CARBOHYDRATES OF *Acetabularia* SPECIES

PART I. *A. crenulata**

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ABSTRACT

The green seaweed *Acetabularia crenulata* is shown to synthesise D-glucose, fructose, sucrose, *ribo*-hexulose, *myo*-inositol and a second alcohol (tentatively identified as *allo*-quercitol), aqueous alcohol-soluble inulin-type oligosaccharides, a water-soluble inulin-type polysaccharide, and a complex acid-containing polymer. Alkali extracts a polydisperse mannan which has an average chain-length of ~14, β-(1→4)-linked D-mannose residues, and some degree of branching.

INTRODUCTION

The carbohydrates of *Acetabularia* (mermaid's wine glass), a unicellular green seaweed, have not previously been studied systematically by chemical means. *A. mediterranea* was reported[1] to contain (paper chromatography) fructose, sucrose, and small amounts of glucose and fructose-containing oligosaccharides similar to those found in higher plants. Du Merac[2] noted that spherocrystals characteristic of inulin could be seen in *Acetabularia* after treatment with alcohol and that these had $[\alpha]_D$ −44° and gave X-ray powder photographs similar to those of inulin. Iriki and Miwa[3] treated *A. calyculus* with alkali, acid, and sodium chlorite, and then dissolved the residual fibres in 50% aqueous zinc chloride. Precipitation with acetone gave a polysaccharide, $[\alpha]_D$ −50.6° (*c* 1.54, 50% aqueous zinc chloride), which gave mannose on hydrolysis. Periodate-oxidation studies indicated a linear (1→4)-linked polysaccharide having a d.p. of 16.

The present study is on the carbohydrates extracted from *Acetabularia* which was harvested both at low tide and in deeper water in Jamaica, West Indies. It was identified as *A. crenulata* by the Marine Biological Station at Discovery Bay, Jamaica, West Indies, but the distinction from *A. mediterranea* is not clear.

DISCUSSION

The alga, which was highly calcified, was dried rapidly in the sun and stored in the dried form. The caps and stalks were separated and extracted sequentially with ethanol, water, and alkali. The percentage weights of carbohydrate in each of the extracts is given in Table I.

*Dedicated to Professor M. Stacey, C.B.E., F.R.S., in honour of his 65th birthday; submitted by Dr. E. Percival.

TABLE I

EXTRACTION OF *A. crenulata*

Extract	*Percentage of total dry weight*[a]	
	Stalks	*Caps*
Ethanol (80%): Carbohydrate content	0.78	2.6
Ketose content	0.7	0.9
Cold water: Fructan	0.08	0.7
Acid polysaccharide	0.46	1.5
Hot water: Fructan	0.4	10.3
Acid polysaccharide	0.8	1.6
4% Sodium hydroxide (mannan)	0.025	0.02
20% Sodium hydroxide (mannan)	0.95	1.1
Residue on hydrolysis gives mannose		

[a]For polysaccharides, these percentages are after dialysis and purification.

From the ethanolic extracts, carbohydrates of low molecular weight and oligosaccharides were separated by paper chromatography. The identity of D-glucose was confirmed with D-glucose oxidase[4] and fructose by conversion into the crystalline 2,3:4,5-di-*O*-isopropylidene derivative. A second ketose-positive spot was characterised as *ribo*-hexulose by comparison (paper chromatography and g.l.c.) with authentic D-*ribo*-hexulose isolated from *Itea virginiana* by the method of Hough and Stacey[5]. This is the first time that this sugar has been isolated from any organism other than *Itea*. *myo*-Inositol was identified by its chromatographic mobility and by g.l.c. of the *O*-trimethylsilyl (TMS) derivative. A trace quantity of a second, crystalline alcohol was tentatively identified as *allo*-quercitol (1,2,3,4/5).

Sucrose and a number of non-reducing oligosaccharides were isolated, and these comprised an homologous series, since a straight line was obtained for the plot of $\log(1/R_F - 1)$ against d.p. The lower homologues up to the hexasaccharide were isolated and each gave increasing proportions of fructose and less glucose on hydrolysis. Methylation of the tetra- and penta-saccharides and characterisation of the methylated sugars showed the presence of end-group glucose and fructose and a larger proportion of (1→2)-linked fructose, showing the presence of a series of inulin-type oligosaccharides.

