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Enzyme Systems in Marine Algae. The Carbohydrase Activities of Unfractionated Extracts of *Cladophora rupestris*, *Laminaria digitata*, *Rhodymenia palmata* and *Ulva lactuca*

By W. A. M. DUNCAN, D. J. MANNERS and A. G. ROSS
Department of Chemistry, University of Edinburgh

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Present knowledge of the metabolism of terrestrial plants and freshwater algae is considerable (cf. Bonner, 1950; Fogg, 1953) and a large number of enzymes and enzyme systems have been detected in plant tissues and extracts; subsequently, many of these have been isolated and purified. In addition, certain freshwater algae (e.g. *Chlorella* and *Scenedesmus*) have been widely used in studies of photosynthesis and intermediary carbohydrate metabolism. By contrast, similar information on marine algae is lacking, and few investigations of the enzyme systems of marine algae have been reported, although the chemical structure of many of the end-products of anabolism has been investigated (cf. Black, 1953).

In co-operation with the Institute of Seaweed Research, a survey of the enzyme systems of marine algae has been commenced, and, in view of our previous interest in the enzymic hydrolysis of glucosides and glucosans (Manners, 1952, 1955), our preliminary experiments have been directed towards the detection of carbohydrases in extracts of marine algae. In the present paper, evidence for the presence of a number of soluble carbohydrases in a member of the Phaeophyceae (*Laminaria digitata*), a member of the Rhodophyceae (*Rhodymenia palmata*) and two species of Chlorophyceae (*Cladophora rupestris* and *Ulva lactuca*) is recorded. A preliminary account of part of this work has already been published (Duncan, Manners & Ross, 1954).

METHODS AND MATERIALS

Analytical methods

Paper chromatography. (*a*) *Sugars.* Descending chromatograms were carried out at room temperature with Whatman no. 1 paper and *n*-butanol–pyridine–water–benzene (5:3:3:1, by vol.) as solvent (De Whalley, Albon & Gross, 1951). An alkaline silver nitrate reagent (Trevelyan, Procter & Harrison, 1950) or aniline oxalate reagent (Partridge, 1949) was used to detect the sugars on the chromatograms. The rate of movement of sugars (R_G) was determined by dividing the distance moved by the sugars from the starting line by the distance moved by D-glucose (R_G 1·0) under identical conditions. The R_G value of a particular sugar was found to vary on different chromatograms, e.g. laminaribiose had R_G 0·65–0·76; hence, preliminary identification of a sugar was carried out by placing the sugar and the appropriate reference compound on the same chromatogram, and not by calculation of the R_G value.

(*b*) *Phosphate esters.* Development and detection was effected by the method of Hanes & Isherwood (1949), with *n*-propanol–ammonium hydroxide–water (6:3:1, by vol.) and glucose 1- and 6-phosphates as reference compounds.

Reducing sugars. Reducing sugars were determined by (*a*) the iodometric Shaffer & Somogyi (1933) reagent as modified by Hanes & Cattle (1938), (*b*) the iodometric Somogyi (1945*a*) reagent, or (*c*) the colorimetric Nelson (1944) reagent as modified by Somogyi (1952). The reagents were calibrated, as required, against glucose and maltose. Deproteinization, when necessary, was effected by $ZnSO_4$–$Ba(OH)_2$ (Somogyi, 1945*b*).

Glucose 1-phosphate. A slight modification of the method of Allen (1940) was used.

Nitrogen. Kjeldahl-N was determined by the method of Chibnall, Rees & Williams (1943).

Soluble protein. Soluble protein was extracted from fresh minced seaweed by gentle stirring at room temperature with a suitable solvent; the extract was centrifuged, and the supernatant solution dialysed, freeze-dried and analysed for Kjeldahl-N.

Viscosity measurements. The decrease in viscosity of solutions of certain polysaccharides was measured with an Ostwald viscometer with a 10 cm. capillary and flow time of 222 sec. for 10 ml. of water at 20°. Results are expressed in terms of the specific viscosity (i.e. relative viscosity − 1).

Substrates

Glycosides and sugar phosphates. Phenyl α- and β-D-glucosides were synthesized by the methods of Helferich & Smitz-Hillebrecht (1933) and Nath & Rydon (1954) respectively. Glucose 1- and 6-phosphates were prepared by Maung Khin Maung by the methods of McCready & Hassid (1944) and Viscontini & Olivier (1953) respectively. Dr D. J. Bell kindly provided specimens of phenyl and *n*-butyl β-D-galactosides. The other glycosides were laboratory or commercial specimens.

