Carbohydrate Research, 72 (1979) 165–176

THE EXTRACELLULAR POLYSACCHARIDES OF *Porphyridium cruentum* AND *Porphyridium aerugineum*

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(Received June 20th, 1978; accepted for publication, July 17th, 1978)

ABSTRACT

The extracellular mucilages from *Porphyridium cruentum* and *P. aerugineum* contain D-xylose, D-glucose, D- and L-galactose, 3-*O*-methylxylose, 3- and 4-*O*-methylgalactose, and D-glucuronic acid in the approximate molar proportions of 3:1:2.5:0.13:0.13:0.8 and 1.7:1:1.1:0.3:0.6:0.5, respectively. In addition, *P. cruentum* mucilage contains 2-*O*-methylhexose (0.13) and 2-*O*-methylglucuronic acid (0.2), whereas *P. aeruginum* mucilage is devoid of these two sugars but contains 2,4-di-*O*-methylgalactose (0.5). Both polysaccharides contain ~10% of half-ester sulphate and appear to be linked to ~5% of protein. Attempted fractionation into homopolysaccharides was unsuccessful. Methylation, periodate oxidation, and partial hydrolysis studies revealed that the glucuronic acid is 1,3-linked and is attached solely to O-3 of D-galactose in both mucilages. The 2-*O*-methylglucuronic acid in *P. cruentum* is linked to O-4 of L-galactose. Xylose, glucose, and galactose are present in both mucilages as end groups, and 1,3- and 1,4-linked residues, with galactose and glucose also present as 1,3,4-linked or sulphated residues. Molecular weight determinations on Sepharose 4B indicate a $\overline{M}_w$ of 4×10^6 for *P. cruentum* mucilage and 5×10^6 for that from *P. aerugineum*.

INTRODUCTION

P. cruentum is a unicellular, marine red alga which occurs as numerous cells embedded in a gelatinous polysaccharide matrix[1]. Chemical analysis of a hydrolysate of this polysaccharide[2,3] revealed the presence of galactose, glucose, xylose, uronic acid, and a wide variety of amino acids. The mucilage contains 6–7% of protein, and 10% of ester-bound sulphate. The uronic acid was shown to be glucuronic acid, and the galactose to be 90% of the L and 10% of the D isomer[3]. In the polysaccharide, the xylose and galactose residues were found[3] to be 1,4-linked with branches at C-3 of xylose and C-3 and C-6 of galactose. The glucose and glucuronic acid are 1,3-linked, with some branching at C-6 of glucose. Viscous solutions of the polymer were obtained at concentrations of 1% or less, which showed good pH and temperature stability. Other studies[4] confirmed some of this work and showed that protein

is linked to carbohydrate *via* serine–xylose and threonine–xylose linkages. The polysaccharide was reported to comprise D- and L-galactose, D-glucose, xylose, D-glucuronic acid and its 2-*O*-methyl derivative, and sulphate in the molar proportions 2.12:1.0:2.42:1.22:2.61, respectively. The hexose, uronic acid, and about half of the xylose residues were shown by their resistance to periodate oxidation to be 1,3-linked. The aldobiouronic acids 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactose, 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, and 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-glucose were isolated from partial hydrolysates.

P. aerugineum is a unicellular, freshwater red alga which is easily grown in artificial culture and which exudes a water-soluble polysaccharide mucilage. Ramus[5] showed that this contained 7.6% of half-ester sulphate and comprised xylose, galactose, and glucose. He tentatively identified small proportions of guluronic acid, galacturonic acid, another pentose, and an anhydrohexose. The molecular weight was reported to be in excess of 5×10^6.

The polysaccharides from *P. cruentum* and *P. aerugineum* [hereinafter called (Pc) and (Pa), respectively] investigated in this communication were supplied by Marine Colloids Inc., Rockland, Maine, U.S.A., as the sodium salts in a total of ~20 g from *P. cruentum* and 24 g from *P. aerugineum*.

RESULTS

The constituents of the two mucilages are given in Table I. The major difference between the two materials is the higher methoxyl content of (Pa).