Examination of a hydrolysate of the aqueous extracts indicated the presence of complex polysaccharide material, comprising mainly fructose. but glucose, galactose, rhamnose, xylose, and uronic acid were also present in each extract. The polysaccharides were fractionated into neutral and charged material, and structural studies on the charged polysaccharide will be published elsewhere. Hydrolysis of each of the neutral polysaccharides gave mainly fructose with traces of glucose. The fructose was characterised as the crystalline 2,3:4,5-di-*O*-isopropylidene derivative. The quantity of neutral polysaccharide obtained from the stalks was too small to permit extensive structural studies, but it appeared to contain a higher proportion of

glucose. This may indicate the presence of a small proportion of a glucan, although a test for starch was negative.

The fructan from the caps, which had a similar rotation to that of inulin, was methylated and gave the same methylated sugars as the tetrasaccharide, thus proving the presence of a (1→2)-linked or inulin-type fructan. This was further confirmed by its infrared spectrum. Estimation of the proportion of glucose to fructose gave a ratio of 1:33 and hence an average chain-length for the fructan of 34.

The 20% alkaline extract was purified by precipitation as the copper complex. It gave mainly D-mannose on hydrolysis (characterised as the phenylhydrazone and as the TMS derivative of the sugar and alditol by g.l.c.) and a trace of glucose. Methylation and analysis of the derived methyl sugars showed that the mannan was mainly (1→4)-linked. A di-*O*-methyl sugar, tentatively identified as the 3,6-derivative, was present in approximately the same proportion as the tetra-*O*-methylmannose, indicating some degree of branching. Tentative evidence for the presence of end-group glucose was obtained. Parallel oxidations of the mannan and ivory-nut mannan gave a reduction of 0.94 and 1.05 moles of periodate/hexose residue, respectively. Mild hydrolysis of the polyalcohol from the oxidised and reduced, algal mannan and chromatographic analysis of the hydrolysate gave spots having the mobility of glycolaldehyde, erythritol, and glycerol, and spots with R_{Man} 0.72 (major) and 0.3. Separation and hydrolysis of the major spot gave mannose and erythritol. Complete, acid hydrolysis of the polyalcohol gave glycolaldehyde, erythritol, glycerol, and mannose. These products are consistent with (1→4)-linkage of the mannose residues. The presence of intact mannose confirmed the presence of branching in the mannan.

The proportion of tri- to tetra-*O*-methylmannose gave an average chain-length of 15. The formic acid released on periodate oxidation corresponded to an average chain-length of 14. Determination of the proportion of erythritol to glycerol in a total hydrolysate of the polyalcohol confirmed this finding.

In an attempt to obtain some estimate of the molecular size, the mannan was eluted from a Sephadex G100 column. The range of fractions in which the mannan was eluted indicates a considerable polydisperisty with an upper limit of molecular weight above 1×10^5, assuming that the mannan behaves in the same way as dextran on the Sephadex.

The overall results show that *Acetabularia* mannan is a β-(1→4)-linked, polydisperse polysaccharide of average chain-length 14 with a certain degree of branching, possibly at C-2. It is probable that some of the polydisperisty is due to degradation during the extraction procedures.

The results show that stalks and caps of *Acetabularia* metabolise the same carbohydrates, but the proportions of fructan and mannan differ in the two organs. The stalks synthesise a relatively higher proportion of mannan, which is to be expected since this appears to be the structural polysaccharide and the stalks require considerable rigidity in order to support the caps. Tentative evidence was obtained for a higher proportion of glucose-containing polysaccharide in the stalks. This could well have been present in the caps also, but the high proportion of fructan in the caps

made detection of a small amount of glucan impossible. Amyloplasts have been reported in *Acetabularia*[21], but all tests for starch in the present experiments were negative.