Di- and oligo-saccharides. Gentiobiose was prepared by the synthetic action of emulsin on glucose as described by Peat, Whelan & Hinson (1952). Isomaltose and isomaltotriose were isolated by charcoal–Celite chromatography of a partial acid hydrolysate of dextran; laminaribiose was prepared by similar means from laminarin. We are indebted to Miss M. Carter for a sample of xylobiose. Maltose was recrystallized thrice from 80% (v/v) aqueous ethanol and further purified by charcoal–Celite chromatography. Dr W. J. Whelan kindly provided a sample of maltotriose. The homogeneity of all other di- and oligo-saccharides was examined by paper chromatography.

Polysaccharides. We are indebted to Dr G. O. Aspinall for samples of esparto xylan (Chanda, Hirst, Jones & Percival, 1950) and ivory-nut mannan A (Aspinall, Hirst, Percival & Williamson, 1953); to Dr D. J. Bell for a sample of leafy-cocksfoot levan (Bell & Palmer, 1952); to Professor C. S. Hanes, F.R.S., for a sample of cellodextrin; to Dr D. H. Northcote for samples of yeast glucan (Bell & Northcote, 1950) and yeast mannan; to Dr B. Lindberg for a sample of pustulan (Lindberg & McPherson, 1954); to Professor I. A. Preece for a sample of barley β-glucosan (cf. Aspinall & Telfer, 1954); to Dextran Ltd. for a sample of dextran; and to Imperial Chemical Industries Ltd. for a sample of Cellofas B (sodium carboxymethylcellulose). Xylan was isolated from *R. palmata* by the method of Barry & Dillon (1940).

Reference carbohydrases

Emulsin. Emulsin (British Drug Houses Ltd., 10·5 g.) was suspended in water (175 ml.) and 0·2M acetate buffer (pH 5·0; 25 ml.), and dialysed against tap water at room temperature for 4 days, to lower the reducing sugar content. After centrifuging, the supernatant liquid was freeze-dried; yield 3·0 g.; N, 11·8%.

Barley 'laminarinase'. Flour (100 g.) from Spratt–Archer barley was extracted with 3% (w/v) KCl solution (350 ml.) by gentle stirring at room temperature for 2 hr. To the supernatant solution, obtained by centrifuging, ammonium sulphate was added to 0·75 saturation. The precipitated protein was suspended in water, and dialysed for 4 days against running tap water. During dialysis a precipitate formed and was removed by centrifuging; yield 1·1 g.; N, 6·4%. The remaining solution was freeze-dried and the laminarinase activity of the resulting powder was determined (Table 4); yield 1·8 g.; N, 10·1%.

Enzymic reactions

Qualitative demonstration of carbohydrase activity. Carbohydrate (approx. 30 mg.) was incubated with freeze-dried extract (approx. 30 mg.) suspended in 0·07–0·20M acetate buffer (pH 5·0; 3 ml.). The digests were examined at intervals by paper chromatography, the results being expressed as follows: (3+) end-products detected within 1–3 days of incubation; (2+) within 3–7 days; (+) after 7 days; (±) very slight activity. By this method, reproducible results have been obtained. Unless otherwise stated, enzymic reactions were carried out at 35°, aseptic conditions being maintained by use of toluene.

Quantitative demonstration of carbohydrase activity. Digests containing carbohydrate (approx. 50 mg.), freeze-dried extract (approx. 50 mg.), 0·2M acetate buffer (pH 5·0) and water to a final volume of 40–50 ml. were set up. The reducing power of 5 ml. portions was determined at intervals; as the extracts (0·1%, w/v) had no reducing power and did not interfere with the Somogyi reagents, deproteinization was unnecessary. In a single experiment (with yeast glucan), 1·3% (w/v) of extract was used, and the samples were accordingly deproteinized. The effect of pH on the 'maltase' and 'laminarinase' activities of the extracts was investigated by incubating substrate (10 mg.), extract (12 mg.), water (10 ml.) and B.D.H. Universal Buffer (pH 4·0–9·0; 10 ml.) at 35°. The reducing power of 5 ml. portions was determined after 24 or 48 hr. In similar experiments on the salicinase activity, the digests comprised salicin (15 mg.), extract (30 mg.), water (7 ml.) and B.D.H. Universal Buffer (pH 4·4–7·0; 5 ml.).

