ltd., Maidenhead, Berks.]; bovine γ -globulin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex);

human serum albumin, trypsin and thyroglobulin (Koch-Light Laboratories Ltd., Colnbrook, Bucks.); human haemoglobin was extracted from fresh blood. The tricalcium phosphate was kindly supplied by Dr D. McGarry.

All other chemicals were of A.R. quality or of the highest commercially available grades.

RESULTS

The properties of the isolated pigments indicated a high degree of purity. With allophycocyanin the visible spectrum was similar to that of material isolated by electrophoresis or tricalcium phosphate chromatography; however, the E_{654}/E_{620} ratio was greater than those obtained by the other two procedures and also higher than those measured from published spectra (Haxo *et al.* 1955; Ó hEocha, 1965). This indicated a more effective removal of C-phycocyanin ($\lambda_{\max}$ 620 m μ). The $E_{1\text{cm}}^{1\%}$ at 620 m μ for allophycocyanin was therefore calculated for material prepared by procedure (1) (see Table 1). The results presented for C-phycocyanin (Table 1) are similar to those reported by Scott & Berns (1965), their material having an E_{620}/E_{280} ratio greater than 4 and $E_{1\text{cm}}^{1\%}$ 60 at 622 m μ . From the spectra of the purified biliproteins (Fig. 1) it was possible to derive an equation for calculating the relative amounts of the pigments present in a mixture of the two. The equation is derived from an expression relating the ratio of the extinctions for the two pigments at different wavelengths, based on the procedure used by Bucke, Leech, Hallaway & Morton (1966). Let E_{620} be the extinction at 620 m μ of the mixture of both pigments, E_{654} be the extinction at 654 m μ of the mixture of both pigments and E_{620}^C be the contribution of C-phycocyanin to the total extinction of the mixture at 620 m μ . Given that the ratio of the extinctions at 620 m μ and 654 m μ for C-phycocyanin is 4.18 and for allophycocyanin is 0.627:

$$E_{620}^C = 1.177 E_{620} - 0.7377 E_{654}$$

The application of the derived equation was tested with known quantities of the purified materials and was found to give results having a mean deviation from the expected of 5.7% (4) for variations in composition between 5 and 20% C-phycocyanin. This procedure was applied to the analysis of the pigment species separated by thin-layer gel filtration.

Examination of the cell-free extracts by thin-layer gel filtration. After development of plates that had been loaded with a sample of cell-free extract applied as a line of small drops, four regions of coloured material were observed. Described in terms of their R_{Hb} values, these were 2.20, 1.50, 1.20 and 1.04 (the R_{Hb} value is a measure of the migration of the protein relative to that of haemo-

Table 1. *Properties of the pigments isolated from Anabaena variabilis*

(1) Pigments isolated by ammonium sulphate fractionation and gel filtration; (2) pigment isolated by electrophoresis; (3) pigment isolated by calcium phosphate chromatography. Sodium phosphate buffer, pH 6.8 (25 mm), was used throughout.

Pigment	E_{654}/E_{620} ratio	E_{620}/E_{280} ratio	$E_{1\text{cm}}^{1\%}$ at 620 m μ
C-phycocyanin (1)	0.24	4.7	73
Allophycocyanin (1)	1.59	1.6	16
Allophycocyanin (2)	1.24	—	—
Allophycocyanin (3)	1.27	—	—

Fig. 1. Visible spectra of C-phycocyanin (---) and allophycocyanin (—) isolated from *Anabaena variabilis* as described in the Materials and Methods section. The proteins were dissolved in 25 mm-sodium phosphate buffer, pH 6.8.

globin; Morris, 1964). Band R_{Hb} 2.20 was yellow-green in colour and identified as chlorophyll *a* containing membrane fragments and was not further examined. Band R_{Hb} 1.50 after elution was identified as C-phycocyanin ($\lambda_{\max}$ 620 m μ) and analysis of the spectrum by using the derived equation did not reveal any contamination with allophycocyanin. The lower two bands did not separate completely from one another, but were distinguished by their difference in colour. Band R_{Hb} 1.20 was blue-green and had a spectrum characteristic of mixed C-phycocyanin and allophycocyanin ($\lambda_{\max}$ 620 and 654 m μ), whereas band R_{Hb} 1.04 only exhibited the absorption maximum at 620 m μ (blue).