EXPERIMENTAL

All solutions were evaporated under diminished pressure below 50°. Specific rotations, unless otherwise stated, were measured in water at 20° in a Perkin–Elmer 141 polarimeter. Paper chromatography was carried out on Whatman No. 1 and 3MM paper with the following solvent systems (v/v): (*A*) butyl alcohol–ethanol–water (40:11:19); (*B*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*C*) butyl alcohol–pyridine–water (6:4:3); (*D*) butanone half-saturated with water +1% ammonia; (*E*) chloroform–acetone–5M ammonium hydroxide (1:8:1); (*F*) benzene–ethanol (20:3). T.l.c. was performed on 0.25-mm thick silica gel. Papers were sprayed with (*1*) saturated, 80% ethanolic aniline oxalate; (*2*) urea–hydrochloride[7] for ketoses; (*3*) 2 parts of 2% sodium metaperiodate +1 part of 1% potassium permanganate or (*4*) dipped in silver nitrate[8]. Ionophoresis was carried out on Whatman No. 3 paper in 0.1M borate buffer at pH 9.8. G.l.c.[9] was performed on columns of acid-washed Celite coated with 7.5% by weight of poly(butane-1,4-diol succinate) (column *1*), 10% of polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] (column *2*), and 10% of poly(ethylene glycol adipate) (column *3*) at an operating temperature of 175° for methylated sugars. Retention times (*T*) are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. For the trimethylsilylated (TMS) sugars and alditols, the liquid phase was 7.5% of Apiezon K (column *4*) at 155° and 175°, and 3% of S.E.30 (column *5*) at 175°, and the retention times (*T*) are expressed relative to that of the TMS derivative of xylitol. All reductions were carried out with potassium borohydride in aqueous solution. Excess borohydride was destroyed and the cations were removed with Amberlite IR-120 (H^+) resin. Boric acid was removed by methanol. The carbohydrate content of the polysaccharides was determined by the phenol–sulphuric acid method and calculated from appropriate graphs[10]. The ketose content was measured by the method of Arni and Percival[11]. Unless otherwise stated, hydrolyses were with 90% formic acid in sealed tubes in an atmosphere of carbon dioxide for 6 h at 100°, followed by hydrolysis of the formic esters by dilution (5 vol.) and further heating for 2 h at 100°. Conversion of methylated sugars into the methylated glycosides was with anhydrous methanol and Amberlite IR-120 (H^+) resin[20].

Extraction of the weed. — The caps and stalks of *Acetabularia* were separated, and each was extracted and investigated separately. Where the constituents were similar, the extracts were combined. The dried, powdered, calcified material [caps (30 g) and stalks (12 g)] was subjected to sequential extraction with (*a*) 80% ethanol at 70° under reflux, (*b*) cold water, (*c*) hot water in an atmosphere of carbon dioxide, (*d*) 4% aqueous sodium hydroxide at room temperature, and (*e*) 20% aqueous sodium hydroxide at room temperature. Both alkaline extractions were under

nitrogen. The percentage yields of carbohydrate isolated from the different extracts are given in Table I.

Ethanolic extract. — Both extracts were concentrated and deionised with Amberlite IR-120 (H^+) and IR-4B (OH^-) resins. The derived, neutral material was separated by preparative chromatography on 3MM paper into mono- and oligosaccharides as follows. The identity of D-glucose, which had the same chromatographic mobility as an authentic sample in all solvents, was confirmed by its response to a D-glucose oxidase spray.

Fructose was identified by paper chromatography and g.l.c., and as 2,3:4,5-di-*O*-isopropylidenefructopyranose[12], m.p. and mixed m.p. 93–95°.

Sucrose was separated as a non-reducing syrup having a chromatographic mobility identical with that of authentic material and giving a blue spot with spray *2*. Hydrolysis of an aliquot gave equal quantities of glucose and fructose.

ribo-Hexulose, R_{Glc} 1.45 (solvent A), 1.74 (solvent *B*), and 1.31 (solvent *C*), had chromatographic properties identical with those of an authentic sample of D-*ribo*-hexulose from *Itea virginiana*[5]. G.l.c. of the TMS derivatives of the unknown and authentic material gave (column *4* at 175°) three peaks having identical retention times of 1.74 (strong), 1.93 (weak), and 2.0 (strong). On column *5*, a single peak with *T* 1.46 was given by both materials.

myo-Inositol had the chromatographic mobility of authentic material in solvents *A*–*C*. G.l.c. (column *5*) of the TMS derivative gave a single peak, *T* 4.55, identical with that of the TMS derivative of authentic *myo*-inositol.

A very small amount of crystalline material, R_{Glc} 1.40 (solvent *B*), was separated in one experiment. It had m.p. 262–263°, undepressed on admixture with *allo*-quercitol, m.p. 263°. Ionophoresis in molybdate buffer[22] gave a spot having a mobility similar to that of *allo*-quercitol. All attempts to isolate more of this alcohol were unsuccessful.

Oligosaccharides. — After separation of the monosaccharides, the oligosaccharides were re-run in solvents (*A*) and (*B*) and sprayed with reagent *2*. A large number of ketose-containing oligosaccharides, including sucrose, was revealed. By plotting log $(1/R_F - 1)$ against d.p., straight lines were obtained for the spots with both solvents.

