Structural Studies on the Extracellular Polysaccharides of the Red Algae *Porphyridium cruentum* and *Porphyridium aerugineum*

by

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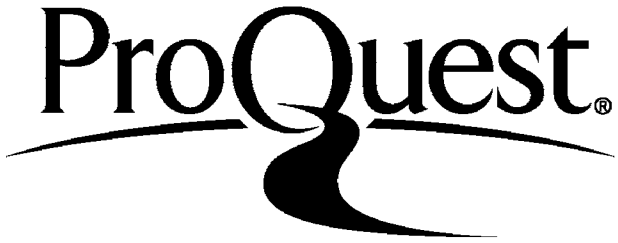

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ABSTRACT

Structural studies on the extracellular polysaccharides of the red algae Porphyridium cruentum and Porphyridium aerugineum

Investigations of the mucilages exuded by the red seaweeds Porphyridium cruentum and P. aerugineum revealed many similarities between them. Both contained D-xylose, D-glucose, D- and L-galactose, 3-O-methylxylose, 3- and 4-O-methylgalactose, and D-glucuronic acid. In P. cruentum the approximate molar proportions are 3:1:2.5:0.13:0.13:0.8 and in P. aerugineum 1.7:1.0:1.1:0.3:0.6:0.5. In addition P. cruentum mucilage contained a 2-O-methylhexose (0.13) and 2-O-methylglucuronic acid (0.2) whereas P. aerugineum mucilage was devoid of these two sugars, but contained 2,4-di-O-methylgalactose (0.3). This is the first time methylated sugars have been reported as constituents of these mucilages. Both polysaccharides contained about 10% half ester sulphate and appeared to be linked to about 5% protein.

Attempted fractionation into homopolysaccharides was unsuccessful. The separation and characterisation of oligosaccharides containing glucose, galactose and xylose; glucose and galactose; and glucose and xylose confirmed that single polydisperse heteropolysaccharides are present.

Methylation and periodate oxidation studies revealed that the glucuronic acid is 1,3-linked and is attached solely to C-3 of D-galactose in both mucilages. The 2-O-methylglucuronic acid in P. cruentum is linked to C-4 of L-galactose. Xylose, glucose and galactose are present in both mucilages as end group 1,3- and 1,4-linked residues with the galactose and glucose also present as 1,3,4-linked branch points.

Desulphation and infrared studies indicate that the half ester sulphate may be located at C-6 of 1,3-linked hexose units and at C-2 and/or C-3 of 1,4-linked units. If this is so then in P. aerugineum mucilage the units must be in the unfavourable $^{1}C_{4}$ conformation.

Molecular weight determinations on Sepharose 4B of the original mucilages, the methylated materials and the polyalcohols before and after cleavage of the acetal linkages indicated a molecular weight of 4×10^{6} for P. cruentum and 5×10^{6} for P. aerugineum. Methylation and periodate oxidation caused considerable degradation of the polymers. The partially hydrolysed polyalcohols have molecular weights (confirmed by high pressure liquid chromatography) of about 30,000.

In spite of the considerable similarity of these exudates, that from P. aerugineum has about ten times the viscosity of that from P. cruentum. It is tentatively suggested that this is connected with the different conformation of some of the sulphated residues which would alter the shape of the macromolecule.

Also included in this thesis are structural studies on a purified fucoidan (Appendix I), methylation of starch extracted from Chroomonas salina (Appendix II) and a preliminary investigation of the polysaccharides from the red algae Constantinea subulifera and C. simplex (Appendix III).

CONTENTS

INTRODUCTION

The majority of algae occur in the sea where they range in size from small unicellular organisms such as Porphyridium cruentum, which occurs as numerous single cells embedded in a gelatinous matrix,[1] to large brown seaweeds, such as Macrocystis pyrifera which may grow up to 50 m in length.[2] The free floating types, most of them unicellular, can be found in the upper layers of the seas, but most of the larger species are attached to rocks. As this type of habitat is very small when compared to the total area of the oceans, by far the greater number of the marine algae occur as unicellular organisms.

Algae also occur in freshwater and in the soil, an example of the latter type being Porphyridium aerugineum.[3]

The principle photosynthetic pigment appears to be the same chlorophyll a as that found in higher plants, but the accessory pigments associated with it in the algae differ, it is this difference which has resulted in the classification of the algae into six main classes,[4] Phaeophyceae (brown), Chlorophyceae (green), Rhodophyceae (red), Cyanophyceae (blue-green), Bacillariophyceae (diatoms) and Chrysophyceae (diatoms).

The majority of the polysaccharides which occur in the algae may be extracted from the cells with water, dilute acid, or alkali. While many of them are quite different from those of land plants, cellulose and starch-type polysaccharides are synthesized by a number of algae. A characteristic feature of seaweed polysaccharides is the presence of half ester sulphate groups linked with at least one of the

polysaccharides in each species. Land plant polysaccharides are devoid of these groups but they are common in animal polysaccharides. The precise function of the sulphate is not fully understood but evidence has been advanced[5] that it is concerned in the ion exchange mechanism whereby cations such as potassium are selectively absorbed from the water. It is also probable that the sulphated polysaccharides, which are hygroscopic and mucilaginous in nature, give seaweeds the flexibility and ease of movement their environment requires and also helps to prevent excessive dehydration when the plant is exposed to air at low tide.

Algal polysaccharides may conveniently be divided into the following groups:-

1 Food reserve material

2 Structural polysaccharides(which may occasionally be sulphated)

3 Sulphated polysaccharides.

1 Food Reserve Material

Several species of the Chlorophyceae contain starches which have been shown to be essentially similar with those found in land plants.[6,7] A number of these have been fractionated into amylose and amylopectin. Recent work on Urospora wormskioldii and its Codiolum phase has shown that amylose and amylopectin are present in the water soluble polysaccharides whilst only amylose appears to be synthesized by U. penicilliformis.[26,26a]

Many of the Rhodophyceae contain floridoside, 2-O-glycerol-α-D-galactoside, which seems to be an end product of photosynthesis and a reserve material.[44] Floridoside was shown to be the major product

of photosynthesis in Gracilaria verrucosa and this floridoside may then be transferred to the parasitic red algae Holmsella pachyderma and accumulated there as floridoside, mannitol and floridean starch, the main food reserve material of the Rhodophyceae.[45]

Floridean starch, originally discovered in members of the Florideae, resembles amylopectin, a 1,4-linked α-D-glucan with 1,6-linked branch points and an average chain length of between 20 and 25 units, and glycogen, a similar polymer but with an average chain length of about 12 units.[8] The glucan in floridean starch occurs essentially in α-1,4-linked chains with 1,6-branch points and an average chain length of 9-19 units,[9] though a very small proportion of α-1,3-linkages has been reported in some species.[8,9]

The brown algae contain laminaran, a β-1,3-linked glucan, which, together with mannitol, serve as their main food reserve. Laminaran occurs in two forms differentiated by their solubility in cold water, they are therefore known as 'soluble' and 'insoluble' laminaran although both are soluble in hot water. The main difference between the two types has been found to be in the degree of branching, the 'soluble' polymer seems to contain more 1,3,6-linked glucose units than the 'insoluble'.[10] Apart from this difference in branching, the two polymers appear to be very similar.[11,12]

In addition to glucose many laminarans also contain a small proportion of mannitol. However as a result of this discovery it was found that laminaran is not a single molecular species, about 50% of the chains in some extracts are terminated by mannitol at the reducing end, while the remaining chains are terminated by glucose units. The mannitol is linked through one of its two primary hydroxyl functions to the adjacent glucose unit,[13] these chains are known as M-chains and the ones which are terminated by 3-linked glucose units at the

reducing end are known as G-chains.

Laminarans are also found as food reserve material in the Chrysophyceae[14] and the Bacillariophyceae,[15] but as yet no chains which are terminated by mannitol have been found in these groups.

2 Structural polysaccharides

Cellulose has been found in the brown, red and green algae.[16,17] The content in the brown seaweeds varies from 0.5 to 10%, whereas in the red weed Gelidium amansii it is as high as 12.5%.[4]

D-mannans and D-xylans have also been found as cell wall polysaccharides in some algae.[4,18,19]

Alginic acid, a polymer consisting of varying proportions of D-mannuronic acid and L-guluronic acid residues, has been found in all the Phaeophyceae so far investigated.[4,20,22] Commercially only a few species are available for use including Macrocystis pyrifera and Ascophyllum nodosum.[22] The alginic acid content of the brown algae varies from 14 to 40% of the dry solids depending on the species and also the season of collection, the content seems to be smaller when the algae undergo rapid growth. The alginate is believed to act as a cation exchanger, the selectivity for different cations being governed to some extent by the varying proportions of mannuronic acid to guluronic acid.[23] The alginate is present as the salt of a mixture of cations, but the calcium content is high enough to keep it insoluble.

Alginates form gels which are not affected by temperature changes, setting is induced by the addition of bivalent ions such as Ca^{2+},

removal of these ions or replacement with an alkali metal such as Na^+ causes the gels to 'melt' and become water soluble.

All attempts to fractionate alginic acid into a D-mannuronan and an L-guluronan have failed. It is now believed that alginic acid consists of regions of mannuronic acid and regions of guluronic acid connected by regions containing both these acids.[63] X-ray work[24] has shown that the mannuronic acid regions form a three-fold helix with the same type of regions from other chains, while the guluronic acid regions form a two-fold rod like helix where the Ca^{2+} ions can replace hydrogen bonds to stiffen the chain, which then aggregate and become junctions in the network. The mannuronan and the alternating segments cannot participate in this type of complex and constitute the flexible part of the network. The mannuronan content of alginates is higher in the tissues undergoing growth and expansion (flexible) whereas the support tissues at the plant base have a greater proportion of the guluronan.[25,81]

Recent investigation[26] of the alginic acid extracts from the brown seaweed Desmarestia ligulata have confirmed that the mannuronic acid is more easily degraded and hydrolysed than the guluronic acid.

3 Sulphated Polysaccharides

(a) Fucoidans or "Fucans"

Fucoidan, like alginic acid, is one of the main polysaccharides found in the Phaeophyceae, it has been found in all the species so far studied, although the amount in some species is extremely small. There is some seasonal variation[27] but the main differences in content are found between species which are permanently submerged, such as the Laminarias and Desmarestias where the fucoidan content is less than

7%, and species which are exposed to the air for long periods such as Pelvetia canaliculata where the fucoidan content may be 23% of the dry weight.[4] It is very hygroscopic and is exuded at the surface of the brown algae as droplets (with alginic acid) and so may help to prevent dehydration of the plant on exposure to the air.[28]

Hydrolysis of fucoidan gives mainly L-fucose, but galactose, mannose, xylose and uronic acids have also been found; a fucoidan containing only fucose has not been isolated so far. Evidence for more than a single molecular species in fucoidan, from Fucus vesiculosus[29] and from Ascophyllum nodosum[30] has been shown using free boundary electrophoresis. These results may just indicate the presence of similar polysaccharides with different degrees of sulphation, or they may show that polysaccharides of different composition are present. In addition three distinct fucose containing polysaccharides have been separated from Ascophyllum nodosum all containing varying amounts of fucose, xylose, glucuronic acid, ester sulphate and protein.[31,32] The protein may be linked chemically with the carbohydrate but cleavage could be effected by heating in the acid form (pH 2.05) at 80° for 20 h.[64]

More recent work on fucans from Desmarestia ligulata and D. firma[26] has shown the presence of ethanol soluble oligosaccharides containing galactose, xylose and mannose; and galactose and xylose which seem to be unique to these algae. They are possibly precursors of the fucans in these weeds and since neither fucose nor glucuronic acid occurs in these extracts it is possible that these oligosaccharides are side chains to be linked on to a fucose/glucuronic acid backbone. Fucans from Himanthalia lorea, Bifurcaria bifurcata

and Padina pavonia were found to contain fucose, xylose, glucuronic acid and only traces of galactose whereas the fucans from D. ligulata, D. firma and D. aculeata[40] were found to contain the same sugars but with larger proportions of galactose. Furthermore the fucans from D. ligulata[26] and Sargassum linifolium[65] were found to contain a fairly high proportion of mannose.

(b) Polysaccharides from the Chlorophyceae

The green seaweeds synthesize mucilaginous sulphated polysaccharides which constitute the major carbohydrates of this class of algae. Those already examined chemically can conveniently be divided into two groups depending upon the constitutent sugars.

The first group contains L-rhamnose, D-xylose, D-glucuronic acid and variable small quantities of D-glucose. They have been obtained from Enteromorpha compressa,[33] Ulva lactuca,[34,82] Acrosiphonia centralis[35] and Urospora penicilliformis.[36] They have all resisted fractionation so may be regarded as a family of hetero-polydisperse polymers. The second group comprises mainly L-arabinose, D-xylose and D-galactose. These polysaccharides have been obtained from Cladophora rupestris,[37] Caulerpa filiformis,[38] Codium fragile[39] and Rhizoclonium implexum and riparium.[40] The polysaccharide from Acetabularia crenulata,[41] on the other hand, is of an intermediate structure as it contains galactose, xylose, rhamnose and glucuronic acid as the main components.

Cladophora[37] was the first algae in the group to be studied in detail. Later work on Chaetomorpha[42] showed that the two species produced polysaccharides which were very similar, the major linkages being galactose 1,3- and 1,6-linked, arabinose 1,4-linked and xylose

1,4-linked and end group. The sulphate was present at C-3 on some arabinose units and at C-6 on some galactose residues. It was also found that blocks of at least eight 1,4-linked arabinose units occurred, linked together by single galactose units.[42,43] The polysaccharide from Codium[39] was similar but no arabinose sulphate was found, and in addition to galactose-6-sulphate, galactose-4-sulphate was also found.[39]

The rhamnans from Ulva,[34] Enteromorpha,[33] Acrosiphonia[35] and three species of Urospora[26] have many similarities. They are all polydisperse heteropolysaccharides which contain L-rhamnose, D-xylose, D-glucuronic acid and half ester sulphate. They all have similar negative rotations and seem to comprise a family of polysaccharides with variable proportions of sugars, extent of branching and sulphation. However the rhamnose residues in Ulva, Enteromorpha and Acrosiphonia appear to be mainly 1,4-linked, with smaller amounts of 1,3-linkages whilst the rhamnose residues in Urospora are mainly 1,3-linked with smaller amounts of 1,2- or 1,4-linkages. Other differences were found in the site of the half ester sulphate and also in the degree of branching.[26a]

(c) Polysaccharides from the Rhodophyceae

The water soluble polysaccharides from the red algae are mainly sulphated galactans consisting of varying proportions of D- and L-galactose, 3,6-anhydro-D- and L-galactose, half ester sulphate and partially methylated galactose. The common structure for many of these galactans is an alternating chain of galactose units of the type -A-B-A-B-.[61] The A-units are linked through C-3 and C-1 with the β-configuration and are either D-galactose, or

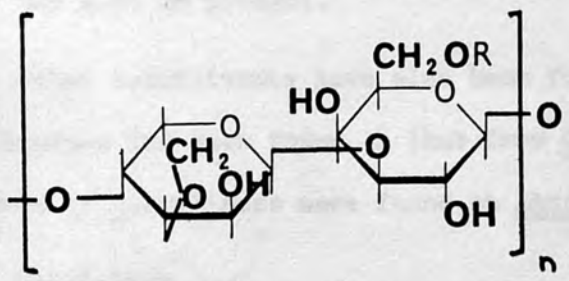
methyl ethers or mono sulphates of this sugar. The B-units are linked through C-4 and C-1 with the α-configuration, but the nature of the unit determines whether the polymer is an agaroid or carrageenan type galactan. In the agaroids the B-unit is always L-galactose or a derivative of L-galactose, but in the carrageenans[62] the B-unit is always D-galactose or a derivative of D-galactose.

These galactans may conveniently be divided into agar-, porphyran-, carrageenan- and furcellaran-type polysaccharides.

Agars

Extensive studies have recently shown that agar is not made up of one neutral (agarose) and one charged (agaropectin) polysaccharide as was previously thought[46] but is composed of a complex series of related polymers ranging from almost neutral to highly charged galactans. They can be expressed as three extremes.[47]

(a) Neutral agarose consists of chains of alternating 1,4-linked-3,6-anhydro-α-L-galactose and 1,3-linked-β-D-galactose with some of the latter units carrying a methoxyl group at C-6, see fig. 1.



R: H, CH_3

Figure 1

(b) Pyruvylated agar with some sulphate. These chains are similar to agarose but about one in twenty of the D-galactose residues are substituted with pyruvic acid as 4,6-O-(1-carboxy-ethylidene)-D-galactose units.[68,69,70] Also a few of the 3,6-anhydro-L-galactose units are replaced by L-galactose-6-sulphate (ca. 2% sulphate), see fig. 2.

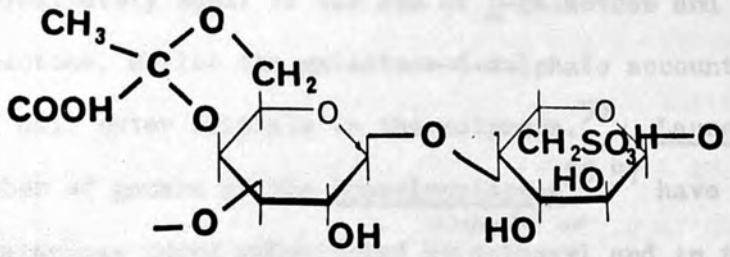


Figure 2

(c) A highly sulphated galactan which contains little, or no 3,6-anhydro-galactose or pyruvic acid but consists of alternating 1,3- and 1,4-linked galactose mono- and di-sulphated. D-glucuronic acid may also be present.

Other substituents have also been found in agars, 4-O-methyl-L-galactose has been found in that from Gelidium amansii[49] and large amounts of L-arabinose were found in Ahnfeltia plicata.[48]

Porphyrans

As the name implies these polymers are named after the galactans extracted from the Porphyra species. They have also been found in Bangia fuscopurpurea and species of the Laurentia and the

Grateloupiaceae genera.[50,51] These polysaccharides are similar to agar in containing 3,6-anhydro-L-galactose or L-galactose-6-sulphate with α-1,4- linkages but differ from agars in their higher sulphate content. These units alternate with β-1,3-linked-D-galactose (or 6-O-methyl-galactose).[52] The proportions of the constituents varies with season and environment[53] but the sum of L-galactose-6-sulphate and 3,6-anhydro-L-galactose is always approximately equal to the sum of D-galactose and 6-O-methyl-D-galactose, whilst the galactose-6-sulphate accounts for nearly all the half ester sulphate in the polymers.[50] Laurentia[55] and a number of genera of the Grateloupiaceae[56,57] have C-2 of the D-galactose units substituted by sulphate or methoxyl and in the galactan from Aeodes orbitosa this is the major methyl sugar.[54] 4- and 6-O-methyl residues are also present, the former being present on the L-galactose units. In Anatheca dentata,[58] a member of the Solieriaceae, 3-O-methyl-galactose has been found as a constituent of the galactan.

More recently a galactan sulphate from Ceramium rubrum has been extracted.[59] It was found to contain an alternating structure with D-galactose or 6-O-methyl-D-galactose as one alternating unit, and L-galactose, 3,6-anhydro-L-galactose, and their respective 2-O-methyl ethers as the other unit. The half ester sulphate is present on C-6 of both D- and L-galactose residues, with smaller amounts on C-2 and C-4 of, probably, D-galactose residues. This polysaccharide differs from others previously examined in that most of the L-galactose residues are non-sulphated.

Another agaroid type galactan, in which 3-linked derivatives of β-D-galactose alternate with 4-linked derivatives of α-L-galactose,

was extracted from Polysiphonia lanosa.[60] It was found to contain D-galactose, 6-O-methyl-D-galactose, a previously unreported unit 6-O-methyl-D-galactose-4-sulphate, L-galactose-6-sulphate, 2-O-methyl-L-galactose-6-sulphate and 3,6-anhydro-L-galactose.

Carrageenans

Carrageenans and furcellarans differ from the agaroids in that they appear to have no methoxyl residues and the L-galactose is replaced by D-galactose,[51] carrageenan has a higher sulphate content than the others.[67] Carrageenan may be fractionated into κ- and λ-carrageenan, the κ- being precipitated as a gel in the presence of potassium ions.[71] κ-Carrageenan consists of chains of alternate 1,3-linked-D-galactose-4-sulphate and 1,4-linked-3,6-anhydro-D-galactose, see fig. 3.

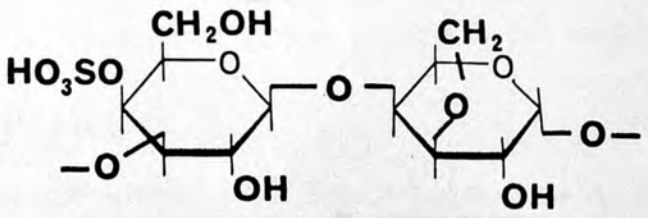


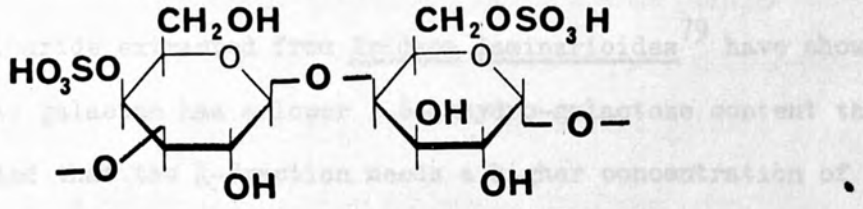
Figure 3

The 3,6-anhydro-residue may be replaced by galactose-6-sulphate.

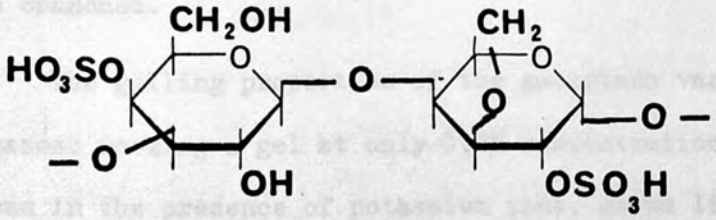
λ-Carrageenan may be thought of as alternately 1,3-linked D-galactose sulphated on some units at C-2 and 1,4-linked-D-galactose-2,6-disulphate, although its composition and structure may vary widely with the source.[72,73] A useful distinction between κ- and λ-carrageenans is that the former has a maximum content of 3,6-anhydro-

galactose and the latter has a minimum. Recent work on Chondrus crispus[74] and Iridaea cordata[75] has suggested that the varying proportions of κ- to λ-carrageenans is due mainly to the various life stages of the algae rather than the season at which the plant is collected, this agrees with work carried out on Gigartina species.[83]

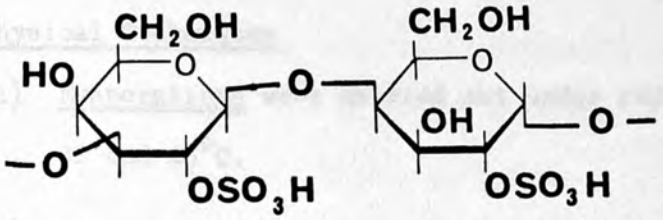
Other carrageenans have also been isolated, notably ν-, ι-, μ- and ξ- fractions.[76,77,78] The idealised structures of some of these are shown in fig. 4.



μ-carrageenan



ι-carrageenan



ς-carrageenan

Figure 4

Most of the carrageenan type polymers are mixtures and deviants of these idealised structures. Studies on the carrageenan type polysaccharide extracted from Iridaea laminarioides[79] have shown that this galactan has a lower 3,6-anhydro-galactose content than usual, and that the k-fraction needs a higher concentration of potassium chloride to effect precipitation than is usually needed.

Furcellarans

Furcellaran has many similarities with k-carrageenan, 56% of it being exactly the same.[80] However it has a lower sulphate content, only 40% of the residues carrying half ester sulphate, and only about half of the D-galactose unit are sulphated at C-4, the molecule may be branched.

The gelling properties of the galactans vary considerably, agarose forming a gel at only 0.1% concentration, whereas μ-carrageenan even in the presence of potassium ions, shows little or no inclination to gel. It is interesting to note how the algae have devised a method to radically alter the properties of their polysaccharides by such simple modifications to the polymer chains.

GENERAL METHODS

I. Physical Techniques

(i) Evaporations were carried out under reduced pressure between 30 and 45°C.

(ii) The water used in all experiments was deionised.

(iii) Melting points were determined on a Gallenkamp micro melting point apparatus.

(iv) Dialysis of solutions was performed in Visking cellophane tubing against distilled water with toluene added as a bacteriostat.

(v) Specific rotations were measured in a 1 dm polarimeter tube using a Perkin-Elmer 141 polarimeter. All measurements were made in aqueous solution using the sodium D-line.

(vi) Unless otherwise stated all resin used was Amberlite.

(vii) Freeze-drying samples were first frozen in a cardice-acetone mixture before being placed on the freeze-drier.

(viii) All solutions and hydrolysates were filtered through millipore filters (0.45 μ) before quantitative determinations.

II. Acid Hydrolysis

(i) Formic Acid method

The sample was dissolved in 90% formic acid and solid carbon dioxide added to give an inert atmosphere. The tube was sealed and heated for 6 hours at 100°C. The hydrolysate was diluted with water (5 vol.) and heated at 100°C for a further two hours, to hydrolyse the formyl esters. The solution was evaporated to dryness and residual formic acid removed by codistillation with methanol.

(ii) Sulphuric acid

The sample was mixed with sulphuric acid in a sealed tube and heated at 100° for the amount of time stated. After cooling the acid was neutralised with barium carbonate.

III. Chromatography

(i) Paper Chromatography using the following solvent systems for descending chromatography (v/v).

(a) Ethyl Acetate : acetic acid : formic acid : water (18:3:1:4).

(b) n-butanol : pyridine : water (6:4:3).

(c) n-butanol : ethanol : water (40:11:19).

Whatman No.1 paper was used for qualitative work. For preparative paper chromatography Whatman No.3 MM or No.17 paper was used.

(ii) Ionophoresis. The Shandon high voltage electrophoresis apparatus L24 was used with Whatman No.3 MM paper and the following electrolytes:

(a) Borate[84]

0.2M-Sodium borate in water adjusted to pH 10 with sodium hydroxide. Electrophoresis was carried out for 1.5 h. at 2.5 kv. The non-migrating marker was 2,3,4,6-tetra-O-methyl-D-glucose.

(b) Pyridine/Acetic acid[85]

Pyridine (1-litre) adjusted to pH 6.8 with 5% acetic acid in water. The electrophoresis was carried out for 2.0 h at 3.0 kv. Glucose was the non-migrating marker.

(c) Borate with Calcium ions[86]

0.01M-Sodium tetraborate (borax) in water containing 0.005M-calcium chloride (pH 9.2). Electrophoresis was carried out for 2.0 h at 0.5 mA/cm.

(d) Molybdate buffer[84]

Sodium molybdate dihydrate (25 g) in water (1200 ml) was brought to pH 5 with conc. H_2SO_4. The voltage applied was 30–60 v/cm for 1–3 h. The non-migrating marker was glucose.

IV. Staining Reagents

(i) Silver nitrate dip.[87]

Three solutions through which the paper was sequentially dipped.

(a) Saturated aqueous silver nitrate solution (2.5 ml) and water (10 ml) in acetone 500 ml.

(b) Sodium hydroxide (20 g) in water (40 ml) and ethanol (960 ml).

(c) 10% aqueous sodium thiosulphate.

(ii) Aniline oxalate spray[88]

Aniline oxalate (25 g) in 50% aqueous ethanol (1L).

(iii) Ninhydrin

A freshly made solution of ninhydrin (2 g) in ethanol (100 ml). A blue colour specific for amino acids developed after 5 min at 70°.

(iv) Glucose oxidase[89]

'Glucostat' kit (Worthington Biochemical Company) made up as directed. A pink colour indicating β-D-glucose develops after 5 min at room temperature.

(v) Galactose oxidase[89]

'Galactose' kit (Worthington Biochemical Company) made up as directed. A pink colour indicating D-galactose develops after 5 min. at room temperature.

(vi) Urea hydrochloride[90]

Urea (10 g) in ethanol (200 ml) and concentrated hydrochloric acid (8 ml) in water (32 ml). A blue colour specific for ketoses develops after 5 minutes at 100°C.

V(a) Gas Liquid Chromatography (g.l.c.)

A. Instrumentation

A Pye 104 gas chromatograph with nitrogen carrier gas and flame ionisation detector with glass columns (3 m x 5 mm). Column (i) for the TMS derivatives of the sugars and alditols. Columns (iii) and (ii) for the partially methylated alditol acetates and alditol acetates.

B. The columns were packed with the following materials

(i) Apiezon K, 7.5% on silane treated Chromosorb W.

(ii) OV 225, 3% on Gaschrom Q.

(iii) ECNSS-M, 3% on HMDS treated Chromosorb W.

C. Gas Chromatography linked to Mass spectrometry

A Pye 104 gas chromatograph with helium carrier gas was coupled to a VG Micromass 12F mass spectrometer with a total ion monitor detector system. For EI spectra the 'ion source' was operated at about 200°C, 70 eV and 20 μa target current under a pressure of 10^{-6} torr.

