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LOW MOLECULAR WEIGHT CARBOHYDRATES AND WATER-SOLUBLE POLYSACCHARIDE METABOLIZED BY THE CLADOPHORALES

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Abstract—*Cladophora rupestris* was found to metabolize glucose, fructose, sucrose, and an homologous series of linear oligosaccharides containing (2→1) linked fructose residues attached to the fructose moiety of sucrose, giving inulin-type polymeric material. Nine other members of the Cladophorales and *Urospora bangioides*, a member of the Acrosiphoniales, have been examined for the presence of these oligosaccharides and for the nature of the water-soluble polysaccharide. The similarities and differences in these carbohydrates in the various species are described.

INTRODUCTION

THE ARTIFICIAL taxonomic boundaries within the green and yellow-green algae are often indistinct and some are in dispute. Very little is known about the carbohydrates metabolized by many of these algae, and it is hoped that a knowledge of them will help to establish affinities between different genera. Since fairly extensive studies[1–4] on the water-soluble sulphated polysaccharide from *Cladophora rupestris* have established its main structural features, it was decided to extend these studies to the low molecular weight carbohydrates metabolized by this alga, and then carry out a preliminary study of these two types of carbohydrate in other species of the Cladophorales. The present paper contains a detailed investigation of the major sugars and oligosaccharides found in *C. rupestris*. Three other species of *Cladophora*, three species of *Rhizoclonium*, two species of *Chaetomorpha* and *Urospora bangioides* have also been examined for these carbohydrates and for the water-soluble sulphated polysaccharide.

RESULTS AND DISCUSSION

It was found that 80% ethanol extracted monosaccharides and a series of fructose-containing oligosaccharides from *C. rupestris*. Concentration to small volume and addition of ethanol to 98% precipitated all the oligosaccharides from the trisaccharide upwards, and these were found to comprise an homologous series, since a straight line was obtained when the value of log $(1/R_f-1)$ was plotted against degree of polymerization of each of the oligosaccharides[5] (including sucrose). The constitution of the oligosaccharide presumed from the graph to be the hexasaccharide was confirmed, by separation, hydrolysis and GLC,[4] to contain glucose to fructose in the ratio of 1:5. That these oligosaccharides are

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[2] E. HIRST, W. MACKIE, and E. PERCIVAL, *J. Chem. Soc.* 2958 (1965).
[3] P. G. JOHNSON and E. PERCIVAL, *J. Chem. Soc.* 906 (1969).
[4] E. J. BOURNE, P. G. JOHNSON and E. PERCIVAL, *J. Chem. Soc.* in press (1970).
[5] E. C. BATE-SMITH and R. G. WESTALL, *Biochim. Biophys. Acta.* 4, 427 (1950).

built up from sucrose by the addition of fructose units to C-1 of the non-reducing fructose end unit (Fig. 1) was confirmed by hydrolysis, methylation and infrared studies. Fructose

$$\underset{\text{sucrose}}{\text{F}(2\rightarrow1)\text{G}} \rightarrow \text{F}(2\rightarrow1)\text{F}(2\rightarrow1)\text{G} \rightarrow \text{F}(2\rightarrow1)\text{F}(2\rightarrow1)\text{F}(2\rightarrow1)\text{G etc.}$$

FIG. 1.

and glucose were the only sugars obtained on acid hydrolysis of this ethanol insoluble material and the fructose was by far the major sugar. Further confirmation of this structure was obtained by separation and methylation of the oligosaccharides which had an R_f of *ca.* 0·1. Analysis of the derived methylated sugars as their glycosides by GLC showed that 3,4,6-tri-*O*-methylfructose was the major sugar with small amounts of 2,3,4,6-tetra-*O*-methylglucose and 1,3,4,6-tetra-*O*-methylfructose. Paper chromatography and paper electrophoresis of an acid hydrolysate of the methylated fraction confirmed this and showed the absence of any lower methylated sugars. The 3,4,6-tri-*O*-methylfructose was separated and further characterized as the 1,2-isopropylidene derivative. From these results it can be seen that the seaweed is gradually building up a linear 2,1-linked fructan of the inulin-type. Infrared studies confirmed this conclusion; the same absorption bands were obtained as those found by Verstraeten[6] for inulin.