Component sugars of the mucilages. — Hydrolysates of each mucilage were analysed as the alditol acetates by g.l.c.–m.s. The main constituents were a pentose and two hexoses. In addition, in the (Pc) hydrolysate, a 3-*O*-methylpentose and 2-, 3-, and 4-*O*-methylhexoses were also detected. The retention times of the methylated hexoses as hexitol acetates, relative to xylitol penta-acetate, were 0.60, 2.02, and 1.64, respectively. The mobility in p.c. and the retention times of the 3-*O*- and

TABLE I

APPROXIMATE PERCENTAGE COMPOSITION OF THE MUCILAGES

Source	*Composition (%)*						
	Moisture	*Ash*	*Carbohydrate*[a]	*Sulphate*[b]	*Uronic acid*[b]	*Methoxyl*[c]	*Protein*[c]
P. cruentum	5.4	13.5	63	11	13	0.62	3.8
P. aerugineum	10.0	8.0	68	9	9	3.6	3.8

[a]Phenol–sulphuric acid method[6]: values read from a standard graph made from a synthetic mixture of xylose, glucose, galactose, and glucuronic acid in the ratios of 3:2:3:2. [b]Expressed as percentage of the carbohydrate. [c]Percentage of total weight.

4-*O*-methylhexoses in g.l.c. indicated that they were galactose derivatives [see later for their characterisation in (Pa) hydrolysate]. The identity of the 2-*O*-methylhexose could not be determined; (Pa) was devoid of this component, but contained material having the retention time and breakdown pattern of a di-*O*-methylhexose.

After separation by p.c. of the constituents of the hydrolysates from the two mucilages, the following sugars were characterised.

Xylose was isolated from both hydrolysates as a reducing syrup that had the same mobilities and retention times as xylose in p.c. and g.l.c. The $[\alpha]_D$ values [+19.5° from (Pc) and +18° (Pa)] confirm that D-xylose (+19°) was present.

Galactose, isolated as a syrup from both mucilages, had the same mobility in p.c. and the same retention time in g.l.c. as galactose. The $[\alpha]_D$ values [+41° from (Pc) and +26° from (Pa); *cf.* +79° for D-galactose] indicated that 75% of the galactose in (Pc), and 67% in (Pa), was the D sugar. The response to D-galactose oxidase confirmed the presence of D-galactose in (Pc). Quantitative determination with D-galactose oxidase[7] showed that 75% of the galactose in (Pa) was the D isomer.

Glucose, isolated as a syrup from both mucilages, had the same mobility in p.c. and the same retention times in g.l.c. (both as the sugar and the alditol) as glucose. The response to D-glucose oxidase confirmed the presence of D-glucose, and the $[\alpha]_D$ value of +56° indicated the absence of any L-glucose.

3-*O*-Methylxylose, isolated as a pure syrup from (Pc), had the same mobility (R_{Glc} 2.1, solvent *B*) and the same retention time in g.l.c. as 3-*O*-methylxylose. The corresponding fraction (R_{Glc} 2.1) from the hydrolysate from (Pa), after demethylation[8], gave spots having the mobility of xylose and galactose in p.c. The retention time of the methylated galactose in g.l.c. indicated a di-*O*-methylgalactose. When analysed as the derived alditol acetate derivatives by g.l.c.–m.s., a 3-*O*-methylpentose and a 2,4-di-*O*-methylhexose were characterised. This fraction therefore contained 3-*O*-methylxylose and 2,4-di-*O*-methylgalactose.

3- and 4-*O*-Methylgalactose. A reducing syrup [R_{Glc} 1.6 (solvent *B*) and 1.3 (solvent *C*)], isolated from the hydrolysate of (Pa), gave only galactose on demethylation (p.c.). Analysis of the syrup by g.l.c.–m.s. of the derived alditol acetates showed that it could be either a 3- or a 4-*O*-methylhexose. Reduction of the syrup with sodium borodeuteride followed by hydrolysis and analysis by g.l.c.–m.s. gave a mass spectrum that showed peaks at *m/e* 189, 190, 261, and 262 characteristic of 3- (*m/e* 190, 261) and 4-*O*-methylhexose (*m/e* 189, 262). The intensities of the peaks indicated approximately equal quantities of each.

Electrophoresis of the hydrolysate at pH 6.7 and 10 indicated the absence of uronic acid and mannose, respectively, and use of the urea hydrochloride spray in p.c. showed the absence of ketose.

Characterisation of aldobiouronic acids. — From the hydrolysates of both mucilages, an oligouronic acid, $[\alpha]_D$ +19°, was isolated. It had d.p. 1.95, R_{Glc} 0.40, R_{GlcA} 0.40 (solvent *B*), and M_{GlcA} 0.65 (pH 6.7). After esterification and glycosidation, reduction, and hydrolysis, equal proportions of glucose and galactose were produced. These were confirmed as the D sugars with D-glucose and D-galactose oxidases.