V(b) Spectrometry

(i) Ultra violet and visible spectroscopy

Quartz cells were used and the absorbances were read in the Unicam SP500 machine.

(ii) Infra-red spectroscopy

Polysaccharide films were made on AgCl plates and KBr discs were made. The Perkin-Elmer 177 Grating Infra-red Spectrophotometer was used.

VI Assays and Analyses

(i) Carbohydrate Content was assayed by the phenol sulphuric acid method.[91] Water (1 ml) containing 10-100 µg sugar was added to 4% phenol (1 ml) and concentrated sulphuric acid (5 ml) added rapidly. The colour developed was read at 487 nm on a Unicam SP500. Standard graphs were prepared for different sugars and mixtures of sugars in the ratios corresponding to those of the particular polysaccharide.

(ii) Uronic acid determination

This was determined by the meta-hydroxydiphenyl method.[92] To a sample solution (0.6 ml) containing from 1.5 to 60 µg uronic acid, a 0.0125M solution of sodium tetraborate in concentrated sulphuric acid (3.6 ml) was added. The solutions were cooled in ice and then mixed and heated at 100° for 5 min. After again cooling in ice the m-hydroxydiphenyl solution (50 µl, 0.15% solution in 0.5% sodium hydroxide) was added. The solutions were shaken and the absorbance measured at 520 nm on a Unicam SP500. Standard graphs were prepared for the different uronic acids.

(iii) Sulphate estimation. The polysaccharide (10 mg) was digested in a sealed tube with analar nitric acid (1 ml plus a few mg of sodium chloride) for 12 h at 100°C. After evaporation to dryness the residual solid was treated with concentrated hydrochloric acid (1 ml) and evaporated to dryness again. The solid was treated with water and evaporated to dryness and the tube was then placed in an oven at 105°C for 2 h. The sample was then ready for sulphate determination and the following modification of the Jones and Lethamm method[93] was used.

To the sulphate solutions (0.5 ml) containing 30-100 μg of sulphate in micro centrifuge tubes the reagent 4-chloro-4'-amino-diphenyl (0.5 ml of 0.19% in 0.1N hydrochloric acid) and a trace of solid hexadecyltrimethylammonium bromide were added. After mixing, the solutions, including a blank, were kept for 2 hours and then centrifuged. Aliquots (0.2 ml) of the supernatants were removed and diluted to 25 ml with 0.1N hydrochloric acid. The optical densities were read at 254 nm on a Unicam SP500. From a standard graph the differences from the blank reading gave the sulphate content (the sulphate contents quoted in this thesis are always based on the carbohydrate content).

(iv) Nitrogen and Protein content. Nitrogen content was measured by A. Bernhardt (W. Germany) and the protein content calculated by multiplying by 6.25.[96]

(v) Molar proportions of sugars were estimated from the peak areas on g.l.c. of (a) the TMS derivatives of derived alditols or (b) alditol acetates or (c) methylated alditol acetates.

(vi) Degree of polymerisation was determined by Timell's modification[94] of Peat, Whelan and Roberts method.[95] Three solutions were prepared as follows:-

1) A blank containing water (0.5 ml) and 2% potassium borohydride solution freshly made (0.5 ml).

2) An aqueous sugar solution (0.5 ml containing 60–80 µg as monosaccharide) and 2% potassium borohydride solution (0.5 ml).

3) A 2N sulphuric acid solution (0.5 ml) containing the same weight of sugar as solution 2) and 2% potassium borohydride solution (0.5 ml).

The mixtures were left at room temperature overnight and the carbohydrate content of each was determined using the phenol/sulphuric method of estimation (this only gives a colour reaction with reducing sugars). The carbohydrate contents were read off suitable standard graphs and the degree of polymerisation was determined by:-

$$D.P. = \frac{(\mu g \text{ in } 3)}{(\mu g \text{ in } 3) - (\mu g \text{ in } 2)}$$

VII. General Reactions and Preparations

(i) Preparation of IR 120H$^+$ dry form in methanol. The resin was washed with water until free of colour. It was then stirred with methanol for 18 h and then filtered. This was repeated three times, the third time with dry methanol. The resin was then stored under dry methanol.

(ii) Preparation of methanolic hydrogen chloride. Hydrogen chloride gas was passed into dry methanol until saturation was reached. The solution was titrated with N-NaOH and diluted with dry methanol as required.

(iii) Preparation of dimethyl sulphinyl carbanion.[97] Sodium hydride (1.5 g, 55% coated with mineral oil) was washed three times with n-pentane (30 ml) which was removed by successive evacuations of the vessel. After each evacuation, dry nitrogen was passed into the vessel. Dry distilled dimethylsulphoxide (15 ml) was added and the contents of the vessel heated at 55°C until evolution of hydrogen ceased. The carbanion was transferred to serum bottles and stored at 0°C under an atmosphere of nitrogen. Its normality ($\sim$2N) was determined by titration with 0.1N-HCl.

(iv) Esterification of uronic acids and glycosidation of sugars. The sugar was dried in a desiccator over concentrated sulphuric acid and then dissolved in dry methanol and a small amount of IR 120H$^+$/ (dry form) added as catalyst. The mixture was refluxed for 18 h, the resin filtered off and the methanol removed by evaporation.

(v) Reduction of sugar to alditol. The sample (25 mg) was dissolved in water (3 ml) or water/methanol (1:1 v/v), and a small spatula tip of sodium borohydride was added to give approximately a 2% borohydride solution. It was left standing for about 6h. If the solution was not still alkaline after this time, more sodium borohydride was added and the mixture left for a further 6 h and then neutralised with IR 120H$^+$ resin. After filtration the filtrate was co-distilled with methanol to remove boric acid and finally evaporated to dryness. Complete reduction was checked with Fehling's solution.

(vi) Trimethyl silyl derivatives.[98] The material (10-15 mg), dried by co-distillation with methanol and dry benzene, was dissolved in dry pyridine (1 ml) and trimethyl chlorosilane (0.1 ml) added followed by hexamethyldisilazane (0.2 ml). After shaking for 5 mins the precipitate of ammonium chloride was removed by centrifugation and the sample evaporated to dryness. The residue was dissolved in dry hexane and analysed on g.l.c. (column (i)).

(vii) Alditol acetates.[99] Samples of sugar alditols or of partially methylated alditols, dried in a desiccator, were dissolved in a pyridine: acetic anhydride (1:1 v/v) mixture and heated for 10 mins at 100°C. After dilution with water the sample was evaporated to dryness. The residue was dissolved in chloroform and analysed by g.l.c. or g.l.c.-m.s.(columns (ii) and (iii)).

(viii) Methylation. The Hakomori method[100] (modified by Bjorndal and Lindberg[101]) was used. The polysaccharide (5-20 mg) was dissolved or swelled in dry DMSO (2 ml) in a serum bottle under nitrogen atmosphere. Dimethylsulphinyl carbanion (1 ml) was injected into the bottle and the mixture shaken for 8 h. For a single methylation freshly distilled methyl iodide was added (1 ml) with cooling and the mixture again shaken for 8 h. For two methylations methyl iodide was added (0.1 ml) with cooling and after 8 h shaking more carbanion (1 ml) was added and after another 8 h shaking methyl iodide was again added (1 ml) with cooling. After the final period of shaking the solution was poured into water (25 ml) and dialysed for three days when the oily layer became solid. The mixture was then evaporated to dryness.

(ix) Periodate Oxidation. Estimation of the extent of oxidation was measured by the spectrophotometric method of Aspinall and Ferrier.[102] An aqueous solution of polysaccharide was added to an equal volume of sodium metaperiodate (0.1N). Aliquots (0.3 ml) were withdrawn at intervals and diluted to 250 ml and the absorbance read at 223 nm. The initial absorbance of the periodate before the addition of polysaccharide and the absorbance of an equimolar solution of sodium iodate were measured and thus the number of moles of periodate reduced at any time was measured. Excess periodate was destroyed with ethylene glycol. The product was reduced by sodium borohydride and obtained pure by dialysis and freeze-drying.

(x) Reduction of uronic acids.[103] A modified Taylor and Conrad method was used.

The polysaccharide (10 mg, 25 μeq/carboxyl groups) was dissolved in 50 ml of water and ethyl-3-dimethyl aminopropyl carbodiimide HCl (25 mg, 0.25 mol) was added, after the pH had been adjusted to 4.75 with 0.1M hydrochloric acid. During the reaction the pH was maintained at 4.75 by adding 0.1M hydrochloric acid. When hydrogen ion uptake had ceased, a sodium borohydride solution (2M, 10 ml) was added and the mixture stirred for 4 h. The mixture was dialysed against running tap water overnight and against distilled water for 3 days. Thereafter the solution was freeze-dried.

(xi) Preparation of a DE-52 ion exchange cellulose column. The cellulose (200 g, D.E.A.E., grade D.E.52 preswollen) was suspended in 0.5M HCl (2l) and deaerated with magnetic stirring under vacuum for about 20 min. After standing another 20 min. the supernatant was decanted and the cellulose filtered. The cellulose was washed with

water to neutrality and thereafter suspended in 0.5M NaOH (21). The alkaline suspension was treated in the same way as the acid suspension. These two operations were repeated, though after the second alkali treatment the alkaline slurry was transferred into a column (internal diameter 3.7 cm and length about 50 cm) and the material washed to neutrality with water.

The column was equilibrated with 0.5M NaCl solution (21) and washed with water till the washings were chloride free.

(xii) Desulphation by methanolic hydrogen chloride. An aliquot (about 50 mg) was suspended in 0.08M-HCl in MeOH (25 ml) and left on a shaker overnight. The solid was filtered off and washed with dry MeOH and resuspended in HCl/MeOH solution (25 ml) and again left on a shaker overnight. After a third similar treatment the solid was redissolved in water and freeze-dried.

(xiii) Desulphation by alkali in the presence of sodium borohydride. Polysaccharide (250 mg) was dissolved in water (250 ml) and sodium borohydride (125 mg) added. The mixture was left at room temperature overnight. Sodium borohydride (250 mg) and 3N sodium hydroxide (125 ml) were then added. The mixture was left at 80° for 8 h and then, after cooling, set aside at room temperature overnight. The borohydride and alkali were removed by dialysis and the desulphated polymer obtained by freeze-drying.

(xiv) Demethylation.[104]

The sugar sample in dry dichloromethane (2 ml) was cooled to -80°. Boron trichloride (1-2 g), cooled to -80°, was added and the mixture kept at -80° under anhydrous conditions for 30 min.

The solution was then allowed to warm up to room temperature and allowed to stand under anhydrous conditions for 16 h. The remaining solvent was removed under vacuum and methanol (3 x 3 ml) was added and evaporated.

This thesis describes the chemical investigation of the extracellular polysaccharides of two species of Porphyridium, the marine cruentum and the freshwater aerugineum.

While this work was in progress two chemical studies on cruentum were published, one by Medcalf et al[113] in late 1975 and the other by Heaney-Kieras and Chapman[1] in late 1976.

Porphyridium cruentum

Introduction

P. cruentum is a unicellular red alga which occurs as numerous cells embedded in a gelatinous polysaccharide matrix.[129] It may readily be cultivated in artificial media. Jones[116] carried out the first structural studies on this alga. He showed that the production of extracellular polysaccharide was directly proportional to the number of P. cruentum cells present. Chemical analysis of the mucilage revealed that besides carbohydrate 6-7% of protein (dry basis) was present and approximately 10% of ethereally bound sulphate. Analysis of a hydrolysate by chromatography showed the presence of galactose, glucose, xylose and uronic acid as the carbohydrate constituents while aspartic acid, glutamic acid, serine, glycine, threonine, alanine, proline, histidine, lysine, arginine, phenylalanine, valine, methionine and leucine were detected as being constituents of the protein moiety.

More recently Williamson[117] has reported that in addition to the above amino acids, isoleucine cysteine and tyrosine were also present in the protein moiety. He also found galactose, glucose and xylose as constituents of the carbohydrate along with two unidentified spots on chromatograms, one of which may have been a methylated or deoxy monosaccharide and the other an acidic disaccharide. Evidence was obtained for the existence of two different types of polysaccharide by a histochemical study of the cells. Oxidation of the polysaccharide with periodic acid followed by staining with Schiff's reagent showed distinct regions. Polymers whose constituent sugars

reacted with periodic acid were stained pink. The extracellular region not stained with Schiff's reagent but subsequently stained with alcian blue, which stains acidic polysaccharides, may therefore be an acidic polysaccharide with 1,3-linked residues. However no fractionation of the extracellular polysaccharide was achieved on an ion exchange column.

More detailed work on the structure of the extracellular polysaccharide has been carried out by Medcalf et al.[113] They found the sulphate content to be 7.4% and the protein content 5-6%. After hydrolysis xylose, L-galactose (1.0:0.9 molar ratio) and glucuronic acid (9%) were the main sugars present but a minor amount of glucose and traces of mannose and rhamnose were also found. These authors showed that the polymer was primarily 1,4-linked with branches on C-3 of xylose and C-2 or C-6 of galactose. The glucose and glucuronic acid were 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.

The most detailed study on this polysaccharide has been by Heaney-Kieras et al.[1,107,118] They reported a polysaccharide-protein complex (1-2% amino acids) with 9% ester sulphate and 8.5% uronic acid. The protein moiety contained the same amino acids as had been reported by Williamson with the exception that cysteine and methionine were absent. The protein was shown to be linked to the carbohydrate via serine-xylose and threonine-xylose linkages. The constituents of the carbohydrate were shown to be D- and L-galactose, D-glucose, xylose, D-glucuronic acid and 2-O-methyl D-glucuronic acid in the molar proportion, 2.12:1.0:2.42:1.22 respectively.

The hexoses and uronic acids were shown, by periodate oxidation, to be joined to other residues by 1,3-linkages, in contrast to the earlier report of Medcalf's. About half the xylose residues were present as 1,3-linkages. D-glucuronic acid was found in the aldobiouronic acid D-glucuronosyl (1 $\xrightarrow{\alpha}$ 3)-L-galactose whilst the 2-O-methyl-D-glucuronic acid was present in the disaccharide units 2-O-methyl-D-glucuronosyl (1 $\xrightarrow{\alpha}$ 3)-D-galactose and 2-O-methyl-D-glucuronosyl (1 $\xrightarrow{\alpha}$ 3)-D-glucose.

EXPERIMENTAL

Porphyridium cruentum

Porphridium cruentum was supplied (September 1974) by Dr F. Williamson from Aberdeen as crude cells suspended in ethanol. The extracellular polysaccharide was extracted by shaking the cells with water and centrifuging the slurry. The supernatant solution of the polysaccharide was extensively dialysed before freeze-drying to yield 4 g of crude polysaccharide. Further material was supplied by Marine Colloids (U.S.A.) as the Na^+ salt of the polysaccharide (2 g, 6.9.75), (2.5 g, 5.5.76), (2 g, 2.10.76), (15 g, 30.1.77).

Expt. 1 Analysis of the different samples

Aliquots (10 mg) of the different samples were hydrolysed [GM II (i)] and analysed by paper chromatography in solvents [GM III (i) (a)(b)(c)] with srpays [GM IV (i) (ii)(iii)].

Expt. 2 Fractionation on a DE52 cellulose column

The crude polysaccharide (500 mg, 27% carbohydrate) was dissolved in water (40 ml) and layered onto a DE52 cellulose column (3 x 42 cm) [GM VII (xi)]. The sample was eluted successively from the column with water (8 l), 0.1M - KCl (3 l), 0.5M - KCl (4 l) and 1.0M - KCl (4 l). Each fraction was dialysed and recovered by freeze-drying.

Expt. 3 Analysis of the different fractions

Aliquots (10 mg) of each of the fractions obtained from the column were hydrolysed [GM II (i)] and analysed by paper chromatography [GM III (i) (a)(b)(c)] with sprays [GM IV (i)(ii)]. The carbohydrate contents [GM VI (i)] and the uronic acid contents [GM VI (ii)] were determined for each of the fractions.

Expt. 4 Purification of the crude polysaccharide

The crude polysaccharide (2.5 g), obtained after extraction from the cells, was extensively dialysed against deionised water using a hollow fibre beaker dialyser b/HFD-1 (Dow Chemicals). After freeze-drying a white polysaccharide (1.15 g) was recovered. The carbohydrate [GM VI (i)], sulphate [GM VI (iii)], nitrogen [GM VI (iv)] and uronic acid [GM VI (ii)] contents were determined for the purified material and also for the samples from Marine Colloids. As no difference was found between the different materials no distinction will be made between them. All subsequent experiments were carried out either on the purified polysaccharide or the samples from Marine Colloids and will be called either the polysaccharide or the mucilage.

Expt. 5 Precipitation with Hexadecyltrimethylammonium bromide (CETAB)

The polysaccharide (1 g) was dissolved in water (500 ml) and a solution of CETAB (1.5 g) in water (10 ml) was added. The mixture was left stirring overnight at room temperature. The resulting precipitate was filtered with a nylon cloth and the excess CETAB

removed by washing with water. The complex was stirred with 2M-NaCl (500 ml) overnight. The precipitate dissolved completely, and the polysaccharide was recovered from solution by precipitation with ethanol. Purification was effected by twice redissolving in water and reprecipitating with ethanol (800mg).

The supernatant was treated with Biodeminrolit resin (carbonate form), and Amberlite IR 120 (H^+) and IR 45 (OH^-) resins to remove the CETAB. The carbohydrate content [GM VI (i)] was determined on the resulting solution. The carbohydrate content was also determined on a standard glucose solution containing CETAB.

Expt. 6 Viscosity Studies

The viscosity of a 0.35% solution of the mucilage in water was measured using a viscometry U-tube (British Standard LAS 3) at 30° and was compared to that of water (relative viscosity). The viscosities were also measured after heating to 70° and cooling, and after autocl aving in a pressure cooker at 120° for 15 min and cooling to 30°. The pH dependence of the solution was determined by measuring the viscosity at pH 3.5, 7.0, 8.9 and 11.0.

Expt. 7 Characterisation of the constituent sugars

An aliquot (50 mg) of the polysaccharide was hydrolysed [GM II (i)]. Part of the hydrolysate was analysed by paper chromatography [GM III (a)(b)(c)] using sprays [GM IV (i)(ii)(iv)(v)(vi)]. A second aliquot of the hydrolysate was analysed by g.l.c.

[GM V (a) B(i)(ii)] as the sugar TMS, the alditol TMS [GM VII (v)(vi)] and the alditol acetate [GM VI (v)(vii)] derivatives. Electrophoresis in borate buffer and in pyridine/acetic acid buffer was carried out on the hydrolysate [GM III (ii)(a)(b)(c)]. The alditol acetate derivatives were also studied by g.l.c.-mass spectrometry.

The methoxyl content of the polysaccharide was determined by A. Bernhardt (W. Germany).

The reducing syrups glucose, galactose, and xylose (separated in expt. 8) were studied by g.l.c. as the derived TMS derivatives [GM VII (vi)] and were compared to standard TMS derivatives of these sugars.

The optical rotations of these syrups [GM I (v)] were measured.

Expt. 8 Hydrolysis and separation of sugars and oligosaccharides

The mucilage (2 g) was hydrolysed [GM II (i)] and the constituents separated by paper chromatography on Whatman 3MM paper. Half the hydrolysate was separated in solvent system [GM III (i)(a)] and half in [GM III (i) (b)]. Side and centre strips were cut from both chromatograms and were developed with spray [GM IV (ii)(a)]. The regions containing the different components were cut from the rest of the chromatograms and were eluted with water. The carbohydrate content [GM VI (i)] were determined for each of the eluted sugars and oligosaccharides using standard graphs made from the appropriate synthetic mixtures.

The proportions of the different sugars present were also calculated from the peak areas of a g.l.c. trace of the derived alditol acetates from a hydrolysate of the mucilage [GM VI (v)].

Expt. 9 Partial hydrolysis

(a) Preliminary Studies

Aliquots (50 mg) of the polysaccharide (carbohydrate content 65%) were dissolved in 0.5M-oxalic acid, 0.25M-oxalic acid and 0.1N-sulphuric acid. They were heated separately in sealed tubes under CO_2 at 100°. Aliquots were withdrawn after 0.25 h, 0.5 h, 1.5 h, 2 h, 4 h and 8 h. The oxalic acid hydrolysates were neutralised with calcium carbonate and the sulphuric acid hydrolysate with barium carbonate. After filtration the different neutral partial hydrolysates were studied by paper chromatography [GM III (i) (a)(b)] with sprays [GM IV (ii)]. The conditions yielding the maximum quantity of oligosaccharides was found to be 0.1N-sulphuric acid at 100° for 4 h.

(b) Large scale hydrolysis

The polysaccharide (2.9 g) was hydrolysed with 0.1N-sulphuric acid (535 ml) under CO_2 in a sealed flask at 100° for 4 h. After neutralisation with barium carbonate and filtration of the resulting barium sulphate, the hydrolysate was layered onto an FF1P ('Deacidite') column (90 x 1.6 cm). The neutral components were eluted with water. The absence of acid fragments was tested for, after concentration, by running an electrophoretogram in pyridine/acetic acid

buffer pH 6.7 [GM III (ii)(b)]. The acidic material was eluted from the column with 2N-formic acid which was removed from the sugars by codistillation with methanol.

Fractionation of the neutral components was initially attempted using a horizontal column packed with cellulose powder.[119] However as preliminary results using mixtures of standard sugars failed to give a satisfactory separation, this method was abandoned. Separation was achieved however on 3MM paper chromatograms in solvent [GM III (i)(a)].

The acidic components were separated on 3MM paper chromatograms in solvent [GM III (i)(a)]. For both chromatograms two side strips and a centre strip were cut and developed with aniline oxalate [GM IV (ii)(a)]. The regions containing the different components were cut from the remaining chromatograms and the sugars were eluted with water.

Expt. 10 Studies on the neutral components

Three neutral oligosaccharides (1) 0.5 mg, R_{glc} 0.30, (2) 5 mg, R_{glc} 0.51 and (3) 3 mg, R_{glc} 1.0 were separated. The chromatographic mobilities are for solvent (b). (3) was reseparated in solvent (a) where it had R_{glc} 0.82. The carbohydrate content [GM (vi)(i)] and the degree of polymerisation [GM VI (vi)] were determined for each oligosaccharide.

Aliquots of each of these oligosaccharides were hydrolysed with 90% formic acid in sealed tubes under CO_2 at 100° for 3 h, followed by dilution with water etc. [GM II (i)]. The hydrolysates were studied by paper chromatography [GM III (i)(b)] and by g.l.c. as the

sugar T.M.S. [GM VII (vi)] and the alditol acetate [GM VII (v)(vii)] derivatives.

Aliquots of (2) and (3) were reduced separately [GM VII (v)] and then hydrolysed with 90% formic acid in sealed tubes under CO_2 at 100° for 1.5 h, followed by dilution with water etc. [GM II (i)]. The resulting aldoses and alditols were studied by g.l.c. as the TMS derivatives [GM VII (vi)] and by paper chromatography [GM III (i)(b)] with spray [GM IV(i),(iv)].

Expt. 11 Linkage studies on the neutral oligosaccharides

The neutral oligosaccharides (2) and (3) were reduced [GM VII (v)] and an electrophoretogram run in molybdate buffer [GM III (ii)(d)]. Oligosaccharide (2) (1 mg) was methylated[105] after drying, by shaking with methyl iodide (0.2 ml), dimethyl formamide (0.2 ml) and dry silver oxide (0.2 g) in the dark at 0° for 2-3 h, then at room temperature overnight. The mixture was filtered and the residue washed with chloroform. The combined filtrate and washings were dried (anhydrous sodium sulphate), filtered and evaporated to dryness under 0.03 mm pressure at room temperature. The resulting methylated oligosaccharide was hydrolysed [GM II (i)] and the derived methylated sugars were studied by g.l.c. - m.s. as the alditol acetate derivatives [GM VII (v)(vii)].

Expt. 12 Studies on the acid oligosaccharides

Four oligouronic acids were separated but two were present in only trace amounts making further study impossible. The major oligouronic

acid (15 mg), R_{glc} 0.40 in solvent system [GM III (i)(a)], was tested for carbohydrate content [GM VI (i)] and for the degree of polymerisation [GM VI (vi)]. The optical rotation [GM I (v)] was measured. An aliquot was esterified [GM VII (iv)], reduced [GM VII (v)] and part was hydrolysed [GM II (i)]. A second aliquot was reduced, esterified, reduced and hydrolysed as above. The two hydrolysates were studied by paper chromatography [GM III (i)(b)] with sprays [GM IV (i)(ii)(iv)(v)] and by g.l.c. [GM V (a) B(i)] as the TMS derivatives [GM VII (vi)].

The remainder of the esterified, reduced oligosaccharide was methylated[105] as described in expt. 11, hydrolysed [GM II (i)] and the hydrolysate analysed by g.l.c. - m.s. [GM V (a)C] as the alditol acetate derivatives [GM VII (v)(vii)].

The optical rotation [GM I (v)] was determined for the other olgiouronic acid (3 mg), R_{glc} 0.67 in solvent system [GM III (a)], which was then esterified [GM VII (iv)], reduced [GM VII (v)] and half was hydrolysed [GM II (i)]. The hydrolysate was studied by paper chromatography [GM III (i)(b)] with sprays [GM IV (ii)(iv)(v)] and by g.l.c. [GM V (a) B (ii)] as the alditol acetate derivatives [GM VII (v)(vii)]. The other half of the esterified, reduced oligosaccharide was methylated[105] as described in expt. 11, hydrolysed [GM II (i)] and the hydrolysate analysed by g.l.c. - m.s. [GM V (a) C] as the alditol acetate derivatives [GM VII (v)(vii)].

Expt. 13. Desulphation of the mucilage and Infra-red Studies

Desulphation of the polysaccharide (250 mg), carbohydrate content 65%, sulphate content 10%, was initially attempted using sodium

hydroxide [GM VII (xiii)]. After freeze-drying (213 mg) the sulphate content [GM VI (iii)] of the derived material was found to be unchanged.

Methanolic hydrogen chloride (0.08M) was therefore used [GM VII (xii)] on the recovered polysaccharide and effected complete removal of the sulphate [yield 152 mg from 203 mg].

Infra-red spectra were run as KBr discs and also as polysaccharide films on silver chloride plates [GM V (b)(ii)] of both the desulphated and the original polysaccharide.

The 3,6-anhydro galactose[110,120] content was determined before and after attempted desulphation with alkali and was compared with a synthetic mixture of sugars, (xylose (30), glucose (20), galactose (30), glucuronic acid (20)).

Expt. 14 Oxidation of the mucilage and desulphated mucilage with sodium metaperiodate

(a) The mucilage (100 mg) and the desulphated mucilage (100 mg) were separately dissolved in water (100 ml) and an equal volume of 10 mM-sodium metaperiodate solution was added to each. Aliquots (3 ml) were withdrawn at intervals and diluted to 250 ml with water, the absorbances were read at 223 nm. The oxidised mucilage was then reduced with borohydride as described in [GM VII (ix)]. A dilute solution of sodium metaperiodate was used so that the change in absorbance as the periodate was used up could be followed more easily. After freeze-drying the derived polyalcohols of the mucilage (69 mg)

and the desulphated mucilage (68 mg) were subjected to a further oxidation using 10 mM-sodium metaperiodate. Further oxidation was negligible, nevertheless reconversion into the polyalcohols was effected.

(b) A second oxidation of the mucilage (500 mg) dissolved in water (250 ml) was achieved with 0.05M-sodium metaperiodate (250 ml). Aliquots (0.6 ml) were withdrawn at intervals, diluted to 250 ml with water and the absorbance read at 223 nm. The recovered polyalcohol (360 mg) [GM VII (ix)] was reoxidised using 5 mM sodium metaperiodate, no further oxidation was detected. Part of the derived polyalcohol (317 mg) was hydrolysed and studied by paper chromatography and g.l.c.. The carbohydrate content [GM VI (i)] was determined after each oxidation.

Expt. 15 Methylation of the mucilage

The mucilage (50 mg) was methylated once by Hakomori's method [GM VII (viii)]. An aliquot (20 mg) of the partially methylated polysaccharide (48 mg) was remethylated by Hakomori's method. In both methylations an ultrasonic bath was used in addition to shaking, both after addition of the 'carbanion' and after addition of the methyl iodide. The methylated polysaccharide was hydrolysed [GM II (i)] and the sugars in the hydrolysate converted into the partially methylated alditol acetates [GM VII (v)(vii)] which were studied by g.l.c. - m.s. [GM V (a) C_1B (ii)(iii)] and by g.l.c. using the same columns.

The sulphate content [GM VI (iii)] was determined for the methylated polysaccharide.

Expt. 16 Studies on the polyalcohols

Aliquots (10 mg) of the polyalcohols derived from the mucilage and from the desulphated mucilage were hydrolysed [GM II (i)]. The hydrolysates were studied by electrophoresis [GM III (ii) (b)], paper chromatography [GM III (i)(b)] with sprays [GM IV (i)(ii)] and g.l.c. [GM V (a) B (i)] as the sugar TMS derivatives [GM VII (vi)]. The polyalcohols were methylated once by the Hakomori method [GM VII (viii)] and hydrolysed [GM II (i)]. The derived partially methylated alditol acetates [GM VII (v)(vii)] were studied by g.l.c. - m.s. [GM V (a) C B(ii)(iii)] and by g.l.c.