The results of 80% ethanolic extraction of samples of *C. sericea*, *C. laetevirens*, *C. glomerata*, *Rhizoclonium implexum*, *R. riparium*, a fresh-water species of *Rhizoclonium*, *Chaetomorpha melagonium*, *Ch. linum* and *Urospora bangioides* can be seen in Table 1. It is of interest that the fresh-water species of *Rhizoclonium*, marine *Ch. melagonium*, *Ch. linum* and *U. bangiodes* were devoid of these inulin-type oligosaccharides. Examination of *Ulva lactuca*, *Enteromorpha compressa*, *Codium fragile* and *Microspora* species showed that these too do not metabolize these oligosaccharides. Sucrose in varying relative proportions was present in all the species examined (see Table 1). Furthermore an unknown fructose-containing oligosaccharide which travelled just behind sucrose on a paper chromatogram in certain solvents was found in *C. laetevirens* and the fresh-water *Rhizoclonium* sp. A detailed structural investigation of this will be published elsewhere.

As a result of studies by other workers[7–9] on four species of the *Dasycladales* it has been suggested[9] that the group as a whole may be labelled as 'fructosan-algae'. Our results indicate that many members of a much larger group comprising the Cladophorales also metabolize fructans and that the occurrence of these in green algae may be much more widespread than hitherto supposed.

All the samples of Cladophorales metabolized sulphated water-soluble polysaccharides resembling that from *C. rupestris* and containing galactose, arabinose and xylose together with small quantities of rhamnose. The proportions of the major sugars vary from species to species (see Table 1). In this respect it should be emphasized that each sample of seaweed was extracted under exactly the same conditions; cold water or more drastic conditions of extraction would probably yield a water-soluble polysaccharide with more variable proportions of constituent sugars.

The polysaccharide from *C. rupestris* is a highly branched polysaccharide with the majority of the arabinose present as blocks of some eight 1,4-linked arabinose units joined

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[7] M. L. RUBAT DU MÉRAC, *C. R. Acad. Sci., Paris* **241,** 88 (1955).
[8] M. L. RUBAT DU MÉRAC, *C. R. Acad. Sci., Paris* **243,** 714 (1956).
[9] B. J. D. MEEUSE, *Acta Botan. Neerl.* **12,** 315 (1963).

together by single galactose units, with all the xylose and some of the galactose on the periphery of the molecule.[4] In the other species so far examined there does not appear to be any fixed difference in the proportion of the sugars in the different genera. In all of them, xylose is the minor sugar and the amount present varied independently of the variation in arabinose and galactose. It is probable that a family of polysaccharides is metabolized all built up on the same general plan but with different degrees of branching in the various species.

TABLE 1. CARBOHYDRATES OF SOME ALGAE

Algal species	Marine or fresh	Time place	80% Ethanol extract: Relative Fructan	Relative sucrose	Relative glucosyl fructose	Water soluble polysaccharides: Approx. propor. sugars	$AgNO_3$ on papers	GLC ratio
Cladophora rupestris	M	various	▬	■	nil	Gal Ara Xyl	+++ +++ +	1·0 1·0 0·4
*Cladophora sericea**	M	Scotland Aug. 1969 April 1970	▬	■	nil	Gal Ara Xyl	++ ++++ ++	1·00 1·27 0·80
Cladophora laetevirens	M	Scotland Sept. 1969 April 1970	▬	■	▬	Gal Ara Xyl	++ +++++ ++	1·00 1·80 0·90
Cladophora glomerata	F	Surrey 1969	■	■	nil	Gal Ara Xyl	++ +++ +	1·00 0·90 0·66
Rhizoclonium implexum	M	Scotland 1969	▬	■	nil	Gal Ara Xyl	++++ ++ ++	1·00 0·63 0·45
Rhizoclonium riparium	M	Scotland 1969	▬	■	nil	Gal Ara Xyl	++++ ++ ++	—
Rhizoclonium sp.	F	Surrey 1969 Scotland 1970	nil	■	▬	Gal Ara Xyl	++ +++ ++	1·00 1·13 0·62
Chaetomorpha melagonium	M	Scotland 1969	nil	▬	nil	Gal Ara Xyl	++ +++ +	1·00 1·20 0·45
Chaetomorpha linum	M	Scotland 1969 1970	nil	▬	nil	Gal Ara Xyl	++ +++ +	—
Urospora sp.	M	Scotland 1969, 1970	nil	■	nil	Polysac. contains xylose, rhamnose and mannose		

Key: Gal = galactose; Ara = arabinose; Xyl = xylose.
* May have been contaminated with *C. laetevirens*.