Reduction, esterification, reduction, and hydrolysis yielded glucose and galactitol, indicating that the aldobiouronic acid is D-glucuronosyl-D-galactose. After esterification and reduction, the derived disaccharide was methylated and hydrolysed. Analysis of the derived methylated sugars by g.l.c.–m.s. of the partially methylated alditol acetates gave 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, thus showing that the aldobiouronic acid is 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. The β linkage was deduced from the $[\alpha]_D$ value (*cf.* 3-*O*-methyl-D-galactose, +87°; methyl α-D-glucosiduronamide, +136°; methyl β-D-glucosiduronamide, −47°.

A second aldobiouronic acid, $[\alpha]_D$ +20°, isolated from the (Pc) hydrolysate, had R_{Glc} 0.76 (solvent *B*) and M_{GlcA} 0.74 (pH 6.7). After esterification and glycosidation, reduction, and hydrolysis, equal quantities of 2-*O*-methyl-D-glucose and -L-galactose were present. G.l.c.–m.s. analysis of the derived alditol acetates showed galactose and 2-*O*-methylglucose to be the main constituents with a lesser amount of xylose. Further chromatography of this fraction gave a small quantity of (1→3)-linked xylobiose in addition to the aldobiouronic acid. The latter disaccharide was subjected to esterification and glycosidation, and reduction followed by methylation and hydrolysis. G.l.c.–m.s. of the derived, partly methylated alditol acetates showed 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol. The aldobiouronic acid is therefore 4-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-L-galactose. The α linkage was deduced from the $[\alpha]_D$ value (*cf.* 4-*O*-

TABLE II

APPROXIMATE PERCENTAGE COMPOSITION OF POLYSACCHARIDE HYDROLYSATES

	T[a]	Porphyridium cruentum		Porphyridium aerugineum	
		From g.l.c. peak areas[b] *(%)*	*From eluted fractions*[c] *(%)*	*From g.l.c. peak areas*[b] *(%)*	*From eluted fractions*[c] *(%)*
Xylose	1.0	32.8	35.0	29	29
Galactose	2.16	20.0	18.0	11	17
Glucose	2.35	12.4	12.0	18	20
3-*O*-Methylxylose	0.58	1.6		5	8[d]
2,4-Di-*O*-methylgalactose	1.35	—	—	11	
3- and 4-*O*-Methylgalactose	2.0	1.6	5.0[d]	5	5[d]
2-*O*-Methylhexose	1.64	1.6		—	—
Oligouronic acid (A)[e]			21		19
Oligouronic acid (B)[e]			4		—
Other oligosaccharides			5		2

[a]Retention time of alditol acetate relative to xylitol penta-acetate on column *6*. [b]Based on 70 and 79% of the carbohydrates as monosaccharides. [c]By phenol–sulphuric acid, after separation of sugars on a paper chromatogram. [d]As no standards were available, the absorptions were read from a standard graph of the free sugar. [e]Contains 50% of galactose.

methyl-L-galactose, −92°; methyl α-D-glucosiduronamide, +136°; methyl β-D-glucosiduronamide, −47°.

Relative proportions of the constituents. — The relative percentages of the constituents present in the hydrolysates were determined by measuring the carbohydrate content of each after elution from a paper chromatogram. At the same time, an aliquot of each hydrolysate, after conversion of the carbohydrates into the corresponding alditol acetates, was analysed by g.l.c. and the peak areas of the monosaccharides were measured. The latter method took no account of the oligosaccharides present in the hydrolysates [30% of the carbohydrate in (Pc), and 21% in (Ps)]. In order to compare the two results, it was necessary to adjust the proportions from the peak areas to correspond to 70% and 79%, respectively. When this adjustment is made (Table II), the results from the two methods are in good agreement.