Expt. 17 Molecular Size studies

The mucilage (1.5 mg) was dissolved in water (3 ml) and was layered onto a column (90 x 1.6 cm) packed with Sepharose 4B. Throughout packing and during elution of the column a flow rate of 1 drop every 15 sec. was maintained using a peristaltic pump. The sample was eluted from the column with 1.0M-KCl solution. Fractions (2 ml) were collected automatically. The void volume (38 ml) was determined with blue dextran of molecular weight in excess of 5×10^6 which is the exclusion limit of Sepharose 4B. Glucose was eluted at 117 ml (Fig.14).

A second column was prepared (16.5 x 1.3 cm) which had a void volume of 10 ml. Glucose was eluted at 31 ml. The derived

polyalcohol (0.5 mg) obtained after periodate oxidation of the mucilage was dissolved in water (0.5 ml) and layered onto the column. Fractions (2 ml) were collected during elution with 1.0M-KCl solution.

Aliquots (0.5 mg each) of the mucilage after one and after two Hakomori methylations were dissolved in water (0.5 ml each) and separately layered onto columns (16.5 x 1.3 cm) and eluted with 1.0 KCl, 2 ml fractions being collected. Each of the fractions collected was analysed for its carbohydrate content [GM VI (i)] and a graph plotted of mls eluted against carbohydrate content (Figs.3,4). A standard graph was made of molecular weight against mls eluted by separately passing standard dextran solutions of known molecular weight (T 2000, T 250, T 70, T 40, T 10) through the columns and analysing the carbohydrate content in each fraction collected. (Figs.18,19)

Expt. 18 Treatment of the polyalcohol with trifluoroacetic acid

The polyalcohol (150 mg) was dissolved in 1.0M-trifluoroacetic acid (28 ml) and left stirring at room temperature.[106] An aliquot (0.1 ml) was withdrawn after 23 h and passed down a Sepharose 4B column (16.5 x 1.3 cm) as described in expt. 17. A second aliquot (0.1 ml) was withdrawn after 43 h and passed down the column. The partially hydrolysed polyalcohol was recovered by freeze-drying (145 mg). An aliquot was redissolved in water and treated with 'Amberlite' IR 45 (OH) resin to remove the last trace of trifluoroacetic acid. The resulting solution was studied by high pressure liquid chromatography on a Waters Associates Liquid Chromatograph ALC 202 and a LiChrosphere Si100 column (Merck).

Acetic acid (0.1%) solution was the solvent. The flow rate was 0.1 ml/min and the column size was 60 x 0.2 cm.

The LiChrosphere column was calibrated by passing standard dextran solutions (T 70, T 40, T 10) of known molecular weight through the column (Fig.5,6).

An aliquot of the partially hydrolysed polyalcohol was hydrolysed [GM II (i)] and the hydrolysate was studied by paper chromatography [GM III (i) (a)(b)] using sprays [GM IV (i)(ii))].

Expt. 19 Carbodiimide reduction of uronic acid

The mucilage (100 mg) was dissolved in water (100 ml) and the pH adjusted to 4.75. The carbodiimide reagent was added [GM VII (x)], however the polymer precipitated immediately and no change in pH occurred. The uronic acid content [GM VI (ii)] was determined after reduction, dialysis and freeze-drying.

Expt. 20 Hydrolysis of the mucilage with hydrochloric acid

The mucilage (300 mg) was hydrolysed with 1.0M-HCl (30 ml) in a sealed flask for 2 h at 100°. The HCl was removed by evaporation under vacuum at 40°. Saponification of lactones was achieved by treatment of the hydrolysate with 1.0M-NH_4OH until pH 8.0 was reached. The mixture was left at room temperature for 2 h and the ammonia was removed by evaporation under vacuum at 40°. The acidic components were separated from the neutral components by passage of the hydrolysate through a Bio-Rad AG 3-X4A column (90 x 1.6 cm, formate form) as described by previous workers.[107] All the acidic

material was eluted with 1.0M-formic acid. This was then fractionated on a Bio-Rad AG 3-X4A column (90 x 1.6 cm, formate form) using 0.3M-formic acid as the eluent, 2 ml fractions being collected. The carbohydrate content [GM VI (i)] was determined for each fraction collected and the appropriate fractions were combined. The formic acid was removed by evaporation and co-distillation with methanol under vacuum at 40°, and the fractions analysed by paper chromatography [GM III (i) (a)] with sprays [GM IV (ii)].

Results and Discussion

It should be pointed out that the first 12 months of this work was restricted to 4 g of crude polysaccharide extracted from cells of P. cruentum supplied by Dr. Williamson. This dictated the type of experiment which could be undertaken and prevented all large scale experiments.

The carbohydrate content of the crude polysaccharide from Aberdeen was 27%. However after purification by the hollow fibre beaker dialyser this had increased to 65%. The samples from Marine Colloids Inc. contained 75% carbohydrate. These measurements were by the phenol/sulphuric acid method and were based on a standard graph made from a synthetic mixture of xylose (30), glucose (20), galactose (30) and glucuronic acid (20).

The purified polysaccharide and the polysaccharide from Marine Colloids Inc. both had uronic acid contents of ca. 13%, sulphate contents of ca. 11%, protein contents of ca. 5% and methoxyl contents of ca. 0.63%. The first two figures are expressed as a percentage of the carbohydrate while the last two are expressed as a percentage of the total weight. Analysis of the mucilage by Marine Colloids is shown in table 1.

Table 1

Analysis of P. cruentum by Marine Colloids

%	mucilage	anhydrous mucilage
Water	5.42	-
Ash	13.55	14.32
Na^+	3.78	4.00
K^+	0.001	0.001
Ca^{2+}	0.02	0.02
Mg^{2+}	0.03	0.03
N	0.217	0.23
Cl^-	0.09	0.095
Total sulphate	11.82	12.50
Free sulphate	0.29	0.31
3,6-anhydrogalactose	1.26	1.33
Uronic acid (carbazole)	14.50	15.33
Carbohydrate (anthrone)	83.0	87.8
PO_4^{3-}	0.039	0.041
Hyamine	-	-

A solution of the polysaccharide was opaque even after filtration through millipore filters so the optical rotation could not be determined. It was decided to investigate any differences between the samples by analysing their hydrolysates by paper chromatography; this showed their essential similarity. Paper chromatograms gave spots which corresponded to xylose, glucose, galactose, traces of two fast spots ($R_{glc.}$ 1.3 and 1.4 in solvent (b)), one major oligouronic

acid ($R_{glc.}$ 0.43), one minor oligouronic acid ($R_{glc.}$ 0.76) both in solvent (a), and faint spots corresponding to neutral oligosaccharides. The absence of fructose was shown by the negative result obtained with urea hydrochloride spray. Electrophoresis in borate buffer confirmed that mannose was also absent (Expt. 7).

Fractionation of the polysaccharide

Such a complex mixture suggested that there could be a number of polysaccharides present, so fractionation of the crude polymer was attempted using a DE 52 cellulose column (expt. 2). The carbohydrate and uronic acid content of the various fractions obtained are shown in table 2 and are compared with those of the original mucilage.

Table 2

Fractions from DE 52 column

	Weight	Carbohydrate content	Uronic acid content*
Original mucilage	500 mg	27%	13%
Fraction eluted with water	51 mg	23%	10%
0.1M-KCl	83 mg	43%	10%
0.5M-KCl	113 mg	43%	9%
1.0M-KCl	87 mg	19%	15%

* Calculated on carbohydrate content.

This shows a 67% recovery by weight but a 83% recovery of carbohydrate. The nitrogen content of all the fractions were very similar giving a protein content of about 5% for each. Hydrolysates

of each fraction were compared by paper chromatography and were found to be practically identical. As there were only minor differences between each fraction it was assumed that no significant fractionation had taken place.

Fractionation of the pure polysaccharide was attempted by complexing the mucilage with CETAB (expt. 5). The complex was obtained as a white flocculent precipitate. Although the yield of polysaccharide recovered from the complex was only 80% by weight of the starting material, no carbohydrate could be detected in the supernatant after removal of CETAB with resin. A hydrolysate of the recovered polysaccharide gave the same chromatographic pattern on paper and g.l.c. as a hydrolysate of the initial material. The 20% loss probably occurred during the dissolution in water and reprecipitation with ethanol to remove the last traces of CETAB from the polymer. At the same time the procedure did remove part of the protein which was reduced from 5% to 1.2%. Otherwise it is concluded that no fractionation, apart from possibly the removal of low molecular weight material, had occurred.

In view of these results no distinction between the different samples is made in subsequent studies.

Viscosity studies

The relative viscosities (compared to that of water) of a 0.35% solution of the polysaccharide under various conditions are shown in table 3.

Table 3

Relative viscosities of a 0.35% solution of *Porphyridium cruentum* mucilage at 30°

pH		Relative viscosity
3.5		44
7.0		12
9.5		10
11.0		16
after heating to 70°	at pH 9.5	16
after autoclaving		11

As can be seen the relative viscosity remains reasonably stable until pH 3.5 when it increases significantly.

Characterisation of sugars

After separation by paper chromatography (expt. 8) the following sugars were characterised.

Xylose isolated as a reducing syrup (expt. 10) which on paper chromatogram and g.l.c. had the same mobility as xylose. The optical rotation +19.5° confirmed that D-xylose was present (cf. D-xylose +19°).

Galactose isolated as a reducing syrup which had the same retention time as galactose on paper chromatograms and g.l.c. The optical rotation +41° indicated that 75% of the galactose was present as the D-sugar (cf. D-galactose $[\alpha]_D$ +79°) and 25% as the L-sugar. Galactose oxidase showed that some D-galactose was present.

For further determination of the proportions of D- and L-galactose see P. aerugineum p.85.

Glucose isolated as a reducing syrup having the same mobility as glucose on paper chromatograms and g.l.c. Glucose oxidase confirmed the presence of D-glucose. The optical rotation +58° indicated that all the glucose was present as the D-sugar (cf. D-glucose +53°).

3-O-methyl xylose isolated as a reducing syrup ($R_{glc.}$ 2.1 in solvent (a)) had the same mobility on paper chromatograms and on g.l.c. as 3-O-methyl xylose. Further proof for the presence of this sugar is given for P. aerurineum (p.99). This sugar has recently also been found and characterised from the red alga Rhodella maculata,[114] and also from the bacteria Myxococcus fulvus.[121]

The alditol acetates from the hydrolysed polysaccharide (expt. 7) were analysed by g.l.c. - m.s. A fully acetylated pentose and two fully acetylated hexoses (xylose, galactose and glucose) were found as the main constituents. In addition to these a 3-O-methyl pentose (3-O-methyl xylose), 3- and 4-O-methyl hexose and a 2-O-methyl hexose were also detected. Their retention times relative to xylitol pentaacetate were 0.60, 2.02 and 1.64 respectively. The retention time of the 3- and 4-O-methyl hexose on paper chromatograms and on g.l.c. indicated that they were galactose derivatives. The 2-O-methyl hexose, however, could not be resolved into the glucose or galactose derivative. Because of the small quantities of these sugars present in this polysaccharide further confirmation was impossible. However more complete characterisation

of both 3- and 4-O-methyl galactose is given in P. aerugineum (p.100).

The relative proportion of the constituents

The relative amounts of the constituent sugars present in a hydrolysate of the polysaccharide were determined by measuring the carbohydrate content of each fraction after elution from a paper chromatogram (expt. 8). At the same time an aliquot of the hydrolysate, after conversion of the carbohydrates into the corresponding alditol acetates, was analysed by g.l.c. and the peak areas of the monosaccharides measured. This latter method took no account of the oligosaccharides present in the hydrolysate which corresponded to 30% of the carbohydrates. In order to compare the two methods it was therefore necessary to adjust the proportions determined from peak areas to correspond to 70% of the carbohydrate. When this adjustment is made it can be seen that the two methods are in good agreement (table 4).

It can be seen from this table that all the uronic acid in the hydrolysate is present as aldobiouronic acids. The proportion of these, 25%, corresponds to a uronic acid content of 12.5% which is in good agreement with the figure of 13% obtained from the uronic acid determination which is based on a carbohydrate content of 75%. No trace of monouronic acid could be detected in hydrolysates carried out under a wide variety of conditions (expts. 7, 9).

Table 4

Percentage Proportions of the constituent sugars

Sugar	$T^{(a)}$	Carbohydrate content of eluted fractions	Adjusted peak areas[(b)]
Xylose	1.0	35%	32.8%
Galactose	2.16	18%	20.0%
Glucose	2.35	12%	12.4%
3-O-methyl xylose	0.58		1.6%
3- and 4-O-methyl galactose	2.0	5%	1.6%
2-O-methyl hexose	1.64		1.6%
Oligouronic acid (1)		21%	-
Oligouronic acid (2)		4%	-
Other oligosaccharides		5%	-

(a) Retention time relative to xylitol penta acetate on column (ii).

(b) Based on 70% of the carbohydrate as monosaccharides.

These results indicate the danger of determining the molar proportions of the monosaccharides in hydrolysates of polysaccharides containing uronic acid residues by peak areas on g.l.c. It is impossible to hydrolyse such polymers to the monouronic acid without excessive degradation of the acid and to some extent the neutral sugars.

The 4.8 - 5% of monomethyl sugars found is in good agreement with the figure of 4.7% obtained from the methoxyl content of 0.63% and based on a carbohydrate content of 75%.

Characterisation of oligouronic acid (1)

As stated previously all attempts to separate a monouronic acid were unsuccessful. However oligouronic acid (1) (17 mg) was separated from a hydrolysate. It had a D.P. of 1.95, an optical rotation of $+19^{\circ}$ and had $R_{glc.}$ 0.40 and $R_{glc.U.A.}$ 0.46 on a paper chromatogram in solvent (a). An electrophoretogram in pyridine/acetic acid buffer pH 6.7 showed its mobility to be $M_{glc.U.A.}$ 0.65. These results indicate an aldobiouronic acid. Aniline oxalate spray gave a yellow colour showing that the reducing end of the molecule was a hexose.

After esterification, reduction and hydrolysis equal proportions of glucose and galactose were found, these were confirmed as the D-sugars by the positive results obtained with glucose and galactose oxidases.

After reduction, esterification, reduction and hydrolysis, glucose and galactitol were found to be present indicating that the aldobiouronic acid is a D-glucuronosyl-D-galactose.

After esterification and reduction the derived disaccharide was methylated (expt. 12) and hydrolysed. Analysis by g.l.c.-m.s. of the derived methylated sugars as the partially methylated alditol acetates gave 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl galactitol and 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl glucitol thus showing that

the original aldobiouronic acid is 3-O(β-D-glucopyranosyluronic acid)-D-galactose. From the rotation, $[\alpha]_D$ +19°, it can be deduced that the linkage is β-(cf. 3-O-methyl-D-galactose $[\alpha]_D$ +87°, α-methylglucuronamide $[\alpha]_D$ +136°, β-methyl glucuronamide $[\alpha]_D$ -47°).

Characterisation of oligouronic acid (2)

The second oligouronic acid (3 mg) had an optical rotation of +20°, $R_{glc.}$ 0.76 in solvent (a). An electrophoretogram in pyridine/acetic acid showed its mobility to be $M_{glc.U.A.}$ 0.74.

After esterification, reduction and hydrolysis D-glucose and L-galactose were found as shown by the reaction with glucose and galactose oxidases. The derived alditol acetates, when analysed by g.l.c.-m.s. showed galactose and a 2-O-methyl hexose as the main constituents with a lesser amount of xylose present.

After esterification and reduction the derived disaccharide was methylated, hydrolysed, and then analysed by g.l.c.-m.s. as the derived partially methylated alditol acetates. This showed the presence of 2,3,4,6-tetra-O-methyl glucose and 2,3,6-tri-O-methyl galactose as their alditol acetates.

In addition minor amounts of 2,3,4-xylose and 2,4-xylose were found when the sample was analysed by g.l.c..

The optical rotation of +20° indicates that the aldobiouronic acid is probably α-linked (cf. $[\alpha]_D$ 4-O-methyl-L-galactose -92°, $[\alpha]_D$ α-methylglucuronamide +138, $[\alpha]_D$ β-methylglucuronamide -47°). The original aldobiouronic acid is therefore 4-O-(2-O-methyl-α-D-glucopyranosyluronic acid)-L-galactose. The presence of xylose in the hydrolysate and of methylated xyloses in the methylated

hydrolysate is considered to be due to 1,3-linked xylobiose being present with the aldobiouronic acid. This was subsequently separated from a formic acid hydrolysate.

Partial hydrolysis

Since all attempts to fractionate the polysaccharide were unsuccessful it was concluded that the mucilage from P. cruentum was a single heteropolysaccharide containing D-xylose, D- and L-galactose, D-glucose, 3-O-methyl xylose, 3- and 4-O-methyl galactose, 2-O-methyl hexose, D-glucuronic acid and 2-O-methyl-D-glucuronic acid. In order to investigate the mutual linkages within this complex polysaccharide it was decided to partially hydrolyse it and to separate and characterise any oligosaccharides present in the hydrolysate (expt. 9).

Preliminary hydrolysis experiments with different concentrations of sulphuric and oxalic acids for various periods of time followed by analysis of the different hydrolysates showed that 0.1N-sulphuric acid at 100° for 4h gave the highest yield of oligosaccharides. These conditions were therefore used in a large scale experiment.

Neutral oligosaccharides

After separation of the neutral and acidic fragments on a deacidite column the following neutral oligosaccharides were separated on 3 MM chromatography paper (table 5).

Table 5

Neutral oligosaccharides

	Mobility in solvent (b)	Weight	D.P.	Colour when developed with aniline oxalate
Oligosaccharide (1)	0.30	0.5 mg	3.7	pink
Oligosaccharide (2)	0.51	5 mg	2.2	yellow
Oligosaccharide (3)	1.0	3 mg	1.7	pink

Oligosaccharide (1), after hydrolysis was found to contain glucose, galactose and xylose in the molar proportions 1:1:2. As this oligosaccharide gave a pink colour with aniline oxalate spray it was assumed that xylose was present at the reducing end. Insufficient material prevented any further study of this oligosaccharide.

Oligosaccharide (2), after hydrolysis was found to contain glucose, galactose and xylose in the molar proportions of 2:3:0.5. After reduction and hydrolysis D-glucose and galactitol were found thus indicating that galactose was present as the reducing sugar. The reduced disaccharide after electrophoresis in molybdate buffer gave one spot with $M_{glucitol}$ 0.56 which is indicative of a 1,4-linked disaccharide.[122] Methylation of the disaccharide followed by hydrolysis and analysis by g.l.c. - m.s. as the derived alditol acetates gave 2,3,4,6-tetra-O-methyl glucose and 2,3,6-tri-O-methyl galactose. This shows that this disaccharide is mainly D-glucosyl (1 → 4) galactose. The presence of additional galactose and xylose was considered to be due to a second oligosaccharide of the same mobility.

Oligosaccharide (3) was reseparated from glucose and galactose in solvent (a), in which it had $R_{glc.}$ 0.82. However even after reseparation some free galactose remained as an impurity. In view of the fact that only a small amount of material was available it was decided to continue analysis of this oligosaccharide with galactose present as a known contaminant. After hydrolysis of the disaccharide, xylose, D-glucose and galactose were found, xylose and glucose being in the molar proportions of 1:1. As the disaccharide was pink when sprayed with aniline oxalate xylose was thought to be present at the reducing end of the molecule. After reduction and hydrolysis xylitol, galactitol and glucose were found. Thus this disaccharide was considered to be a D-glucosyl-xylose. Insufficient material remained for methylation studies.

Another oligosaccharide (4 mg) was separated from a formic acid hydrolysate (expt. 8). This oligosaccharide had a D.P. of 1.8, gave a pink colour when developed with aniline oxalate and on hydrolysis gave only xylose. It had $R_{glc.}$ 0.81 in solvent (a). Comparison with standard 1,3- and 1,4-linked xylobiose showed that this disaccharide was xylosyl (1 → 3) xylose, xylosyl (1 → 4) xylose having $R_{glc.}$ 0.55 in solvent (a).

Besides showing some of the linkages between the different sugars in this mucilage, oligosaccharide (1) also confirms the presence of glucose, galactose and xylose in a single polysaccharide.

Desulphation studies (expt. 13)

The sulphate content was unaffected by alkali in the presence of borohydride thus indicating that trans elimination via an epoxide ring

does not occur. Therefore the sulphate is not present in a trans position relative to an adjacent free hydroxyl group. The absence of any new sugar or the presence of 3,6-anhydro galactose in the hydrolysate of the alkali treated polysaccharide confirms this result, at the same time eliminating the possibility of a 6-sulphated hexose with a free hydroxyl at C-3. The polysaccharide was recovered in 85% yield from the alkali treatment.

Treatment with 0.08M-methanolic HCl effected complete removal of the sulphate, the desulphated material was recovered in 71% yield.

Infra-red spectra of the polysaccharide before desulphation gave absorbances at 1250-60 cm^{-1}, 820 and 830 cm^{-1} which are characteristic of ester sulphate groups in polysaccharides. A band occurring at 1240 cm^{-1} is characteristic of S = O stretching frequency and an absorption band at 820 cm^{-1} is characteristic of a primary half ester sulphate group and that at 830 - 840 cm^{-1} is indicative of an equatorial half ester sulphate group.[111] After desulphation the absorption band at 1250 - 60 cm^{-1} and the bands at 820 and 830 cm^{-1} were greatly reduced. No band at 930 - 940 cm^{-1}, which is characteristic of 3,6-anhydro galactose, was seen in either the sulphated or desulphated polysaccharide.

Unlike most other polysaccharides the sulphate is removed during methylation and it was therefore impossible to determine the site of sulphate by methylation before and after desulphation.

The conclusions that may be drawn from the stability to alkali about the position of the sulphate are that it cannot be present in a trans position relative to an adjacent free hydroxyl group i.e. it is

not present on C-2 or C-3 of 1,4-linked glucose, galactose, xylose or glucuronic acid, although these units may be disulphated at C-2 and C-3. The absorption band at 820 cm^{-1} indicates that the sulphate may be present on C-6 of 1,3-linked glucose and/or galactose. The absorption band at 830 cm^{-1} indicates that the sulphate may be present on C-2 or C-3 of glucose, galactose, glucuronic acid or xylose that are 1,2- or 1,3-linked. Later work on the linkages within the polysaccharide (p. 70) indicate that no 1,2-links are present therefore the sulphate must be present on C-2 of 1,3-linked glucose, galactose, glucuronic acid or xylose.

Periodate oxidation (1) (expt. 14)

Periodate oxidation of the mucilage (carbohydrate content 70%) and the desulphated mucilage (carbohydrate content 80%) with 5 mM sodium metaperiodate gave a reduction per anhydro unit of 0.25 and 0.35 moles of periodate respectively (Fig.1). The derived polyalcohols were recovered in yields of 69 and 68% respectively by weight (carbohydrate contents 48% and 52% respectively). The increased reduction of periodate after desulphation means that additional glycol groups have resulted from the desulphation supporting evidence of sulphate on C-2 and C-3 of 1,4-linked units. The hydrolysates of the two polyalcohols were very similar, each giving glucose, galactose, xylose, glycerol, erythritol and threitol, 1,3-linked aldobiouronic acid and a trace of 2-O-methylglucuronic acid. No small acidic fragments were detected. When the hydrolysate was analysed by g.l.c.-m.s. as the derived alditol acetates, small amounts of methylated hexoses were also found. The

E.R.

Figure 1

Periodate oxidation curves for F.cruentum with 5 mM periodate

× = Desulphated mucilage

⊙ = Original mucilage

Moles/anhydro unit

0.5
0.4
0.3
0.2
0.1
0

0 4 8 12 16 20 24 28 32 36 40

Time hs.

relative proportions of the different sugars present, as shown by the peak areas from g.l.c. of the alditol acetates, are given in table 6.

Table 6

Molar proportions and percentages of the sugars present in *P. cruentum* and its derived polyalcohol

Sugar	Original mucilage		Derived polyalcohol	
	% based on 75% carbohydrate	Molar proportion	% based on 48% carbohydrate	Molar proportion
Xylose	23.2	2.65	6.4	1.03
Galactose	14.1	1.61	11.0	1.75
Glucose	9.0	1.0	6.3	1.0
3-O-methyl xylose	1.2	0.13	1.1	0.17
3-and 4-O-methyl galactose[a]	1.2	0.13	1.1	0.17
2-O-methyl hexose	1.2	0.13	1.1	0.17
Uronic acid[b]	13	1.44	21	3.2

(a) m.s. indicates that equal quantities of the two monomethyl sugars were present.

(b) as determined by the m-hydroxydiphenyl method.

The methyl hexoses must be immune to oxidation by periodate and their relative increase in the polyalcohol support this. The above figures (table 6) clearly indicate that a large proportion of the xylose units are cleaved during oxidation but they must be viewed with a certain amount of caution when it is remembered that about 30% of the

mucilage is lost during the oxidation and reduction. Nevertheless the presence of glycerol in the hydrolysate confirms the loss of 1,4-linked xylose. Since the polyalcohol only contains 48% of uncleaved carbohydrate and the hydrolysate contains threitol and erythritol, some of the galactose and glucose must have been cleaved by the periodate and were therefore present as 1,4-linked units.

The occurrence of the majority of the uronic acid as an aldobiouronic acid agrees with the presence of 3-O-(β-D-glucopyranosyl-uronic acid)-D-galactose. It also indicates that the glucuronic acid residue itself is 1,3-linked or is present as a 1,2,4- or 1,3,4-linked branch point. The presence of a small amount of 2-O-methylglucuronic acid is in agreement with the earlier finding of 2-O-methyl-D-glucuronosyl (1 → 4)-L-galactose. The 2-O-methyl-D-glucuronic acid residue being 1,3- or 1,4-linked. The relative increase in uronic acid in the polyalcohol and the absence of any small acidic fragments such as glyceric acid confirms that the uronic acid residues are 1,3-linked and are not attached by periodate.

The only difference between the hydrolysates of the polyalcohols derived from the mucilage and the desulphated mucilage was the larger quantity of xylose present, relative to glucose and galactose, in the latter. This would indicate that more glucose and galactose had been cleaved by the periodate in the desulphated polyalcohol than in the sulphated polyalcohol which confirms that the majority of the sulphate occurs in these two sugars in the polysaccharide. That periodate had not been hindered by acetal formation[112] was shown by the fact that a second treatment of the polyalcohols caused no further oxidation.

Methylation of the polyalcohols

Methylation of the derived sulphated and desulphated polyalcohols (expt. 16) gave the following partially methylated sugars, after hydrolysis, which were analysed by g.l.c.-m.s. as their alditol acetate derivatives.

Table 7

Sugar derivatives found in both methylated polyalcohols

Sugar	Linkage indicated
2,3,4-tri-O-methyl xylose	end group xylose
2,4-di-O-methyl xylose	1,3-linked xylose
2,3-di-O- methyl xylose	1,4-linked xylose
2,4,6-tri-O-methyl glucose	1,3-linked glucose
2,4,6-tri-O-methyl galactose	1,3-linked galactose
2,6-di-O-methyl glucose	1,3,4-linked glucose
2,6-di-O-methyl galactose	1,3,4-linked galactose

The presence of end group and 1,4-linked xylose could have arisen from the 3-O-methylxylose which would not be cleaved by periodate and in the polyalcohol could have risen to at least 5% of the carbohydrate.

The methylated sulphated polyalcohol also contained some 2,3,6-tri-O-methylhexose. Such residues should have been cleaved by periodate unless the free hydroxyl groups were masked by sulphate residues. This seems very probable as the sulphate is cleaved during methylation, and is further evidence that the sulphate groups are located at C-2 and C-3 of the 1,4-linked hexoses.

Periodate oxidation 2 (expt. 14 (b))

The derived polyalcohol was recovered in 72% yield from oxidation of the mucilage with 25 mM-sodium metaperiodate which reduced 0.6 moles of periodate per anhydro unit (Fig.2). This increase in reduction of periodate when compared with the previous oxidation indicates that incomplete oxidation may have taken place with 5 mM-periodate. At the same time the stronger periodate removed the sulphate from the polysaccharide during oxidation so no comparison could be made between the sulphated and desulphated mucilages in this experiment.

Analysis of the hydrolysate of the derived polyalcohol (carbohydrate content 42%) by paper chromatography, electrophoresis and g.l.c. showed no differences from the hydrolysate of the polyalcohol obtained after oxidation of the mucilage with 5 mM-periodate.

Two separate samples of the polyalcohol derived after oxidation with 25 mM-periodate were methylated and were analysed, after hydrolysis, by g.l.c.-m.s. as the derived alditol acetates on columns (ii) and (iii) respectively. The results are shown in table 8.