By comparison with a series of proteins of known molecular weight a relationship between R_{Hb} and $\log(\text{mol.wt.})$ was established for the system (Fig. 2). Assuming that this linear relation held for the biliproteins, it was possible to assign molecular weights to the phycocyanin species. The faster-migrating C-phycocyanin had mol.wt. 200 000, mean deviation $\pm 15 000$ (16), and the overlapping bands R_{Hb} 1.20 and 1.04 had average mol.wt. 104 000, mean deviation $\pm 14 000$ (16). This would be consistent if the phycocyanin in the system were present in the hexameric (mol.wt. 180 000) and trimeric (mol.wt. 90 000) configurations (Scott & Berns, 1965). The trimer and allophycocyanin (mol.wt. 134 000) (Hattori & Fujita, 1959) would be incompletely resolved. The presence of other

aggregate forms of C-phycocyanin was not detected by this system.

Analysis of phycocyanin throughout the growth cycle. The application of the described formula to the results obtained from thin-layer gel filtration permitted a rapid assessment of the proportions of C-phycocyanin existing in its various association states, and the amount of allophycocyanin relative to the other biliproteins could also be assayed. Analysis at five stages throughout the growth cycle (0.1–2.5 mg. dry wt. of material/ml. of growth media) revealed that the major biliprotein present was C-phycocyanin, representing $82 \pm 3\%$ (5) of the total extinction at 620 m μ , and that the proportion of this material to allophycocyanin did not significantly alter during the growth cycle. Both chlorophyll *a* and total phycocyanin concentrations increased with respect to the dry weight, achieving maximum values of 1.7% and 17% of the dry weight respectively; however, these values decreased if determinations were continued into the stationary phase of the growth curve. The C-phycocyanin state found to predominate in the analyses was that of the hexamer, its contribution to the total C-phycocyanin concentration having a value of 60%, mean deviation $\pm 4.5\%$ (5). All determinations during this experiment were carried out in duplicate.

Effect of decreasing the light-intensity. By decreasing the light-intensity, accomplished by the introduction of neutral-density filters in front of the growth vessel, the mean generation time of a culture was more than doubled with reference to the control. Pigment analysis revealed an increase in both the total biliprotein and chlorophyll *a* concentrations, but analysis with thin-layer gel filtration revealed only very slight changes in the contribution of allophycocyanin to the total biliproteins (see Table 2).

Comparison between Anabaena variabilis and Anacystis nidulans. When extracts of *Anacystis nidulans* were subjected to gel filtration on thin-layer plates, a difference in pigment distribution became apparent compared with the pattern obtained with *Anabaena variabilis*. The fast-migrating band of C-phycocyanin ran, as before, ahead of allophycocyanin, but in this case no

Fig. 2. Calibration curve of the R_{Hb} value as a function of $\log(\text{mol.wt.})$ for protein samples run on Sephadex G-200 thin-layer plates as described in the Materials and Methods section. The proteins used were: A, cytochrome *c*; B, pepsin; C, serum albumin; D, γ -globulin; E, thyroglobulin. (1) R_{Hb} value observed for the fast-migrating C-phycocyanin; (2) R_{Hb} value calculated from the mean distance travelled by the overlapping C-phycocyanin and allophycocyanin regions observed on analysis of *Anabaena variabilis* extracts as described in the text.

Table 2. *Pigment variation after growth of Anabaena variabilis in decreased light-intensity*

Decrease of light-intensity was effected as described in the text and determinations of chlorophyll *a* and dry weight were performed as described in the Materials and Methods section. Total biliproteins were estimated on the basis that the total extinction at 620 m μ was contributed by C-phycocyanin alone.

