Structural Studies on the polysaccharides of the green seaweed *Urospora wormskioldii* and of the brown seaweeds *Desmarestia ligulata* and *D. firma*

by

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THIS THESIS IS DEDICATED

TO MY FAMILY

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Abstract

From investigations of two life stages of the green seaweed Urospora wormskioldii (U. wormskioldii and Codiolum pusillum) it was found that both weeds synthesise the following ethanol soluble carbohydrates: glucose, fructose, sucrose, myo-inositol, glyceric acid and a number of glucose containing oligosaccharides. Both weeds also synthesise starches comprising amylose and amylopectin as well as highly branched 1,3-linked glucans. Evidence for a 1,4-linked homoxylan with C-2 branch points was found in U. wormskioldii and indications of a homoxylan both 1,3- and 1,4-linked was found in Codiolum. Small amounts of an α-1,3-linked mannan and large amounts of a β-1,4-linked mannan branched at C-6 and sulphated at C-2 were found in Codiolum, although U. wormskioldii contained only trace quantities of a mannan.

Polydisperse heteropolysaccharides ("rhamnans") containing L-rhamnose, D-xylose and D-glucuronic acid and half ester sulphate linked to the rhamnose were found in both weeds. The glucuronic acid was found to be 1,4-linked and end group and the xylose to be 1,4-linked and end group and in U. wormskioldii it was also 1,2,4-linked. The rhamnose residues were mainly 1,3-linked and sulphated at C-2 or C-4 and to a lesser extent 1,4-linked with sulphate groups at C-2 or C-3. A small amount of 1,2-linked and end group rhamnose units was also found. The "rhamnans" metabolised by Codiolum appear to be basically linear while those from U. wormskioldii appear to be highly branched with possibly 1,3,4- or 1,2,4-linked rhamnose branch points.

The structural cell-wall polysaccharide in both weeds was found to be cellulose admixed with protein and "rhamnan".

A "rhamnan" from the green seaweed Ulva lactuca was found to be a linear polysaccharide consisting of 1,4- (major), 1,3-linked and end group rhamnose, 1,4-linked xylose and 1,4-linked and end group glucuronic acid.

The "fucans" (polydisperse heteropolysaccharides) from the brown seaweeds Himanthalia lorea and Padina pavonia were found to consist of fucose, glucuronic acid, xylose and traces of galactose (H. lorea). The xylose and glucuronic acid were found to be 1,4-linked and end group while the fucose was found to be 1,3-linked with sulphate groups at C-2 or C-4 and 1,4-linked with sulphate groups at C-3. Indications were obtained for a linear fucose backbone for these "fucans".

The two brown seaweeds Desmarestia ligulata and Desmarestia firma were both found to have a high content of free sulphuric acid in the vacuolar sap. The free sulphuric acid in D. firma was found to be 6% of the dry weight of the weed. Consequently new methods to avoid degradation of the polysaccharides had to be devised. Both weeds were found to synthesise mannitol as their main ethanol soluble material though oligosaccharides containing xylose and galactose (D. firma) and xylose, galactose and mannose (D. ligulata) as well as myo-inositol (D. ligulata) and indications of glucose-mannitol di- and tri-mers (D. firma) were also found in this extract. Both weeds were found to synthesise laminaran, D. firma in comparatively

large amounts. The "fucans" extracted from the two species were found to contain fucose, xylose, galactose, and glucuronic acid and mannose was also found in the "fucans" from D. ligulata. The alginic acid from D. ligulata was found to contain 33% of mannuronic acid and the mannuronic acid in both alginic acids was found to be more easily degraded and hydrolysed than the guluronic acid.

The structural polysaccharide in D. ligulata was found to be cellulose.

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Chapter I

INTRODUCTION

Most of the vegetation in the sea belongs to the class known as algae. They are of the most primitive plant groups and morphologically they have changed very little with time. Although they also grow in soil and fresh water they occur mainly in salt water.

Algae are essentially photosynthetic organisms and they are therefore restricted to those parts of the sea where light is available. They have a great diversity of form, ranging from unicellular free floating organisms to gigantic seaweeds such as Macrocystis pyrifera which may reach a length of sixty metres.

The algae have mainly been classified according to the nature of their pigments, as this generally coincides with important morphological distinctions. This has resulted in four main classes of algae, namely brown (Phaeophyceae), green (Chlorophyceae), red (Rhodophyceae) and blue-green (Cyanophyceae). As more species are being studied chemically the presence or absence of a particular type of metabolite as the "marker" for a phylogenetic classification is being sought. This appears to be consistent with respect to the Phaeophyceae, the Rhodophyceae and to some extent to the Chlorophyceae, but the number of species investigated chemically is still small and hence classification of algae based at least on the nature of the particular carbohydrate may be regarded as tentative.

A characteristic feature of seaweed polysaccharides is the presence of half ester sulphate groups linked with at least one polysaccharide. Land plant polysaccharides are devoid of these groups but they are common to animal polysaccharides. The functions of these half ester sulphates is probably to produce polysaccharides which are hygroscopic and mucilaginous in nature and thereby giving seaweeds the

flexibility and ease of movement their environment requires. Another function is probably to prevent excessive dehydration when the plant is exposed to air at low tide.

Few chemical studies have been carried out on the carbohydrates from species of the blue-green algae and these will therefore not be discussed further.

The carbohydrates present in seaweeds can conveniently be divided into the following groups:

1 Low molecular weight carbohydrates (soluble in 80% ethanol).

2 Other mainly water soluble carbohydrates

a) Food reserve material

b) Other soluble polysaccharides.

3 Structural polysaccharides.

On the following pages the carbohydrates of the three main groups will be described under these headings.

Rhodophyceae

1. Low molecular weight carbohydrates

Floridoside, 2-O-glycerol-α-D-galactoside is found in many species of the Rhodophyceae[1] and it seems to be an end product of photosynthesis and a reserve material in these algae[2]. In some species 2-D-glyceric acid-α-D-mannopyranoside is more important[3]. In addition to these 3-O-floridoside-α-D-mannopyranoside[4] and isofloridoside(1-O-glycerol-α-D-galactopyranoside) have been isolated from several red algae[5] and various sugar alochols and inositols have also been found in some species.