The lower homologues up to the hexasaccharide were separated by preparative chromatography on Whatman 3MM paper in solvent A. On hydrolysis with 2% oxalic acid at 80° for 2 h, each gave mainly fructose with less glucose. The tetra- and pentasaccharides were methylated by one Haworth[13] and two Purdie[13] procedures. G.l.c. of the derived methyl glycosides[20] on columns *1*, *2*, and *3* gave peaks corresponding to those given by methyl 2,3,4,6-tetra-*O*-methylglucosides, 1,3,4,6-tetra-*O*-methylfructosides, and 3,4,6-tri-*O*-methylfructosides. The tri-*O*-methylfructose was separated by paper chromatography in solvent *D*, On ionophoresis at 3000 volts for 1 h, it gave a single spot M_{Glc} 0.52 (*cf.* 3,4,6-tri-*O*-methylfructose, 0.52; 1,3,4-tri-*O*-methylfructose, immobile).

Cold and hot water extracts. — Each of the extracts from the caps and stalks

was dialysed separately and freeze-dried. An aliquot of each was hydrolysed and analysed by paper chromatography. Spots with the mobility of fructose (major), glucose, galactose, rhamnose, xylose, and glucuronic acid were detected in each of the hydrolysates. The only discernible difference was a higher proportion of glucose in the hydrolysate of the stalk extract. Each extract was separated into neutral and acid polysaccharides on a DEAE-cellulose (Cl^-) column (See Table I). The neutral polysaccharides were eluted with water, and the acidic polysaccharides with M potassium chloride. Each was dialysed, concentrated, and freeze-dried. Little neutral material was obtained from the stalk extracts, and no further work was done on these polysaccharides except for hydrolysis and paper chromatography which revealed spots having the mobility of glucose and fructose.

The neutral polysaccharide from the caps had $[\alpha]_D$ −36.3° (*c* 0.5, water) (*cf.* inulin $[\alpha]_D$ −40°) and a carbohydrate content of 80%. It gave (paper chromatography) only fructose (major) and glucose on hydrolysis. The average ratio between glucose and fructose was determined by measuring the total carbohydrate[10] and total fructose[11] contents. After hydrolysis with 2% oxalic acid at 80° for 2 h, the fructose was isolated by preparative, paper chromatography and converted into the 2,3:4,5-di-*O*-isopropylidene derivative, m.p. and mixed m.p. 93–95°. Methylation of an aliquot as for the oligosaccharides, followed by hydrolysis and glycosidation[20] of the methylated material, gave the same methylated sugars as for the oligosaccharides, except that the tri-*O*-methylfructose was present in larger proportion.

The i.r. spectrum (KBr) of the fructan gave bands at 930, 890, and 815 cm^{-1}. An identical spectrum was given by inulin[14].

20% Sodium hydroxide extracts. — On hydrolysis, these extracts gave only mannose and traces of glucose (paper chromatography). The respective extracts were purified by precipitation as the copper complex. After filtration and washing with water, the mannan was recovered as a white powder by treating the complex with 1% ethanolic hydrogen chloride. The mannan from the stalks had a carbohydrate content of 100%, and that from the caps 93%. The cap mannan had $[\alpha]_D$ −38° (*c* 0.25, 90% formic acid) (calculated on the carbohydrate content) (*cf.* the mannan[15] from *Codium fragile*, $[\alpha]_D$ −41°). Hydrolysis gave mainly mannose, $[\alpha]_D$ +17.5° (*c* 0.4), and traces of D-glucose identified by a D-glucose-oxidase spray. The mannose was isolated, and characterised as the phenylhydrazone[16], m.p. and mixed m.p. 198–200°, and by g.l.c. as the TMS derivatives of the sugar and alditol. On column *4* at 175°, peaks with *T* 1.65 and 2.83 were given by mannose, and a peak at *T* 2.60 by the alditol.

Methylation of the mannan. — The method followed was that of Unrau[17] which consists of one Haworth methylation[13] and one Sandford and Conrad[18] methylation. T.l.c. then indicated that the methylation was complete[17]. T.l.c. (solvent *F*) of a hydrolysate of the methylated material revealed three spots having R_F 0.33, 0.16 (major), and 0.05, respectively. G.l.c. of the derived methyl mannosides[20] on columns *1*, *2*, and *3* gave the same peaks as an authentic mixture of methyl 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-mannosides. A small peak for methyl 2,3,4,6-tetra-*O*-methyl-

β-D-glucoside (*T* 1.0) could be detected, but any α-D-glucoside was masked by the mannosides. An additional peak was observed on columns *2* and *3* (*T* 4.48 and 6.50, respectively). This is in the region of peaks given by di-*O*-methylmannosides. All the methyl di-*O*-methylmannosides, except the 3,6-di-*O*-methylmannoside, were available, but none of them gave peaks having the same retention times as the unknown, nor did it correspond to any of the methyl di-*O*-methylglucosides. The respective areas of the peaks given by the methyl tetra- and tri-*O*-methylmannosides indicated a ratio of ~1:15.