RESULTS

Extraction of soluble protein from marine algae

Although in exploratory experiments a variety of methods were used in attempts to disintegrate algal fronds and stipes (e.g. by homogenization, freezing at −35° and milling, freeze-drying and milling), mechanical mincing in ice water proved to be the most satisfactory and has been used throughout this study. A variety of solvents have been used to extract protein from the minced algae; the results obtained with *R. palmata* are recorded in detail in Table 1; essentially similar results were obtained with *L. digitata* stipe.

Since extraction with 0·25% (w/v) Na_2CO_3 gave material with the highest 'protein' content, this solvent has been used in later extractions, even though extraction with distilled water and 20% ethanol gave a greater total yield of crude protein.

Preparation of whole extracts of marine algae

Several extracts from the four algae have been prepared during the past 3 years. The method of preparation has been modified slightly on occasions, but a typical preparation is described below.

Fresh hand-pressed algae (2–3 kg.), minced with ice water, was extracted by stirring with 0·25% (w/v) Na_2CO_3 (3–4 l.) at room temperature for 15–18 hr. The pH of the solution, originally approx. 9, fell slowly to a final value of 6–7. (If the pH of a *L. digitata* extract falls below 5, co-precipitation of protein and alginate occurs.) After centrifuging (2000 *g* for 20–30 min.), the extract was dialysed against running tap water for 3–4 days to remove free sugars, amino acids, peptides and other material. Ammonium sulphate was added to 0·7–0·8 saturation, and the precipitated protein was collected by centrifuging at 0°. No further precipitate was formed on increasing the $(NH_4)_2SO_4$ above 0·8 saturation. (If the dialysis stage is omitted, the precipitation of protein by $(NH_4)_2SO_4$ is retarded and is incomplete.) The precipitated protein was redissolved in water, and reprecipitated with $(NH_4)_2SO_4$ at approx. 0·7 saturation. The resulting precipitate was centrifuged, dissolved in water, dialysed until free of $(NH_4)_2SO_4$ and freeze-dried.

The yields of crude protein extracted from the four marine algae, in one series of experiments, are recorded in Table 2; the weights of extracts used in the following experiments refer to these freeze-dried materials. In some extracts, a precipitate of inert material appeared during the dialysis and was discarded; in other experiments, a proportion of the material precipitated by $(NH_4)_2SO_4$ was found to be insoluble in water, and since it had only weak carbohydrase activity it was also discarded. Experiments with *L. digitata* fronds showed that although the residue from the original extraction still contained appreciable amounts of nitrogenous material (N, 1·9%), it showed little or no carbohydrase activity. Control experiments have shown that the carbohydrase activity of the extract (with the exception of that towards starch) is unaffected by common inorganic ions, and that dialysis does not appreciably diminish the activity.

Table 1. *Kjeldahl*-N *content of extracts of* Rhodymenia palmata

Minced algae (50 g.) was extracted with 100 ml. of solvent at room temperature for 18 hr. The extracts were then centrifuged, freeze-dried and analysed for Kjeldahl-N.

Solvent	Weight of material extracted (g.)	N content (%)
Distilled water	0·75	3·0
0·25% (w/v) Na_2CO_3	0·44	3·7
3% (w/v) KCl	0·69	2·1
20% (v/v) Ethanol	1·21	1·6
Water, saturated with *n*-butanol	1·00	1·4
0·2M Borate buffer, pH 9·0	0·41	2·3
0·2M Phosphate buffer, pH 8·0	0·47	3·1
Solvent A*	0·63	1·0
Solvent B†	1·10	0·9

* Solvent A: 0·95% (w/v) borax solution–ethanol–ether (10:4:1, by vol.).

† Solvent B: 0·2M Borate buffer (pH 9·0)–water–ethanol–*n*-butanol (5:5:4:1, by vol.).

Specificity of carbohydrase activity

The seaweed extracts were tested for activity towards various groups of similar carbohydrates. The results, obtained by paper chromatography, were essentially similar for all four extracts; those for *C. rupestris* are as follows, activity being expressed as detailed under Methods.

α-Glucosides. (3+) Isomaltose, isomaltotriose, maltose, maltotriose, phenyl α-glucoside; (2+) sucrose, trehalose; (±) methyl α-glucoside.

β-Glucosides. (3+) Aesculin, amygdalin, arbutin, cellobiose, gentiobiose, helicin, laminaribiose, phenyl β-glucoside, salicin; (±) methyl β-glucoside, phloridzin.