2. Other mainly water soluble carbohydrates

a) Food reserve material

Floridean Starch

This reserve polysaccharide is universally present in the red algae. It consists solely of D-glucose units and a close relationship to amylopectin of land plants has been established[6]. It is essentially an α-1,4-linked glucan with 1,6-branch points and an average chain length of 9-15,[7] though a small proportion of α-1,3-linkages has been reported in some species[6,7].

b) Other soluble polysaccharides

The water soluble polysaccharides from the red algae are mainly galactans, consisting of varying proportions of D and L-galactose (some carrying half ester sulphate), 3,6-anhydro-D and L-galactose, and 6-O-methyl-D-galactose.

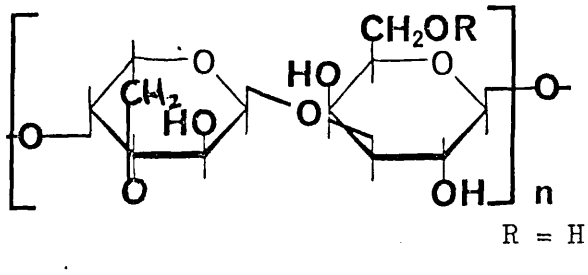
These units offer a broad spectrum of polysaccharides comprising chains of alternate 1,3- and 1,4-linked galactose or modified galactose units[8]. The individual polymers differ in their finer details of structure possibly due to a particular environment[8]. These polysaccharides occur in nature as hydrated viscoelastic gels probably serving both structural functions and also as anti-desiccants[9].

The galactans can be divided into three groups: agar-, porphyran- and carrageenan-type polysaccharides although polysaccharides related to more than one of these types have been found[10,11].

Agars

Comprehensive recent studies have indicated that agar is not made up of one neutral (agarose) and one charged polysaccharide (agaropectin) as was previously thought[12] but is composed of a complex series of related polysaccharides ranging from an almost neutral polysaccharide to a highly charged galactan. The extremes of structure can be defined as follows[13]

a) neutral agarose which consists of chains of alternating 1,4-linked 3,6-anhydro-α-L-galactose and 1,3-linked-β-D-galactose, see fig. 1.



R = H, CH_3

Figure 1

b) Pyruvylated agarose with a little sulphate. These molecules consist of the same alternating pattern with about one in twenty D-galactose residues substituted by pyruvic acid as 4,6-O-(1-carboxy-ethylidene)-D-galactose units and a few of the 1,4-linked residues present as L-galactose-6-sulphate (ca. 2% sulphate). These two residues are shown in fig. 2.

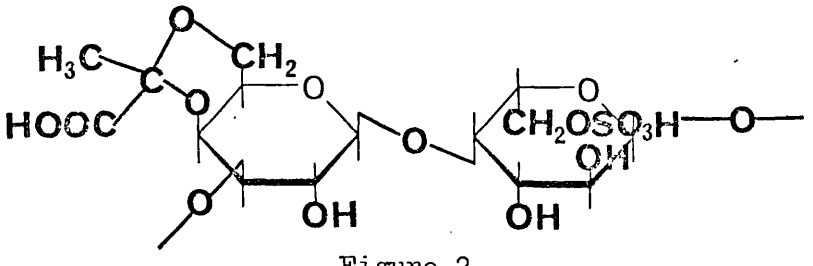


Figure 2

c) A highly sulphated galactan. These molecules contain little or no 3,6-anhydro galactose or pyruvic acid but consist of alternating 1,3- and 1,4-linked galactose residues mono- and di-sulphated.

All the intermediate forms between these extremes are thought to be present in agar.

Carrageenan

Carrageenan differs from agar mainly in that 3,6-anhydro-D-galactose replaces the 3,6 anhydro-L-galactose of agar and in that it has a higher content of mainly alkali stable half ester sulphates[8]. The repeating unit of K-carrageenan a fraction obtained from carrageenan by precipitation with potassium chloride, consists of chains of alternate 1,3-linked-D-galactose-4-sulphate and 1,4-linked-3,6-anhydro-D-galactose, see fig. 3.

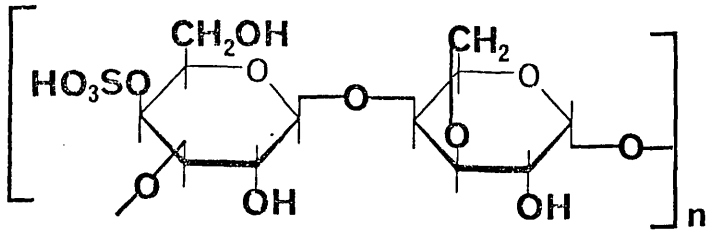


Figure 3

Porphyran

This polymer consists mainly of β-1,3-linked-D-galactose (or 6-O-methyl-galactose) units alternating with α-1,4-linked-L-galactose-6-sulphate (or 3,6-anhydro-L-galactose)[14]. The proportions of the sugars again varying with the season and the environment[15]. The mucilage obtained from Anatheca dentata (a member of the Grateloupiaceae) is one example of a polysaccharide not belonging to any of the three just mentioned groups. This polysaccharide has the "back-bone" structure of a type (c) agar, but the high sulphate content (ca. 30%) and the presence of xylose (and smaller amounts of 3-O-methyl-galactose and D-glucuronic acid) appear to place it outside the region of the true agars[16].

A recent investigation of the extracellular mucilage produced by Rhodella maculata[17], a microscopic unicellular red alga, showed that it comprised a sulphatedheteropolysaccharide containing mainly xylose and glucuronic acid together with smaller proportions of 3-O-methyl-xylose, rhamnose, galactose and glucose. The xylose units are 1,3- and 1,4-linked, and glucuronic acid is 1,3-linked. Rhamnose is present as end-groups together with 1,3- and 1,4-linked galactose and 1,2- and 1,4-linked glucose, while branch points are occupied by 1,3,4- and 1,2,4-linked galactose and/or glucose units.

Though water soluble xylans and mannans have been obtained these will be discussed under the structural heading.

3. Structural polysaccharides

Cellulose

Cellulose in small proportions, has been reported in different species of red algae[18] and the structure of the cellulose from Gelidium amansii was found to be similar to that of cotton except for its colloidal properties[19].

Mannan

A β-1,4-linked mannan with an average chain length of about twelve has been extracted from the cell wall of Porphyra umbilicalis[20], while a sulphated α-1,3-linked mannan was found in the aqueous extract of a Nemalion vermiculare Sur sample[21].