The polyalcohol was subjected to a single Hakomori methylation which resulted in considerable under methylation, greater in the first case. However a second Hakomori methylation would have resulted in considerable degradation (see p.68). When the initial mucilage was subjected to two Hakomori methylations no monomethylhexose persisted and the proportion of 2,6-di-O-methyl hexose was considerably less (see p.67). Nevertheless ignoring the lower methylated sugars certain conclusions can be drawn from the methylation results in Table 8. The presence of the small amount of 2,3-di-O-

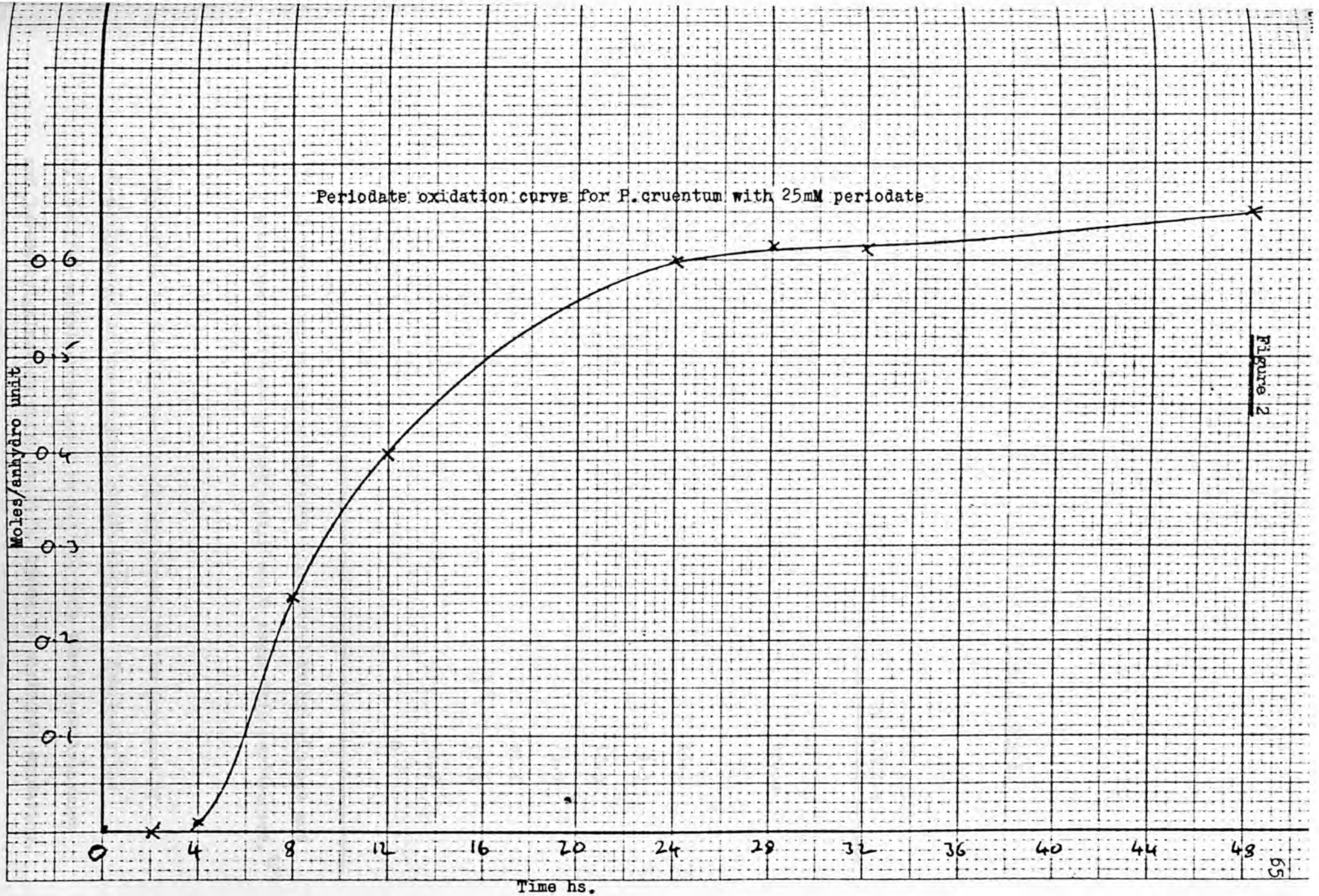

Periodate oxidation curve for P.cruentum with 25mM periodate
Moles/anhydro unit
0·6
0·5
0·4
0·3
0·2
0·1
0
4
8
12
16
20
24
28
32
36
40
44
48
Time hs.

Figure 2

Table 8

Percentage of sugars found in the methylated polyalcohol derived from P. cruentum mucilage

Sugar	Column (ii) 3% OV 225	Column (iii) 3% ECNSS-M
2,3,4-tri-O-methyl xylose		
2,3,4,6-tetra-O-methyl glucose	trace	trace
2,3,4,6-tetra-O-methyl galactose		
2,4-di-O-methyl xylose	28.0	24.3
2,3-di-O-methyl xylose	4.9	4.3
2,4,6-tri-O-methyl glucose	16.0	12.3
2,4,6-tri-O-methyl galactose		3.0
2,3,6-tri-O-methyl hexose(a)	5.6	10.6
2,6-di-O-methyl hexose	12.9	13.6
6-mono methyl hexose	15.8	31.9
unmethylated	16.8	0

(a) It proved impossible to separate 2,3,6-tri-O-methyl-glucose and galactose and hence to distinguish which sugar this represented.[123]

methylxylose is due, as previously explained, to the presence of 3-O-methylxylose in the original polysaccharide. Similarly the 2,3,6-tri-O-methylhexose could be derived from the 2-O-methylhexose and 3-O-methylgalactose which would be immune to periodate oxidation.

At the same time the percentage 10.6 of this seems rather high and some of it may have resulted from the incomplete removal of sulphate residues from the hexoses during the oxidation and their removal during methylation.

Methylation of the mucilage (expt. 15)

The methylated polysaccharides, after one and after two Hakomori methylations, were analysed, after hydrolysis, by g.l.c.-m.s. as the alditol acetate derivatives. The percentages of the partially methylated sugars found are shown in table 9.

The proportions of xylose and 3-O-methylxylose, galactose, and glucose in the original mucilage is 2.78:1.61:1.0 (table 4). The proportion of xylose and 3-O-methyl xylose, galactose and glucose after methylation is 2.31:0.33:1.0 (table 9).

Table 9

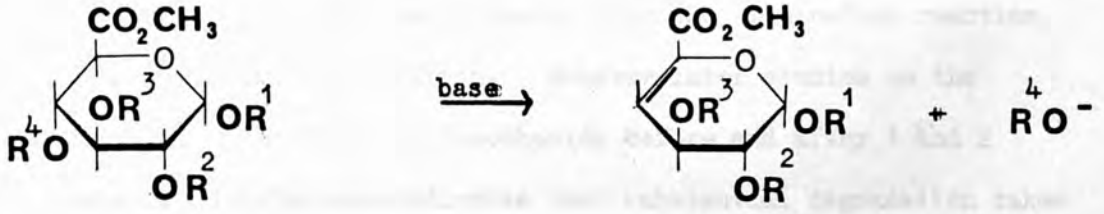
Percentage of Methylated sugar from P. cruentum

Sugar	After 1 Hakomori methylation	After 2 Hakomori methylations
2,3,4-tri-O-methyl xylose	6.6	17.5
2,3,4,6-tetra-O-methyl glucose	12.5	11.1
2,4-di-O-methyl xylose		15.5
2,3,4,6-tetra-O-methyl galactose	10.7	4.9
2,3-di-O-methyl xylose		16.1
2,4,6-tri-O-methyl glucose 2,4,6-tri-O-methyl galactose	6.1	12.1 *
2,3,6-tri-O-methyl hexose	6.4	17.1
2,6-di-O-methyl hexose	0	5.7
non-methylated sugars	57.7	0

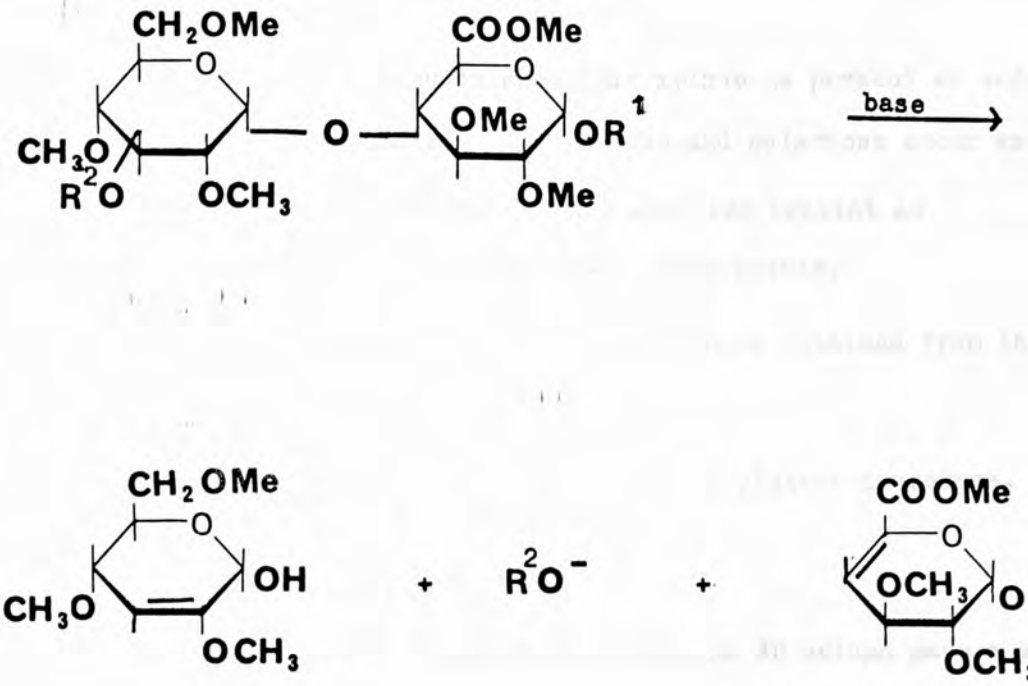
* It was assumed that 10.0% and 2.1% of this figure was due to the glucose and galactose derivatives respectively from the figures obtained from the methylated polyalcohol as the proportion of these sugars should be unaltered by periodate.

Adjusting the figures to the value for xylose as it was in the initial mucilage (i.e. 2.78) the proportions of xylose, galactose and glucose become 2.78:0.40:1.20. These figures, when compared to the proportions of the sugars in the original mucilage i.e. 2.78: 1.61: 1.0, show that most of the 2,3,6-tri-O-methyl and 2,6-di-O-methyl hexose (22.8%, i.e. 1.3 parts) in the methylated polysaccharide must be the galactose derivative. This would make the proportions of the xylose, galactose and glucose derivatives from the methylated polysaccharide 2.78:1.70:1.20 which is in reasonable agreement with the corresponding proportions of the sugars in the initial mucilage.

The high proportion of end group to branch point sugars (which should be equal) after 2 Hakomoris may be explained by the fact that degradation of the uronic acid and hydrolysis of the polysaccharide occurs during the methylation process.[109]



If R^4 is part of the polysaccharide chain and is linked through position 3 further degradation occurs via a β-elimination reaction.



This process can continue until a linkage through a position other than C-3 is encountered. This degradation process is considered to take place only during the second Hakomori methylation as the methyl iodide reacts faster than the elimination reaction during the first methylation. However later studies on the molecular size of the polysaccharide before and after 1 and 2 Hakomori methylations indicates that substantial degradation takes place during the first methylation process as well (expt. 17).

Analysis of a hydrolysate of the methylated polysaccharide by electrophoresis showed that some of the uronic acid was still present as an aldobiouronic acid.

The methylation results show that xylose is present as end group, 1,4- and 1,3-linked units. The glucose and galactose occur as end group and as 1,3- linked units, they are also present as 1,4-linked units and as 1,3,4-linked branch points.

These results are in agreement with those obtained from the periodate oxidation studies.

The sulphate is removed during the methylation procedure.

Molecular size studies (expt. 17)

Elution of the mucilage from a Sepharose 4B column gave a single peak which corresponded to a molecular weight in the order of 4×10^6. The polysaccharide after 1 Hakomori methylation had a wide molecular weight distribution but with two distinct peaks occurring (Fig. 3), one corresponding to a molecular weight of 4×10^6 (40%) and the other corresponding to a molecular weight of 30,000 (60%).

After 2 Hakomori methylations all the methylated polysaccharide was eluted in a band which corresponded to a molecular weight of about 30,000 (Fig. 3).

The derived polyalcohol obtained after oxidation of the polysaccharide with 25 mM-periodate had a wide molecular weight distribution, 60% occurring within the range 4×10^6 — 50,000 and 40% occurring between 50,000 — 200 (Fig.4).

After treatment of the polyalcohol with 1.0M-trifluoroacetic acid at room temperature (expt. 18) the molecular weight distribution was centred around 30,000 (Fig. 4). Analysis of this sample by HPLC confirmed this figure. (Fig.5)

These results indicate the possibility of a repeating unit occurring in the polysaccharide with a molecular weight of about 30,000 (i.e. about 150 sugar units). They also show that a large amount of degradation occurs both during 1 and 2 Hakomori methylations and also during periodate oxidation.

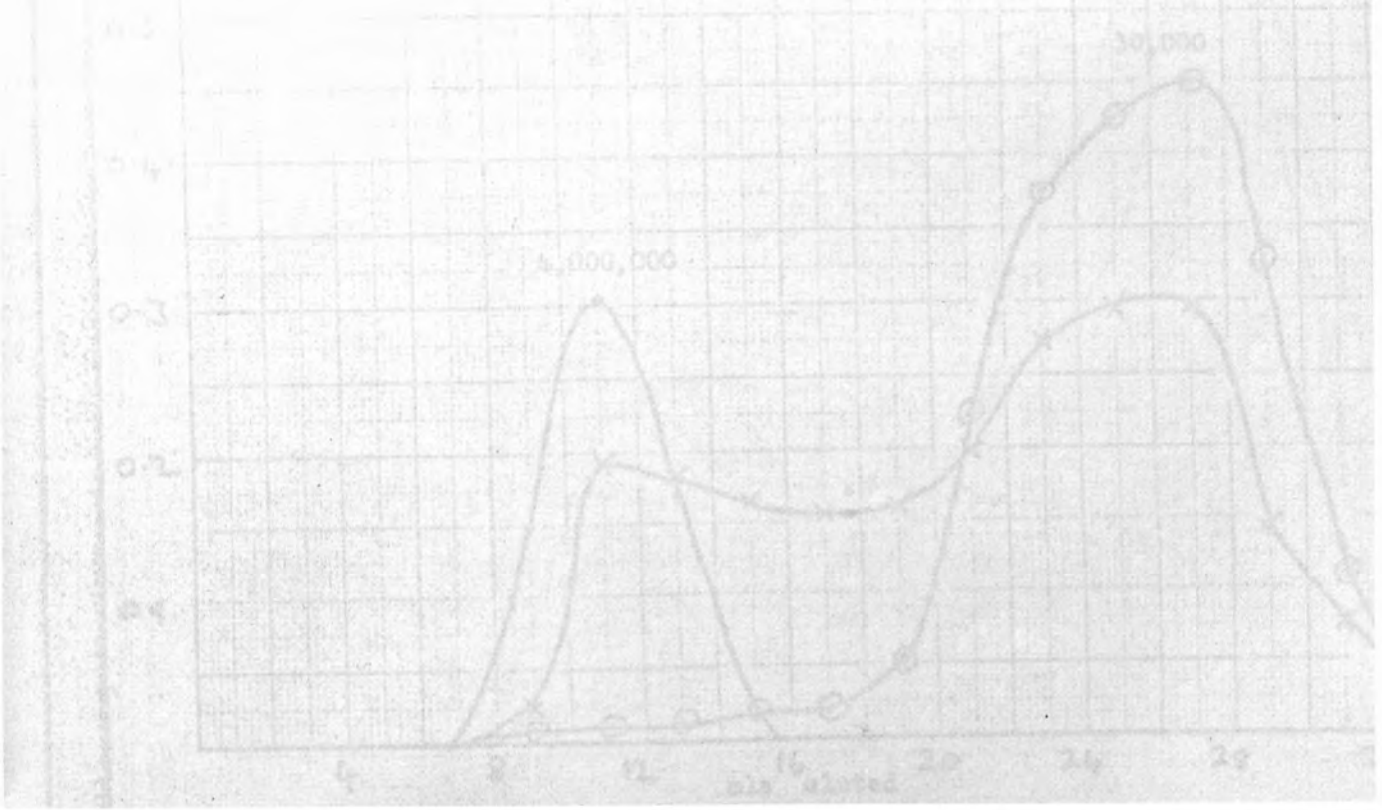


Figure 3

Molecular size of the methylated mucilage from P.cruentum

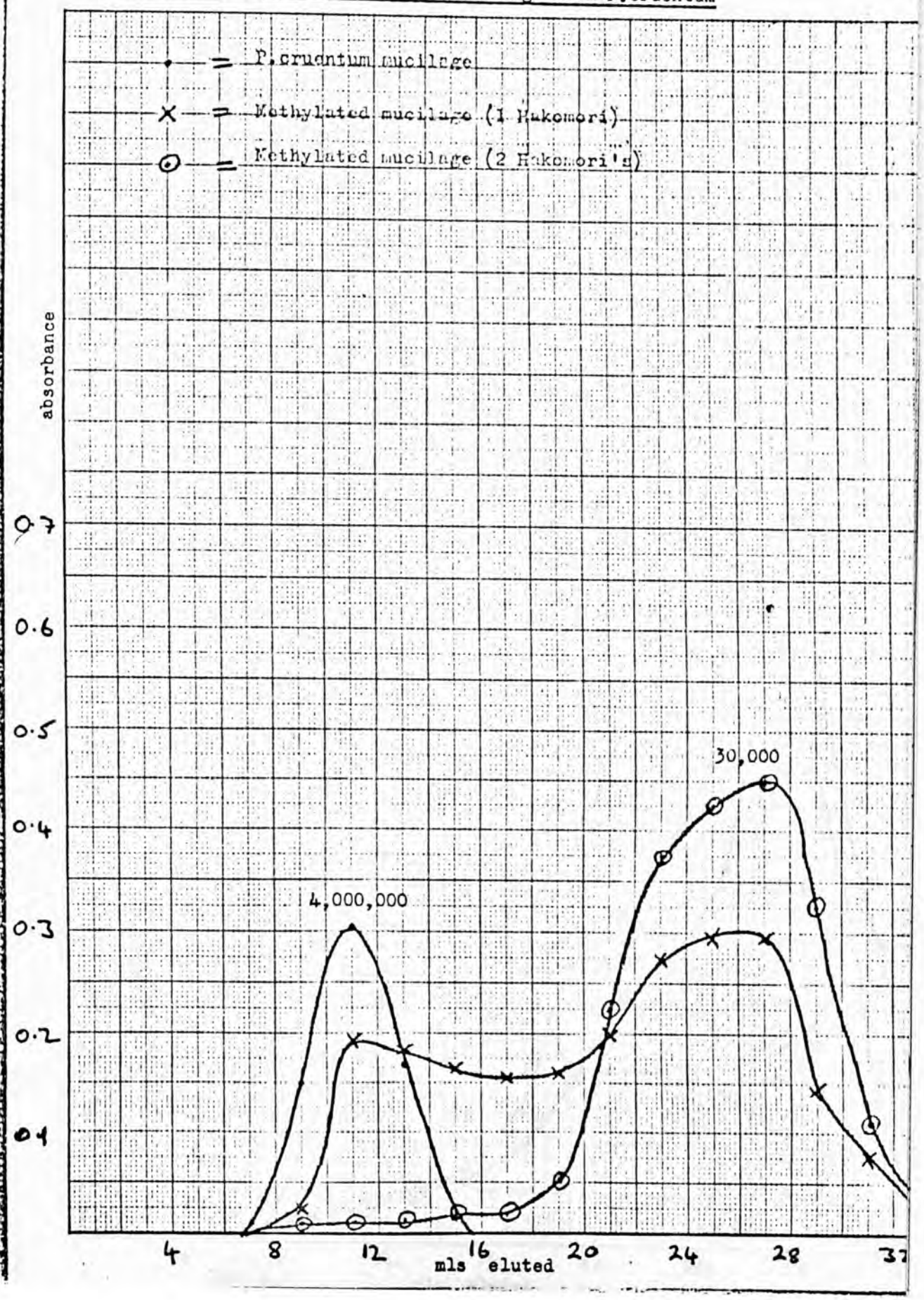
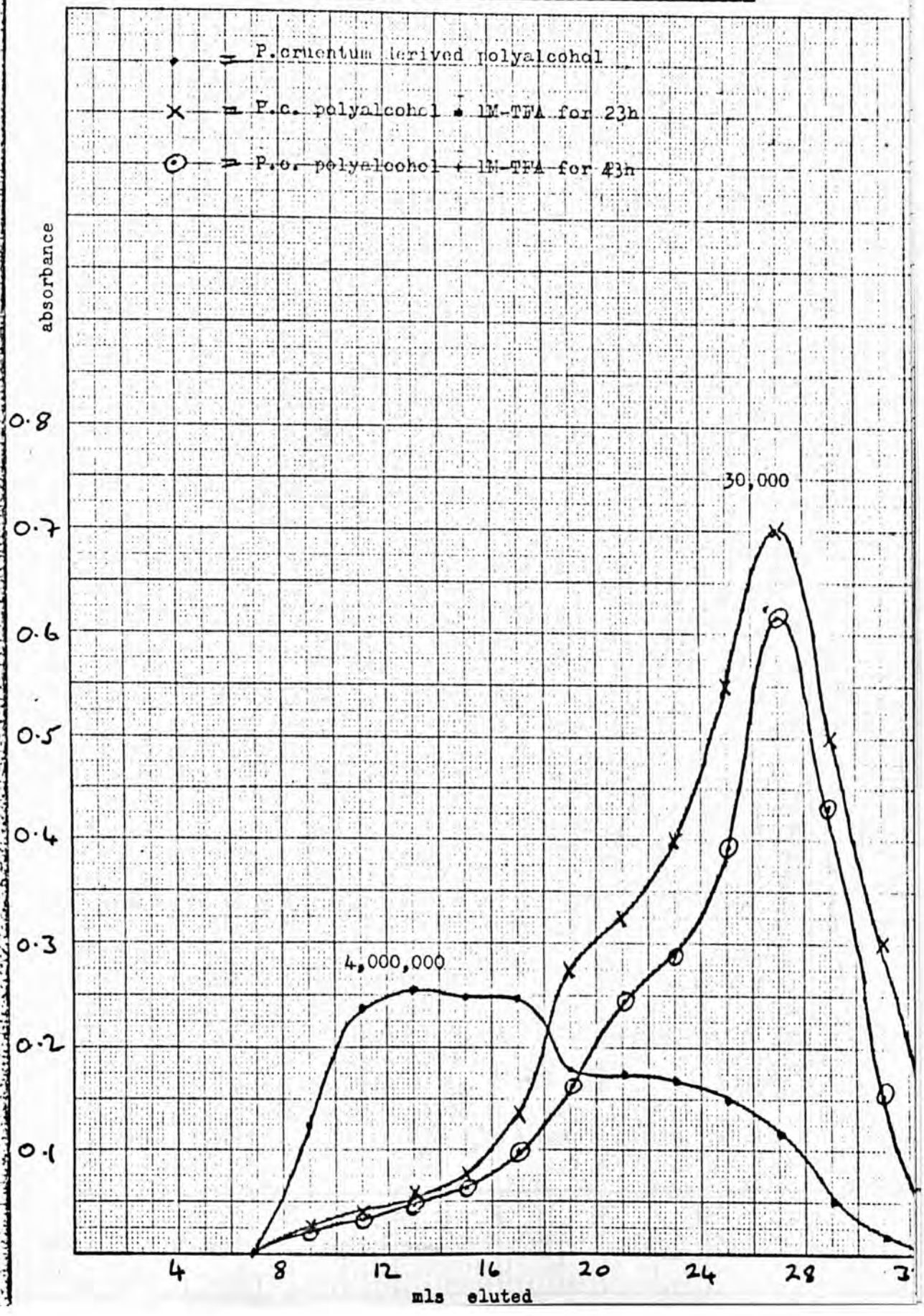


Figure 4

Molecular size of the polyalcohols from P.cruentum mucilage



E.R.

Figure 5

H.P.L.C.elution patterns

- - - = T.F.A. treated polyalcohols

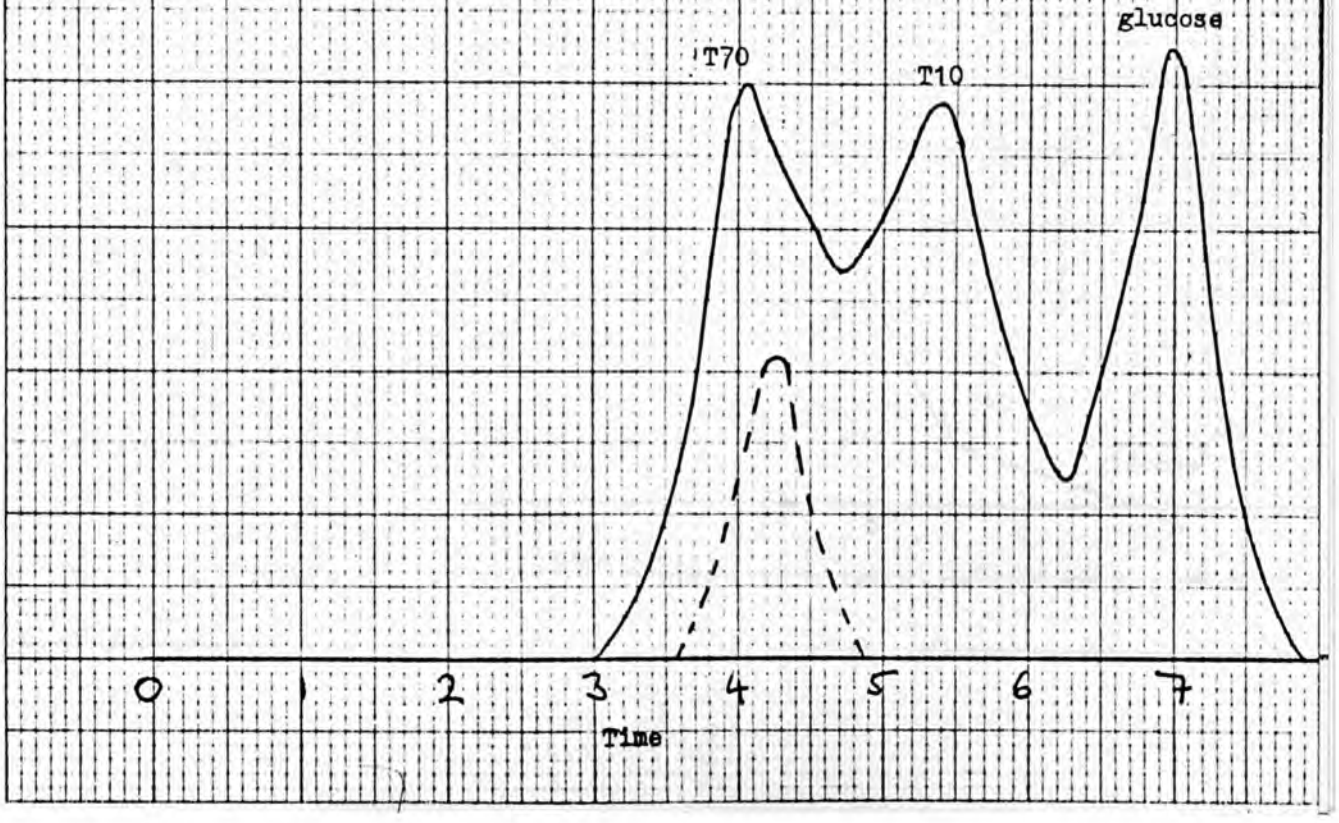
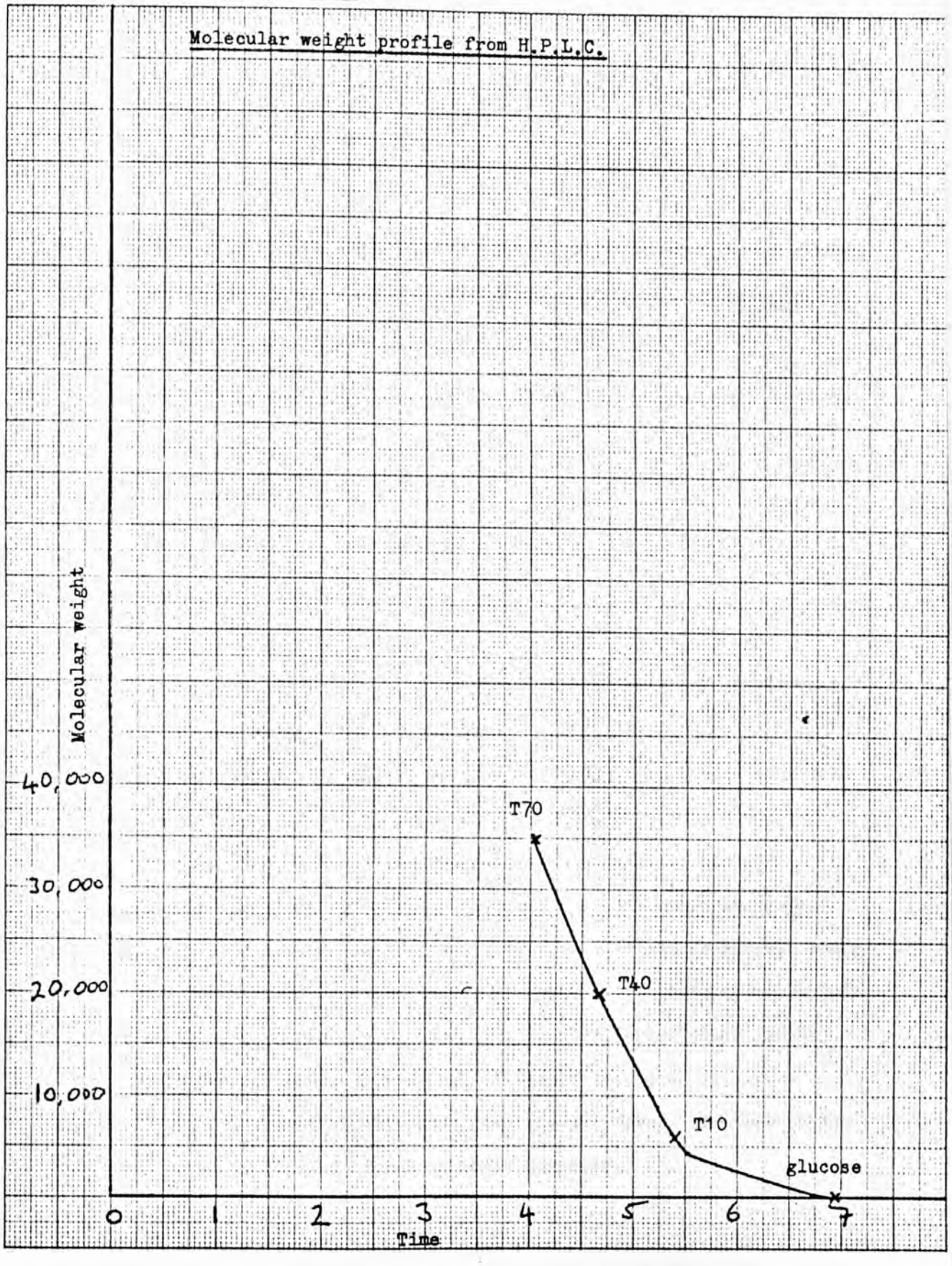


Figure 6


Molecular weight profile from H.P.L.C.
Molecular weight
40,000
30,000
20,000
10,000
T70
T40
T10
glucose
0
1
2
3
4
5
6
7
Time

Trifluoroacetic acid treated polyalcohol (expt. 18)

After complete hydrolysis of the polyalcohol (mol. wt. ca. 30,000) and analysis of the hydrolysate, the same sugars were found, in approximately the same proportions as those found in a hydrolysate of the original polyalcohol. This adds additional evidence for the presence of a repeating unit of molecular weight 30,000.