Partial hydrolysis. — Various methods of partial hydrolysis, including autohydrolysis of the free-acid form of (Pa), were performed in order to maximise the yield of oligosaccharides. In every case, a complex mixture of neutral oligosaccharides together with the two aldobiouronic acids from (Pc) and the glucuronosyl-(1→3)-galactose from (Pa) were obtained. Repeated fractionation of the mixtures led to the isolation and tentative identification of the following disaccharides from both mucilages: D-glucosyl-(1→4)-galactose, D-glucosyl-D-xylose, and D-xylosyl-(1→3)-D-xylose. In addition to the neutral oligosaccharides isolated from the dialysate of the autohydrolysis of (Pa), xylose, glucose, galactose, 3-*O*-methylxylose, 3- and 4-*O*-methylgalactose, 2,4-di-*O*-methylgalactose, and a sulphated L-galactosyl-D-xylose, $[\alpha]_D$ −31°, were also isolated, but no uronic acids or their oligomers were present. The residual polymer (PaR) recovered in 60% yield by weight after autohydrolysis had a carbohydrate content of 80%, and was devoid of sulphate. The uronic acid and methoxyl contents had increased from 9 and 3.6% in the original polysaccharide to 20 and 4%, respectively, in (PaR). The increased carbohydrate content (68→80%) in (PaR) is explained by the loss of 9% of sulphate and its accompanying cations. The absence of free uronic acid, together with the doubling of the acid content in the residual polymer, is strong evidence that these residues are all present in the inner part of the molecules.

A hydrolysate of (PaR) contained all the sugars, all the derivatives, and the aldobiouronic acid present in a similar hydrolysate of the initial polysaccharide, but the proportions of glucose and galactose were considerably smaller, indicating that a large proportion of these units occurred on the periphery of the macromolecules.

Elution of the polysaccharides from a column of Sepharose 4B gave single peaks which corresponded to a molecular weight of the order of 4×10^6 for (Pc) and 5×10^6 for (Pa) (Fig. 1). (PaR) had a wide range of molecular weight distribution with a maximum concentration at 1×10^6 (Fig. 1). Since ~30% of the carbohydrate was lost during the autohydrolysis, it follows from the relatively small change in molecular size that most of the loss occurred from the periphery of the molecules and that few cleavages occurred in the inner regions of the macromolecules.

TABLE III

APPROXIMATE PERCENTAGES OF METHYLATED SUGARS FROM METHYLATED POLYSACCHARIDES

Sugar	P. cruentum	P. aerugineum	*Linkages present*
			Xylose
2,3,4-Tri-*O*-methylxylose	17.5	14	end group
2,4-Di-*O*-methylxylose	15.5	7	1,3-linked
2,3-Di-*O*-methylxylose	16.0	12	1,4-linked
			Glucose
2,3,4,6-Tetra-*O*-methylglucose	11.0	5	end group
2,4,6-Tri-*O*-methylglucose	10.0[a]	21[b]	1,3-linked
			Galactose
2,3,4,6-Tetra-*O*-methylgalactose	5.0	10	end group
2,4,6-Tri-*O*-methylgalactose	2.0[a]	[b]	1,3-linked
			Glucose/galactose
2,3,6-Tri-*O*-methylhexose	17.0[c]	15	1,4-linked
2,6-Di-*O*-methylhexose	5.5	12	1,3,4-linked
6-*O*-Methylhexose	0	5	1,2,3,4-linked

[a]The combined figure for the 2,4,6-tri-O-methylhexose was 12%. This was separated into percentages of glucose and galactose obtained from the methylated polyalcohols. [b]Contains both glucose and galactose. [c]It proved impossible to separate 2,3,6-tri-*O*-methyl-glucose and -galactose quantitatively.

The results of partial hydrolysis support the view that the mucilages consist of heteropolysaccharides containing a complex mixture of sugars. This view was confirmed by the failure to isolate different entities by elution from a DE52-cellulose column[9] or by precipitation with Cetrimide[10].

Methylation studies. — It was necessary to methylate twice by the Hakomori method, since a single methylation resulted in a high proportion of unmethylated sugars, particularly with (Pc). Table III lists the approximate proportions of the various methylated sugars and the linkages present, as determined by g.l.c. of the alditol acetates. Although the linkages were the same for the two polysaccharides, the relative proportions of the derived methyl sugars were different. (Pa) contains relatively less 1,3- and 1,4-linked xylose than (Pc) and appears to be much more highly branched. Analysis of hydrolysates of the methylated polysaccharides by electrophoresis showed that some uronic acid was still present as an aldobiouronic acid, which probably accounts for the low proportion of 1,3-linked galactose.

The high proportion of end groups to branch points may be explained by degradation of the polysaccharides during methylation. This view is substantiated by the change in molecular size that occurs (Fig. 1) for both polysaccharides.