Xylan

The xylans found in the red seaweeds are either cell wall material or water soluble. The first type is essentially linear and consists of either β-1,3-linked[20] or β-1,4-linked[22] xylose residues. The water soluble types are not skeletal material and are possibly food reserve polysaccharides. They consist of chains of both β-1,3- and β-1,4-xylosidic linkages in a structure that may be branched or linear and they may either be pure xylans or heteropolysaccharides[22].

Chlorophyceae

1. Low molecular weight carbohydrates

Of the three main classes of algae the green seaweeds have been the least investigated, but the main low molecular weight carbohydrate seems to be sucrose[23]. Sucrose, glucose and fructose have been found in most of the species investigated. In addition small quantities of myo-inositol and other polyalcohols as well as other

sugars have been found in some species[24]. In Acetabularia crenulata[25] for instance allulose, myo-inositol, allo-quercitol as well as D-glucose, D-fructose and a homologous series of fructose containing oligosaccharides were found.

2. Other mainly water soluble carbohydrates

a) Food reserve material

Starch

Most of the species so far investigated chemically were found to have starch-type glucans similar to that of land plants comprising both amylose and amylopectin[26]. Aqueous extraction yields these glucans together with sulphated heteropolysaccharides. They can be separated as starch-iodine complexes or as glucans left in the aqueous solution after complexing the sulphated polysaccharides with cetyltrimethylammonium hydroxide. The structures of the glucans have been established through enzymic, periodate oxidation, methylation and X-ray studies[26]. The main differences between the starches from the green seaweeds and that from land plants seem to be that the former has a lower molecular weight and hydrolyses more easily than the latter[27].

Fructans

Fructans have been found in various green algae and they are thought to be the food reserve material[28]. In Acetabularia crenulata[25] the fructan was shown to be of the inulin type, i.e. a linear 2,3-linked polysaccharide with average molecular size varying from 33 to 62 units.

b) Other soluble polysaccharides

As in the case of the Rhodophyceae, characteristic mucilaginous polysaccharides constitute the major polysaccharides of this class of algae. They can roughly be divided into two groups depending on their sugar units.

The first group contains mainly galactose, arabinose and xylose units and has been obtained from species of Cladophora[29], Chaetomorpha[30], Caulerpa[31], Codium[32] and Rhizoclonium[33]. The second group comprises mainly rhamnose, xylose and glucuronic acid and has been obtained from Enteromorpha compressa[34], Ulva lactuca[35], Acrosiphonia arcta[36] and Urospora penicilliformis[37]. The polysaccharide from Acetabularia crenulata[25] on the other hand is of an intermediate structure as it contains galactose, xylose, rhamnose and glucuronic acid as the main components.

The half ester sulphate content of the polysaccharides belonging to the first group, is found to be fairly constant (15-20%) and galactose and arabinose are the major sugars in most samples. The majority of the polysaccharides contain appreciable amounts of xylose, and traces of rhamnose have been found. In spite of the number of different sugars present all attempts to fractionate the polysaccharides into homo-polysaccharides have been unsuccessful.

Cladophora rupestris[29] was the first alga in this group to be studied in detail. These studies established the major linkages, galactose 1,3- and 1,6-linked, arabinose 1,4-linked and xylose end group and 1,4-linked. Later studies confirmed these findings and showed that some of the arabinose units are sulphated at C-3 and some of the galactose residues are sulphated at C-6[30]. Autohydrolysis of this water soluble sulphated polysaccharide yielded a number of hetero-oligosaccharides containing galactose and xylose, arabinose

and galactose and a large quantity of a sulphated oligosaccharide with a high proportion of arabinose to galactose (4:1)[38]. These results proved not only the heteropolymeric nature of the mucilage but also the wide diversity of the structural units present. From these and related studies it was possible to deduce that the polysaccharide contains blocks of at least eight 1,4-linked arabinose units, some sulphated at C-3 and linked together by single galactose units.

Studies on the water soluble starch free polysaccharides from two Chaetomorpha species[30] indicates very similar structures to that found in Cladophora, the major difference being a higher galactose content in the latter.

The polysaccharide from Codium fragile[32] contains galactose and arabinose as the major sugars but it appears to differ from the above mentioned polysaccharides on the siting of the sulphate groups. No arabinose sulphate was found in the Codium polysaccharide though galactose-6-sulphate and galactose-4-sulphate were both isolated together with a 1,3-linked galactose dimer and a 1,3-linked arabinose dimer.

As studies of Urospora comprise a part of this thesis they will be discussed in more detail in the Chapter III (p. 44). The water soluble polysaccharides obtained from the other species mentioned in the second group contain the same sugars and have many similar properties. The polysaccharides were found to be heteropolymers with L-rhamnose and D-xylose as the major sugars and smaller quantities of D-glucose. The uronic acid content was found to be around 20% while the sulphate content varied, about 17% in Ulva[35] and Enteromorpha[34] and 8% in Acrosiphonia[36].

Partial acid hydrolysates from all three polysaccharides gave a relatively high proportion of an aldobiouronic acid, 3-O-β-D-glucopyruronosyl-L-rhamnose.

The polysaccharide from Ulva lactuca has been examined in greatest detail and the following results have been obtained. Methylation studies[39] of the desulphated and carboxyl reduced polymer has indicated 1,4- and 1,3,4-linked rhamnose, 1,4-linked and end group xylose and 1,3-linked glucose/glucuronic acid. Tentative evidence for 1,3-linked and 1,4-linked glucose was also obtained.

The position of the sulphate ester has been deduced from alkali desulphation and from periodate oxidation experiments to be mainly at C-2 of rhamnose and to a lesser extent at C-2 of xylose[40].

An acidic heptasaccharide was isolated in high yield after Smith degradation and from this it was deduced that the following structure represents a repeating unit in the polysaccharide[39].

$$\text{GlcA}(1\rightarrow 4)\underset{2S}{\text{Rha}}(1\rightarrow 4)\text{GlcA}(1\rightarrow 3)\underset{2S}{\text{Xyl}}(1\rightarrow 4)\underset{2S}{\text{Rha}}(1\rightarrow 3)\underset{6S}{\text{Glc}}(1\rightarrow 4)\text{Xyl}$$

Although the investigations on the mucilage from Ulva lactuca have advanced much further than those on the polysaccharides from the other two species it is believed that they have a similar structure.