Miscellaneous glycosides. (3+) Xylobiose; (+) lactose; (±) melibiose; no activity towards *n*-butyl and phenyl β-galactosides, hesperidin, methyl α-galactoside, methyl α-mannoside, quercitrin, raffinose and rutin.

Polysaccharides. (3+) Barley β-glucosan, cellodextrin, Floridean starch, glucan (yeast), glycogen, laminarin, lichenin, soluble starch, xylan (*Rhodymenia*); (2+) mannan (ivory nut); (+) carob gum, xylan (Esparto); no activity towards fucoidin, inulin, levan, mannan (yeast) and pustulan.

Table 2. *Extraction of soluble protein from marine algae*

The method of extraction is described in the Results section.

Species	Place and date of collection	Weight of algae extracted (g.)	Weight of soluble extract (g.)	N content (%)
*Cladophora rupestris**	Dunbar (22. i. 54)	2900	42·2	8·8
Laminaria digitata†	North Berwick (1. xii. 53)	3550	2·7	5·5
*Rhodymenia palmata**	North Berwick (24. ix. 53)	2600	2·4	7·2
*Ulva lactuca**	North Berwick (29. vi. 54)	2500	16·0	7·7

* Whole plant minced. † Stipes only minced.

The extracts thus contain α-glucosidase, β-glucosidase, xylobiase, lactase, amylase, mannanase, xylanase and β-glucosanases capable of hydrolysing both 1:3- and 1:4-glucosidic linkages.

Examination of the paper chromatograms from the above experiments has provided evidence for the type of polysaccharase action, since step-wise hydrolysis ('exo' action) of a polysaccharide yields only the constituent monosaccharide(s), whereas random hydrolysis ('endo' action) gives rise, at intermediate stages, to a series of oligosaccharides. During the hydrolysis of laminarin by *L. digitata* extract a series of reducing sugars was produced; the sugar with the highest chromatographic mobility had the same R_G value as authentic glucose. Random hydrolysis of β-1:3-glucosidic linkages had therefore occurred. The xylanase activity of the extracts is also 'endo' action since pentoses with the R_G values of xylose, xylobiose, xylotriose (and higher xylosaccharides) are produced; these di- and oligo-saccharides are not reversion products since incubation of xylose (7 %) with *C. rupestris* or *U. lactuca* extract under identical conditions did not result in the synthesis of xylosaccharides within 21 days.

The enzyme action on maltose, laminarin, starch and xylan does not involve phosphorolysis. After incubation of *C. rupestris* extract with maltose, laminarin or starch, 0·1 M phosphate buffer (pH 6·7), 0·2 % ammonium molybdate (glucose 1-phosphatase inhibitor) and 1·4 mM mercuric chloride or 0·01 M sodium fluoride (phosphoglucomutase inhibitors), for 4–7 days, glucose 1-phosphate could not be detected. Furthermore, paper chromatographic analysis of a digest of *R. palmata* extract, xylan (*Rhodymenia*) and phosphate buffer showed sugar phosphates to be absent.

In control digests from which the algal extract had been omitted, or in those containing boiled extract, free monosaccharides were not liberated on incubation for periods up to 28 days. The observed activity is therefore due to thermolabile factors in the algal extract and not to the adventitious presence of toluene-resistant carbohydrase-producing micro-organisms in the buffer and carbohydrate solutions.

Action on α-glucosides and α-1:4-glucosans

The four algal extracts contain enzymes catalysing the hydrolysis of maltose and other α-glucosides, and of glycogen and starch. Qualitatively, maltose was hydrolysed more readily than the other α-glucosides, and, in dilute solution, enzyme action was essentially complete. Thus, *R. palmata* extract (0·04 %) caused a slow hydrolysis of maltose (0·04 %) at pH 5·1 and 35°, a constant level of 91 % conversion being attained in 10 days.