Periodate oxidation. — Oxidation (5mM periodate) of (Pc) and desulphated (Pc) reduced 0.25 and 0.35 mol of periodate, respectively. The increased reduction of periodate by the desulphated mucilage indicates either depolymerisation or the presence of additional glycol groups, and that the sulphate had been linked to C-2 and/or C-3 of 1,4-linked units. Periodate oxidation of (Pa) slowly removed the

sulphate groups. The polyalcohol recovered in 69% from (Pc) had 48% of uncleaved sugars, and that (83%) from (Pa) had 44% of uncleaved sugars. Hydrolysis of the polyalcohols gave glucose, galactose, xylose, 3-*O*-methylxylose, 3- and 4-*O*-methylgalactose, glycerol, erythritol and threitol, and 1,3-linked aldobiouronic acid, together with a trace of 2-*O*-methylglucuronic acid from *P. cruentum*, and 2,4-di-*O*-methylgalactose from *P. aerugineum*. The erythritol and threitol were distinguished by ionophoresis in molybdate buffer[11]. No acidic fragments or glucuronic acid were detected. This fact and the presence of unoxidised aldobiouronic acid indicated that the uronic acid was linked through O-3, and confirmed that it was linked to O-3 of the galactose. The formation of glycerol confirmed the presence of 1,4-linked xylose, and erythritol and threitol indicated 1,4-linked glucose and galactose, respectively. Oxidation with 25mM periodate gave a similar pattern of cleaved sugars, but also hydrolysed the sulphate groups. The polyalcohols were resistant to periodate, proving that acetal formation[12] had not hindered the initial oxidation. Comparison of the proportions of galactose, glucose, and xylose in the hydrolysates of the polyalcohols derived from (Pc) and desulphated (Pc) showed that a larger quantity of xylose was present relative to glucose and galactose in the latter. More of the hexoses must have been cleaved by the periodate in the desulphated polysaccharide, and therefore these two sugars are linked to the majority of the sulphate. Methylation of the polyalcohols confirmed the oxidation results and also the earlier methylation results.

Some degradation of the mucilages occurred during periodate oxidation, but a substantial proportion of material of high molecular weight was present in the polyalcohols (Fig. 1). However, after cleavage of the acetal linkages by trifluoroacetic acid, the molecular weight of the majority of the material had diminished to 30,000, as in the methylated products (Fig. 1). This was confirmed by high-pressure liquid chromatography, the polyalcohol being eluted at the position of T70. It is tempting to suggest that this figure represents the weight of a repeating pattern in the polysaccharides, which still contains all the sugars and derivatives present in the initial materials.

Desulphation and infrared studies. — The sulphate content in (Pc) was unaffected by alkali[14], indicating that trans elimination *via* an epoxide ring does not occur. In contrast, the sulphate content of (Pa) decreased from 10 to 4% on similar treatment, and the partially desulphated polysaccharide was recovered in 87% yield. No new sugars or 3,6-anhydrogalactose could be detected in the hydrolysate, although the presence of initial sugars could have masked the colorimetric detection of the latter[15].

Treatment of both mucilages with methanolic hydrogen chloride completely removed the sulphate, and the partly degraded polysaccharides were recovered in 71% yield from (Pc) and 69% from (Pa). The infrared spectra of the polysaccharides showed absorbances at 1250–1260 cm^{-1} characteristic of the S=O stretching frequency, and absorbances for (Pc) at 820 and 830 cm^{-1}, and for (Pc) at 820 and 850 cm^{-1}. Absorbances at 820, 830, and 850 cm^{-1} are characteristic[16] of primary, equatorial, and axial half-ester sulphate groups, respectively. Partial removal of

TABLE IV

RELATIVE VISCOSITIES OF 0.35% SOLUTIONS OF *P. cruentum* AND *P. aerugineum* POLYSACCHARIDES AT 30°

	Relative viscosities	
	P. cruentum *(pH 9.5)*	P. aerugineum *(pH 8.9)*
Unheated	10	79
After heating to 70°	16	103
After autoclaving	11	59

sulphate from (Pa) by alkali considerably decreased the intensity of the absorption at 850 cm^{-1}. Complete desulphation of the polysaccharides removed all the above absorption bands. No absorption at 930–940 cm^{-1}, characteristic of 3,6-anhydro-galactose, could be detected in the sulphated or desulphated polysaccharides.