Urospora occupies a somewhat precarious taxonomic position in the Acrosiphoniales.[10] In the present study it was found that the water-soluble polysaccharide of *Urospora* is quite unlike those of the Cladophorales or the Acrosiphoniales[11] so far studied (see Table 1). It consists mainly of xylose, rhamnose and mannose, and structural studies of this polymeric material are in progress.

It is surprising that the relatively labile fructose-containing oligomers found in these seaweeds persisted, in apparently their original concentration, in samples of dried *Rhizoclonium* and *Cladophora* which had been stored for 11 and 8 yr respectively. The rest of the extractions were made on fresh samples of algae collected at different seasons from March to September 1969 and again in April 1970 (see Table 1). Each collection was extracted separately and no seasonal variation could be detected either in the 80% ethanolic or the aqueous extract. Neither did there appear to be any difference in an alga collected from sites as far apart as Nova Scotia, the south of England and north west Scotland.

Given then the standard conditions of extraction, it follows that the derived water-soluble polysaccharide has a definite constitution for a given species and this is constant and independent of the biological age, storage age and geographical location in which the weed grows. Studies of this kind should therefore be of help to the taxonomist.

EXPERIMENTAL

Algal Material

The marine algae were collected as follows: *Rhizoclonium implexum* from St. Margaret Bay, Nova Scotia, September 1958; *Cladophora rupestris*, east coast of Scotland, near Dunbar 1961; (both air-dried); *C. rupestris*, Churchill Rocks, Bexhill, England, December 1969. *C. sericea*, *C. laetevirens*, *Rhizoclonium implexum*, *R. riparium*, *Chaetomorpha melagonium*, *Ch. linum* and *Urospora bangioides* were all collected from rock pools, Farr Bay, Sutherland, Scotland, during April, May and September 1969 and *C. sericea*, *C. laetevirens*, *Chaetomorpha linum* and *Urospora* were collected again from the same site in April 1970. Fresh-water samples of *C. glomerata* were collected in Surrey in 1969 and of *Rhizoclonium* sp. in Surrey in 1969 and in Scotland in 1970.

General Methods

Evaporations were done at 40° under reduced pressure. Solvents for paper chromatography on Whatman No. 1 filter paper were: (1) *n*-BuOH–EtOH–H_2O (40:11:19), (2) EtCOMe half sat. with water contg. 1% NH_4OH, (3) *n*-BuOH–pyridine–H_2O (6:4:3), (4) *iso*-PrOH–EtCOMe–$HCONMe_2$–H_2O (50:25:5:20),[12] (5) *n*-BuOH–H_2O,[13] and the reducing aldoses were located by (a) aniline oxalate, the ketoses by (b) urea-hydrochloride[14] and by (c) *p*-anisidine reagent[14] and both reducing and non-reducing sugars with (d) $AgNO_3$/NaOH.[15] All the sugars and their derivatives were characterized by co-chromatography. R_s values refer to the distance migrated relative to sucrose. Carbohydrates were assayed by the phenol-sulphuric acid method.[16] Paper electrophoresis was conducted in Whatman No. 1 filter paper at 3000 V 60 mA for 1 hr using borate buffer (pH 10)[17] and acetic acid–pyridine buffer at pH 6·5 at 2000 and 30mA. GLC (Pye-Argon gas chromatograph) of mixtures of *O*-methyl sugars was carried out on columns of (1) 15% by weight of butan-1,4-diol succinate polyester on 80–100 mesh Celite at 175° and (2) 10% polyphenyl ether *m*-bis (*m*-phenoxy-phenoxybenzene). Retention times (T) of methylated sugars are given relative to that of 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside, and of the trimethylsilyl (TMS) ethers of the sugars relative to xylitol. Galactose, arabinose and xylose were estimated as the TMS ethers. The polysaccharides (30 mg) were hydrolyzed (N-H_2SO_4, 4 hr at 100°) and the hydrolysates were neutralized with $BaCO_3$, filtered, deionized with Amberlite IR-120(H^+) resin and evaporated and dried at 60° for 10 min *in vacuo* and further dried by evaporation with ethanol, ethanol and benzene, and benzene to ensure complete removal of water.