The polysaccharide from the unicellular alga Acetabularia crenulata[25] was found to belong to neither of the groups so far discussed as the main sugar in this polymer was found to be galactose with lesser amounts of glucuronic acid and rhamnose, and still smaller amounts of xylose and 4-O-methyl-galactose present. This alga also seems to metabolise a family of related polysaccharides all built on a similar plan. The main features of these polymers were found to be 1,3-linked galactose units sulphated mainly on C-4 and to a lesser extent on C-6, rhamnose is mainly present as end groups and 1,2-linked or 2-sulphated units, while xylose, glucuronic acid and galactose all were found to be present as end groups. The branch points were found

to consist of galactose and rhamnose units. Due to the evidence for a relative high proportion of end groups, the polysaccharide is thought to be highly branched with very short branches.

3. Structural polysaccharides

Mannan

Mannans have been shown to be present in the cell wall of certain green algae[41]. The mannans from Codium fragile[42] and Acetabularia crenulata[25] have been extensively studied and found to be β-1,4-linked mannans with indication of some degree of branching at C-2.

Xylan

The xylans constitute the main skeletal polysaccharide of a number of green algae. Methylation, periodate oxidation and enzymatic studies showed these xylans to be composed of β-1,3-linked D-xylose units[41,43]. These linkages result in an open wire spring like helical structure. The strong fibrous structure required by a skeletal polysaccharide is achieved by three chains forming a triple helix[44].

Glucan

X-ray diffraction studies[45] have suggested that cellulose is present in various families of the Chlorophyceae but due to difficulties in isolating the pure cellulose no structural studies have been carried out.

Phaeophyceae

1. Low molecular weight carbohydrates

D-mannitol appears to be universally present in the brown algae, in some species and at some seasons in large quantities[46]. Mannitol is believed to be a storage product and also a substrate for respiration in these plants[47]. The seven carbon atom alcohol, D-volemitol has been found in Pelvetia canaliculata[48], and 1-O-D mannitol-β-D-glucopyranoside and 1,6-O-D-mannitol-di-(β-D-glucopyranoside) have been reported in several brown algae[49]. A C-methyl-inositol, laminitol, has been found in Laminaria species and trace quantities of sucrose, galactose and mannose have been reported in Cladostephus species[50].

2. Other mainly water soluble carbohydrates

a) Food reserve material

Laminaran

Laminaran, a β-1,3-linked glucan is present in most brown algae[51]. Its proportion of the dry weight varies both with the species and with the season.

Laminaran occurs in two forms distinguished by their solubility in cold water. They are therefore referred to as "soluble" and "insoluble" laminaran although both forms dissolve in hot water. The main difference between the two forms has been found to be the presence of branch points, the "soluble" polymer appears to contain more 1,3,6-linked glucose units than the "insoluble"[52]. Laminarans have been found to have a fairly low molecular weight (normally in the region of 4-5000), and evidence for 1,6-linked glucose within the chains has been reported[52].

Complete hydrolysis of laminaran gives glucose as the sole sugar. With better separation and monitoring techniques mannitol was found to be a constituent of the laminaran from some species[53]. Further studies showed that these mannitol residues terminate some of the polymer chains through a glycosidic linkage to one of mannitol's primary hydroxyl groups. The molecules of laminaran terminated by mannitol residues are called M-chains, while the remaining molecules terminated by reducing glucose residues are called G-chains. By determining the yield of formaldehyde liberated by periodate oxidation of the laminaran before and after reduction the M-chain and G-chain content can be calculated[54].

It seems that laminaran is not a single molecular species but that the name covers a whole range of essentially linear β-1,3-linked glucans, in some of which the reducing end is terminated by a mannitol residue while others have reducing glucose units. Laminaran from some species is a mixture of these two types while other species are devoid of mannitol. Furthermore the presence of C-6-linkages within the chains and C-6 branch points seems to determine the solubility of the polysaccharide in cold water.

b) Other soluble polysaccharides

Fucoidan

Fucoidan like alginic acid is one of the principal polysaccharides of brown algae. Hydrolysis of fucoidan gives mainly fucose, but galactose, xylose and uronic acids have also been found. A fucoidan free from any other sugar units has so far never been isolated. In a highly purified sample from Himanthalia lorea[55] for instance, the fucose content was found to be 57%, sulphate 38%, galactose 4%, xylose 1.5% and uronic acid ca. 3%.

Alkali treatment of a fucoidan from Fucus vesiculosus[56] (fucose 38% and sulphate 33%) indicated that about 10% of the ester sulphate groups were alkali labile, thus showing that only a small proportion of the sulphate groups were linked to C-2 or C-3 of a 1,4-linked fucose residue. It shows that the main bulk of the L-fucopyranose units cannot be 1,4-linked as this would render sulphate groups alkali labile. Methylation studies of the material indicated that the main structural feature of this polysaccharide is 1,2-linked fucose units mono-sulphated at C-4 (mainly) but also disulphated at C-3 and C-4.

Later work on fucoidan confirmed this structure but at the same time indicated that the other sugar residues present might constitute an integral part of the fucoidan macromolecule. The heterogeneity of the fucoidan has been shown by free boundary electrophoresis on the material extracted from Fucus vesiculosus[57] and from Ascophyllum nodosum[58], two bands were observed in the former case and the latter showed three components.

Extracts from Ascophyllum nodosum[58] and other brown algae suggested that the amount of other sugar residues present in the fucoidans not only varied from species to species but also from sample to sample in the same species depending on the method of extraction or fractionation.

Dilute sodium hydroxide treatment of Ascophyllum nodosum[58] after removal of the dilute acid soluble polysaccharides, gave a mixture of alginic acid and glucuronoxylofucans.

Glucuronoxylofucans

These polysaccharides contain varying proportions of fucose, xylose, fairly high amounts of glucuronic acid and half ester sulphate.

The alginic acid in the above mentioned experiment was removed by

pH adjustments, and the glucuronoxylofucans were recovered by fractional precipitation of the supernatant. The major fraction, called ascophyllan, contained approximately 25% fucose, 26% xylose, 19% uronic acid, 13% sulphate and 12% protein. Two smaller equal fractions F_1 and F_2 differ from ascophyllan only in the relative proportion of the constituents, all three fractions gave similar chromatographic patterns on hydrolysis although variation in the intensity of various products was apparent.

Attempts to fractionate the polypeptide from the carbohydrate in ascophyllan were unsuccessful and this suggested a chemical linkage between the two moieties. Mild hydrolysis of ascophyllan with 0.5M oxalic acid cleaved peptide as well as glycosidic linkages of the polysaccharide giving a mixture of dialysable mono- and oligo-saccharides and non dialysable degraded polysaccharide. The latter contained almost all the uronic acid present in the polysaccharide but was almost devoid of fucose, xylose and half ester sulphate. These results strongly indicated that ascophyllan is comprised of a glucuronic acid back-bone to which relative long side chains of sulphated fucose and xylose residues are attached.