Viscosity measurements. — The relative viscosities of 0.35% aqueous solutions compared to that of water were measured under various conditions (Table IV). The polysaccharides are reasonably stable to sterilisation.

DISCUSSION

This is the first time that methylated sugars have been characterised in the hydrolysates of these two polysaccharides, where they comprise ~5% of the monosaccharides in (Pc) and as much as 20% of those in (Pa). Although *P. cruentum* is a marine alga and *P. aerugineum* a freshwater alga, their extracellular polysaccharides are very similar. They are both polymers of high molecular weight, their major sugars are the same, and these are linked together by the same linkages. The major differences are the presence of 2-*O*-methylglucuronic acid in (Pc) and 2,4-di-*O*-methylgalactose in (Pa). The half-ester sulphate groups in (Pc) are stable to alkali, whereas 60% of these groups in (Pa) are cleaved by alkali.

The results on (Pc) are mainly in agreement with those of Heaney-Kieras and Chapman[4], except that their polysaccharide contained a much higher proportion of L-galactose and their 1,3-linked aldobiouronic acid contained L-galactose, whereas in our studies it was the D sugar. The 2-*O*-methylglucuronic acid is linked to O-4 of L-galactose, whereas the earlier studies reported 3-linked D-galactose as the other residue.

The mucilage from *P. cruentum* investigated by Medcalf *et al.*[3] differs considerably from that examined in the present study, both in the proportions of the sugars present and in the overall structure. It is possible that these differences arise from different culture conditions or from hybridisation of the alga.

EXPERIMENTAL

The general methods[17], ionophoresis procedures, and methods for methylation of polysaccharides and their analysis have been described previously[10]. G.l.c.–m.s. was performed with a Pye 104 gas chromatograph coupled to a VG Micromass 12F mass spectrometer operating at ~200°, 70 eV, a trap current of 20 μA, and a pressure of 10^{-6} Torr. For the chemical-ionisation spectra, the ion source was operated at 150°, 50 eV, and an emission current of 1000 μA with isobutane at 0.4–0.5 Torr as the reactant gas. The molecular weight of methylated sugars was determined by this means[9]. Uronic acid was determined by the *m*-hydroxybiphenyl method[18]; standard graphs were prepared for the different uronic acids. Sulphate (expressed as percentage of carbohydrate) was determined by the method of Jones and Letham[19] after digestion of the polysaccharide[20].

Analysis of the mucilages. — The moisture, ash, sulphate, uronic acid, and carbohydrate contents of each mucilage are recorded in Table I. Aliquots (50 mg) were hydrolysed, and the constituent sugars were identified by p.c. (solvents *A–C*; sprays 1, 2, 4, 6, and 10), and by g.l.c. of the trimethylsilylated sugar and alditol derivatives and g.l.c.–m.s. of the alditol acetates (columns *4* and *6*), and by electrophoresis in (*a*) borate buffer, (*c*) pyridine–acetic acid at pH 6.7, and (*e*) borate in the presence of Ca^{2+}.

Characterisation of the constituent sugars. — The sugars and oligosaccharides in the hydrolysates (from 1–2 g) were fractionated by chromatography on Whatman 3MM paper with solvent *B*, and where necessary purified by using solvent *C*. The proportion of D- and L-galactose in each of the galactose fractions was determined with D-galactose oxidase[7]. Glucose, galactose, and xylose were isolated as pure, reducing syrups, and their p.c. mobilities and the g.l.c. retention times of suitable derivatives were compared with those of authentic sugars and derivatives. The methylated sugars were demethylated[8], and the products identified by p.c. They were also analysed by g.l.c.–m.s. of the derived alditol acetates. The d.p. of each acidic oligosaccharide was determined, together with the mobility in electrophoresis and the optical rotation. Each was esterified and reduced, a portion hydrolysed, and the hydrolysate examined by p.c. A second aliquot was reduced, esterified, again reduced, and examined by p.c. after hydrolysis. The remainder of the esterified, reduced oligosaccharides were methylated and hydrolysed, and the hydrolysates analysed by g.l.c.–m.s.

Relative proportions of the constituents. — The relative proportions of the constituents were determined by p.c. of a hydrolysate as described above. The carbohydrate content was determined for each of the eluted sugars and oligosaccharides by using standard graphs made for the appropriate synthetic mixtures (Table II). The relative proportions of the sugars in the hydrolysates were also calculated from the peak areas of a g.l.c. trace for the derived alditol acetates (Table II).