Carbodiimide reduction of the polysaccharide failed to give any change in the uronic acid content of the polymer (expt. 19). The polysaccharide precipitated as soon as the carbodiimide reagent was added.

Hydrolysis of the mucilage with hydrochloric acid

This experiment was carried out in order to see whether 2-O-methyl-D-glucuronic acid could be isolated from the hydrolysate of the present mucilage as had been reported previously.[107] Three aldobiouronic acids were separated from a hydrolysate by the previous workers, namely 3-O-(α-D-glucopryanosyluronic 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. However in the present study only two aldobiouronic acids were present and separated from this hydrolysate, both of which had already been characterised from a formic acid hydrolysate namely 3-O-(β-D-glucopyranosyluronic acid)-D-galactose and 4-O-(2-O-methyl-α-D-glucopyranosyluronic acid)-L-galactose. Neither of the two uronic acids were found as their monomers.

Conclusions

The polysaccharide exuded by the microscopic marine red alga Porphyridium cruentum, as supplied to us by Marine Colloids Inc., is a complex mucilage containing D-xylose, D- and L-galactose, D-glucose, D-glucuronic acid and its 2-O-methyl derivative, 3- and 4-O-methyl galactose, 3-O-methyl xylose and a 2-O-methyl hexose in the approximate molar proportions of 3:2.5:1:1:0.13:0.13:0.13. Half ester sulphate (ca. 10%) and protein (ca. 3.8%) are also present in the mucilage which appears to be linked to protein. This is the first time that methylated sugars have been reported in this polysaccharide.

Attempts to fractionate this mucilage into separate polysaccharides were unsuccessful and the isolation and characterisation of a number of hetero-oligosaccharides have confirmed the existence of a hetero-polymer containing the above wide variety of units.

These results apart from the methyl aldoses agree in the main with those of Heaney Kieras and Chapman.[1] The mucilage Medcalf[113] examined had a smaller proportion of glucose together with traces of rhamnose and mannose. Both groups of workers reported about 85% of L- and 25% of D-galactose units. In the present study these figures appear to be reversed approximately 75% of D-galactose being present.

The glucuronic acid appears to be solely linked to C-3 of D-galactose and the 2-O-methylglucuronic acid to C-4 of L-galactose. This is in contrast to the results of Heaney Kieras and Chapman. Their 1,3-linked aldobiouronic acid contained L-galactose and the

2-O-methylglucuronic acid was reported to be linked to C-3 of D-galactose.

Exact repetition of these authors' hdyrolysis conditions and separation of the hydrolysate failed in our experiments, in contrast to theirs, to yield any free glucuronic acid or its 2-O-methyl derivative, although the two aldobiouronic acids previously characterised were separated.

Methylation and periodate oxidation studies showed that all three sugars are present as end group, although this may be the result of some degradation. Xylose and galactose are present as 1,3- and 1,4-linked units, and glucose as 1,3-linked and 1,4-linked units. In addition both may be present as 1,3-linked branch points and the galactose as methylated units. The uronic acid appears to be solely 1,3-linked, in agreement with the findings of other workers,[1,113] but the linkages found for the neutral sugars differ somewhat from those reported previously.

It is difficult to explain the different results of other investigators. It is possible that these arise from different culture conditions or from hybridisation of the alga.

Infra-red studies and the stability to alkali of the sulphate groups indicate that they may be located at C-6 of 1,3-linked hexose units and at C-2 and C-3 of 1,4-linked galactose residues.

Molecular weight studies indicate at least 4×10^6 for the initial material, but this high value might be due to aggregation of the molecules and linkage to protein.

Methylation, periodate oxidation, Smith degradation and partial hydrolysis studies degrade the macromolecule to approximately 30,000 molecular weight. In every case the degraded material contained the same complex mixture of sugars with, as would be expected, a high proportion of the glucuronosyl (1 → 3) galactose moiety. It is tempting to suggest that this residual material represents a repeating unit in the polysaccharide, but more evidence is necessary to establish this.

Porphyridium aerugineum

Introduction

Porphyridium aerugineum is a unicellular red alga easily grown in artificial cultures. It exudes an extracellular, water soluble, polysaccharide mucilage. Only very limited work on the structure of this mucilage has been carried out.

Ramus[124] showed that the polysaccharide components were xylose, glucose, galactose and two unidentified sugars. He found that the polymer was polyanionic, demonstrated by precipitation in the presence of cetyl pyridinium chloride (a quarternary ammonium compound). Part of the anionic character may be attributed to the fact that 7.6% of half sulphate ester is present. Attempts to determine the molecular weight of the polysaccharide by column chromatography were unsuccessful. The mucilage was viscous at concentrations as low as 1 mg/ml, indicating that it is of high molecular weight.

Subsequent work by Ramus[128] indicated that the main constituent sugars were xylose, galactose and glucose and in addition he tentatively identified small amounts of guluronic acid, galacturonic acid, another pentose and an anhydrohexose. The molecular weight was found to be in excess of 5×10^6. This seemed rather high and was explained by the fact that polysaccharide chains may have been forming cross-linkages, possibly via the anionic functional groups (carboxyl and half ester sulphate) with divalent cations, resulting in a polymer of infinite molecular weight. However in the absence of half ester sulphate cross-linking should be at a minimum so the sulphate was removed by mild hydrolysis and the molecular weight was

determined on the desulphated mucilage using a Sephadex G200 column. The resulting elution profile was very broad indicating a molecular weight range from $1.3 \times 10^3 - 2 \times 10^5$. However the possibility that the mild hydrolysis conditions may have cleaved some labile glycosidic linkages as well as the sulphate cannot be ruled out. From data obtained from the molecular weight determinations and from electrophoresis experiments it was concluded that the polysaccharide is heterogeneous.

The production of this encapsulating polysaccharide is mainly dependent on the particular life phase the cell is in.[124] The production of the extracellular mucilage, when measured on a per cell basis, drops off rapidly during the first few cell divisions and stays depressed during log phase (rapid cell division), then increases slowly during the stationary phase. Therefore during the log phase very little or no encapsulating polysaccharide is present as the rate of solubilisation is greater than the rate of production, the capsule builds up again during the stationary phase. The production of polysaccharide is also light dependent as cells maintained in the dark produce the mucilage at a greatly reduced rate.

The rate of production of chlorophyll a and Floridean starch are similar to each other. The quantities of both per cell drops significantly for the first few divisions, then begins to build up slowly. In the dark the levels of both slowly decrease.

The already polymerised polysaccharide is transported from within the cell to and through the cell wall by Golgi vesicles indicating that the Golgi complex plays a crucial role in the production of the mucilage.

Further work by Ramus and Groves[125,126,127] using $^{35}SO_4^{2-}$ as a label, has shown that the cells accumulate the label and then excrete the sulphate labelled polysaccharide in amounts which are easily monitored. The sulphate fixation is rapid and direct, 50% of the sulphate absorbed by the cells was incorporated into the polysaccharide as half ester sulphate thus indicating that polysaccharide esterification is a major metabolic pathway for sulphate. Inorganic sulphate (and/or thiosulphate) is necessary for growth, the cells lysing when denied adequate amounts of this nutrient. 3'-Phosphoadenosine-5'-phosphosulphate was tentatively identified as the donor for sulphate transfer reactions. Molybdate was shown to be a competitive inhibitor for active transport sites for sulphate, however the inhibition is reversible. The molybdate is transported into the cells and inhibits the sulphation of the polysaccharide.

Experimental Porphyridium aerugineum

The polysaccharide from this alga was supplied as the Na^+ salt by Marine Colloids Inc., Rockland, Maine, U.S.A., (2.5 g, 6/9/75), (5 g, 5/5/76), (2 g, 2/10/76), (15 g, 30/1/77).

Several experiments with the mucilage from P. aerugineum were performed in parallel with the mucilage from P. cruentum, in these instances reference will be made to these experiments by using the following nomenclature:-
P.c. 8, for example, would mean the experimental section for Porphyridium cruentum, experiment 8.

Experiment 1 Precipitation with hexadecyltrimethylammonium bromide (CETAB)

The polysaccharide (1 g) was dissolved in water (500 ml) and a solution of CETAB (1.5 g) (Eastman Kodak) in water (10 ml) was added. The resulting white precipitate was treated as described previously (P.c. 5). The recovery of polysaccharide after twice redissolving in water and reprecipitating in ethanol was 750 mg.

The supernatant was treated as previously described (P.c.5).

Experiment 2 Viscosity studies

The viscosity of a 0.35% solution of the polysaccharide in water was measured under the same conditions described in P.c.6.

Experiment 3 Preliminary analysis of the mucilage

The polysaccharide (20 mg) was hydrolysed [GM II(i)] and analysed by paper chromatography [GM III(i)(a)(b)(c)] with sprays [GM IV (i)(ii)(iii)].

The carbohydrate content [GM VI (i)], the sulphate content [GM VI(iii)], the uronic acid content [GM VI(ii)], the nitrogen content [GM VI(iv)] and the methoxyl content (A. Bernhardt, W. Germany) were determined for the polysaccharide.

Experiment 4 Characterisation of the constituent sugars

An aliquot (50 mg) of the polysaccharide was hydrolysed [GM II(i)]. Part of the hydrolysate was analysed by paper chromatography [GM III(i)(a)(b)(c)] using sprays [GM IV(i)(ii)(iv)(v)(vi)]. Separate aliquots of the hydrolysate were subjected to electrophoresis in borate buffer [GM III (ii)(a)] and in pyridine/acetic acid buffer [GM III(ii)(b)]. The remainder of the hydrolysate was studied by g.l.c. as the sugar TMS [GM VII(vi)], the alditol TMS [GM VII(v)(vi)] and as the alditol acetate [GM VII(v)(vii)] derivative. The alditol acetate derivatives were further analysed by g.l.c.-m.s. [GM V (a) B(ii) C]. The proportions of the sugars present were determined from the peak areas obtained from g.l.c. of the alditol acetate derivatives.

Experiment 5 Separation of sugars and oligosaccharides from a hydrolysate

The mucilage (900 mg) was hydrolysed [GM II (i)] and the constituents separated by paper chromatography on Whatman 3MM paper

in solvent system [GM III (i)(b)]. Side and centre strips were cut from the chromatogram and were developed with spray [GM IV(ii)(a)] and the regions containing the different sugars were cut from the chromatogram and the sugars eluted from the paper with water. The carbohydrate content was determined [GM VI (i)] for each fraction using standard graphs made from the appropriate sugars. Those fractions that were still mixtures, as shown by paper chromatography [GM III (i)(a)], were reseparated on Whatman 3MM paper in that solvent. The optical rotations [GM I (v)] of the xylose, glucose and galactose fractions were measured.

Experiment 6 Determination of the proportion of D- to L-galactose

The optical rotation [GM I (v)] of a solution of galactose (2.8 mg), isolated from the hydrolysate of the mucilage (expt. 5), in water (1 ml) was determined. This solution was then diluted to 10 ml. A standard solution of D-galactose (10 mg) in water (100 ml) was made and a graph was plotted of weight against absorbance using the phenol/sulphuric method [GM VI (i)]. Galactose oxidase reagent (enzyme and chromagen) was added to the standard solution and the mixture was incubated at 30° for 2 h, after which time the reaction was complete. Aliquots were withdrawn and the absorbance was measured (520 nm) and plotted against the weight of D-galactose present.

The weight of sugar present in the solution of galactose from the hydrolysate was determined using the phenol/sulphuric method. The same proportion of galactose oxidase reagent was added and the mixture incubated at 30° for 2 h. Aliquots were withdrawn and their

absorptions measured and compared to the standard graph. The difference between the weight of galactose present as shown by the phenol/sulphuric method and the weight shown by the galactose oxidase method indicates the amount of L-galactose present as the galactose oxidase enzyme only oxidises the D-galactose.

Experiment 7 Characterisation of the methylated hexoses

Two unknown fast moving spots were present in the hydrolysate of the mucilage. One of these, R_{glc} 1.3 in solvent (b), R_{glc} 1.6 in solvent (a), was separated from a paper chromatogram. The second spot, R_{glc} 2.1 and 2.2 in the above respective solvents proved to be a mixture.

Aliquots of these unknown sugars were separately reduced with borohydride and then acetylated [GM VII (v)(vii)]. The resulting alditol acetates were studied by g.l.c.-m.s. [GM V (a) B (ii) C].

A second aliquot of the first unknown (R_{glc} 1.3 and 1.6) was reduced with borodeuteride, then acetylated and studied by g.l.c.-m.s. as above.

Both unknowns were separately demethylated [GM VII (xiv)] and the resulting sugars were analysed by paper chromatography in solvent [GM III (i)(a)(b)] with sprays [GM IV (ii)(iv)(v)].

Experiment 8 Partial hydrolysis studies

(a) Preliminary studies

The same conditions were used for this polysaccharide as for P. cruentum [P.c. 9(a)]. In the case of P. aerugineum the conditions yielding most oligosaccharides were 0.25M-oxalic acid at 100° for 2 h.

(b) Large scale hydrolysis

The polysaccharide (1 g) was hydrolysed with 0.25M-oxalic acid (70 ml) at 100° for 2 h. After neutralisation with $CaCO_3$ and filtration of the resulting calcium oxalate the uncleaved polysaccharide (290 mg) was precipitated with ethanol. This polymer was redissolved in 0.25M-oxalic acid (20 ml) and was further hydrolysed at 100° for 2h. After neutralisation as above the uncleaved polymer (54 mg) was again precipitated with ethanol, hydrolysed and studied by paper chromatography. The two partial hydrolysates were studied by paper chromatography [GM III (i)(a)(b)] and were then combined as no difference could be seen between them. The constituent sugars and oligosaccharides were separated on Whatman 3MM paper in solvent [GM III (i)(a)] [P.c. 9(b)].

Experiment 9 Neutral oligosaccharides from formic acid and oxalic acid hydrolysis

Three neutral oligosaccharides (A, B and C) were separated from the oxalic acid hydrolysate (expt. 8). The degree of polymerisation [GM VI (vi)] was determined for each and each was hydrolysed [GM II (i)] and the hydrolysate analysed by paper chromatography [GM III (i)(b)] and by g.l.c. [GM V (a) B(i)] as the sugar TMS derivatives [GM VII (vi)].

The oligosaccharides were reduced [GM VII (v)] and the derived oligosaccharide alditols were studied by electrophoresis in molybdate buffer [GM III (ii)(d)]. A fourth oligosaccharide (D) was isolated from a formic acid hydrolysate (expt. 5) and was compared with authentic 1,4- and 1,3-linked xylobiose by paper chromatography [GM III (i)(a)(b)].

Experiment 10 The acid oligosaccharide

One acid oligosaccharide was separated from the formic acid hydrolysate (expt. 5). This was subjected to electrophoresis in pyridine/acetic acid buffer, pH 6.7 [GM III (ii)(b)]. An aliquot was esterified, reduced [GM VII (iv)(v)] and part was hydrolysed [GM II (i)]. Another aliquot was reduced, esterified, reduced and hydrolysed as above. The two hydrolysates were studied by paper chromatography [GM III (i)(b)] with sprays [GM IV (i)(ii)(iv)(v)] and by g.l.c. [GM V (a) B (i)] as the TMS derivatives [GM VII (vi)]. The remainder of the esterified, reduced oligosaccharide was methylated[105] (P.c. 11), hydrolysed [GM II (i)] and the hydrolysate reduced and acetylated [GM VII (v)(vii)].

The resulting partially methylated alditol acetates were analysed by g.l.c. - m.s. [GM V (a) B(ii) C].

Experiment 11 Desulphation of the mucilage and Infra-red studies

Desulphation of the polysaccharide (250 mg) was initially attempted using alkali [GM VII (xiii)], this method only resulted in the partial loss of sulphate from the polysaccharide (205 mg). An aliquot (5 mg) of the partially desulphated polysaccharide was hydrolysed [GM II (i)] and the hydrolysate studied by paper chromatography [GM III (i)(a)(b)] and by g.l.c. as the alditol acetate derivatives [GM VII (v)(vii)].

Methanolic hydrogen chloride (0.08M) was used to effect complete removal of the sulphate [GM VII (xii)] from the polysaccharide (200 mg, yield 130 mg).

Infra-red spectra were run of both the desulphated, partially desulphated and the original polymers as polysaccharide films on AgCl plates and also as KBr discs]GM V (b)(ii)[.

The 3,6-anhydrogalactose content[110,120] was determined before and after desulphation and was compared with a synthetic mixture of sugars xylose (30), glucose (20), galactose (30), glucuronic acid (20).

Experiment 12 Autohydrolysis of the mucilage

The mucilage (2 g) was dissolved in water (600 ml) and the solution was poured into a dialysis sac with prewashed IR 120 H^+ resin (20 g) to convert the polysaccharide into its free acid form. The sac was supported in an upright position in a beaker of deionised water (3 l). The solution was stirred by a mechanical stirrer and the dialysate by a magnetic stirrer, both were kept at 70°. As soon as the concentration of sugars outside the sac reached 20 ug/ml, as determined by the phenol/sulphuric method [GM VI(i)], five drops of 880 ammonia were added to the dialysate which was then changed. The ammonia was added to ensure that any acidic or sulphated fragments present in the dialysate would not hydrolyse further when the dialysate was concentrated. Five dialysates were collected each of 3 l over a period of 3 weeks. Each was concentrated and analysed by paper chromatography [GM III (i)(a)(b)] which showed that they were very similar to each other so they were combined. Electrophoresis in pyridine/acetic acid buffer pH 6.7 [GM III (ii)(b)] however showed that the first dialysate contained a small amount of a negatively charged sugar derivative which was absent from the other fractions.

Therefore the experiment was repeated three more times but only the first dialysate was retained. The residual polymer (1.2 g) in the sac after the initial experiment was recovered by filtering off the resin through glass wool and freeze-drying the solution. This polymer will hereinafter be called the residual polymer and all further experiments on it were carried out in parallel with the original polysaccharide from P. aerugineum.

Experiment 13 Neutral oligosaccharides from the autohydrolysate

Three neutral oligosaccharides were separated from the dialysate, after autohydrolysis of the mucilage, by paper chromatography [GM III (i)(a)] on Whatman 3MM paper. The degree of polymerisation [GM VI (vi)] and the optical rotations [GM I (v)] were determined for each except that the amount of oligosaccharide II was too small to measure the rotation. No uronic acid containing fragments could be detected.

The neutral oligosaccharides were each reduced [GM VII (v)] and studied by electrophoresis in molybdate buffer [GM III (ii)(d)]. The reduced oligosaccharides were hydrolysed [GM II (i)] and were studied by paper chromatography [GM III (i)(a)(b)] and by g.l.c. as the TMS derivatives [GM V (a) B(i), VII (vi)]. The oligosaccharides were hydrolysed [GM II (i)] and analysed by paper chromatography [GM III (i)(a)(b)] with sprays [GM IV (i)(ii)(iv)] and by g.l.c. as the alditol acetate derivatives [GM V (a) B(ii), VII(v)(vii)]. Oligosaccharides I and III (p.110) were methylated and analysed as the derived alditol acetates.

Experiment 14 The charged oligosaccharide from the autohydrolysate

The first dialysate from each of the three autohydrolysis experiments were combined and concentrated. The charged molecule (900 µg) was separated by electrophoresis in pyridine/acetic acid buffer [GM III (ii)(b)] on Whatman 3MM paper.

An aliquot was hydrolysed [GM II (i)] and the hydrolysate studied by paper chromatography [GM III (i)(a)(b)] with sprays [GM IV (ii)(iv)(v)] and by electrophoresis in pyridine/acetic acid buffer [GM III (ii)(b)]. The sulphate [GM VI (iii)] was determined qualitatively on the hydrolysate.

An infra-red spectrum was run of the oligosaccharide on a AgCl plate [GM V (b)(ii)]. Similarly an infra-red spectrum was run after desulphation of the oligosaccharide by alkali [GM VII (xiii)]. The optical rotation [GM I (v)] was determined for the oligosaccharide.

The desulphated oligosaccharide was methylated[105] (P.c. 11) and the derived alditol acetates [GM VII (v)(vii)] were analysed by g.l.c. [GM V (a) B (ii)].

Experiment 15 Preliminary studies on the residual polymer

The sulphate content [GM VI (iii)], the uronic acid content [GM VI (ii)] and the methoxyl content (A. Bernhardt, W. Germany) of the residual polymer were determined.

The viscosities of 0.1% solutions of the residual and initial polymers in water were determined at 30° and after heating to 70° and allowing to cool to 30° in a viscometry U-tube (BSS No.2). The viscosities were compared to that of water. The time taken for water to pass through this tube was 17 secs.

An aliquot of the residual polymer was hydrolysed [GM II (i)] and the hydrolysate analysed by paper chromatography [GM III (i)(a)(b)] with sprays [GM IV (i)(ii)(iv)(v)], by electrophoresis in pyridine/acetic acid buffer pH 6.7 [GM III (ii)(b)] and by g.l.c. as the alditol acetate derivatives [GM V (a) B (ii), VII (v)(vii)].

Experiment 16 Oxidation of the mucilage, desulphated mucilage and residual polymer with periodate

(a) The polymers (100 mg) were each separately dissolved in water (100 ml) and an equal volume of 10 mM-sodium metaperiodate solution was added to each (P.c. 14). After freeze-drying, aliquots from the derived polyalcohols [mucilage (64 mg), desulphated mucilage (70 mg), residual polymer (65 mg)] were hydrolysed [GM II (i)] and analysed.

(b) The mucilage (500 mg) and the residual polymer (450 mg) were separately dissolved in water (250 ml) and were oxidised with an equal volume of 50 mM-periodate [as in P.c. 14 (b)] and then reduced to the polyalcohols. The recovered polyalcohols (372 mg, 301 mg respectively) were reoxidised with 10 mM-periodate. Negligible further oxidation could be detected. Aliquots of the derived polyalcohols (340 mg, 281 mg respectively) were hydrolysed [GM II (i)] and the hydrolysates analysed.

Experiment 17 Analysis of the polyalcohols

Aliquots of the polyalcohols derived from the mucilage, desulphated mucilage and the residual polymer (expt. 16a) were separately hydrolysed [GM II (i)] and the hydrolysates analysed by paper chromatography [GM III (i)(a)(b)] with sprays [GM IV (i)(ii)],

by electrophoresis in pyridine/acetic acid buffer pH 6.7 [GM III (ii) (b)] and in molybdate buffer pH 5.0 [GM III (ii)(d)], and by g.l.c. as the alditol acetate derivatives [GM V (a) B (ii), VII (v)(vii)].

Part of the polyalcohols derived from the mucilage and the residual polymer (expt. 16b) were methylated once [GM VII (viii)], hydrolysed [GM II (i)], reduced and acetylated [GM VII (v)(vii)] and the resulting partially methylated alditol acetates were analysed by g.l.c. - m.s. [GM V(a) B(ii)(iii)C].

Experiment 18 Methylation of the mucilage and the residual polymer

(a) Hakomori methylation

Aliquots (50 mg) of the mucilage and the residual polymer were separately methylated once [GM VII (viii)]. The methylated polysaccharides (39mg, 37 mg) were remethylated [GM VII (viii)]. Aliquots (5 mg) of the methylated polymers after one and after two methylations were hydrolysed [GM II (i)], reduced and acetylated [GM VII (v)(vii)] and the derived partially methylated alditol acetates were analysed by g.l.c.-m.s. [GM V(a) B(ii)(iii)C]. The sulphate content [GM VI (iii)] was determined for each methylated polysaccharide.

(b) Haworth methylation[108]

The mucilage (500 mg) formed a slurry in acetone (50 ml). Dimethyl sulphate (20 ml) and 30% NaOH (40 ml) were added simultaneously, dropwise, with stirring over a period of 2 h. Excess dimethyl sulphate was decomposed and the acetone removed by raising the temperature to 85°. The partially methylated mucilage was filtered

and washed with methanol, then dissolved in water, dialysed and freeze-dried. The partially methylated polysaccharide was dissolved in dimethyl sulphoxide (30 ml) (insoluble in THF, acetone and 1,4-dioxan) and 30% NaOH (20 ml) and dimethyl sulphate (6 ml) were added in seven portions over 1 day. The mixture was refluxed for 1 h, and then mixed with water. The solution was dialysed and the polysaccharide recovered by freeze-drying (302 mg). The sulphate content [GM VI (iii)] and the methoxyl content (A. Bernhardt, W. Germany) were determined. The methylated polysaccharide was hydrolysed [GM II (i)], reduced and acetylated [GM VII (v)(vii)] and the resulting partially methylated alditol acetates were analysed by g.l.c. [GM V (a) B(ii)].

Experiment 19 Alkali degradation of the methylated mucilage[109]

An aliquot (50 mg) of the mucilage after one Hakomori methylation [GM VII (viii)] was dissolved in DMSO (3 ml) and 2.0M-carbanion (2 ml) [GM VII (iii)] was added. The mixture was left stirring overnight and then 50% acetic acid (6 ml) was added to neutralise the carbanion. The degraded methylated polysaccharide was repeatedly extracted with chloroform until no DMSO was left in the chloroform extract. The chloroform solution was taken to dryness and treated with 50% acetic acid (10 ml) at 100° for 1 h. This solution was evaporated under reduced pressure at 40° and the resulting syrup redissolved in water. Sodium borodeuteride (50 mg) was added and the solution left overnight. The mixture was then treated with IR 120 H^+ resin, filtered and the boric acid removed by repeated codistillation with methanol. An aliquot (25%) of the

recovered material was hydrolysed [GM II (i)], reduced and acetylated [GM VII (v)(vii)] and the resulting alditol acetates analysed by g.l.c.-m.s. [GM V (a) B(ii) C]. The remaining material (75%) was remethylated once using trideutero methyl iodide [GM VII (viii)]. The remethylated material was again extracted with chloroform, hydrolysed [GM II (i)], reduced and acetylated and analysed by g.l.c.-m.s..

Experiment 20 Molecular size studies

The mucilage and residual polymer (1.5 mg each) were separately dissolved in water (3 ml) and layered onto a Sepharose 4B column (90 x 1.6 cm) as described previously (P.c. 17).