	Mean generation time (hr.)	Total biliprotein (% of dry wt.)	Chlorophyll <i>a</i> (% of dry wt.)	Allophycocyanin (%) contribution to E_{620}
Control	24	18	1.6	17
Light-restricted	70	22	2.0	15

distinct separation occurred. The slower-moving C-phycoerythrin was observed as a discrete region, showing no evidence of the overlap exhibited by the *Anabaena variabilis* system.

Analysis of the pigment proportions indicated that, under extraction procedures identical with those utilized in experiments with *Anabaena variabilis*, the fast-migrating C-phycoerythrin represented 30–45% of the total and that allophycoerythrin contributed about 9% of the biliprotein extinction at 620m μ . Also resolved by the system was a low-molecular-weight compound whose absorption properties were characterized by $\lambda_{\max}$. 410m μ and therefore probably identical with the pteridine reported in this organism by Forrest, Van Baalen & Myers (1957). The possibility that the slower migration of the C-phycoerythrin species in *Anacystis nidulans* was due to individual variation between plates was investigated with extracts of both algae run on the same plate. Subsequent analysis of the stained filter-paper 'prints' with a Beckman–Spinco Analytrol indicated that inter-species variance was present (Fig. 3).

DISCUSSION

The results obtained from thin-layer gel filtration of *Anabaena variabilis* extracts support those of other workers using different species of blue-green algae and other analytical procedures in demonstrating that the C-phycoerythrin consists, at neutral or slightly acid pH, mainly of trimer and hexamer aggregates based on a monomer of mol.wt. about 30 000. It is apparent that the charge characteristics of the different associates are very similar, as demonstrated by the inability of electrophoresis or calcium phosphate chromatography to separate them. The mol.wt. 200 000 (mean deviation $\pm$ 15 000) determined for the hexamer species by gel filtration is closer to that (180 000) obtained by Scott & Berns (1965) than to that (270 000) obtained by Hattori *et al.* (1965); both these groups used analytical ultracentrifugation for their measurements. However, the value obtained for the trimer aggregate (104 000, mean deviation $\pm$ 14 000) could be interpreted as supporting a monomer size of 45 000 as suggested by Hattori *et al.* (1965). The molecular weight calculated for the trimer species is based on the migration distance of the overlapping bands of C-phycoerythrin and allophycoerythrin and therefore greater emphasis should be placed on the value calculated for the hexamer. Exact comparison of information on the relative preponderance of the various association states from the two techniques is rendered difficult by the differences in pretreatment of samples. Extracts

Fig. 3. Trace recordings, obtained with a Beckman–Spinco Analytrol, of the bromocresol green-stained filter-paper 'prints' taken from thin-layer Sephadex-gel-filtration experiments. The upper trace was obtained with an *Anacystis nidulans* extract and the lower trace with an *Anabaena variabilis* extract. O, Origin; M.F., position of the peak due to membrane fragments totally excluded from the gel. The hexameric C-phycoerythrin peak for *Anabaena variabilis* is indicated by the arrow.

used for thin-layer gel filtration were prepared by high-speed centrifugation of the crushed cell suspension at 0° without further purification procedures, in an attempt to maintain as far as possible correlation with conditions *in vivo*. The gel filtration was performed at 4° in darkness to prevent denaturation of the labile pigments. Scott & Berns (1965) used C-phycoerythrin prepared by ammonium sulphate precipitation from extracts of *Plectonema calothricoides*; when they analysed their preparation at 25° in a buffer similar to that used in gel filtration they found the prevalent C-phycoerythrin species at pH 6 was that of the hexamer (68%) but that at pH 7 the proportion of this material had fallen to 32%. The major aggregate present in our studies (pH 6.8) was the hexamer, possibly indicating that the internal conditions of *Anabaena variabilis* favour this configuration. Experiments designed to ascertain the internal H⁺ ion concentration of the alga by recording the change in pH of a concentrated cell suspension after disruption, though not yielding an exact figure, indicated a value between pH 6.0 and 6.5. This would be consistent with the high concentrations of hexamer noted in the extracts of *Anabaena variabilis* and with the view that the hexamer is the important form of C-phycoerythrin *in vivo*.

