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A COMPARISON OF THE SOLUBLE PROTEINS IN VARIOUS SPECIES OF ALGAE BY DISC ELECTROPHORESIS IN POLYACRYLAMIDE GELS*

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Abstract—Aqueous saline extracts of fresh specimens of twelve species of marine algae, representative of the Chlorophyceae, Rhodophyceae and Phaeophyceae, have been analyzed by disc electrophoresis in polyacrylamide gels. The pattern of proteins in the green algae was the most complex with ~12 bands, that in the reds less complex with ~9 bands and least in the browns with ~5 bands. Fractionation of the extracts with $(NH_4)_2SO_4$ indicated the probable presence of several globulins and chromoproteins, and two albumins, but such fractions were grossly heterogeneous. Fractionation of the extract from *Rhodymenia palmata* indicated the presence of allophycocyanin, R-phycocyanin and R-phycoerythrin mainly from spectrometric data. Trials with gradient gels and isoelectric focusing were less informative than by the conventional method of electrophoresis in 7·5 % gels. Comparison of six stains for algal proteins indicated Coomassie Brilliant Blue and Woolfast Blue as preferable.

INTRODUCTION

THE CHROMOPROTEINS of algae, especially the phycoerythrins and phycocyanins in many species of Cyanophyceae, some Rhodophyceae, a few of the unicellular Chlorophyceae and Cryptomonads, have been investigated extensively since the early work of Kylin.[1] Other algal proteins have been examined *in toto* as to their composition of amino acids.[2-5] A few have been fractionated by way of isolation.[6-9] The technique of disc electrophoresis should be a tool with which to determine semi-quantitatively the distribution of soluble proteins in algal extracts. It has been so used recently with twelve species of blue-green algae.[10] The results indicated up to twenty components in some extracts but only minor differences between species. Phycocyanin was present in all and phycoerythrin in several of those examined. The technique however showed some promise for wider application.

In this communication disc electrophoresis in polyacrylamide gels has been applied to compare aqueous extracts of twelve representative species of green, red and brown marine algae, and the fractions obtained therefrom with ammonium sulphate. In one red species, *Rhodymenia palmata*, the fractions have been analyzed spectrometrically for bilichromoproteins.

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[1] H. KYLIN, *Z. Physiol. Chem.* **69**, 169 (1910).
[2] L. FOWDEN, *Ann. Botany* **18**, 257 (1954).
[3] E. J. LEWIS and E. A. GONZALVES, *Ann. Botany* **26**, 301, 317 (1962).
[4] Y. KOTT and A. M. WACHS, *Appl. Microbiol.* **12**, 292 (1964).
[5] C. B. COWEY and E. D. S. CORNER, in *Some Contemporary Studies in Marine Science* (edited by H. BARNES), pp. 225–231, Allen and Unwin, London (1966).
[6] E. G. YOUNG and D. G. SMITH, *J. Biol. Chem.* **233**, 406 (1958).
[7] Y. KOBAYASHI, Y. IWAI and T. ANDO, *Seikagaku* **35**, 81 (1963).
[8] E. I. MEDVEDEVA and E. F. SELICH, *Biokhimiya* **33**, 635 (1968).
[9] E. I. MEDVEDEVA and E. B. KAGANOVICH, *Izv. Vyssh. Ucheb. Zaved., Pishch. Tekhnol.* **1**, 179 (1969).
[10] E. DERBYSHIRE and B. A. WHITTON, *Phytochem.* **7**, 1355 (1968).

RESULTS

Extraction

The choice of the initial solvent between water and dilute saline solutions, such as acetate, Tris hydrochloride, orthophosphate or pyrophosphate at pH 7–8·5 did not appear to make much difference. Repeated extraction with the same solvent dissolved little more nitrogenous material as exemplified by *Rhodymenia* at (1st) 0·13%, (2nd) 0·04%, (3rd) 0·01%. However, further extraction with buffered 10% NaCl (pH 8·5) gave different results with different botanical groups as shown in Table 1. More protein was extracted from the three species of Chlorophyceae than by Tris and less protein from the species of Rhodophyceae and Phaeophyceae than by phosphate, which may be taken to indicate distribution of