After prolonged incubation (15 days) of maltose (1 %) with an extract of *L. digitata*, in addition to glucose, the formation of a reducing sugar (hereafter designated M 1) with a chromatographic mobility lower than maltose was observed. In further experiments with *C. rupestris* and *U. lactuca* extracts and with higher concentrations of maltose, the synthesis of two additional reducing sugars (M 1 and M 2) has been shown. For example, in more concentrated solution (7 % maltose) glucose, maltose and traces of M 1 were detected within 4 days, and, after 8 days, glucose, maltose, M 1 and M 2 were present, whilst in a digest containing 17 % maltose chromatographic evidence for the synthesis of M 1 and M 2 was obtained after only 24 hr. incubation at 35°. Control experiments have shown that no such synthesis takes place in digests containing boiled seaweed extracts. On incubation with glucose (7 %) no synthesis of any sugars occurred; the formation of M 1 and M 2 from maltose is therefore due to trans-α-glucosylase activity. A comparison of the R_G values of M 1 and maltotriose, and of their corresponding *N*-benzylglycosylamines (cf. Bayly & Bourne, 1953) suggests that M 1 is maltotriose; this suggestion is supported by the fact that M 1 is completely hydrolysed to glucose and maltose by unpurified salivary amylase. Attempts to induce transglucosylase activity by using maltose as glucosyl donor, and methanol, fructose or galactose as glucosyl acceptors have not yet been successful.

Transglucosylation of α-1:6-linkages has also been demonstrated; in a digest consisting of isomaltose (7 %) and *C. rupestris* extract (0·3 %), chromatographic evidence for the formation of a higher saccharide with R_G 0·14 was obtained after 6 days incubation. On continued incubation, the amount of this sugar slowly increased. Isomaltotriose has R_G 0·14 (cf. maltotriose R_G 0·29).

The effect of pH on the 'maltase' activity of the extracts from *R. palmata*, *C. rupestris* and *U. lactuca* has been investigated, and reducing-sugar estimations show that activity is optimum at about pH 6.

The extracts show marked activity towards α-1:4-glucosans. On incubating soluble starch (0·1 %) with *C. rupestris* extract (0·15 %) at pH 5·1, slow hydrolysis to give 41 % conversion into glucose was observed in 70 hr. Under similar conditions, potato amylose and glycogen gave 32 and 13 % glucose, respectively. In the course of purification of the algal amylase, we have noted that the amylase activity may be differentiated from the α-glucosidase activity. On addition of Cl^- to an extract, the amylase activity is selectively increased; in addition, the amylase is more thermolabile than the α-glucosidase.

Action on β-glucosides and β-glucosans

The preliminary experiments have shown the extracts to possess group-specific hydrolytic activity towards β-glucosidic linkages since they readily hydrolyse aromatic β-glucosides, and disaccharides containing β-1:3-, β-1:4- and β-1:6-glucosidic linkages. In these digests (excepting that with cellobiose) glucose was the sole end product of enzymic action; with cellobiose, however, a series of reducing sugars were produced which will be referred to as C 1, C 2, C 3 in order of decreasing chromatographic mobility. From paper chromatographic evidence, C 1 (R_G 0·76) is tentatively identified as laminaribiose, and C_3 (R 0·17) as cellotriose. C_2 has R_G 0·33 (cf. cellobiose, R_G 0·55; gentiobiose, R_G 0·40). Since enzymic synthesis of higher sugars from glucose does not occur under these conditions, the formation of laminaribiose and the cellosaccharides must be ascribed to trans-β-glucosylase activity by the algal extracts.

β-Glucosidase action on salicin has been used to standardize the activity of various β-glucosidase preparations (Veibel, 1950); accordingly, quantitative measurements of the hydrolysis of salicin by the most active seaweed extract (from *C. rupestris*) have been made, and compared with a dilute solution of emulsin under the same conditions. On the basis of equal weights of protein N, the salicinase activity of emulsin was approx. 600 times greater than that of the extract of *C. rupestris*.

The effect of pH on the 'salicinase' activities of the unfractionated extracts from *C. rupestris*, *U. lactuca* and *R. palmata* has been studied; reducing power estimations show the activities to be greatest in the range pH 6–7.

In view of the pronounced hydrolytic activity of the *C. rupestris* extract towards β-glucosans (laminarin, lichenin, glucan), further experiments on the mode of enzyme action have been carried out. Thus in order to decide whether 'laminarinase' activity was due to a single enzyme (capable of hydrolysing both laminaribiose and, in random fashion, laminarin) or to a number of enzymes, the activity of *C. rupestris* towards laminarin (and cellodextrin) under various conditions was examined. Digests containing laminarin or cellodextrin, phosphate buffer (pH 2·9–9·0) and normal, or heated extract were incubated at 35° and examined chromatographically at intervals (Table 3). It is concluded that laminarin is hydrolysed by multi-enzyme action, one component catalysing random scission of β-1:3-linkages, and a second component catalysing stepwise hydrolysis; these components may be differentiated by the greater heat lability of the former. Cellodextrin is also hydrolysed in random fashion, but the enzyme involved differs from the endo-β-1:3-glucosanase since it is relatively heat stable. The extracts of *C. rupestris* thus contain at least three enzymes which can hydrolyse β-glucosidic linkages, viz. an endo-β-1:3-glucosanase, an endo-β-1:4-glucosanase (belonging, therefore, to the cellulase class of hydrolases) and an exo-β-glucosanase. This latter enzyme is probably responsible for the group-specific β-glucosidase activity previously noted.