The failure to observe a band of phycoerythrin in *Anacystis nidulans* corresponding to the hexamer of

Anabaena variabilis could indicate either the lack of this species in the former algae, or that the molecular configuration of the aggregate protein is such that it possesses a different observed molecular weight under the conditions of gel filtration. The latter concept is more probable in light of the report by Fujimori & Pecci (1966) of unusually low sedimentation coefficients for the C-phycoerythrin isolated from *Anacystis nidulans*.

The marginal changes in the allophycoerythrin concentration and in particular the lack of its accumulation towards the end of the growth cycle in *Anabaena variabilis* add further evidence favouring the idea that it is not a degradation product. The relative proportions of biliproteins, when calculated from the $E_{1\text{cm}}^{1\%}$ at values 620m μ , indicated a C-phycoerythrin/allophycoerythrin ratio about 2:1.

Although the absolute value for the allophycoerythrin $E_{1\text{cm}}^{1\%}$ at 620m μ should be interpreted with caution, as only minimal quantities of the purified protein were available for analysis, the results still suggest that in *Anabaena variabilis* allophycoerythrin constitutes more than a minor pigment species.

Further information about the molecular structure of these biliproteins will be necessary before their exact role (or roles) in algal metabolism can be understood.

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REFERENCES

- Berns, D. S. & Edwards, M. R. (1965). *Arch. Biochem. Biophys.* **110**, 511.
- Berns, D. S. & Morgenstern, A. (1966). *Biochemistry*, **5**, 2985.
- Berns, D. S., Scott, E. & O'Reilly, K. T. (1964). *Science*, **145**, 1054.
- Bogorad, L. (1962). In *Physiology and Biochemistry of Algae*, p. 386. Ed. by Lewin, R. A. New York: Academic Press Inc.
- Bucke, C., Leech, R. M., Hallaway, M. & Morton, R. A. (1966). *Biochim. biophys. Acta*, **112**, 19.
- Carr, N. G. & Hallaway, M. (1965). *J. gen. Microbiol.* **39**, 335.
- Forrest, H. S., Van Baalen, C. & Myers, J. (1957). *Science*, **125**, 699.
- Fujimori, E. & Pecci, J. (1966). *Biochemistry*, **5**, 3500.
- Hattori, A., Crespi, H. L. & Katz, J. J. (1965). *Biochemistry*, **4**, 1225.
- Hattori, A. & Fujita, Y. (1959). *J. Biochem., Tokyo*, **46**, 633.
- Haxo, F. T., Ó hEocha, C. & Norris, P. (1955). *Arch. Biochem. Biophys.* **54**, 162.
- Kratz, W. A. & Myers, J. (1954). *Amer. J. Bot.* **42**, 282.
- Lemberg, R. & Bader, G. (1933). *Liebigs Ann.* **505**, 151.
- MacKinney, G. J. (1941). *J. biol. Chem.* **140**, 315.
- Morris, C. J. O. R. (1964). *J. Chromat.* **16**, 167.
- Nolan, D. N. & Ó hEocha, C. (1967). *Biochem. J.* **103**, 39P.
- Ó hEocha, C. (1965). In *Chemistry and Biochemistry of Plant Pigments*, p. 175. Ed. by Goodwin, T. W. London: Academic Press (Inc.) Ltd.
- Scott, E. & Berns, D. S. (1965). *Biochemistry*, **4**, 2597.
- Swingle, S. M. & Tiselius, A. (1951). *Biochem. J.* **48**, 171.