TABLE 1. CONCENTRATION OF PROTEINS AND NUMBER OF BANDS IN AQUEOUS EXTRACTS

Species	Protein in 0·1 M tris or PO_4 (%)	Number of bands	Protein in 10% NaCl (%)	Number of bands
Chlorophyceae				
Ulva lactuca	0·14	11 (2)	0·28	9 (2)
Enteromorpha sp.	0·05	13 (2)	0·20	7 (1)
Spongomorpha arcta	0·06	12 (2)	0·24	6 (1)
Rhodophyceae				
Chondrus crispus	0·14	9 (3)	0·08	3 (1)
Polysiphonia spp.	0·07	9 (3)	0·03	5 (2)
Porphyra umbilicalis	0·25	9 (4)	0·06	9 (3)
Corallina officinalis	1·60	7 (4)	0·54	5 (3)
Rhodymenia palmata	0·15	8 (3)	0·03	5 (2)
Phaeophyceae				
Chorda filum	0·04	5 (1)	0·013	4 (1)
Chordaria flagelliformis	0·09	7 (1)	0·035	3 (1)
Fucus filiformis	0·03	3 (1)	0·018	4 (1)

Figures in brackets indicate major bands.

globulins. The highest concentration was achieved with *Corallina* at 1·6% in 0·01 M pyrophosphate. One additional extraction of *Rhodymenia* with a solution of 1% sodium carbonate and 0·02% sodium dodecyl sulphate showed no new characteristic bands. This result also applied to the use of 0·5% Triton X-100 with *Porphyra*.

Electrophoresis

The exact number of bands was hard to assess as many were very faint and may represent traces of proteins present. Many of the bands were quite sharp in outline but others were diffuse. The total numbers listed in Table 1 must be taken as approximate. However, certain conclusions are possible. It is evident that the pattern of proteins in the Chlorophyceae is the most complex and that in the Phaeophyceae the least complex with only one dense band visible. An interesting fact was the presence in almost all of the initial and later extractions of two dense bands which migrated rapidly with R_p values of between 0·9 and 1·0 and were presumably albumins in nature. This conclusion is supported by the salting-out procedure.

While these components appeared in most of the electrophoretic diagrams they did not always have the same R_p values.

Extraction with 10% NaCl as a solvent for globulins was most effective in the Chlorophyceae as more protein was dissolved than by 0·1 M tris (pH 7·8). In the Rhodophyceae and the Phaeophyceae only about one-third the amount dissolved by 0·1 M phosphate was solubilized by 10% NaCl. The electrophoretic diagram was also simpler. Photographs of the tubes for several species are shown in Fig. 1. Trials for the presence of basic proteins were always negative for the five species examined.

Fractionation

By way of possible identification of the bands, the extracts were adjusted to pH 5 and any precipitate recovered. Then ammonium sulphate in small increments was added to the extracts until saturated. Any precipitate forming was redissolved and examined electrophoretically.

R_p values are not reported because of the varying ionic strengths in the samples and lack of strict comparability. The qualitative results are shown in Table 2. All of the species of brown algae gave precipitates at half and full saturation. In the red species the concentrations causing precipitation were different except at the level of saturation.

Extracts of *Rhodymenia* were the most complex with many different fractions indicative of various chromoproteins, confirmed by their colors and absorption spectra. The results are shown in Tables 2 and 3. The dry powder contained 3·21% N prior to extraction and the residue afterwards 6·39% N. Some insoluble proteinaceous material was thus concentrated during extraction. Absorption maxima of the initial water extract were at 555 and 660 nm while those of the NaCl extract were at 510 and 560 nm. The relatively large amorphous precipitate obtained at approximately 9% $(NH_4)_2SO_4$ was only partially soluble and the solute tended to aggregate spontaneously. The solution gave an unusual spectrum with one broad major band with peak at 480 nm and a very minor one at 612 nm. If due to denaturation of phycoerythrin the major peaks should have been at 498 and 556 nm.[11]

Figure 2 shows the composition of successive precipitates below the spacer gel with increasing concentration of $(NH_4)_2SO_4$ from 49 to 52% for a water extract of *Rhodymenia*. The complexity of such precipitates is apparent, especially at 9–16%, 23% and 40–47% while at 5 and 52% only one or two components are present. The presence of two rapidly migrating bands of differing density in all precipitates up to saturation is peculiar but indicative of albumins. Figure 3 shows a similar series for a pyrophosphate extract of *Corallina* and one of *Fucus*.