Examination of the same cellodextrin digests after 7 and 14 days also revealed the presence of a sugar having R_G 0·71 (? laminaribiose) which had presumably arisen by trans-β-glucosylase action similar to that previously observed with cellobiose.

Quantitative estimations of the 'laminarinase' activity (endo and exo activity) of three seaweed extracts have been made, and compared with the

Table 3. *Effect of* pH *and heat on the 'laminarinase' and 'cellodextrinase' activities of* Cladophora rupestris *extract*

Substrates (200 mg.), 0·07 M phosphate buffer (4 ml.), and extract (15 mg.) were incubated at 35°. In digests 5 and 10, the buffer and extract were heated at 60° for 15 min. before addition of the substrate. The digests were examined by paper chromatography after 2 days (cellodextrin) or 7 days (laminarin).

Digest no.	Substrate and conditions	Reducing sugars present*				
		Glucose	R_G 0·65	R_G 0·33	R_G 0·46	R_G 0·08
1	Laminarin, pH 2·9	2+	2+	2+	—	—
2	Laminarin, pH 5·1	3+	2+	+	—	—
3	Laminarin, pH 7·0	2+	+	+	—	—
4	Laminarin, pH 9·0	+	+	+	—	—
5	Laminarin, pH 5·1 (heated)	2+	—	—	—	—
6	Cellodextrin, pH 2·9	2+	—	—	2+	2+
7	Cellodextrin, pH 5·1	3+	—	—	3+	3+
8	Cellodextrin, pH 7·0	2+	—	—	2+	2+
9	Cellodextrin, pH 9·0	+	—	—	+	+
10	Cellodextrin, pH 5·1 (heated)	2+	—	—	2+	2+

* 3+, Intense spot on chromatogram; 2+, moderate spot; +, weak spot; —, no detectable spot.

Table 4. *Enzymic hydrolysis of laminarin*

Digests contained laminarin (16–24 mg.), freeze-dried extract of *R. palmata*, *C. rupestris* or *U. lactuca* (20 mg.) or of barley (5 mg.) and 0·2 M acetate buffer (pH 5·1) and water to 35–50 ml. After incubation at 35°, 5 ml. portions were analysed for reducing sugar.

Time (days)	Apparent percentage conversion into glucose			
	*R. palmata**	*C. rupestris*	*U. lactuca*	Barley
1	49	52	28	70
2	68	74	47	87
3	75	90	62	—
4	—	—	84	91

* After 7 and 12 days' incubation, the percentage hydrolysis was 84 and 90 respectively.

'laminarinase' activity of an extract of Spratt–Archer barley (Table 4). The 'laminarinase' activity of the barley extract is seen to be several times greater than that of the seaweed extracts, on the basis of equal weights of protein N, although in all digests approx. 90 % conversion into glucose was eventually obtained. The 'laminarinase' activities of the extracts of *C. rupestris*, *R.palmata* and *U. lactuca* are optimum at about pH 5·5, 6·0 and 6·3 respectively.

To show that the above activity was not due to microbial contamination of the freeze-dried extracts, the laminarinase activity of an aqueous extract was quantitatively compared with that of extracts from which micro-organisms had been removed. An aqueous solution of *U. lactuca* extract (1 mg./ml.) was divided into three portions which were treated as follows: (1) filtered by gravity, using a Whatman no. 1 paper; (2) filtered through a Seitz bacteriological filter; (3) centrifuged at 90 500 *g* for 30 min. (by courtesy of Dr C. T. Greenwood). Each solution (10 ml.) was then incubated with 0·2 M acetate buffer (pH 5·0; 5 ml.) and an aqueous solution of laminarin (1·7 mg./ml.; 5 ml.) at 35° for 48 hr. The reducing powers of 5 ml. portions of the digests were equivalent to 3·32, 3·35 and 3·35 ml. of 0·01 N sodium thiosulphate respectively, showing that no loss of laminarinase activity occurred during the procedures carried out to remove micro-organisms from the extracts. The above experiment, together with the control experiments previously reported, show that the digests are not contaminated with carbohydrase-producing micro-organisms.