The methylated mucilage (0.5 mg) after one and after two Hakomori methylations, and after two Haworth methylations were separately dissolved in water (0.5 ml) and were separately layered onto a Sepharose 4B column (16.5 x 1.3 cm) as described previously (P.c. 17).

The derived polyalcohols of the mucilage and residual polymer (0.5 mg each) after oxidation by periodate were separately dissolved in water (0.5 ml) and were separately layered onto a Sepharose 4B column (16.5 x 1.3 cm) as described previously (P.c. 17). The columns were calibrated as described in P.c. 17 (Figs. 18, 19).

Experiment 21 Treatment of the polyalcohols of the mucilage and residual polymer with trifluoroacetic acid

The two derived polyalcohols (150 mg) were separately dissolved in 1.0M-trifluoroacetic acid (28 ml) and were left stirring at room

temperature. They were then treated and analysed as described in (P.c. 18).

Experiment 22 Carbodiimide reduction of uronic acid

The mucilage and residual polymer (100 mg each) were separately dissolved in water (100 ml) and the pH adjusted to 4.75. However on addition of the carbodiimide reagent, both polymers precipitated as with P. cruentum (P.c. 19). The uronic acid content was determined [GM VI (ii)] after addition of borohydride, dialysis and freeze-drying.

Results and discussion

The extracellular polysaccharide exuded from the red alga P. aerugineum, supplied as the Na^+ salt by Marine Colloids Inc., had a carbohydrate content of ca. 68%, a uronic acid content of ca. 9%, a sulphate content of ca. 9%, a protein content of ca. 5% and a methoxyl content of ca. 3.6%. The uronic acid and sulphate percentages are based on the carbohydrate content and the others are expressed as percentages by weight. The analysis of the mucilage as determined by Marine Colloids is shown in table 10.

Viscosity experiments

The viscosity of a 0.35% solution of the mucilage under various conditions (expt. 2) are shown in table 11. The viscosity remains reasonably stable until an acidic pH is reached when it increases considerably.

Fractionation of the polysaccharide

This was attempted using CETAB (expt. 1), however analysis of the supernatant showed that no carbohydrate was present so it was assumed that no fractionation had taken place. Chromatographic analysis of a hydrolysate of the recovered polysaccharide after CETAB precipitation when compared to a hydrolysate before precipitation showed no differences between them.

A solution of the polysaccharide was opaque, even after filtration through millipore filters, so the optical rotation could not be determined.

Table 10

Analysis of P. aerugineum by Marine Colloids

%	mucilage	anhydrous mucilage
H_2O	9.95	-
Ash	8.08	8.97
Na^+	2.03	2.25
K^+	0.001	0.001
Ca^{2+}	0.25	0.28
Mg^{2+}	0.03	0.033
N	1.51	1.68
Cl^-	0.39	0.43
SO_4^{2-}(total)	3.53	3.92
SO_4^{2-}(free)	0.12	0.13
3,6-anhydrogalactose	2.31	2.56
Uronic acid (carbazole)	8.50	9.44
Carbohydrate (anthrone)	85.0	94.4
PO_4^{3-}	0.33	0.36
Hyamine	-	-

Table 11

Relative viscosity of a 0.35% solution of the mucilage

0.35% solution	relative viscosity at 30°
Normal solution (pH 8.9)	79
After heating to 70° and cooling to 30°	103
After autoclaving	59
pH 3.5	225
pH 7.0	77
pH 8.9	79
pH 11.0	57

Characterisation of the constituent sugars

The following sugars were separated as syrups from a hydrolysate of the mucilage.

xylose - a reducing syrup which had the same mobility on paper chromatograms and g.l.c. as xylose. The optical rotation $+18^{\circ}$ confirmed that D-xylose was present (cf. D-xylose $+19^{\circ}$).

galactose - a reducing syrup which on paper chromatograms and g.l.c. had the same retention time as galactose. The optical rotation of $+26^{\circ}$ (cf. D-galactose + 79°) indicated that 67% was present as D-galactose and 33% as L-galactose. When galactose oxidase was used (expt. 6) to determine the proportions, 75% was found as D-galactose and 25% as the L-sugar.

glucose - a reducing syrup which had the same retention time on paper chromatograms and g.l.c. as glucose. The optical rotation $+56^{\circ}$ showed that D-glucose was present (cf. D-glucose + 53°). Glucose oxidase confirmed the presence of D-glucose.

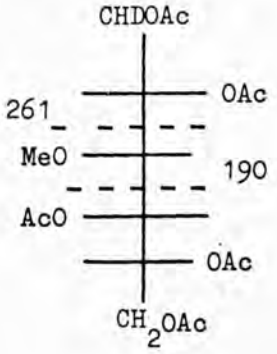
Methylated sugars

3-O-methylxylose and 2,4-di-O-methylgalactose - a reducing syrup which on paper chromatograms (R_{glc} 2.1 in solvent (a)) and g.l.c. had the same mobility as 3-O-methyl xylose. In addition to 3-O-methyl xylose this fraction also contained a reducing hexose. After demethylation (expt. 7) of the mixture xylose and galactose were found thus indicating the presence of a methylated xylose and a methylated galactose. The retention time of the methylated galactose shows that it is probably a di-O-methyl galactose. When analysed by g.l.c.-m.s. as the derived alditol acetate derivatives a 3-O-methyl

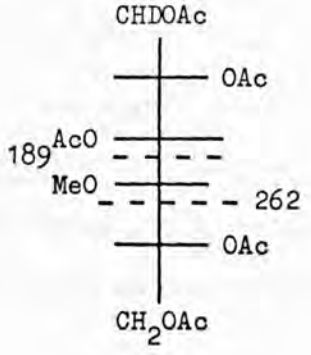
pentose and a 2,4-di-O-methyl hexose were characterised. Therefore it can be concluded that this fraction contains 3-O-methyl xylose and 2,4-di-O-methyl galactose.

An unknown reducing syrup R_{glc} 1.6 (in solvent a) and 1.3 (in solvent b) was also separated. After demethylation (expt. 7) only galactose could be detected on a paper chromatogram. Analysis of the syrup by g.l.c.-m.s. as the derived alditol acetate showed that it could be either 3- or 4-O-methyl galactose. To show which of these was present the methylated galactose was reduced with sodium borodueteride instead of borohydride and the product was then analysed as its derived alditol acetate by g.l.c.-m.s.. If 3-O-methyl galactose was the initial sugar then fragments of m/e 261 and 190 should be found, and if 4-O-methyl galactose was present fragments of m/e of 262 and 189 should be found (Fig. 7).

1,2,4,5,6 penta-O-acetyl-
3-O-methyl galactitol



1,2,3,5,6 penta-O-acetyl-
4-O-methyl galactitol



Fragmentation of 3- and 4-O-methylgalactose

Figure 7

Analysis of the mass spectrum showed peaks at m/e 189, 190, 261 and 262. This showed that both 3- and 4-O-methyl galactose were present. The intensities of the peaks indicated that approximately equal quantities of each sugar were present (Fig. 8).

Mannose was shown to be absent in the hydrolysate from the results obtained after electrophoresis in borate buffer. Fructose was also absent as shown by the negative result obtained after development of a paper chromatogram with urea hydrochloride spray.

Relative amounts of the constituent sugars.

These were determined by analysing each fraction of a hydrolysate, after separation on paper chromatograms, for its carbohydrate content and also by measuring the peak areas from a g.l.c. trace of the derived alditol acetates from a hydrolysate (expt. 4 and 5). The results from the two methods and the retention times relative to xylitol pentaacetate (T) are given in table 12.

The methoxyl content (3.6% by weight, 5% by carbohydrate) shows that about 20% of the residues contain a methoxyl group which is in reasonable agreement with the figures in the table.

The main differences between the constituent sugars in the polysaccharides from P. aerugineum and P. cruentum is that the former contains considerably more methylated sugars and glucose than the latter, and no 2-O-methylglucuronic acid could be detected in P. aerugineum polysaccharide.

Figure 8

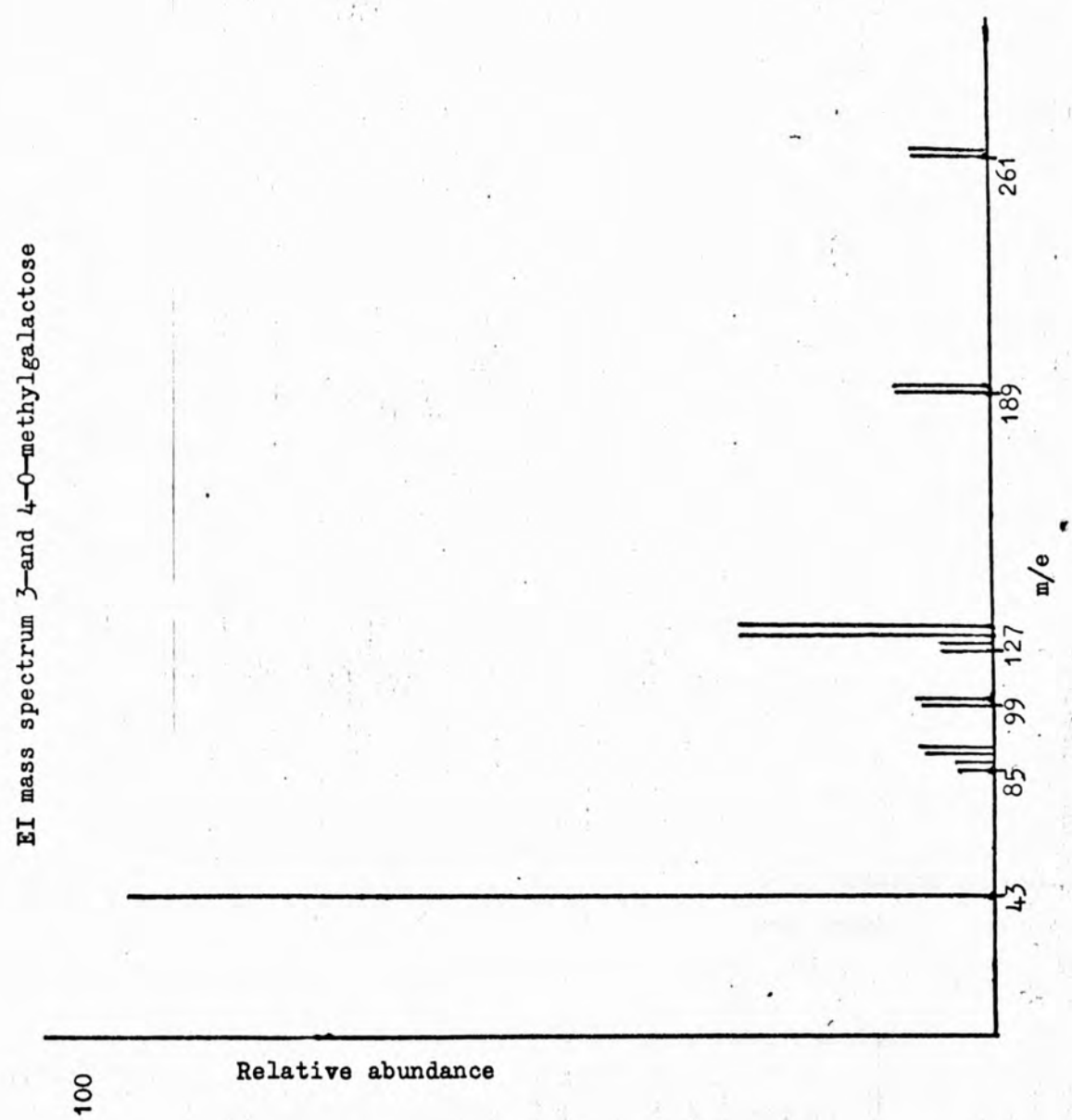

EI mass spectrum 3-and 4-O-methylgalactose
261
189
127
99
85
43
m/e
Relative abundance
100

Table 12

Percentage proportions of the constituent sugars in *P. aerugineum*

Sugar	T	Peak area[a]	Carbohydrate content of eluted fractions
xylose	1.0	29	29
galactose	2.16	11	17
glucose	2.35	18	20
3-O-methylxylose	0.58	5	8[b]
2,4-di-O-methylgalactose	1.35	11	
3- and 4-O-methylgalactose	2.0	5	5[b]
oligouronic acid A[c]	-	-	19
other oligosaccharides	-	-	2
uronic acid[d]	-	9	-

(a) Based on 79% of the carbohydrate.

(b) No standard sugars were available so the absorption was read from a standard graph of the free sugar.

(c) Contains 9.5% galactose.

(d) As determined by the m-hydroxydiphenyl method.

Characterisation of aldobiouronic acid (A) (expt. 10).

Oligouronic acid (A) (20 mg) (Table 12) was separated from a formic acid hydrolysate. It had a specific rotation of + 18.5° (cf. +19° for the aldobiouronic acid from P. cruentum). All other experiments carried out on th s aldobiouronic acid produced identical results to those obtained for oligouronic acid (1) separated from a hydrolysate from P. cruentum (p.53). Therefore this oligouronic acid (A) can be said also to be 3-O-(β-D-glucopyranosyluronic acid)-D-galactose.

Partial hydrolysis

Various conditions for hydrolysis were tried in order to determine which would yield the maximum amount of oligosaccharides. The only acidic oligosaccharide found was the aldobiouronic acid glucuronosyl-galactose which has already been isolated and characterised. Therefore only the amount of neutral oligosaccharides found determined which conditions were used, 0.25M-oxalic acid gave the greatest amount. The neutral oligosaccharides separated (A, B, C), together with another isolated from a formic acid hydrolysate (D), are shown in table 13. The uncleaved polymer (54 mg) recovered after precipitation of the hydrolysate with ethanol, showed the same sugars, after complete hydrolysis, as were present in the initial mucilage.

Table 13

Neutral oligosaccharides isolated from a partial hydrolysate of P. aerugineum mucilage

Oligosaccharide	Mobility in solvent (b)	Weight	D.P.	Colour when developed with aniline oxalate
A	0.45	1.5 mg	2	yellow
B	0.68	1.5 mg	2	pink
C	0.84	2.0 mg	2	pink
D	1.06	2.0 mg	2	pink

Oligosaccharide (A) was found to contain, after hydrolysis, D-glucose and galactose in the molar proportions 1:1. The oligosaccharide, after reduction and ionophoresis in molybdate buffer, had a mobility of $M_{glucitol}$ 0.49 which indicates a 1 → 4 linkage.[122] The hydrolysate of the disaccharide alditol contained D-glucose and galactitol, thus showing that the original oligosaccharide was D-glucosyl (1 → 4) galactose.

Oligosaccharide (B), after hydrolysis, contained D-glucose and xylose. Ionophoresis of the reduced disaccharide gave one spot $M_{glucitol}$ 0.48 which is indicative of a 1 → 4 linkage. Analysis of the hydrolysed disaccharide alditol showed the presence of D-glucose and xylitol. The original disaccharide may tentatively be assigned as D-glucosyl (1 → 4) xylose.

Oligosaccharide (C) contained D-glucose and xylose after hydrolysis. Ionophoresis of the reduced disaccharide gave a spot $M_{glucitol}$ 1.05. This would seem to indicate that hydrolysis of the disaccharide had occurred during the reduction process as a mobility of 1.05 is too fast for a disaccharide. The original disaccharide was tentatively identified as being a D-glucosyl xylose.

Oligosaccharide (D) showed only the presence of xylose after hydrolysis. Comparison by paper chromatography of this disaccharide with authentic 1,3- and 1,4-linked xylobiose showed that this disaccharide was xylosyl (1 → 3) xylose which had been found previously in P. cruentum polysaccharide.

Desulphation studies

Desulphation of the mucilage with alkali (expt. 11) reduced the sulphate content from 9% to 3.7%, the partially desulphated polysaccharide was recovered in 87% yield. However no new sugars could be detected in a hydrolysate of the partially desulphated material. Methanolic hydrogen chloride (0.08M) effected complete removal of the sulphate (69% recovery). No 3,6-anhydrogalactose was detected either before or after desulphation (with alkali or with methanolic hydrogen chloride). However it should be pointed out that the colourimetric method[110,120] for estimation of 3,6-anhydro-galactose is masked to a large extent by some of the other sugars present in the mucilage as shown by comparison with a synthetic mixture (expt. 11).

Infra-red spectra of the mucilage and the partially desulphated mucilage showed absorbances at 1250 - 1260 cm^{-1}, (which is characteristic of S = O stretching frequency) and at 850 and 820 cm^{-1}.

The absorption at 850 cm^{-1} is considered to be due to half ester sulphate group in an axial position,[111] this band was decreased by about half in the spectrum obtained from the mucilage after partial desulphation. The band occurring at 820 cm^{-1} is thought to be due to primary half ester sulphate. Complete desulphation removed all three absorption bands. No band at 930 - 940 cm^{-1} was observed in any of the spectra, this absorption is characteristic of 3,6-anhydrogalactose.

Like P. cruentum and Rhodella maculata[115] and unlike most other sulphated polysaccharides the sulphate is removed by methylation thus making it impossible to determine the site of the sulphate by analysis of the derived methylated polysaccharide before and after desulphation.

From the desulphation studies it may be concluded that the sulphate which is alkali labile occurs mainly in an axial position which means that the sulphate must be present on C-4 of 1,3-linked galactose, assuming that the sugars take up the preferred $^{4}C_{1}$ conformation (Fig. 9).

The rest of the sulphate is present on C-6 of glucose and/or galactose.

These results are difficult to explain. The only sulphate which should be labile to alkali would be on C-2 or C-3 of 1,4-linked xylose, glucose or galactose and unless these are present in the unfavourable $^{1}C_{4}$ conformation such sites would not be axial.

Conformations of glucose and galactose

Figure 9

galactose glucose

$^4C_1$ conformation

galactose glucose

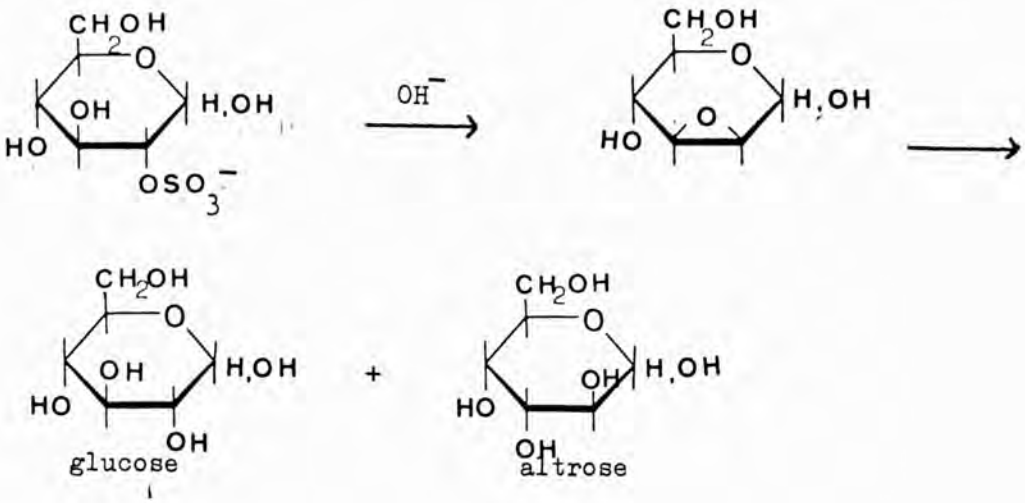
$^1C_4$ conformation

However glucose and galactose 2-sulphates have been shown[133] to give anomalous peaks, and it is possible therefore that the sulphate may be present on C-2 of 1,4-linked units.

In addition the intermediate epoxide ring which should be formed during desulphation with alkali should be cleaved on both sides to give additional sugars arabinose from xylose, altrose from glucose and idose from galactose (Fig. 10).

Figure 10

Alkaline desulphation



No evidence of these new sugars could be detected on paper chromatography or g.l.c.. However steric factors in the polysaccharide may have prevented cleavage of the epoxide ring except on the side giving the original sugars.

Autohydrolysis

The dialysate, obtained by autohydrolysis of the free acid form of the mucilage in a dialysis sac, contained glucose, galactose, xylose, 3-O-methyl xylose, 3- and 4-O-methylgalactose, 2,4-di-O-methylgalactose and three oligosaccharides. Small amounts of a charged oligosaccharide were also found but no oligouronic acids or acidic fragments were present. Details of the three neutral oligosaccharides are shown in table 14.

Table 14

Neutral oligosaccharides obtained from the autohydrolysate

	Mobility in solvent (a)		(b)	Weight	D.P.	Colour when developed with aniline oxalate
oligosaccharide I	0.35	;	0.40	8 mg	2	yellow
oligosaccharide II	0.52	;	0.74	0.8 mg	2	pink
oligosaccharide III	0.76	;	0.90	4.4 mg	2	pink

Oligosaccharide I had $[\alpha]_D$ + 20° and after hydrolysis gave D-glucose and galactose. The mobility of this disaccharide and the presence of D-glucose and galactose in a hydrolysate suggested that this was the same as disaccharide (A) which had been found earlier after partial hydrolysis studies (p. 105). Hydrolysis of the disaccharide alditol gave D-glucose and galactitol. Unfortunately the methylation results could not be interpreted. It would seem that this disaccharide is indeed the same as that found earlier, namely D-glucosyl (1 → 4) galactose.

Oligosaccharide II, after hydrolysis, gave glucose and xylose. After reduction and hydrolysis, glucose, glucitol and xylitol were found. No material was available for any further study but it may be concluded that this is a glucosyl-xylose which during reduction is partially hydrolysed and both sugars reduced. The mobility in solvent (b) suggest that this disaccharide is probably the same as oligosaccharide B isolated earlier.

Oligosaccharide III had $[\alpha]_D$ + 8.2° and after hydrolysis gave D-glucose and xylose. The mobility of this disaccharide in solvent (b)

suggested that this too was the same as the disaccharide (C) isolated earlier namely D-glucosyl xylose. Unfortunately the methylation results could not be interpreted so the linkage could not be determined.

The charged oligosaccharide from the autohydrolysate

The charged oligosaccharide was separated by electrophoresis (expt. 14). It had $[\alpha]_D$ -31° and on hydrolysis gave galactose and xylose. Electrophoresis of the hydrolysate showed that no charged carbohydrates were present. Glucose oxidase and galactose oxidase on the hydrolysate both gave negative results. The rotation indicated the presence of L-galactose and this is confirmed by the enzymic results. The oligosaccharide gave a pink colour when developed with aniline oxalate and its mobility in solvents (a) R_{glc} 0.34 and (b) R_{glc} 0.32 and its electrophoretic mobility (at pH 6.8) $M_{glc.U.A.}$ 0.9 suggest that it is probably a disaccharide. It gave a positive test for free sulphate, after hydrolysis, therefore this oligosaccharide is tentatively identified as a sulphated L-galactosyl-xylose.

Infra-red spectra of this sulphated disaccharide showed a small absorption band at 830 cm^{-1} which indicates that the sulphate is present in an equatorial position, in contrast to infra-red studies on the polysaccharide which indicated axial and primary sulphate groups.

Desulphation of the disaccharide by alkali in the presence of borohydride resulted in the loss of the absorption band at 830 cm^{-1}.

From this it can be deduced that the sulphate was present on C-2 or C-3 of the sugar units which in the disaccharide have the $^4C_1$ conformation, but which, as suggested earlier, may exist in the $^1C_4$ conformation in the polysaccharide.

Methylation of the disaccharide was inconclusive, tetra-O-methylgalactose being the only partially methylated sugar detected.

The analysis of this disaccharide remains incomplete but it is probably a sulphated L-galactosyl-xylose.

The residual polymer

The residual polymer (carbohydrate content 80%; 60% by weight of the initial mucilage) obtained after autohydrolysis of the mucilage contained no sulphate but the uronic acid and methoxyl contents had increased from 9% and 3.6% in the original mucilage, to 20% and 4% respectively in the residual polymer. The increased carbohydrate content, 80% from 68%, is explained by the loss of 9% sulphate and its accompanying metals.

The viscosity of a 0.1% solution compared to that of water (17 secs) was 1.13, that of a similar solution after heating to 70° and cooling to 30° was 1.14. A 0.1% solution of the original mucilage had a relative viscosity of 6 and after heating to 70° its viscosity was 6.5 (expt. 15).

After hydrolysis the following sugars were found as shown by paper chromatography ionophoresis and g.l.c.:- xylose, D-glucose, galactose, 3-O-methyl xylose, 3- and 4-O-methyl galactose, 2,4-di-O-methyl galactose and the aldobiouronic acid, 1,3-linked

D-glucuronosyl-D-galactose, which has been characterised previously (p.104). The proportions of the sugars, as determined by peak areas from a g.l.c. trace of the derived alditol acetals, are shown in table 15.

Table 15

Constituent sugars found in hydrolysates of the residual polymer and the original mucilage

Sugar	Approximate percentage as determined by peak areas. Residual polymer[b]	Original mucilage[c]
xylose	27.5	29.5
galactose	7.5	11.0
glucose	7.0	18.5
3-O-methylxylose	4.5	5.0
2,4-di-O-methylgalactose	10.0	11.0
3- and 4-O-methylgalactose	3.5	5.0
uronic acid[a]	20.0	9.0

(a) As determined by the m-hydroxydiphenyl method. This is holding 20% and 9% of galactose in linkage from the respective polysaccharides.

(b) Neutral sugars should be 60%

(c) Neutral sugars should be 80%.

Since the residual polymer represents only 60% of the initial polysaccharide and the uronic acid content in it has risen to 20%, it follows that the 40% lost is made up entirely of neutral sugars. The table gives the relative percentages of the monosaccharides present in the hydrolysates of the residual polymer and the original

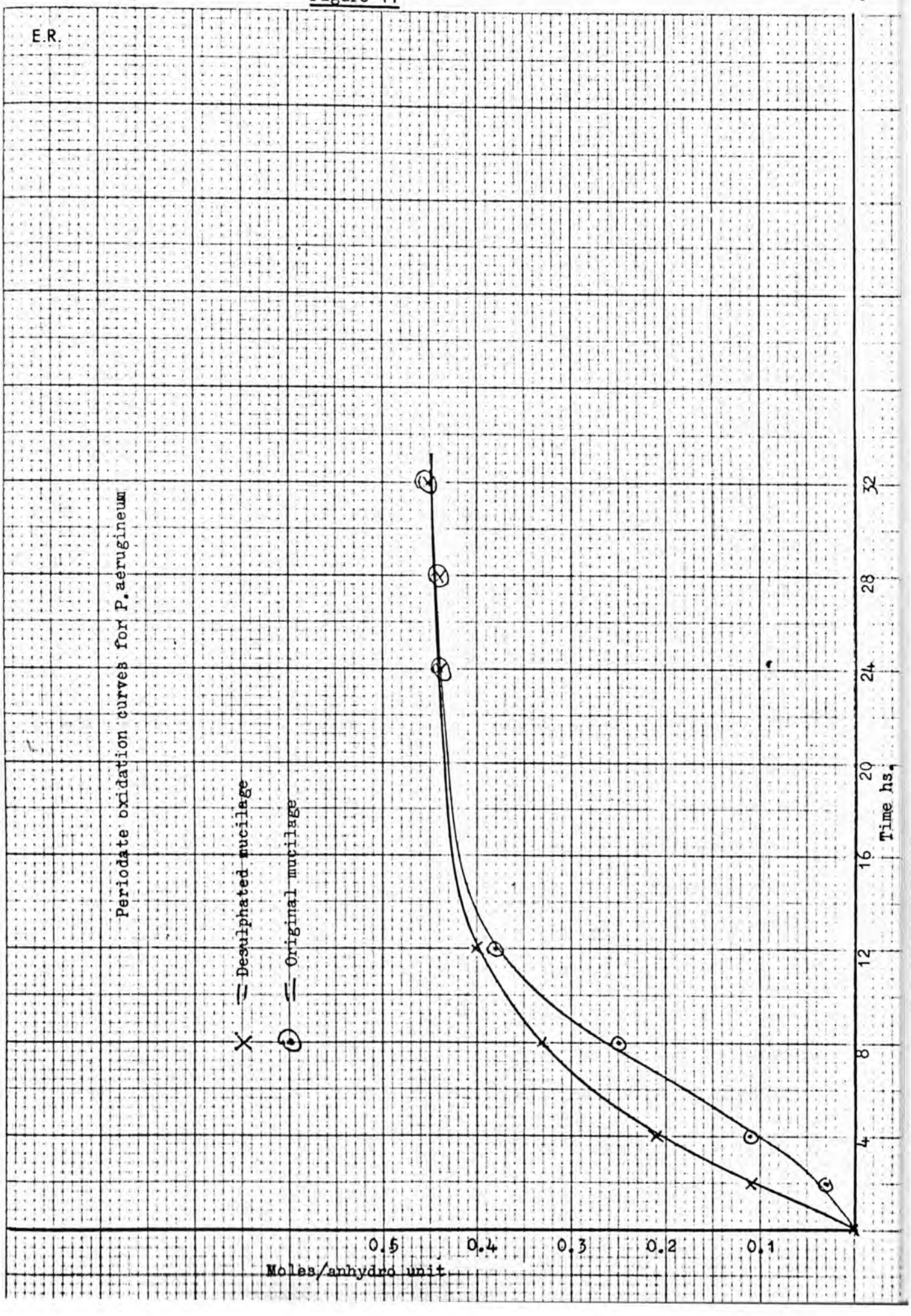
mucilage. From the above facts it is clear that, apart from the uronic acid, a proportion of each of the sugars has been cleaved during autohydrolysis. This was confirmed by paper chromatography of the dialysate. However a greater proportion of the glucose and galactose were split off indicating that a higher proportion of hexoses than pentoses occurs at the periphery of the polymer. Their removal apparently exposed pentose units as new non-reducing end groups since the proportion of tri-O-methylxylose is considerably higher in the methylated residual polymer than in the methylated original mucilage (p.122).