Gel Gradients

Two modifications of the polyacrylamide technique were tried on several algal extracts to determine whether better resolution could be attained. One was on a gradient of 2·5, 5·0, 7·5 and 10% gels as described by Slater.[12] Results with extracts of *Chondrus*, *Polysiphonia*, *Porphyra*, *Corallina*, *Chorda* and *Chordaria* were very similar to those with 2·5 and 7·5% gels but with a tendency for bands to form at each new gel strength.

[11] C. O'hEocha and P. O'Carra, *J. Am. Chem. Soc.* **83**, 1091 (1961).
[12] G. G. Slater, *Anal. Biochem.* **24**, 215 (1968).

TABLE 2. CONDITIONS OF PRECIPITATION OF ALGAL PROTEINS

Group and species	pH 5	Concentration of $(NH_4)_2SO_4$ 4·9*	9·7	14·4	19·0	23·2	27·5	32	36	39	43	46	52
		0·10†	0·19	0·28	0·36	0·45	0·52	0·62	0·70	0·75	0·82	0·90	1·0
(A) From phosphate extracts													
Rhodophyceae													
Chondrus crispus			+						+			+	+
Polysiphonia sp.	+			+			+						+
Porphyra umbilicalis	+			+		+		+			+		+
Corallina officinalis	+		+			+		+		+		+	+
Rhodymenia palmata		+	+	+	+	+	+	+		+		+	+
Phaeophyceae													
Chorda filum							+					+	+
Chordaria flagelliformis							+					+	+
Fucus filiformis		+					+					+	+
(B) From sodium chloride extracts													
Rhodophyceae													
Chondrus						+					+		+
Polysiphonia			+			+					+		+
Porphyra		+				+					+		
Corallina		+				+		+			+		+

* %, w/v at 5°.
† Decimal of saturation.

TABLE 3. PROPERTIES OF FRACTIONS FROM *Rhodymenia palmata*

Conc. $(NH_4)_2SO_4$ at ppt. (%, w/v at 5°)	Fluorescence	Color of soln.	Absorption maxima (mμ)	Probable identity
4·9	yellow	pink	491, 528 (?), 562, 648	R-phycoerythrin allophycocyanin
8·8	pink	blue	480, 612	?
14·4	yellow-pink	violet	550, 612	R-phycocyanin
16·2	orange-pink	violet	553, 613	R-phycocyanin
19·8	orange-yellow	purple	491, 530, 558	R-phycoerythrin
23·2	orange-yellow	purple-red	491, 528, 558	R-phycoerythrin
26·6	orange-yellow	red	492, 529, 558	R-phycoerythrin
30·9	orange-yellow	red	492, 531, 564	R-phycoerythrin
39·4	orange-yellow	red	492, 531, 563	R-phycoerythrin
47·2	orange-yellow	red	492, 531, 564	R-phycoerythrin
51·7	gray-pink	pink	492, 531, 564	R-phycoerythrin

Isoelectric Focusing

Another technique on a different principle is that of isoelectric focusing as developed at the LKB-Produckter AB in Sweden. The microprocedure of Catsimpoolas[13] was used with Ampholine pH 3–10 as the carrier ampholyte in 7% acrylamide. The Ampholine was leached out with 5% TCA to prevent precipitation of the dye. Coomassie Blue was the stain applied after fixation. Over a length of 70 mm the gradient in pH was ~0·1/mm between 2·7 and 8·1. As applied to two fractions from *Porphyra* which showed 3 and 12 bands respectively in electrophoresis at constant pH, isoelectric focusing exhibited 3 bands with pI 4·0, 4·7 and 6·2, and 5 bands with pI 3·8, 4·2 (2) and 6·4 (2). As this technique appeared to be less informative than the conventional no further samples were run. The pI for R-phycoerythrin and R-phycocyanin have been listed as 4·25 and 4·5 or 4·85 respectively.[14]