The hydrolysis of yeast glucan by *C. rupestris* extract has also been examined; after incubation of glucan (0·13 %) with extract (1·3 %) at pH 5·1, 73 and 76 % conversion into glucose was observed after 3 and 6 days respectively.

In view of the marked hydrolytic activity of the extracts towards cellodextrin, it was of interest to examine the activity towards a high-molecular-weight cellulose derivative. Sodium carboxymethylcellulose (Cellofas B) was therefore incubated with freeze-dried extracts of *C. rupestris* and *U. lactuca*, and the change in specific viscosity was determined (Fig. 1). Reducing power estimations at 18 hr. indicated an apparent percentage hydrolysis (as glucose) of 8 and 10 respectively. Chromatographic examination of the digests showed the presence of a series of reducing sugars varying from glucose (R_g 1·0) to zero R_g value, providing further evidence for the presence of endo-type β-1:4-glucosanases in the extracts.

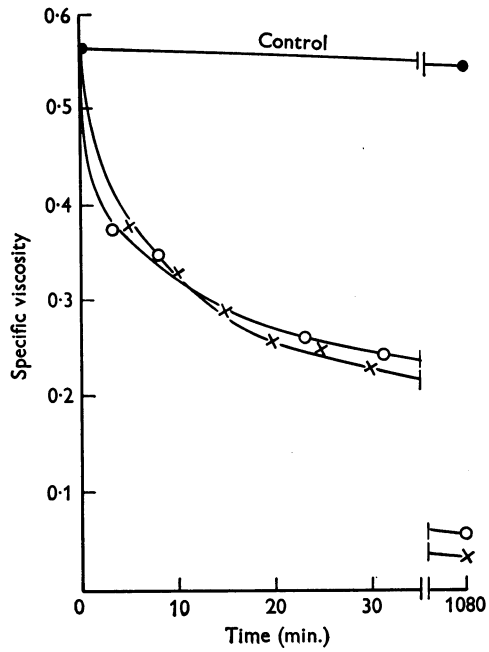

Fig. 1. Viscosity reduction of sodium carboxymethylcellulose by extracts of *C. rupestris* (×) and *U. lactuca* (○). The digests contained 20 ml. of 0·25 % (w/v) Cellofas B and 20 ml. of 0·2 M acetate buffer (pH 5·0) in which 50 mg. of extract was dissolved, and were incubated at 20°. Viscosity measurements were made at intervals.

DISCUSSION

The present preliminary investigation has shown that unfractionated extracts of *L. digitata*, *R. palmata*, *C. rupestris* and *U. lactuca* contain essentially similar complements of carbohydrases, including an α-glucosidase, β-glucosidase, amylase, β-1:3- and β-1:4-glucosanase, xylanase and mannanase; in addition, weak hydrolytic activity towards lactose has been demonstrated. The carbohydrase complements are therefore very similar to those of cereal extracts, although the relative activities are much lower. Attempts (unreported) to detect hydrolytic activity towards alginic acid and pectin by viscometric methods have not been completely successful, although a small decrease in the specific viscosity of the polyuronides after incubation with an extract of *C. rupestris* or *U. lactuca* for 2–7 days has been observed. During this incubation, free uronic acids were not produced. Qualitatively, the activity of the extracts towards the majority of substrates tested increased in the order *L. digitata* < *R. palmata* < *U. lactuca* < *C. rupestris*, and paralleled that of the observed yield of crude protein extracted from the algae (Table 1). It is probable that in *L. digitata*, extraction of protein was retarded by the presence of alginic acid; attempts to remove alginic acid selectively from the extract by precipitation as the calcium salt were unsuccessful, and resulted in coprecipitation of calcium alginate and protein. Smith & Young (1953) observed that under more drastic conditions (0·33 % sodium hydroxide, pH 13; 55°) only about 50 % of the protein-N of *Fucus vesiculosus* could be extracted, and suggested that extraction of protein was impeded by alginate.