Extraction of Ascophyllam nodosum[59] with ammonium oxalate/oxalic acid at pH 2.8 (after acid and alkali extraction) gave a mixture of alginic acid and a glucuronoxylofucan containing 49% fucose, 10% xylose, 12% uronic acid, 21% sulphate and 4% protein. After removing the alginic acid as the insoluble calcium salt, fractionation of the glucuronoxylofucan proved impossible. The polysaccharide was auto-hydrolysed in a dialysis tube and the degraded polymer was found to contain fucose, xylose and glucuronic acid in the molar proportions of 3.5:1:2.5. It was therefore concluded that this polysaccharide

does not have a glucuronic acid back-bone. A major oligosaccharide present in a partial acid hydrolysate from this polysaccharide was characterised as 3-O-(β-D-glucopyranosyl)-L-fucose.

Recent studies on Himanthalia lorea, Bifurcaria bifurcata and Padina pavonia[60] in this laboratory indicate as do previous experiments that the glucuronoxylofucans comprise a whole family of polysaccharides. Fractionation of different extracts on DE-cellulose columns led to the isolation of highly sulphated materials having a high content of fucose, polymers having a high content of glucuronic acid and low content of fucose and sulphate, and polysaccharides with proportions of sugars and sulphate between the two extremes. In future fucoidans and glucuronoxylofucans are called "fucans".

Alginic acid

Alginic acid, a β-1,4-linked polyuronic acid, is located mainly in the middle lamella and in the primary cell wall of most brown algae[61,62]. It consists of from 14-40 per cent of the weeds' dry weight depending on the species and the season, the content seems to be smaller when the algae undergo rapid growth. The alginate is believed to act as a cation exchangerand as such it quickly comes into equilibrium with the salts in the surrounding sea water[63]. The alginate is present as the salt of a mixture of cations, but the calcium content is high enough to keep it insolbule. Alginates form gels which, unlike the agars, are not formed or melted by temperature changes but by a change in the counter-ion. Setting is induced by the addition of Ca^{2+}-ions whereas removal of these ions or replacement with an alkali-metal ion such as Na^{+} "melts" the gel. Alginic acid is normally extracted with dilute sodium carbonate and purified by precipitation either in alcohol or as the calcium salt. Although the presence of D-mannuronic acid and its lactone[64] and the fact that

alginic acid was found to have an uronic acid content of 100%[65] was established nearly 50 years ago, it was not till comparatively recently that the structure was more fully understood[66]. This was mainly due to the alginates marked resistance to hydrolysis under conditions that do not destroy the products and the fact that methylation techniques were difficult to use due to the presence of the carboxyl groups.

It was discovered in 1955 that the D-mannuronic acid 5-epimer L-guluronic acid, was a second component of alginic acid[67]. Periodate and bromine oxidation of alginic acid followed by hydrolysis gave both threaric and erythraric acid, confirming that both uronic acid residues are 1,4-linked. That both acids were present in a single molecule was shown by partial hydrolysis of a sample in which the uronic acids had been reduced to the corresponding sugars and the isolation of mannosylgulose[68]. Information about the sequence of uronic acid residues in the alginate molecule was obtained by heterogeneous hydrolysis. Treatment of sodium alginate from *Laminaria digitata*[69] with M-oxalic acid for 10 hours at 100°C hydrolysed part of the polysaccharide to oligosaccharides leaving an insoluble residue. The hydrolysis was repeated and the derived residue was hydrolysed a third time. From the fact that the first hydrolysis gave a more rapid depolymerisation than the other two it was concluded that the molecule was made up of parts with different structures. The final resistant polymer was separated into a soluble and an insoluble fraction by adjusting the pH to 2.85 in dilute sodium chloride solution. Both fractions had DP values of about 20, but the soluble fraction was made up almost entirely of mannuronic and the insoluble fraction of guluronic acid residues. The first hydrolysate contained both mannuronic and guluronic residues together

with diuronides. The major diuronides of the first ten-hour period was thought to be a mannuronic-guluronic acid dimer while the major diuronides of the last two periods were thought to be composed of dimannuronic and diguluronic acid residues.

The conclusion drawn from this experiment was that the alginic acid is built up of blocks of mannuronic and guluronic acid units, separated by sections of alternating guluronic and mannuronic acid residues. The proportion of the two components depend on the species and on the region of the plant from which the alginate is extracted as well as the season. X-ray work[66] 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 chains which then aggregate and become junctions in the network. The mannuronan or alternating segments cannot participate in this type of complex and constitute the flexible part of the network. This observation correlates with the biological occurrence of these polymers. The mannuronan content of alginates is higher in the tissues undergoing growth and expansion whereas the support tissues at the plant base have a greater proportion of the rigid guluronan[70].

3. Structural polysaccharides

Cellulose

A small proportion of cellulose seems to be a general structural polysaccharide in the brown algae. Evidence for β-1,4-linked glucose units has been obtained and periodate studies excluded the presence of

1,3- and 1,6-linkages[71]. From the same experiments a chain length of about 160 was found. X-ray diffraction studies[72] gave the same pattern as that of normal cellulose which shows the cellulose chains to form a stack of parallel chains. The stacks associate to form microfibrils by hydrophobic contacts and intermolecular hydrogen bonding.

G.l.c. Mass spectrometry as used in the Identification of Methylated Sugar Derivatives

The routine method for analysing the alditol acetates of partially methylated sugars is by g.l.c. - m.s.[73]. The method of ionisation normally used is the electron impact (EI) process which is the method used throughout most of the present work. In this process an electron beam is interacting with the organic molecules at low pressure. The electron beam has an electron energy which is sufficient to cause ionisation of the molecule and, in many cases, degradation to smaller fragment ions. Because of this degradation the molecular ions can seldom be observed in EI spectra of partially methylated alditol acetates. This problem can be overcome by using a different ionisation method; chemical ionisation (CI). CI mass spectra results from the ion-molecule reaction that occurs between a low pressure sample gas and the primary ions of a high pressure reactant gas. Typical pressures are 10^{-6} and 0.3-3 Torr respectively. Both gases are introduced into the ion chamber where they are bombarded by an electron beam, but because of the very low abundance of the sample, initially all primary ionization occurs to the reactant gas. The ionised reactant gas undergoes ion-molecule reactions with itself to form a steady-state plasma which

in turn reacts chemically with the dilute sample gases. The process results in fragment and product ions characteristic of the unknown sample. In the experiments described in this thesis the reactant gas is isobutane and the ion-molecule reaction results in the transfer of a proton to the sugar derivatives. In the CI mass spectra of these compounds the molecular weight plus a proton ion (M+1) and not the molecular weight ion (M) will therefore be observed. As an example the mass spectrum of the alditol acetate of 2,3,4-tri-O-methylxylose is shown in fig. 4. Spectrum (a) is recorded by the EI and spectrum (b) by the CI method.