Comparison of Stains

A comparison of the various dyes recommended to stain protein discs in this technique was carried out on the same fraction of an extract of *Polysiphonia* with the same loading of 50 μl per tube. The stains employed were Amido Black-10B (0·2% in methanol) for 60 min, Coomassie Brilliant Blue R-250 (0·25% in methanol–water–acetic acid as 5:5:1) for 20 min and (0·05% in 12·5% TCA) for 60 min, Toluidine Blue O (0·01% in 1% acetic acid) for 16 hr, Woolfast Blue BL (0·5% in methanol–water–acetic acid as 5:5:1) for 5 min, Light Green SF Yellowish (0·25% in 25% acetic acid) for 60 min and Nigrosine WS (1% in water) for 60 min. All were destained by washing if necessary.

Nigrosine stained too densely as with the usual 0·5–1% Amido Black and required electrophoretic destaining. Woolfast Blue and Coomassie Blue (0·05%) gave the best results, especially for photography. Light Green stained all four bands evident by other dyes but only faintly. The coloration with Toluidine Blue was not as clear as with the other blues and only two bands were evident. An important observation was made in this experiment on the relative density of staining of various bands. Two rapidly migrating bands were present in all tubes. With Amido Black and Woolfast Blue the upper band was denser, with Toluidine Blue the lower and with Coomassie Blue and Light Green they were visually equal. Such an observation throws some doubt on the validity of quantitation by densitometer.

DISCUSSION

There is probably enough evidence from observations of solubility, mobility and precipitation by ammonium sulphate to conclude that there are at least two albumins which occur in most if not all twelve seaweeds examined. Evidence for the distribution of globulins is not so clear but from Table 2 and the electrophoretic patterns the presence of globulins in all the species is indicated by the precipitates obtained at approximately half saturation.

Chromoproteins are, of course also present and migrate as visible pigmented discs with R_p values of between 0·4 and 0·55; often two are easily recognizable. However only the extracts of *Rhodymenia* were examined spectroscopically and biliproteins tentatively identified as listed in Table 3. Variations in recorded spectra[14–16] and the probable occurrence of many such biliproteins in algae make identification difficult without further chromatographic

[13] N. CATSIMPOOLAS, *Anal. Biochem.* **26**, 480 (1968).
[14] R. LEMBERG and J. W. LEGGE, *Hematin Compounds and Bile Pigments*, p. 146, Interscience, New York (1949).
[15] E. GUÉRIN-DUMARTRAIT, *Ann. Biol.* **36**, 171 (1960).
[16] C. O'HEOCHA, *Ann. Rev. Plant Physiol.* **16**, 415 (1965).

purification. Even under such conditions the exact positions of the absorption maxima are reported at some variance.

Rhodophycean genera most studied have been *Porphyra*, *Porphyridium* and *Ceramium*. Few studies have been made with *Rhodymenia*. Haxo *et al.*[17] identified R-phycoerythrin in *R. pacifica* based on absorption maxima of 497, 537 and 564 nm and O'hEocha[18] in *R. palmata* based on 499, 540 and 568 nm to be compared with 492, 531 and 564 nm in Table 3. O'hEocha[19] lists R-phycocyanin and allophycocyanin as also present in *R. palmata*. This is confirmed by the absorption maxima in Table 3 for R-phycocyanin at 550 and 612 nm vs. 552–555 and 610–617 nm as previously recorded. Allophycocyanin is indicated by the distinctive absorption at about 650 nm but this value has been variously reported.

Basic proteins were not detected although they are probably present but in quantities inadequate for the techniques employed.

Microelectrophoresis in polyacrylamide gels is thus a sensitive technique with which to examine both extracts and precipitates of proteins and requires only small amounts of material (*ca.* 50 μg). It is very probable from the experience of others that many more proteins are present in the algal cells than here demonstrated. The residues were never extracted with dilute acid for basic proteins. The earliest examination possible of extracts is desirable subject only to the dissolution of sufficient amounts as are detectable. Autolysis may be a disturbing factor. As in chromatograms the identification of discs with specific proteins still presents difficulties. The technique does, however, indicate state of purification.