Periodate oxidation of the mucilage, the desulphated mucilage

Oxidation of the mucilage and the desulphated mucilage (expt. 16(a)) with 5 mM-periodate solution resulted in a reduction of 0.43 moles of periodate per anhydro unit by each. However the oxidation of the sulphated polymer took longer to reach completion (Fig. 11). The sulphate content of the derived polyalcohol showed that almost all the sulphate had been removed from the initial polymer during the oxidation process. Analysis of the dialysate during the purification of the derived polyalcohol showed that free sulphate was present in the dialysate. It can be concluded that the longer time taken for complete oxidation of the sulphated mucilage is due to the slow removal of the sulphate during the oxidation process.

The hydrolysates of the two polyalcohols were identical, each containing the following sugars:- xylose, glucose, galactose, 3-O-methyl xylose, 3- and 4-O-methylgalactose, 2,4-di-O-methylgalactose, an aldobiouronic acid, glycerol, erythritol and threitol. Although

Figure 11


E.R.
Periodate oxidation curves for P.aerugineum
= Desulphated mucilage
= Original mucilage
0.5
0.4
0.3
0.2
0.1
Moles/anhydro unit
4
8
12
16
20
24
28
32
Time hs.

erythritol and threitol could not be separated in any of the solvents used for paper chromatograpy, electrophoresis in molybdate buffer effectively distinguishes the two. No acidic fragments or monouronic acids were detected.

Glycerol showed that 1,4-linked xylose was present in the original polymer and erythritol and threitol showed the presence of 1,4-linked glucose and galactose. The absence of any acidic fragments and the occurrence of an aldobiouronic acid indicated that the uronic acid was linked through position C-3 and that it was linked to C-3 of the next sugar unit. This confirms that the uronic acid is present in the polysaccharide as D-glucuronosyl (1 → 3) D-galactose with C-3 of the uronic acid involved in linkage.

Periodate oxidation of the mucilage and the residual polymer

Oxidation of the mucilage and the residual polymer with 25 mM-periodate (expt. 16b) resulted in a reduction of periodate per anhydro unit of 0.55 and 0.59 moles respectively (Fig. 12). After reduction the derived polyalcohols were recovered in yields of 74% and 67% respectively by weight. Allowing for the loss of 9% sulphate from the mucilage this brings the recovery of organic material to 83%. The two polyalcohols had carbohydrate contents of 44% and 45% respectively.

Reoxidation of the polyalcohols with 5 mM-periodate resulted in no further reduction of periodate proving the absence of acetal formation during the initial oxidation.[112]

Figure 12

E.R.
Periodate oxidation curves for P.aerugineum and its residual polymer
X = Original mucilage
⊙ = Residual polymer
Moles/anhydro unit
0.6
0.5
0.4
0.3
0.2
0.1
4
8
12
16
20
24
28
32
Time hs.

The increased reduction of periodate resulting from the use of 25 mM-periodate instead of the 5 mM solution was probably due to incomplete oxidation of the polysaccharide with the weaker solution.

Hydrolysates of the two above polyalcohols gave the same sugars and fragments as the previous polyalcohols. The relative proportions of each of the monosaccharides were determined from the peak areas of a g.l.c. trace of the derived alditol acetates from the polyalcohol obtained after the more complete oxidation (tables 16,17) and these are compared with the relative proportions of the sugars present in hydrolysates of the original mucilage and the residual polymer.

Table 16

Approximate relative percentages and molar proportions of the sugars present in P. aerugineum mucilage and its derived polyalcohol

Sugar	Mucilage		Polyalcohol	
	percentage	molar proportion	percentage	molar proportion
xylose	29	1.93	7	0.47
galactose	11 (20)[d]	1.11	28(42)[d]	2.8
glucose	18	1.0	15	1.0
3-O-methylxylose	5	0.28	4	0.27
2,4-di-O-methylgalactose	11	0.61	8	0.53
3- and 4-O-methylgalactose	5	0.27	10	0.67
uronic acid[a]	9[b]	0.50	14[c]	0.93

(a) As determined by the m-hydroxydiphenyl method.

(b) Based on 68% carbohydrate, and linked to 9% of the galactose.

(c) Based on 44% carbohydrate, and linked to 14% of the galactose.

(d) Figures in brackets include the galactose retained linked to the uronic acid.

It must be remembered that bound up with the uronic acid is an equal quantity of galactose which is not hydrolysed to the individual units. Allowance has been made for this in the molar proportions in the table by adding 9% and 14% to the galactose contents in the original mucilage and the derived polyalcohol respectively. It must also be remembered that ca. 20% of the material has been lost during the oxidation and reduction. Nevertheless it is clear that a much higher proportion of xylose has been oxidised than the other sugars and more glucose than galactose. This is to be expected as about half the galactose is present in the aldobiouronic acid glucuronosyl (1 → 3)galactose.

Table 17

Approximate relative percentages and molar proportions of the sugars present in the residual polymer and its derived polyalcohol

Sugar	residual polymer		polyalcohol	
	percentage	molar proportion	percentage	molar proportion
xylose	27.5	3.9	2.5	0.55
galactose	7.5 (27.5)[d]	3.93	14.0(49)[d]	10.8
glucose	7.0	1.0	4.5	1.0
3-O-methylxylose	4.5	0.64	2.0	0.44
2,4-di-O-methylgalactose	10.0	1.43	3.5	0.77
3- and 4-O-methylgalactose	3.5	0.5	3.5	0.77
uronic acid[a]	20.0[b]	2.86	35.0[c]	7.8

(a) As determined by the m-hydroxydiphenyl method.

(b) Based on 80% carbohydrate, linked to 20% galactose.

(c) Based on 45% carbohydrate, linked to 35% galactose.

(d) Figures in brackets include the galactose found in glucuronosyl linkage.

Again it must be remembered that the uronic acid is bound to equal quantities of galactose, therefore in the molar proportions 20% and 35% have been added to the galactose figure in the residual polymer and its polyalcohol respectively. During the oxidation and reduction process 33% of the material has been lost, however it is clear that a substantial proportion of the xylose has been oxidised and much more glucose than galactose.

These results, as in P. cruentum confirm that much of the xylose was present as end group and 1,4-linked units.

The absolute galactose content cannot have increased so the glucose as well as xylose must have decreased in the polyalcohols. Later work on the molecular size of the polyalcohols (p.129) showed that considerable degradation occurs during the oxidation and reduction processes so it is possible that during oxidation the other sugars are preferentially released either as monomers or low molecular weight oligosaccharides and are subsequently lost during dialysis of the derived polyalcohol thus leaving relatively more galactose in the polyalcohol.

Aliquots of the polyalcohols derived from the mucilage and the residual polymer were methylated and were analysed as the derived partially methylated alditol acetates (expt. 17). The results are shown in table 18.

Table 18

Percentages of sugars found in the hydrolysates of the methylated polyalcohols from the mucilage and the residual polymer

Sugar	mucilage[a]	residual polymer[b]
2,4-di-O-methyl xylose	4	8
2,3,4,6-tetra-O-methyl galactose	23	25
2,4,6-tri-O-methyl hexose	27	16
2,3,6-tri-O-methyl hexose	19	18
2,6-di-O-methyl hexose	19	28
6-O-methyl hexose	8	5

(a) Polyalcohol recovered in 83% yield, carbohydrate content 44%.

(b) Polyalcohol recovered in 67% yield, carbohydrate content 45%.

When analysing these results it must be remembered that about some 20 and 30% respectively of the materials are lost during the oxidation process. The presence of 2,3,4,6-tetra-O-methyl galactose may be partially due to 3-O-methyl galactose and 2,4-di-O-methyl galactose occurring as end groups in the original polymers. Similarly part of the 2,3,6-tri-O-methyl hexose may be due to the presence of 1,4-linked 3-O-methylgalactose. However during the methylation process a large amount of degradation occurs and it is this which probably accounts for a large amount of the 2,3,4,6-tetra-O-methyl-galactose, indicating that a large number of non-reducing galactose units are exposed as end groups during this degradation.

Methylation of the mucilage and residual polymer

The mucilage and the residual polymer were methylated (expt. 18(a)), and were analysed, after hydrolysis, by g.l.c.-m.s. as the derived

alditol acetate derivatives. The percentage relative proportions of the partially methylated sugars are shown in table 19.

Table 19

Partially methylated sugars found in the hydrolysate of *P. aerugineum* mucilage and the residual polymer after methylation

Sugar	mucilage %	residual polymer %
2,3,4-tri-O-methyl xylose	14	24
2,3,4,6-tetra-O-methyl glucose	5	13
2,4-di-O-methyl xylose	7	
2,3,4,6-tetra-O-methyl galactose	10	26
2,3-di-O-methyl xylose	12	
2,4,6-tri-O-methyl hexose	21	14
2,3,6-tri-O-methyl hexose	15	7
2,6-di-O-methyl hexose	12	15
6-monomethyl hexose	5	1

No methylated uronic acid could be detected on g.l.c., however ionophoresis indicated the presence of a methylated aldobiouronic acid in a hydrolysate of the methylated polysaccharide.

The proportion of 1,3-linked to 1,4-linked units is approximately equal.

The 2,3,4,6-tetra-O-methyl galactose could have originated from either of the methylated galactoses or galactose itself as end group. However the high proportion of end group to branch point may be explained as for *P. cruentum* (p. 68) i.e. that a certain amount of

alkaline degradation occurs during methylation. This is substantiated by the change in molecular size which occurs during methylation (Fig. 13).

These results show that xylose is present in the polysaccharide and residual polymer as end groups, 1,3- and 1,4-linked units; glucose and galactose are present as end groups, 1,3-linked and 1,4-linked units and branch points.

The only major difference between the hydrolysates of the two methylated polymers (Table 19) is the relative increase in end group xylose and a decrease in the proportions of 1,3- and 1,4-linked hexoses in the residual polymer. As 40% of the polysaccharide was hydrolysed during autohydrolysis the above results indicate that glucose and galactose were preferentially cleaved from xylose units leaving the latter as end groups. The results also indicate that the residual polymer has shorter chains than the mucilage, this was later borne out by studies on the molecular size of the two (Fig. 14).

The sulphate was removed during methylation thus making it impossible to determine the site of the sulphate by studying the methylated polysaccharide obtained before and after desulphation. As a result of this a different methylation procedure was used to try and methylate the polysaccharide without removing the sulphate (expt. 18(b)). However after two Haworth methylations the methoxyl content indicated that only 47% of the residues were completely methylated, analysis of the hydrolysate by g.l.c. confirmed that a large number of the units were unmethylated. The molecular size showed that considerable degradation had occurred (Fig. 15). The sulphate content had decreased

from 9% to 3% so no more information as to the site of the sulphate could be obtained from this experiment.

Alkali degradation of the methylated mucilage

After alkali degradation of the methylated polysaccharide (expt. 19) and analysis by g.l.c.-m.s. the same partially methylated sugars were detected as were present in the polysaccharide after one Hakomori methylation and in approximately the same proportions. After remethylation with trideutero methyl iodide no deuterated partially methylated sugars were detected. This may be because the proportion of these sugars are so small when compared to the methylated sugars present after the first methylation. It is probable that considerable alkali degradation takes place during the first Hakomori methylation, as shown by molecular size (fig.13), and that the subsequent degradation process has little further effect on the polymer. This is confirmed by studying the molecular size of the polysaccharide after a second Hakomori methylation (which should alkali degrade the partially methylated polysaccharide). This indicates that further degradation of the polymer does occur but it is only small when compared to the degradation which occurs during the first methylation procedure.

Carbodiimide reduction

As for P. cruentum the polysaccharide precipitated as soon as the carbodiimide reagent was added with no change in pH occurring (expt. 22). After treatment with borohydride, dialysis and freeze-drying no change in the uronic acid content was detected.

Molecular size studies

Elution of the mucilage and residual polymer from a Sepharose 4B column (figs. 18, 19) (expt. 20) showed that the former had a molecular weight of 5×10^6 and the latter had a wide molecular weight distribution with a maximum concentration occurring at 1×10^6 (fig. 14). Since 40% of the carbohydrate (by weight) was lost during the autohydrolysis it follows from the relatively small change in molecular size that a considerable proportion of the loss occurred from the periphery of the molecule and that an average of only about four cleavages of the macromolecule occurred along its backbone.

Molecular size studies of the mucilage after one and after two Hakomori methylations (Fig. 13) showed that extensive degradation had occurred even after one methylation. After two methylations the majority of the polymer had a molecular weight of only 30,000.

After two Haworth methylations a very broad molecular weight distribution is found (Fig. 15).

The polyalcohols derived from P. aerugineum and the residual polymer are shown, after passage down a Sepharose 4B column (Figs. 16, 17). Some degradation of the mucilage has occurred during periodate oxidation but there is still a substantial amount of high molecular weight material. The residual polymer polyalcohol, on the other hand, shows that a large amount of degradation has taken place and the majority of the material has a molecular weight of around 10,000.

After treatment of the two polyalcohols with 1.0M-trifluoroacetic acid at room temperature (conditions which should hydrolyse the acetal linkages)[112] the molecular weight of the polyalcohol derived from the

residual polymer remained constant at about 10,000 but the polyalcohol derived from the mucilage had decreased from 3 x 10^6 to 30,000 (as shown by a Sepharose 4B column and also by HPLC) (Figs. 16, 17, 5).

These experiments appear to indicate that P. aerugineum polysaccharide has a repeating unit of 30,000 although the results from the residual polymer suggest a repeating unit of 10,000 (i.e. about 50 sugar units). They also show that a large extent of degradation occurs during the Hakomori methylation process.