Partial hydrolyses. — (*a*) Aliquots (50 mg) of each of the mucilages were dissolved in (*i*) 0.5M oxalic acid, (*ii*) 0.25M oxalic acid, and (*iii*) 50mM sulphuric acid,

and heated separately in sealed tubes under CO_2 at 100°. Aliquots were withdrawn after 0.25, 0.5, 1.5, 2, 4, and 8 h, neutralised ($CaCO_3$ for oxalic acid and $BaCO_3$ for sulphuric acid), and subjected to p.c.

(*b*) The polysaccharide [2.9 g of (Pc) and 1.0 g of (Pa)] were partially hydrolysed with 50mM sulphuric acid for (Pc) and 0.25M oxalic acid for (Pa) at 100° for 4 h. After neutralisation and concentration, each hydrolysate was added to a column (90 × 1.6 cm) of FFIP Deacidite. The neutral components were eluted with water. The absence of acidic fragments was demonstrated by electrophoresis in pyridine–acetic acid buffer (pH 6.7). Acidic oligouronic acids were eluted from the columns with 2M formic acid. The neutral and acidic oligosaccharides were purified on Whatman 3MM paper, and characterised in the usual way.

(*c*) *Autohydrolysis.* A solution of (Pa) (2 g) in water (600 ml) was mixed with prewashed Amberlite IR-120 (H^+) resin (20 g) to convert (Pa) into the free-acid form. The mixture was then subjected to dialysis in a closed system at 70°. The material inside the dialysis sac was stirred with a mechanical stirrer, and the dialysate by a magnetic stirrer. When its carbohydrate content reached 20 μg/ml, the dialysate was replaced by fresh deionised water and then neutralised with ammonia. Five dialysates (each of 3 litres) were collected during 3 weeks. Concentration and electrophoresis showed that only the first dialysate contained any charged sugar derivative. The quantity was very small, and so the experiment was repeated three times to the first stage. The combined first dialysates were concentrated. The charged fraction (~900 μg) was isolated by electrophoresis (pH 6.7) on Whatman 3MM paper. The optical rotation was measured, and an infrared spectrum recorded by using a AgCl plate. An aliquot was hydrolysed and the hydrolysate examined by p.c. and by electrophoresis at pH 6.7. The hydrolysate was tested for free sulphate, and for D-galactose with D-galactose oxidase.

The residual polymer (PaR) (1.2 g), after autohydrolysis for 3 weeks, was recovered by freeze-drying the solution after removal of resin by filtration. The carbohydrate, sulphate, uronic acid, and methoxyl contents were measured. An aliquot was hydrolysed, and the products were analysed by p.c., electrophoresis at pH 6.7, and g.l.c. of the derived alditol acetates.

Desulphation and infrared studies. — The polysaccharides (250 mg each) were treated separately with sodium hydroxide in the presence of sodium borohydride[14]. The excess of borohydride and alkali were removed by dialysis, and the polysaccharides were recovered by freeze-drying. The sulphate contents were measured.

Each polysaccharide (200 mg) was suspended in 0.08M methanolic hydrogen chloride (100 ml), and the mixture shaken overnight. The solid polysaccharides were filtered off and washed with dry methanol. The process was repeated twice and solutions of the residual solids in water were freeze-dried.

Infrared spectra (for KBr discs and also for polysaccharide films on silver chloride plates) were recorded for the desulphated and the original polysaccharides. The 3,6-anhydrogalactose contents of the alkali-treated polysaccharides were determined[21].