EXPERIMENTAL

Materials

Specimens were collected locally in July or October in lots of 100 g, and comprised the following species, Chlorophyceae: *Ulva lactuca*, *Enteromorpha* sp., *Spongomorpha arcta*; Phaeophyceae: *Chorda filum*, *Chordaria flagelliformis*, *Fucus filiformis*; Rhodophyceae: *Chondrus crispus*, *Polysiphonia* sp., *Porphyra umbilicalis*, *Corallina officinalis*, *Rhodymenia palmata*.

The plants were transported to the laboratory on ice and carefully cleaned of contaminating macroscopic epiflora and fauna. They were cut into fragments about 1 cm long and homogenized in a Vertis "45" at 0° for short intervals in the proportion of about 25 g to 50 ml of solvent. The mixture stood for 24–48 hr at 4° and was then centrifuged at 12,000 *g* for 30 min in a Servall refrigerated RC-2 centrifuge. The insoluble residue was re-extracted with the same or other aqueous solvent. Extracts were passed through Millipore 0·3 μ filters if necessary, preserved by adding merthiolate (1:10,000) and later fractionated with $(NH_4)_2SO_4$. Aliquots of 50 μl were analyzed electrophoretically.

Methods

Electrophoresis was carried out in the apparatus (model 6) of Canal Co. with a power pack (model 3-1014 A) of Buchler Instruments. The technique was essentially that of Reisfeld, Lewis and Williams[20] with solutions formulated by Davis.[21] The small- and large-pore gels were respectively 7·5 and 2·5% acrylamide photopolymerized in glass tubes (75 × 4 mm). Tray buffer was tris-glycine, pH 8·5. A constant current of 2 mA per tube and about 280 V was maintained for 1·5–5 hr. The gels were fixed in 12·5% trichloroacetic acid in 30 min and usually stained in 30–60 min with 1% Coomassie Brilliant Blue R-250 (Colab.) diluted 1:20 with 12·5% TCA.[22]

Total protein was determined by the micro-Kjeldahl method when possible; in the presence of ammonia

[17] F. Haxo, C. O'hEocha and P. Norris, *Arch. Biochem. Biophys.* **54**, 162 (1955).
[18] C. O'hEocha, in *Comparative Biochemistry of Photoreactive Systems* (edited by M. B. Allen), pp. 181–203, Academic Press, New York (1960).
[19] C. O'hEocha, in *Physiology and Biochemistry of Algae* (edited by R. A. Lewin), pp. 421–435, Academic Press, New York (1962).
[20] R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature* **195**, 281 (1962).
[21] B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
[22] A. Chrambach, R. A. Reisfeld, M. Wyckoff and J. Zaccari, *Anal. Biochem.* **20**, 150 (1967).

the quantitative biuret reagent of Gornall, Bardawill and David,[23] or latterly the more sensitive Folin–Ciocaltu phenol reagent[24] were used.

Extraction

The solvent for extraction was usually 0·1 M phosphate (pH 7·0) as recommended by Steward and Barber[25] and this was followed by a solution of 10% NaCl and 0·1% Na borate (pH 8·5). Other solvents tested were 0·1 M Tris buffer (pH 7·8), 0·1 M Na acetate (pH 4·6), a solution of 1% Na_2CO_3 and 0·02% sodium dodecyl sulphate and 0·5% Triton X-100 (pH 4·5). An effective initial solvent was found to be 0·01 M Na pyrophosphate (pH 8·35).

Fractionation of extracts was carried out by the addition of $(NH_4)_2SO_4$ in steps of 5% to saturation at 5° (~52%, w/v). Precipitates were centrifuged down, redissolved in the initial solvent and examined electrophoretically.

Photography

Gels were photographed in a glass box held in position by a Lucite or Bakelite mask with 70 × 5 mm slots, and covered by 5% TCA. This was placed on a milk glass plate, 4 mm thick, lighted from below with two No. 2 photo-flood bulbs 12 cm below the plate. Film, Ilford FP3, 4/5; ASA 125; 1 sec exposure f32; developer D-11 in 4 min.

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[23] A. GORNALL, C. J. BARDAWILL and M. M. DAVID, *J. Biol. Chem.* **177**, 751 (1949).
[24] O. H. LOWRY, N. J. ROSEBOROUGH, A. S. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[25] F. C. STEWARD and J. T. BARBER, *Ann. N.Y. Acad. Sci.* **121**, 525 (1964).