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[11] J. J. O'DONNELL and E. PERCIVAL, *J. Chem. Soc.* 2168 (1959).
[12] H. H. SCHLUBACH and H. O. A. KOEHN, *Ann. Chem.* **614,** 126 (1958).
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[14] L. HOUGH, J. K. N. JONES and W. H. WADMAN, *J. Chem. Soc.* 1702 (1950).
[15] W. E. TREVELYAN, D. R. PROCTER and J. S. HARRISON, *Nature* **166,** 444 (1950).
[16] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH, *Anal. Chem.* **28,** 350 (1956).
[17] D. J. BELL and D. H. NORTHCOTE, *Chem. & Ind.* 1328 (1954).

Hexamethyldisilazane (0·2 ml), chlorotrimethylsilane (0·1 ml) and pyridine (1 ml) were added, the solutions were shaken for 10 min, kept for 30 min and then centrifuged. The centrifugate was concentrated to dryness and the residue, dissolved in *n*-hexane, was examined by GLC using a column of 7·5% Apiezon K on 80–100 mesh Celite at 177°. The ratios of galactose to arabinose to xylose were estimated from the peak areas obtained on GLC by comparison with calibration graphs.[4]

Extraction of Carbohydrate

The fresh weeds and the two dried samples were each frozen in liquid N_2 and ground to a powder and the latter extracted with 80% aq. EtOH (2 × 24 hr) at 20–50° followed by thorough washing of the residual weed. The combined ethanolic extracts and washings were concentrated to a dark green syrup. To this, ethanol (200 ml) was added with stirring and the mixture set aside for 20 hr. The precipitated pale green solid was removed by filtration and quickly transferred to cold water (8 ml) and thoroughly stirred. Residual dark green particles were removed by filtration and the pale green filtrate poured into ethanol (240 ml, i.e. 96%). The cloudy precipitate was left to settle overnight. The supernatant was decanted off and the precipitate triturated with ethanol and then filtered. The derived pale buff hygroscopic powder was dissolved in the minimum of cold water and freeze-dried to an off-white very hygroscopic powder which hereinafter is known as the crude fructose-containing oligosaccharides (A). In a typical experiment 1·1 g was obtained from 35 g of powdered dried *C. rupestris*. The first ethanolic filtrate was concentrated to a syrup (B).

The residual weed remaining after ethanolic extraction was treated with hot water (2 hr at 100°), filtered, and thoroughly washed. The combined filtrate and washings was concentrated to small volume and poured into ethanol with stirring. The precipitate (hereinafter called the water-soluble polysaccharide) from *C. rupestris* had a carbohydrate content of 50%. (Found: SO_4^{2-}, 21%, N_2, 2·0%.)

Characterization of the Fructose-containing Oligosaccharides

The crude precipitate (A) on paper chromatography, eluted for 5 days with solvent (1) and sprays (b) and (c), showed a number of spots from R_s 0·42 (trisaccharide) right back to the starting line. The value of log $(1/R_f-1)$ of the spots was plotted against degree of polymerization. The material with mobility R_s 0·18 was separated on a preparative scale and hydrolyzed. The proportion of the derived glucose to fructose was determined by GLC by measurement of the respective peak areas[4] and found to be 1:5.

In some species (see Table 1) an additional strong ketose-staining spot R_s 0·78 (solvent 1), R_s 0·80 (solvent 3), R_s 0·98 (solvent 4) and R_s 0·63 (solvent 5) was obtained.

Hydrolysis of an aliquot of (A) with either N-H_2SO_4 (1 hr at 100°) or 2% oxalic acid (4 hr at 80°), neutralization with $CaCO_3$ and deionization with IR 120 (H^+) and IR 45B(OH^-) gave on concentration a syrup. Paper chromatographic analysis of this syrup revealed the presence of fructose and glucose. The latter was characterized with glucose oxidase spray.[18]