Periodate oxidation of the mannan. — Cap mannan and ivory-nut mannan [a linear β-(1→4)-linked mannan[20]] (0.2 mmole of each) were treated with 0.5mM sodium metaperiodate in acetate buffer (pH 3.6, 10 ml) in the dark at 2°. The reaction was complete after 20 h, when 0.94 mole of oxidant per "anhydrohexose" unit had been reduced by both mannans. At room temperature, the ivory-nut mannan continued to reduce periodate to a value of 1.05 mole, whereas the cap-mannan value remained constant. The reaction was stopped with ethylene glycol, and the mixture was set aside for 2 h and then dialysed and reduced. After removal of the boric acid by evaporation with methanol, the polyalcohols were precipitated with ethanol. The cap-mannan polyalcohol gave a positive test for carbohydrate and that from ivory nut a negative test. Paper and thin-layer chromatography (solvent *E*, spray *3*) of hydrolysates of the two polyalcohols revealed the presence of glycolaldehyde, glycerol, and erythritol. The cap-mannan polyalcohol also gave traces of mannose. Glycerol and erythritol were also confirmed by g.l.c. of the TMS derivatives on column *4* at 155°.

An aliquot (11 mg) of the polyalcohol from the cap mannan was hydrolysed with 0.5M sulphuric acid (1 ml) for 5 h at room temperature. Paper chromatography (solvent *C*) gave, in addition to spots having the mobility of glycolaldehyde, erythritol, and glycerol, two spots R_{Man} 0.72 (major) and 0.3. The 0.72 component was isolated by paper chromatography and hydrolysed to give (paper chromatography) erythritol and mannose.

Average chain length. — The areas of the peaks given by erythritol and glycerol on g.l.c. were measured, and the amounts present read from standard graphs of these two substances. Periodate oxidation was carried out under the same conditions as before, but in aqueous solution in the absence of buffer. The release of formic acid was measured by titration with 5mM sodium hydroxide (carbonate-free) and corresponded to 72 mmoles per "anhydrohexose" unit.

Molecular weight determination. — A solution of the mannan (5 mg) in 10% aqueous sodium hydroxide was freed from excess alkali by dialysis and concentrated. The residue was applied to a column (2.5 × 30 cm) of Sephadex G100 which had a void volume of 50 ml (Blue dextran). Fractions (2 ml) were collected and monitored[10].

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REFERENCES

1 H. CLAUSS AND K. KECK, *Planta*, 52 (1959) 543.
2 M. L. RUBAT DU MERAC, *Rev. Gen. Bot.*, 60 (1953) 689.
3 Y. IRIKI AND T. MIWA, *Nature (London)*, 185 (1960) 178.
4 M. R. J. SALTON, *Nature (London)*, 186 (1960) 966.
5 L. HOUGH AND B. E. STACEY, *Phytochem.*, 2 (1963) 315.
6 E. C. BATE-SMITH AND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
7 R. DEDONDER, *Bull. Soc. Chim., Biol.*, 34 (1952) 44.
8 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444.
9 G. O. ASPINALL, *J. Chem. Soc.*, (1963) 1676.
10 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
11 P. C. ARNI AND E. G. V. PERCIVAL, *J. Chem. Soc.*, (1951) 1827.
12 J. E. CADOTTE, F. SMITH, AND D. SPRIESTERSBACH, *J. Amer. Chem. Soc.*, 74 (1952) 1501.
13 E. L. HIRST AND E. PERCIVAL *Methods Carbohyd. Chem.*, 2 (1963) 146.
14 L. M. J. VERSTRAETEN, *Anal. Chem.*, 36 (1964) 1070.
15 J. LOVE AND E. PERCIVAL, *J. Chem. Soc.*, (1964) 3345.
16 E. BOURQUELOT AND H. HERISSEY, *C. R. Acad. Sci.* (Paris), 129 (1899) 339.
17 A. M. UNRAU AND J. M. CHOY, *Carbohyd. Res.*, 14 (1970) 151.
18 D. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508.
19 G. O. ASPINALL, R. B. RASHBROOK, AND G. KESSLER, *J. Chem. Soc.*, (1958) 215.
20 G. N. BOLLENBACK, *Methods Carbohyd. Chem.*, 2 (1963) 327.
21 D. C. SHEPHARD, *Biology of Acetabularia*, Academic Press, New York, 1970, p. 189.
22 E. J. BOURNE, D. H. HUTSON, AND H. WEIGEL, *J. Chem. Soc.*, (1960) 4252.