For the present discussion, the hydrolytic activity of the extracts towards a number of α- and β-glucosides will be referred to as 'α-glucosidase activity' or 'β-glucosidase activity' respectively; the available data do not yet permit a distinction to be made between activity due to a single group-specific α- (or β-)glucosidase and that of a mixture of closely related α- (or β-)glucosidases with a higher degree of specificity.

The α-glucosidase activity resembles that of yeast α-glucosidase in that the rate of hydrolysis of α-glucosides increases in the order methyl α-glucoside, phenyl α-glucoside and maltose, and is most active at approx. pH 6 (Gottschalk, 1950). The hydrolysis of α-1:6-linked glucosaccharides by the algal extracts is an interesting feature of their activity since 'isomaltase' activity has been reported in only a limited number of biological sources, e.g. cell-free extracts of *Clostridium acetobutylicum* (French & Knapp, 1950) and extracts of brewers' yeast (Manners & Khin Maung, 1955). The specificity of β-glucosidase activity follows that reported for β-glucosidases from other biological sources, methyl β-glucoside being hydrolysed at a lower rate than phenyl β-glucoside or salicin (Veibel, 1950). The algal β-glucosidases differ from emulsin in that they show no synthetic activity towards glucose (cf. Peat, Whelan & Hinson, 1952). Several examples of carbohydrases from plant and mould sources which show both hydrolytic and transglycosylase activity have been reported recently (e.g. Pazur & French, 1952; Buston & Jabbar, 1954), and it is now clear that the algal carbohydrases likewise show dual activity.

Although separation of the 'cellodextrinase' and 'laminarinase' activities of the *C. rupestris* extract has not yet been attempted, evidence for the presence of two distinct endo-type enzymes has been obtained (Table 3); the endo-β-1:3-glucosanase activity is inactivated at 60°, whereas the endo-β-1:4-glucosanase and β-glucosidase are only partly inactivated. Dillon & O'Colla (1950) have noted the similar extreme thermolability of wheat laminarinase.

Hydrolytic activity towards laminarin has been reported with almond emulsin, which yields glucose as the initial and sole product of a step-wise action, and with carbohydrases from wheat, barley and rye, which catalyse random hydrolysis of the laminarin, yielding glucose, laminaribiose and higher β-1:3-glucosaccharides as the initial products (cf. Peat, Thomas & Whelan, 1952; Dillon & O'Colla, 1950, 1951; Manners, 1952, 1955). The algal extracts therefore resemble the cereals in type of laminarinase action. Dillon & O'Colla (1951) reported that extracts of barley, active towards laminarin, had no action on yeast glucan, and would only partially hydrolyse (20 %) an oxidized water-soluble glucan. In contrast, Manners (1952) observed that glucan, although insoluble, was slowly hydrolysed by a barley extract, yielding over 70 % glucose; our extract from *C. rupestris* thus shows a similar 'glucanase' activity.

The activity of the algal extracts towards hemicelluloses is of interest, in view of the limited occurrence of hemicellulases. The algal extracts produce xylosaccharides from xylan, and also slowly hydrolyse xylobiose, although the available evidence does not differentiate between single- and multi-enzyme action. The extracts of the two members of the Chlorophyceae also show appreciable mannanase activity, and hydrolyse the β-1:4-mannosidic linkages in ivory-nut mannan and carob gum; in contrast, no action on the α-mannosidic linkages in the highly branched yeast mannan has been detected.

The separation and purification of the several carbohydrases present in the extract of *C. rupestris*

is now being investigated. Preliminary experiments have shown that, on a micro scale, a partial separation of activities can be effected by paper electrophoresis, and, on a larger scale, by fractionation with ammonium sulphate and organic solvents at low temperatures. Later communications in this series will describe the properties of the purified carbohydrases, the chemical characterization of the oligosaccharides produced by transglucosylase action, and the phosphatase and proteinase activity of the *C. rupestris* extract.

SUMMARY

1. A survey of the carbohydrase activity of unfractionated extracts of four species of marine algae has been carried out.

2. The extracts show hydrolytic activity towards a number of α- and β-glucosides, lactose, mannan, xylan, starch, glycogen, laminarin, lichenin, glucan, cellodextrin and sodium carboxymethylcellulose.

3. Under certain conditions, enzymic synthesis of higher saccharides from maltose, isomaltose and cellobiose has been observed.

4. The hydrolytic activity of an extract of *C. rupestris* towards cellodextrin and laminarin has been shown to be due to separate enzymes.

5. The effect of pH on the hydrolytic activity of the extracts towards maltose, salicin and laminarin has been examined.

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