While mass/charge i.e. m/e = 161 is the highest recorded mass ion in the EI spectrum, m/e = 279, the M+1 ion, can be observed in the CI spectrum.

The breakdown pattern of the two spectra can be explained as follows:

The EI spectrum.- It is a well known fact that the primary fragments from alditol acetates are formed by α-cleavage, resulting in fission between the carbon atoms in the alditol chain and that the intensities of the primary fragments decrease with increasing molecular weight. Fission between an acetoxylated and a methoxylated carbon atom occurs rather than fission between two acetoxylated carbon atoms and the methoxylated carbon atom carries the positive charge. Thus, fission (a) and (b) in fig. 5 are more important than fission (c) and (d).

Mass spectra of 1,5-di-O-acetyl-2,3,4-tri-O-methyl xylitol

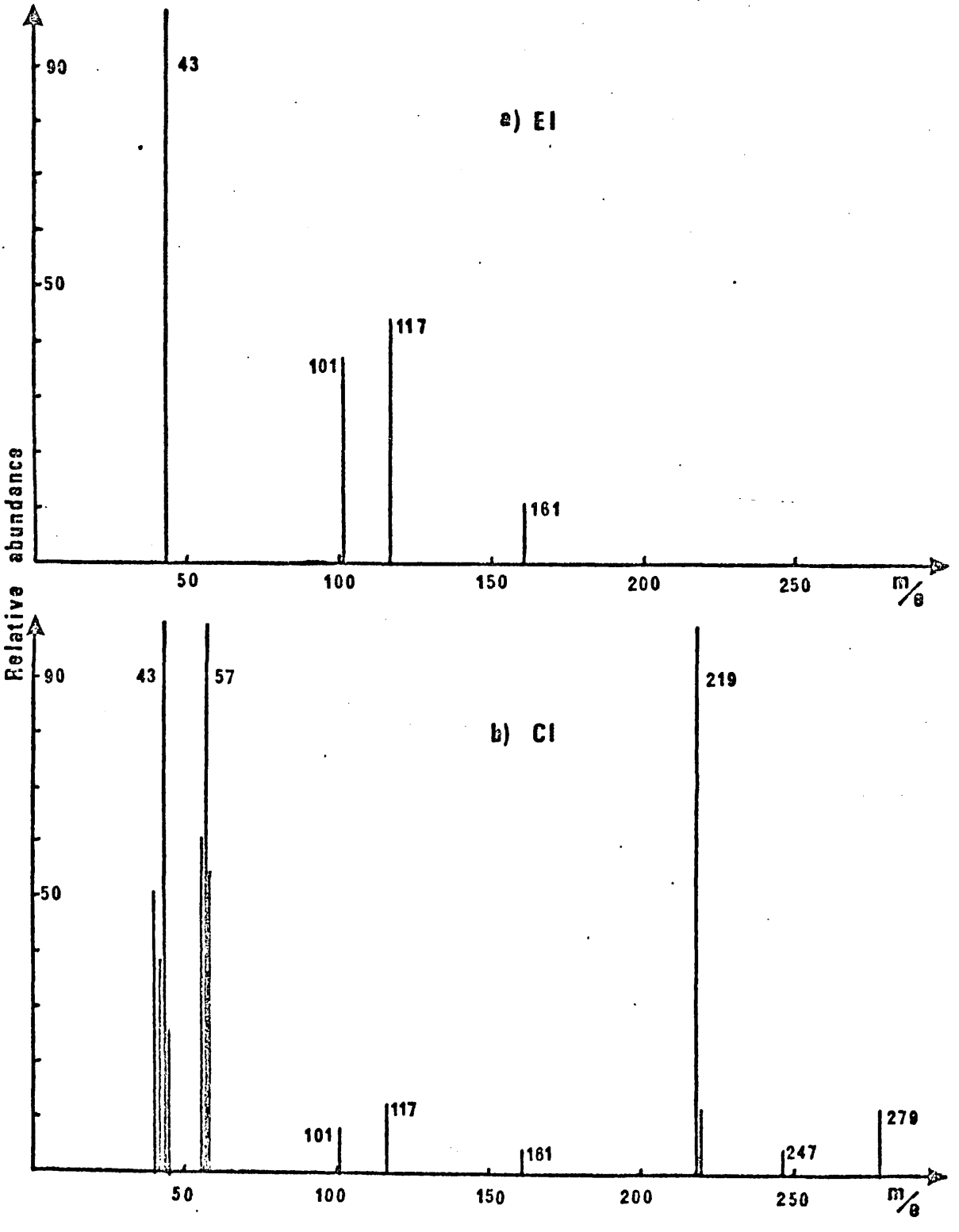


Figure 4

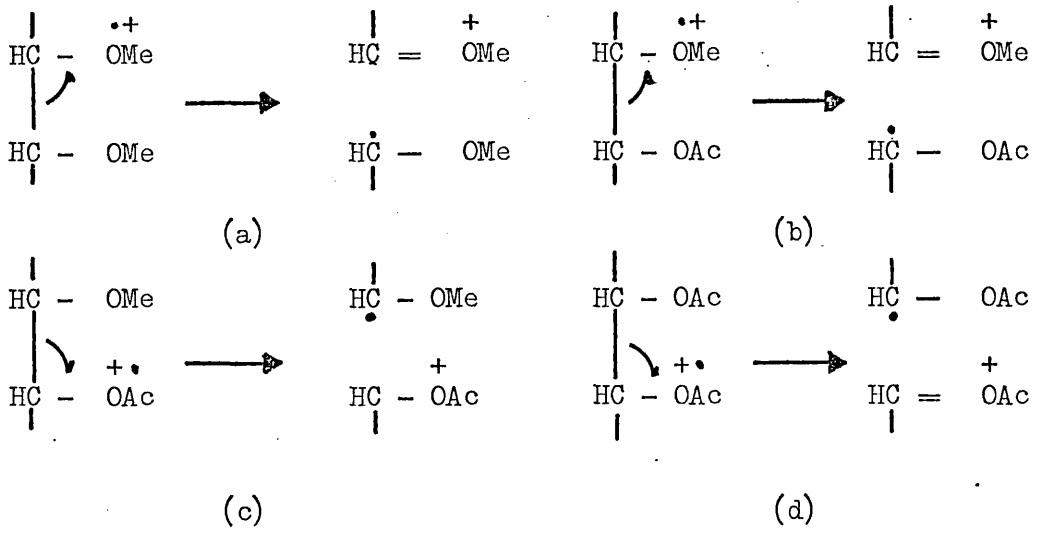
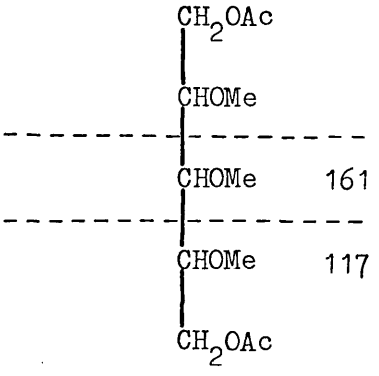
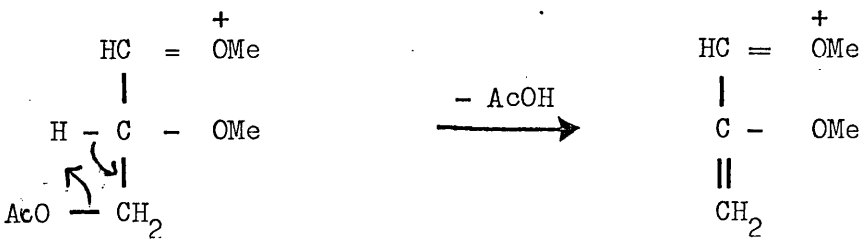


Figure 5

As the methoxylated radical formed in (a) has been shown to be more stable than the acetoxylated radical formed in (b) the former fission is favoured. This is well illustrated in the present example, the primary fragmentations observed in the EI spectrum are shown in the formula below