Figure 13

Elution patterns from Sepharose 4B of methylated mucilage from P.aerugineum

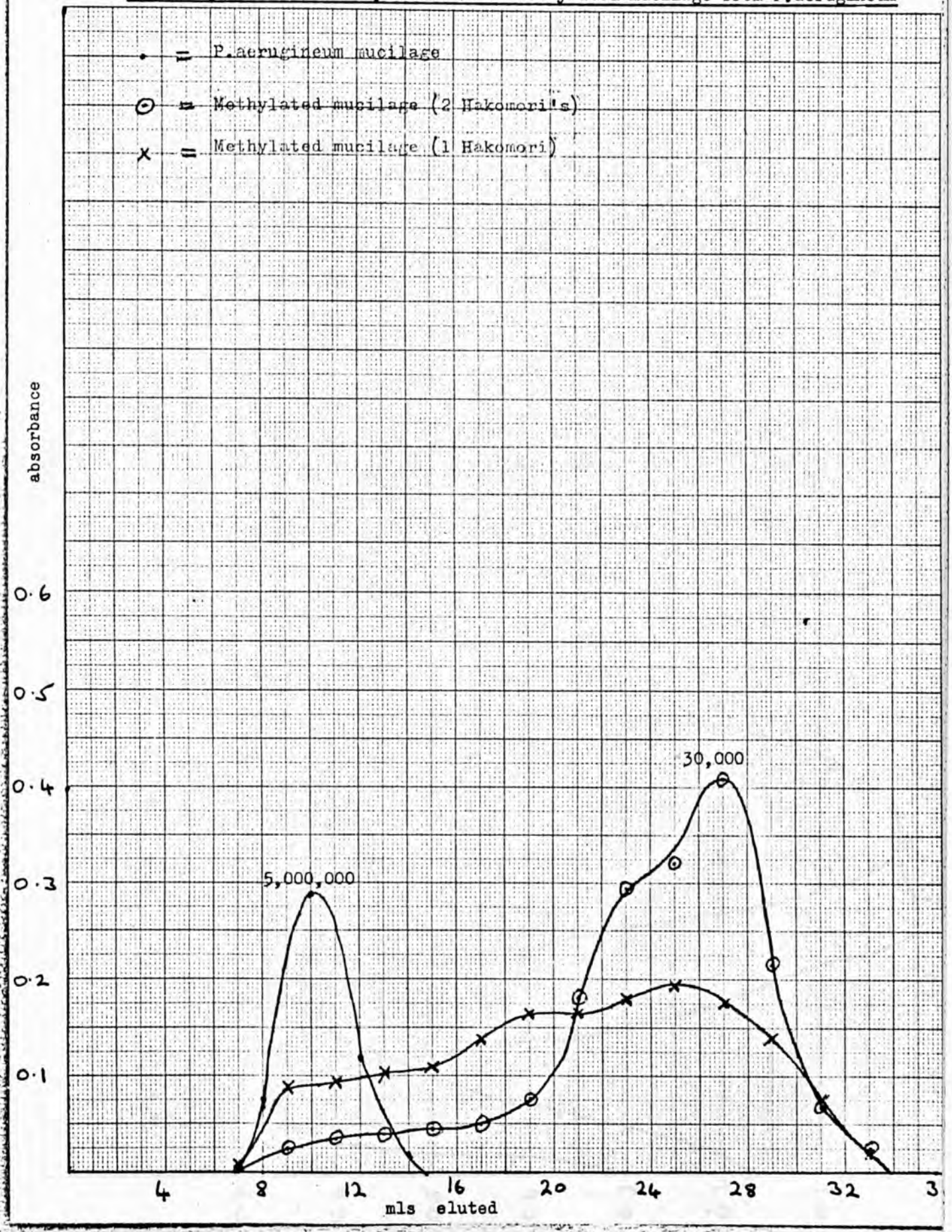


Figure 14

Elution patterns from Sepharose 4B of P.cruentum. P.aerugineum, residual polymer

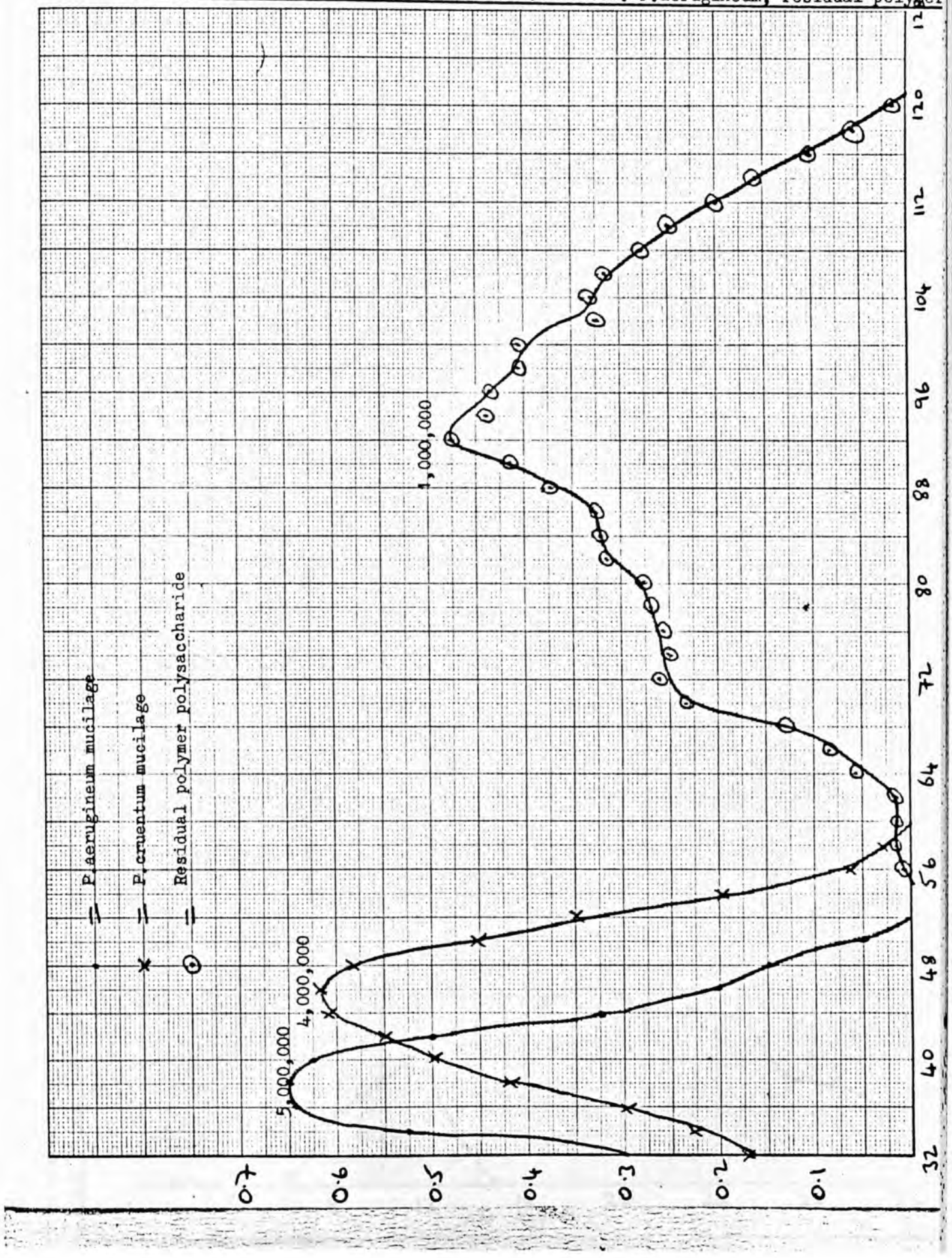


Figure 15

Elution patterns from Sepharose 4B of methylated P.aerugineum mucilage

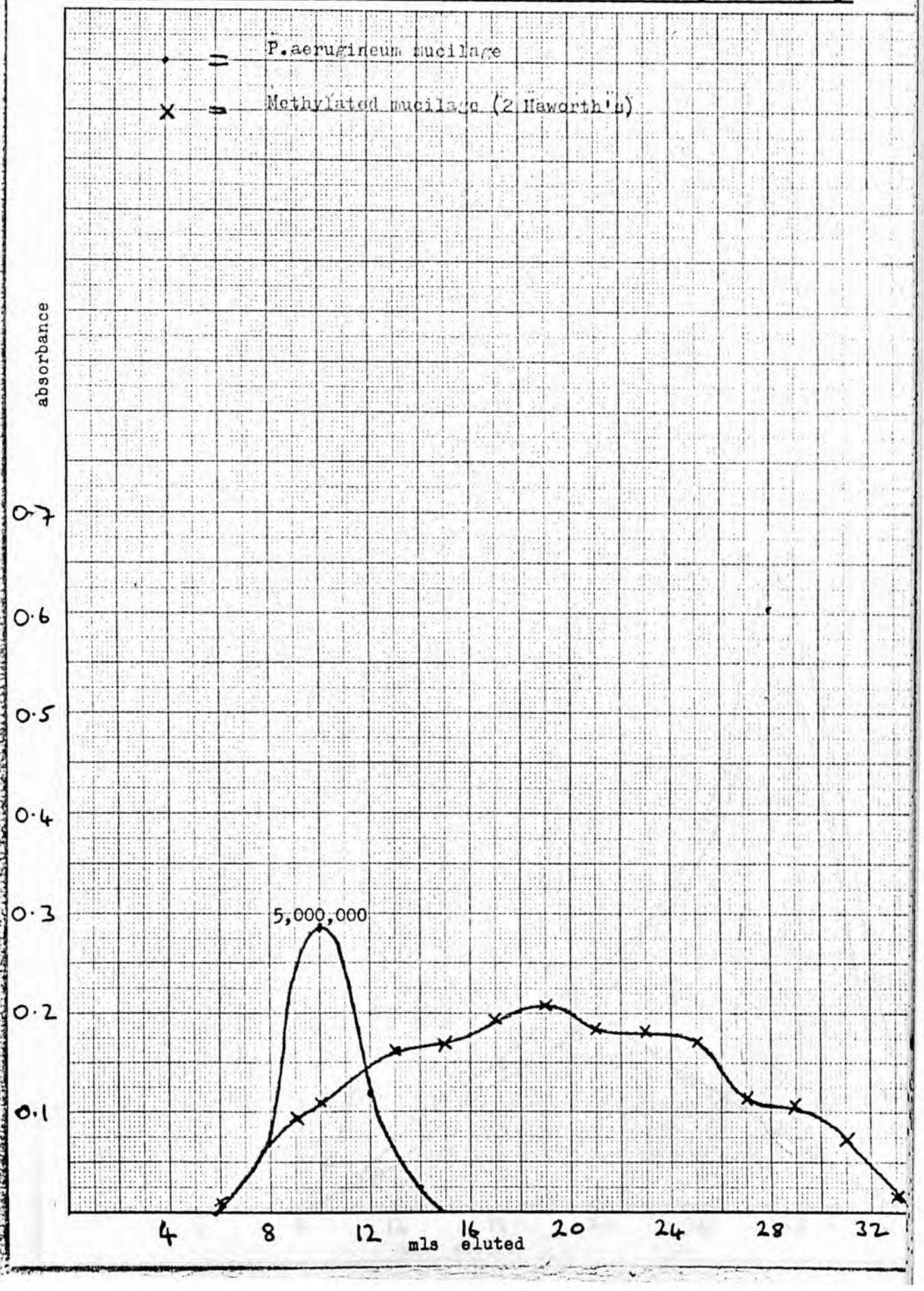


Figure 16

Elution patterns from Sepharose 4B of P.aerugineum polyalcohols

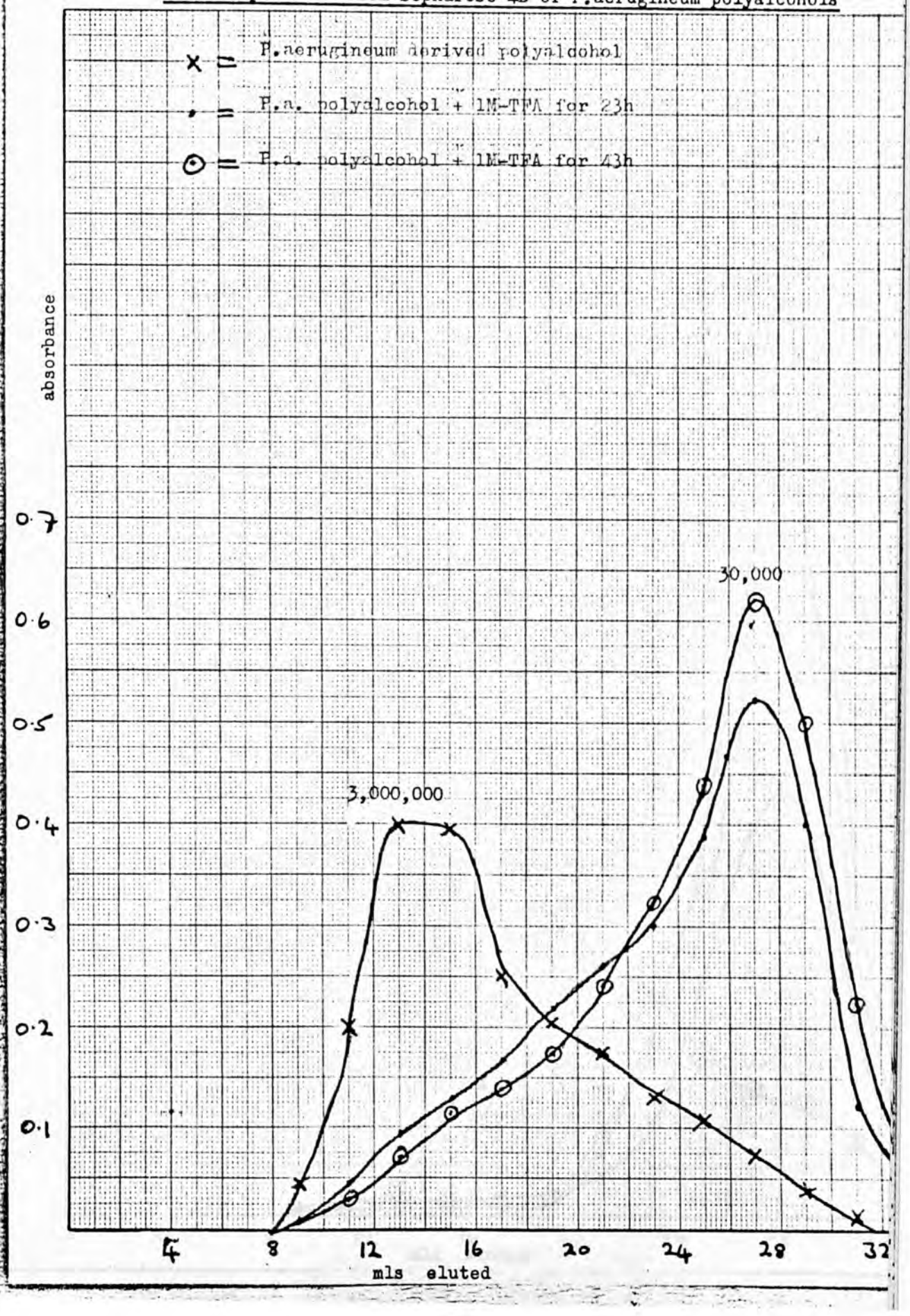


Figure 17

Elution patterns from Sepharose 4B of residual polymer polyalcohol

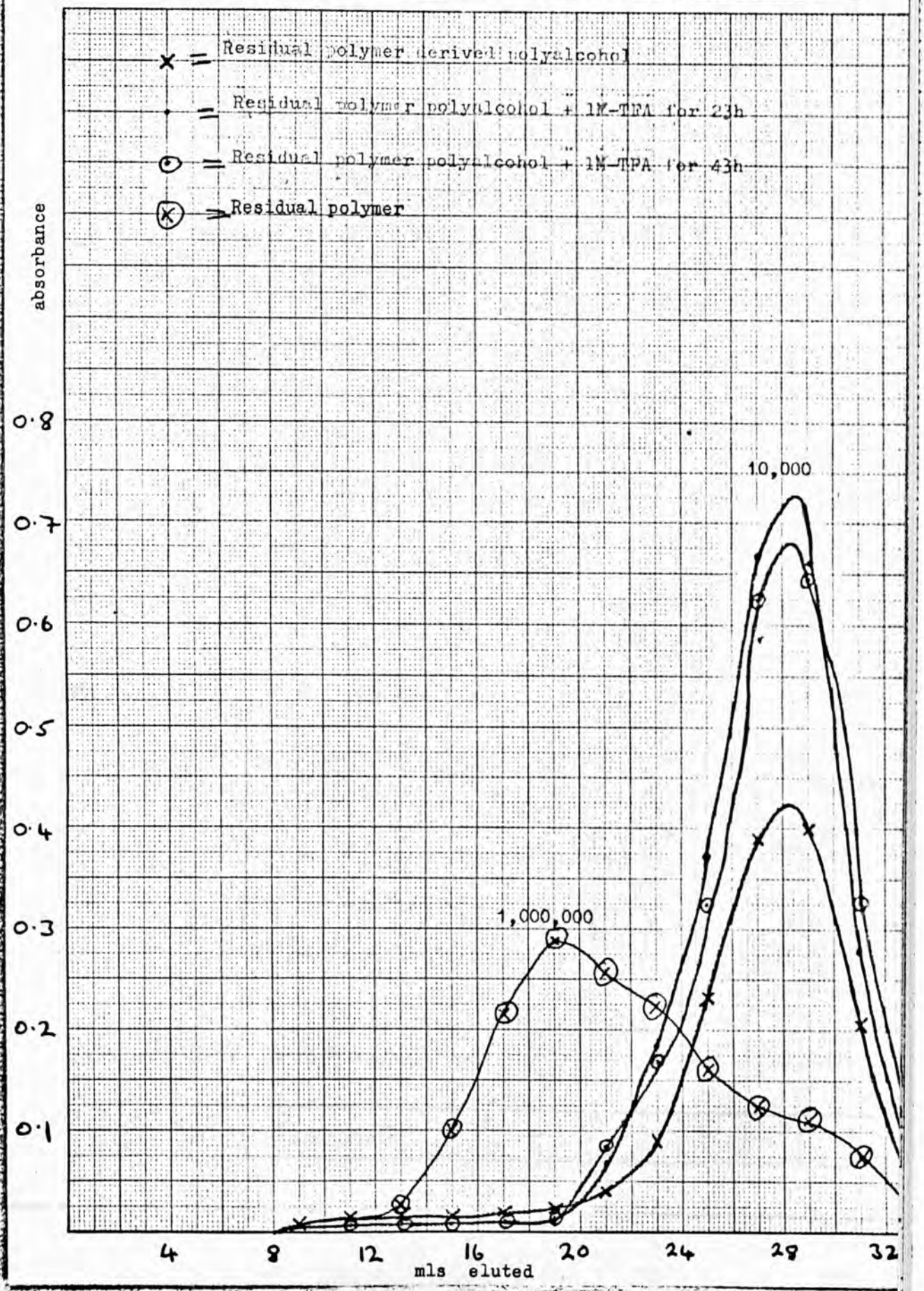


Figure 18

Elution profiles from Sepharose 4B column

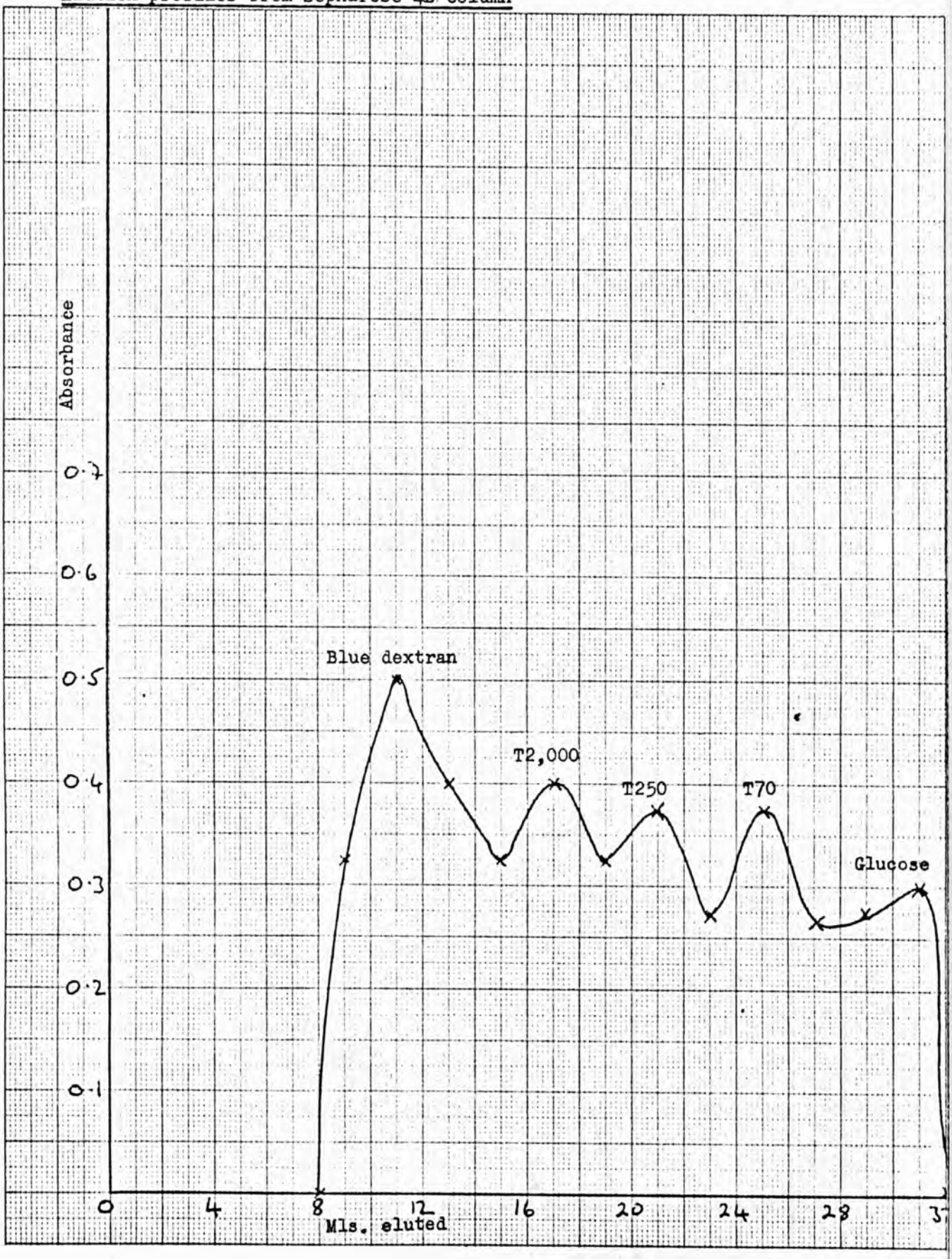
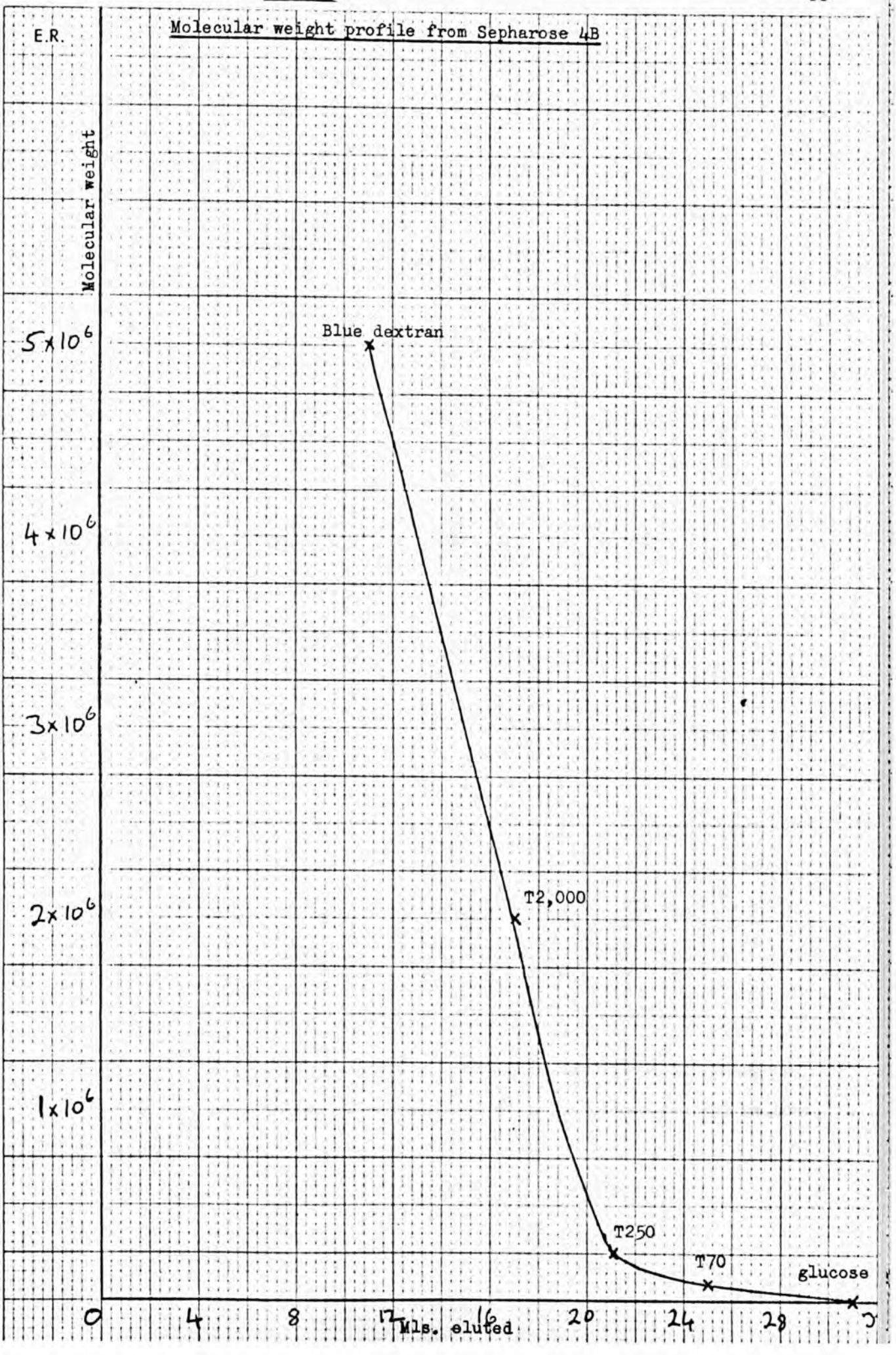


Figure 19


E.R.
Molecular weight profile from Sepharose 4B
Molecular weight
5x10^6
4x10^6
3x10^6
2x10^6
1x10^6
Blue dextran
T2,000
T250
T70
glucose
0
4
8
12
16
20
24
28
Mls. eluted

Trifluoroacetic acid treated polyalcohols

Analysis of hydrolysates of the two polyalcohols after treatment with 1.0M-trifluoroacetic acid showed that the same sugars were present in each and, as expected, the proportions of these sugars were the same as before trifluoroacetic acid hydrolysis. Although there were also trace quantities of glycerol, erythritol and threitol these were very small indicating that the acid had hydrolysed the majority of the acetal linkages.

It is difficult to explain the apparent lack of change in size of the molecules of the polyalcohol from the residual polymer on treatment with trifluoroacetic acid. It can only be assumed that the periodate oxidised units were on the periphery of the molecules and that a molecule of molecular weight 10,000 represents a portion of the original material which is immune to periodate.

Conclusions

The polysaccharide exuded by the red alga Porphyridium aerugineum has an apparent molecular weight of 5×10^6. This high value however could be due to aggregation of molecular chains.[128] It contains the following sugars:- D-xylose, D- and L-galactose, D-glucose, D-glucuronic acid, 3- and 4-O-methylgalactose, 2,4-di-O-methylgalactose, 3-O-methylxylose in the molar proportions 1.7: 1.1: 1.0: 0.5: 0.6: 0.3: 0.3. It also contains 9% sulphate, 9% glucuronic acid, 3.6% methoxyl and 6% protein. The galactose is present as 75% in the D-form and 25% as the L-sugar.

Methylation and periodate studies gave essentially similar results and showed that xylose is present as end groups, 1,3- and 1,4-linked units, glucose and galactose are present as end groups, 1,3- and 1,4-linked units and also as 1,3,4-linked branch points. The uronic acid is 1,3-linked and only occurs linked to C-3 of D-galactose, either in chains or at branch points.

Some of the sulphate was shown to be present on L-galactosyl-xylose, possibly on C-2 or C-3 of the galactose residue. In contrast infra red studies on the mucilage indicated that sulphate is present on C-4 and C-6 of the hexose units with these in the $^{4}C_{1}$ conformation. If, however these units are held in the $^{1}C_{4}$ conformation in the polysaccharide then the sulphate could be located on C-2 or C-3 (in addition to C-6) of the hexose units. This would agree with the infra red results and also with alkali lability of the sulphate.

Autohydrolysis removed 40% of the neutral sugars, however the composition of the residual polymer, apart from the uronic acid content which had doubled, was similar to the initial mucilage. The molecular weight was reduced from 5×10^{6} to 1×10^{6} indicating the hydrolysis of a minimum of four glycosidic links at regular intervals. However many more linkages could have been hydrolysed had the hydrolysis occurred at, for example, only the periphery of the molecule. From this evidence it may be concluded that the glucuronic acid (as glucuronosyl-galactose) only occurs on the interior of the molecule and not on the side chains.

Comparison of the mucilages from P.cruentum and P.aerugineum

It is clear from these studies that the polysaccharides from P. aerugineum and P. cruentum mucilages are branched molecules which have no simple repeating unit. However certain structural features have emerged which are common to both. All the glucuronic acid is glycosidically linked to C-3 of galactose and this aldobiouronic acid appears to be a fundamental structural unit in both polysaccharides. Other structural features are xylose linked to C-3 of a second xylose unit, 1,4-linked xylose, 1,4-, 1,3- and 1,3,4-linked glucose and galactose units.

The main differences between the two mucilages are that the mucilage from P. aerugineum contains a much higher proportion of methylated sugars, the sulphate in contrast to that of P. cruentum, is partially alkali labile and it forms much more viscous solutions than the mucilage from P. cruentum. Furthermore no 2-O-methylglucuronic acid could be detected in P. aerugineum mucilage. The difference in the viscosity may be due to the different conformations of the two polysaccharides which was indicated by studies on the position of the sulphate groups.

Finally it must be remembered that all the analyses are based on the average of the molecules present and that these polysaccharides probably belong to two families of polymers each with the same general plan but differing in fine details.

Appendix I Structural studies on a purified fucoidan

A sample of a partially desulphated fucoidan (carbohydrate content 73%, sulphate content 8%, yield 30%) obtained after treatment of the original purified fucoidan A (carbohydrate content 54%, sulphate content 31%) with dimethylsulphoxide[130] was methylated once by the Hakomori method. The partially methylated sugars present were characterised as their alditol acetates by g.l.c.-m.s.. Comparison of these sugars with the partially methylated sugars found in the methylated original fucoidan A (one Hakomori, table 20) show that a large proportion of the unmethylated fucose has disappeared

Table 20

Partially methylated sugars found in a fucoidan and its partially desulphated derivative after methylation

Sugar	Fucoidan	Desulphated fucoidan
2,3,4-tri-O-methylfucose	5.0%	18.4%
2,3-di-O-methylfucose	7.4%	26.2%
2,4-di-O-methylfucose	trace	trace
2-O-methylfucose	8.9%	14.0%
3-O-methylfucose	33.5%	41.4%
unmethylated fucose	45.2%	trace

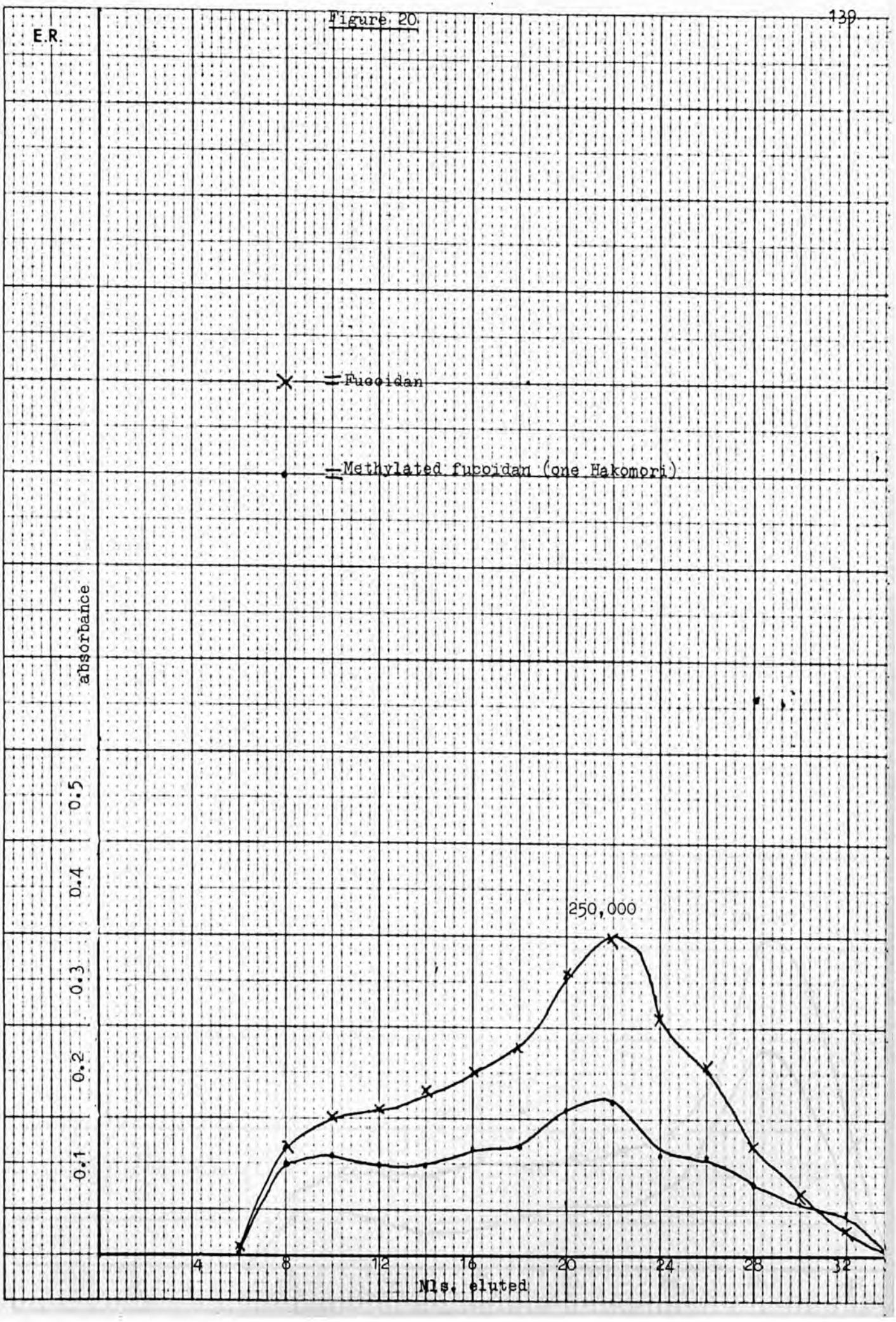
and the proportion of 2,3-di-O-methyl fucose has increased considerably in the hydrolysate from the desulphated fucoidans. This is the first time this dimethyl sugar has been characterised as a product from methylated fucoidan providing definite proof of 1,4-linkages.

Earlier work[132] had indicated that the fucose units were mainly 1,2-linked with sulphate on C-4. Later Cotè[131] isolated both 1,2- and 1,4-linked disaccharides together with traces of the 1,3-linked disaccharide from a partial hydrolysate of a fucoidan. These studies confirm the results of both previous investigations. The 3-O-methyl fucose could be derived from 1,2-linked units sulphated or branched at C-4 or from 1,4-linked units sulphated or branched at C-2. The 2,3-di-O-methyl sugar indicates a reasonable proportion of 1,4-linked residues.

In neither of the hydrolysates does the proportion of end group correspond to the proportion of branches but this could be explained by loss of the tri-O-methyl fucose during concentration of the hydrolysate as this is very volatile.

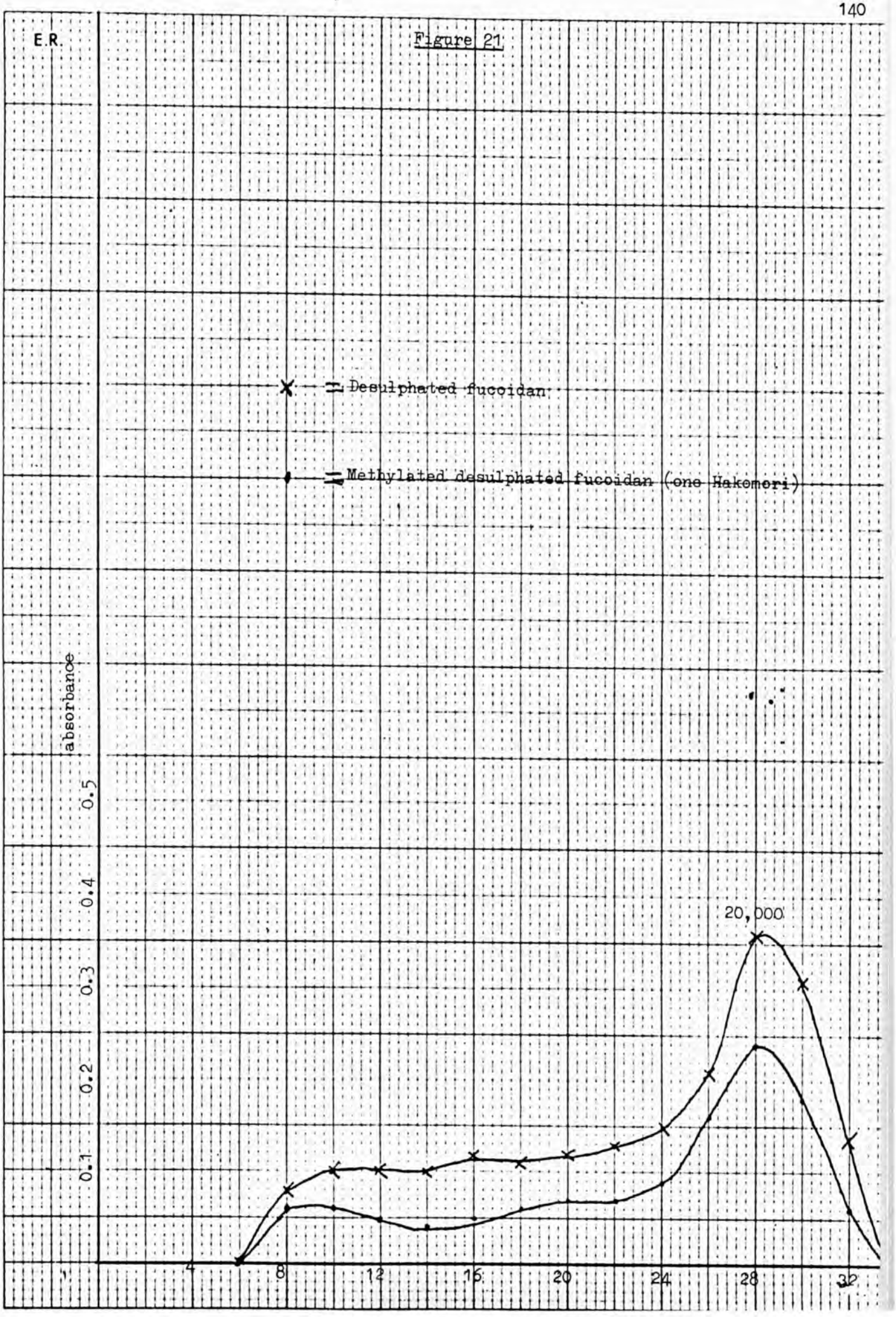
Molecular size studies of the two polysaccharides before and after methylation on a Sepharose 4B column (Figs. 20,21) show that the methylation procedure does not degrade the molecules to any appreciable extent. However desulphation had reduced the molecular weight from 250,000 to 20,000 indicating that some degradation occurs during the desulphation process and this is supported by the increase in end group in the methylated desulphated polysaccharide.

Figure 20.


E.R.
Fucoidan
Methylated fucoidan (one Hakomori)
absorbance
0.5
0.4
0.3
0.2
0.1
250,000
4
8
12
16
20
24
28
32
Mls. eluted

E.R.

Figure 21


= Desulphated fucoidan
= Methylated desulphated fucoidan (ono Hakomori)
absorbance
0.5
0.4
0.3
0.2
0.1
20,000
4
8
12
16
20
24
28
32

Appendix II

Methylation of starch extracted from Chroomonas salina

A sample of purified starch from the alga Chroomonas salina was obtained from Dr. N.J. Antia, Fisheries Research Board, Vancouver, Canada. The starch was methylated once by the Hakomori method. The resulting methylated polymer was analysed as the derived partially methylated alditol acetates by g.l.c.-m.s. This showed the presence of the following sugars in the methylated starch; 2,3,4,6-tetra-O-methylglucose, 2,3,6-tri-O-methylglucose and 2,3-di-O-methylglucose. The ratio of 1,4-linked glucose to 1,4,6-linked branch points was 15:1.

Appendix III

Preliminary studies on the polysaccharides elaborated by the red algae Constantinea subulifera and Constantinea simplex

These two algae, members of the Dumontiaceae family, were reported to have antiviral properties, C. simplex being more active than C. subulifera. The 'inside' and 'outside' of the dried frond (as this tends to peel and divide) of C. subulifera and the respective anionic polysaccharides from both weeds were supplied for investigation by Marine Colloids Inc. U.S.A.

Preliminary studies on the hydrolysates of the 'inside' and 'outside' fronds by paper chromatography showed no major differences between them or between the hydrolysates of the respective polysaccharides.

Hydrolysates of the respective anionic polysaccharides from C. subulifera and C. simplex when analysed by paper chromatography and by g.l.c.-m.s. as the derived alditol acetates indicated that the following sugars were present in both, xylose, galactose, glucose and arabinose in the approximate molar proportions 0.75: 4.1: 1.0: 0.22 and 4.0 : 7.3 : 0.3 : 0.7. In addition the polysaccharide from C. simplex also contained a 3- and/or 4-O-methylhexose (1.0) and a 3,4-di-O-methylhexose (0.23). Traces of the monomethyl hexose were also found in the polysaccharide from C. subulifera. Paper chromatograms of both hydrolysates indicated the presence of a small proportion of aldobiouronic acid.

Treatment of hydrolysates from each of the two polysaccharides with glucose and galactose oxidase showed that D-glucose and L-galactose were present.

About 18% of half ester sulphate was present in each polysaccharide.

Methylation of both polysaccharides by a single Hakomori methylation followed by hydrolysis and chracterisation of the sugars as the derived alditol acetates by g.l.c.-m.s. showed that the major product was 2,3,6-tri-O-methyl hexose. In view of the relative proportions of galactose and glucose in the polysaccharide it is reasonable to assume that this is the galactose derivative, consequently 1,4-linked galactose must be a major structural unit in both polysaccharides. 2,3,4-tri-O-methylxylose, 2,3,4,6-tetra-O-methyl glucose and galactose were also characterised indicating that all three sugars were present as end groups. 2,3,-di-O-methylhexose was also found to be present.

An unusual feature in both polysaccharides was the presence of a relatively large proportion of unmethylated galactose and xylose (greater in C. simplex). Although the polysaccharides had been subjected to only one Hakomori methylation this is unusual and might indicate that these two sugars were carrying an unidentified substituent. This is the only unusual feature detected in the polysaccharides which might account for the antiviral properties of the two red algae.

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