A portion of higher molecular weight material in (A) was separated from the smaller oligosaccharides on 3 MM papers and the part just in front of the starting line eluted. This (100 mg) in water (2 ml) was methylated with Me_2SO_4 (2·5 ml) and 30% NaOH (6·5 ml) added portionwise at 0° with stirring in N_2 during 2 hr. Stirring was continued overnight at room temp. The mixture was neutralized with 8 N-H_2SO_4 and the pH adjusted to 9 with aq. NaOH. Extraction with $CHCl_3$ and removal of the latter gave a pale yellow syrup. This was remethylated with MeI (8 ml) and Ag_2O (3 g) under reflux for 8 hr. The silver salts were removed by filtration and extracted with hot $CHCl_3$. The combined filtrate and $CHCl_3$ extracts were concentrated to a clear yellow syrup. An aliquot was refluxed with 4% MeOH–HCl for 6 hr, neutralized with Ag_2CO_3 and the derived mixture of glycosides examined on GLC on columns (1) and (2). The major peaks corresponded with the retention times of 3,4,6-tri-*O*-methylfructose (col. 1, T, 1·88, 1·49, 2·47, 2·0; col. 2, T, 1·03, 1·22, 0·85, 1·4) and small peaks for 2,3,4,6-tetra-*O*-methylglucose (col. 1, T, 1·0, 1·43; col. 2, T, 1·0, 1·36) and 1,3,4,6-tetra-*O*-methylfructose (col. 1, T, 0·98, 1·19; col. 2, T, 1·01, 1·22) were also obtained. A second aliquot of the methylated material was warmed in 2·25% oxalic acid (4 ml) and methanol (4 ml) at 25° for 90 min. Additional 2·25% oxalic acid (2 ml) was added, the temperature raised to 98° for 1 min (to remove the methanol) and then heated at 85° for 9 hr.[19] The mixture was neutralized with $CaCO_3$ and then extracted with $CHCl_3$. Removal of the solvent gave a yellow oil. Paper chromatographic analysis in solvents (1) and (2) confirmed the presence of the above methylated sugars and the absence of any lower methylated sugars. Paper electrophoresis in borate buffer[19] showed that the major methylated sugar was mobile whereas 1,3,4-tri-*O*-methylfructose and the tetra-*O*-methyl sugars are immobile. The 3,4,6-tri-*O*-methylfructose was separated from a portion of the methylated hydrolysate on several electrophoretograms (borate, 0·1 M). It was eluted from the electrophoretogram with acetone and the eluate concentrated to 1 ml. Conc. H_2SO_4 (0·01 ml) was added and mixture stored at −18° for 48 hr.[20] It was then made alkaline with 50% 0·880–NH_4OH (*ca.* 2 ml), filtered and concentrated to a syrup, which was taken up in ethanol. Paper chromatographic analysis in solvent (1) with authentic 3,4,6-tri-*O*-methylfructose (R_f 0·76) and 1,2-iso-propylidene

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3,4,6-tri-*O*-methylfructose (R_f 0·92) gave two spots R_f 0·92 (major) and 0·76 (trace). The authentic isopropylidene derivative also showed a trace of the trimethylfructose. Elution of the immobile material from the electrophoretograms and analysis on a paper chromatogram (solvent 1) gave only the tetra-*O*-methylsugars and showed no trace of 1,3,4-tri-*O*-methylfructose.

The mixture of oligosaccharides (A) was further purified by repeated dissolution in water and precipitation with ethanol until what appeared to be a white microcrystalline powder was obtained. This was examined by i.r. spectroscopy in a KBr-disk; absorption bands at 820, 877 and 933 cm^{-1} were obtained.

Examination of Syrup (B)—Low Molecular Weight Carbohydrate

Paper chromatography in solvents 1–4 of syrup (B) revealed the presence of glucose, fructose and sucrose. The glucose was confirmed with glucose oxidase.[18]

Examination of the Water-soluble Polysaccharide

An aliquot of each of the polysaccharides extracted by hot water and precipitated with ethanol was hydrolyzed with N-H_2SO_4 at 100° for 4 hr and the cooled hydrolysate was neutralized with $BaCO_3$ and the concentrated filtrate analysed by paper chromatography and on GLC as the TMS derivatives. The peak areas of galactose (T, 2·1; 2·9), arabinose (T, 0·8; 1·0) and xylose (T, 1·4, 1·9) were measured and related to standard graphs of known weights of these sugars[4] and from these the relative proportions of the sugars in each polysaccharide was calculated.

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