This means that only the fissions between methoxylated carbon atoms can be observed in this spectrum. The ion m/e = 101 is formed by loss of acetic acid in a secondary fragmentation as shown below



m/e = 161 m/e = 101

The base peak in the spectrum as in all alditol acetate spectra is the acetylium ion m/e = 43 ($CH_3C \overset{+}{\cdots} O$).

The CI Spectrum.- The main fragmentation pattern in this example is the loss of acetic acid from the M + 1 ion, followed by the loss of another acetic acid molecule as shown in fig. 6.

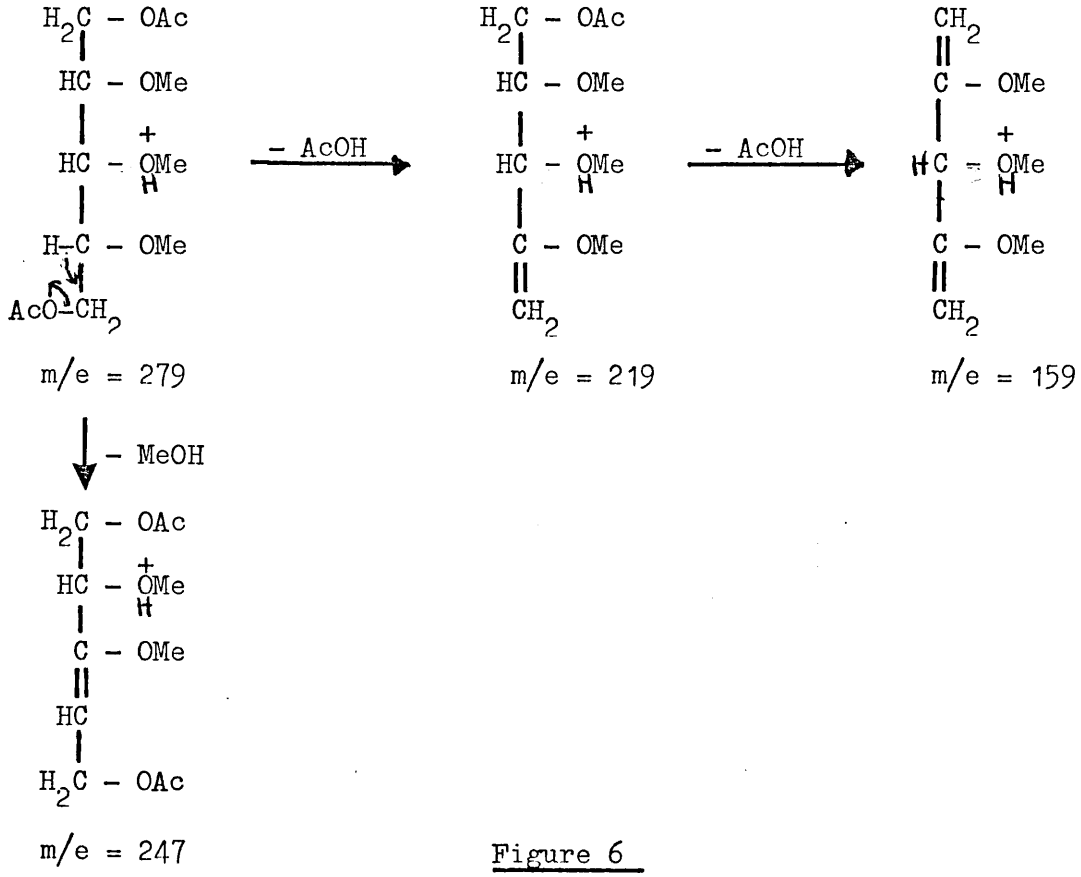


Figure 6

Other fragmentation patterns are the loss of methanol from the M + 1 ion, as well as those observed in the EI spectrum. The ions present in greatest quantity m/e = 43 and 57, are mainly due to the isobutane gas.

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Chapter II

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 (0.45μ) 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) 72% Sulphuric Acid[1]

Cold 72% sulphuric acid was added to the sample and the mixture left at room temperature for one hour. Water (10 vols) was added with cooling and the solution heated at 100°C for 4 hours. 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[2]

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[3]

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[4]

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.