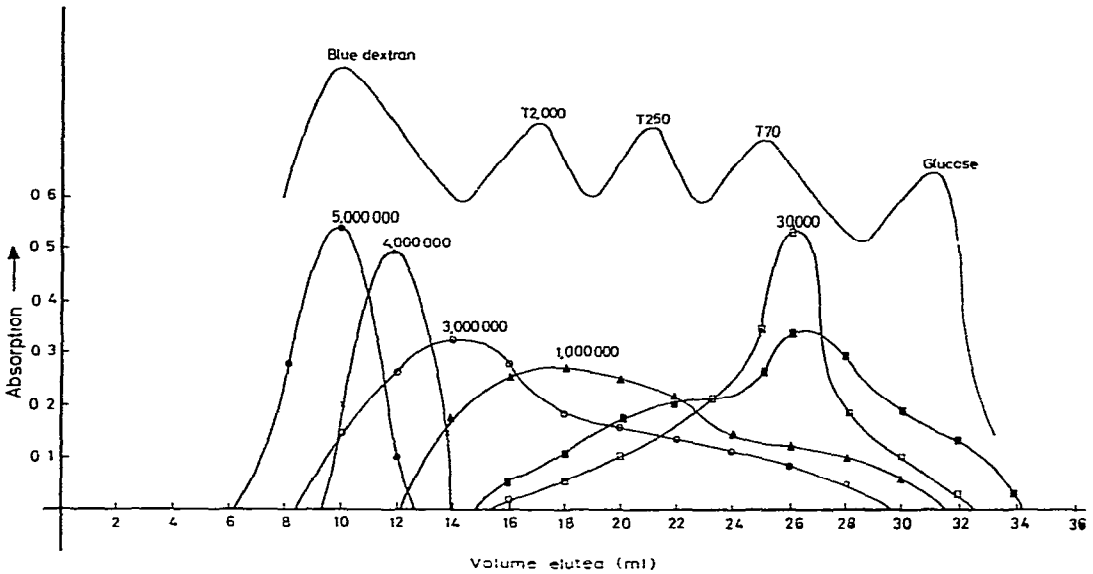

Fig. 1. Elution patterns from Sepharose 4B columns: ●, *P. aerugineum* mucilage; ×, *P. cruentum* mucilage; ▲, residual polymer; ■, methylated mucilages; ○, polyalcohols; □, polyalcohols after treatment with M trifluoroacetic acid for 43 h.

Periodate oxidations. — (*a*) Solutions of each polysaccharide (100 mg; before and after desulphation) in water (100 ml) were treated with an equal volume of 10mM sodium metaperiodate. Aliquots (3 ml) were withdrawn at intervals and the extent of oxidation was measured by the spectroscopic method[22]. Oxidation was complete in 24 h and the solutions were then treated as previously described[17]. The derived polyalcohols [69 and 68 mg from (Pc) and desulphated (Pc); 64 and 70 mg from (Pa) and desulphated (Pa), respectively] were isolated by freeze-drying. Each polyalcohol was methylated and hydrolysed, and the hydrolysate examined by g.l.c.

(*b*) The two polysaccharides [(Pc) and (Pa)] and (PaR) (500 mg each) were oxidised with 25mM periodate as in (*a*), and the derived polyalcohols [360 mg from (Pc), 372 mg from (Pa), and 334 mg from (PaR)] were isolated by freeze-drying. They were each subjected to a second oxidation with 10mM periodate. Each of the polyalcohols was hydrolysed, the hydrolysates were analysed by p.c. and by ionophoresis in molybdate buffer[11], and the proportions of the uncleaved sugars determined.

Molecular size studies. — Aliquots of (Pc), (Pa), (PaR), the methylated mucilages, and the derived polyalcohols before and after treatment with M trifluoroacetic acid were dissolved in water, and the solutions layered on to a column (16.5 × 1.3 cm) of Sepharose 4B. Throughout packing and during elution of the column, a flow rate of 1 drop/15 sec was maintained. The void volume (10 ml) was determined with the dextran having molecular weight in excess of 5×10^6, which is the exclusion limit of Sepharose 4B. Glucose was eluted at 31 ml (Fig. 1). A standard graph was made by separately passing dextran solutions of known molecular weight through the column

and analysing their elution patterns (Fig. 1). The column was eluted with M KCl, 2-ml fractions being collected. Each fraction was analysed for carbohydrate content, and a graph was plotted of the volume eluted against carbohydrate content (Fig. 1).

The polyalcohols recovered after treatment with trifluoroacetic acid were studied by high-pressure liquid chromatography on a Waters Associates Liquid Chromatograph ALC 202 and a Lichrosphere Si 100 column (Merck), with elution by 0.1 % acetic acid. The flow rate was 0.1 ml/min, and the column size was 60 × 0.2 cm. The column was calibrated by using solutions of standard dextrans (T70, T40, and T10) of known molecular weight.

ACKNOWLEDGMENTS

The authors thank Marine Colloids Inc. for the gift of the polysaccharides and for helpful discussions, and Royal Holloway College for the award of a Tutorial Fellowship (to R.A.J.F.).

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