IV. Staining Reagents

(i) Silver nitrate dip.[5]

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) (a) Aniline oxalate spray[6]

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

(b) Aniline oxalate spray[7]

Aniline oxalate (25 g) in glacial acetic acid (1L).

(iii) Heptose Spray[8]

The paper is sprayed successively with the following:-

(a) 0.03M sodium periodate in pH 3.6 0.2M acetate buffer.

(b) after 1 min, 1% aqueous inositol.

(c) ammonium acetate (15 g) acetic acid (0.2 ml) acetylacetone (1 ml)

in methanol (50 ml).

A yellow colour develops after 30 minutes at room temperature.

(iv) Glucose oxidase[9]

'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[9]

'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[10]

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.

(vii) Ninhydrin

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

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

A. Instrumentation

(i) A Pye Argon gas chromatograph with an Argon ionisation detector, and dry argon as carrier gas was used for the methylated methylglycosides. Glass columns (1 m x 5 mm) (i) and (ii).

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

(iii) A Perkin-Elmer F11 gas chromatograph with flame ionisation detector and helium gas with a glass column (4 m x 1.6 mm) (Column v).

B. The columns were packed with the following materials

(i) Butane 1,4 diolsuccinate polyester, 15% on silane treated Celite.

(ii) Polyphenyl ether (P.P.E.) m-bis(m-phenoxy)-benzene 10% on silane-treated Celite.

(iii) Apiezon K, 7.5% on silane treated chromosorb W.

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

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

C. Gas Chromatograpy linked to Mass spectrometry

Two different instruments have been used during these investigations.

(i) The F11 gas chromatograph was coupled via an all glass system through a Watson-Biemann separator to a Hitachi RMS-4 mass spectrometer. Mass spectra of partially methylated alditol acetates were obtained by operating the 'ion source' at 230°C, 50 eV and 80 μa target current.

(ii) 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. For CI spectra the 'ion source' was operated at about 150°C, 50 eV and 1000 μA under a pressure of 0.4 - 0.5 torr with isobutane as the reactant gas.

VI. Assays and Analyses

(i) Carbohydrate Content was assayed by the phenol sulphuric acid method[11]. 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 was carried out by three different methods.

(a) The modified carbazole reaction[12]

A saturated aqueous solution (1 ml) of benzoic acid, containing 4–40 μg uronic acid was layered onto concentrated sulphuric acid (5 ml) containing 0.025 M sodium tetraborate in an ice bath. Keeping the temperature below 4°C the two layers were mixed. The mixture was warmed to room temperature and then heated in a boiling water bath for 20 minutes. After cooling 0.125% carbazole in analar methanol (200 μl) was added and the tubes heated at 100°C for a further 15 minutes. The colour which developed was read at 530 nm on a Unicam SP500. Standard graphs for different acids were prepared.

(b) A second modified carbazole reaction[13]

This method was used for determining the composition of uronic acid mixtures. The samples (containing 10–70 μg of uronic acid in 1 ml, the test solution) were reacted at 55° and 100°C with and without borate solutions.

(1) Reaction without borate (at 55° and at 100°).

The test solution (1 ml) was cooled in ice. Concentrated sulphuric acid (6 ml) was added and the solution re-cooled. After mixing the sample was heated for 20 min at the appropriate temperature and again cooled in ice. After addition of the carbazole solution (200 μl 0.1% solution in ethanol) the solutions were separately mixed and allowed to stand for 3 h for colour development.

(2) Reaction with borate at 100°C.

A 0.1M-boric acid solution in concentrated sulphuric acid (6 ml) was cooled in ice and thereafter the test solution (0.7 ml) was layered on top. After mixing and re-cooling the solution was heated at 100°C for 15 min and then cooled in ice. Thereafter the carbazole reagent

(200 μl) was added. The solution mixed and heated for another 10 min and cooled.

(3) Reaction with borate at 55°C.

The same procedure as above was used except heating before the addition of the carbazole reagent was omitted and afterwards the solution was heated for 30 min at 55°C. The colours which developed were read at 530 nm on a Unicam SP500. Standard graphs for different acids under each of the conditions were prepared.

(c) The meta-hydroxydiphenyl method[14]

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 Lethan method[15] 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 over 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 [16].

(v) Molar proportions of sugars were estimated from the peak areas on g.l.c. of the TMS derivatives of derived alditols or methylated alditol acetates. Standard graphs were prepared to check the response of different sugars to the g.l.c. detection system.

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[17]. 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 (~2N) was determined by titration with 0.1N-HCl.

(iv) Methyl glycosides 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 6 h. 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[18]. 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 (iii)).

(vii) Alditol acetates[19]. 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 (iv) and (v)].

(viii) Methylation. The Hakomori method[20] (modified by Bjorndal and Lindberg[21]) 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[22]. An aqueous solution of polysaccharide was added to an equal volume of sodium metaperiodate (0.03N). Aliquots (0.1 - 1 ml) were withdrawn at intervals and diluted 250 times 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. The product was reduced by sodium borohydride.

(x) Reduction of uronic acids[23]. A modified Taylor and Conrad method was used.

The polysaccharide (40 mg, 25 μeq /carboxyl groups) was dissolved in 50 ml of water and ethyl-3-dimethyl aminopropyl carbodiimide HCl (25 mg, 0.25 mmol) 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 (2l). 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 (2l) 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.

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CHAPTER III

The carbohydrates synthesized by the green seaweeds Urospora wormskioldii and Codiolum pusillum

INTRODUCTION

The genus Urospora (once known as Hormiscia or Conferva) is placed in the order Acrosiphoneaceae with the closely related genera Spongomorpha and Acrosiphonia[1]. These three genera were originally placed in the Cladophoraceae, but unlike the rest of the Cladophoraceae studied until then, it was established that they possess a unicellular "Codiolum" stage in their life history. It was on the basis of this and other criteria that the new family, the Acrosiphoneaceae, was founded. The discovery of a "Codiolum"-like stage in the life-cycle of a culture of Cladophora rupestris[2] has now thrown doubt on the validity of this distinction. However, that a fundamental division does in fact exist is suggested by the different constituents of the sulphated mucilages metabolised by the Cladophora and Acrosiphonia sections of the old Cladophorales, since the former synthesises an arabinogalactan (p.9) while the latter synthesises a complex glucuronoxylorhamnan as does Urospora[3]. A survey of nine members of the Cladophoraceae and Urospora species revealed that only two, Chaetomorpha and Urospora species failed to produce fructosides other than sucrose, further emphasises the above mentioned distinction[4].

Cultivation has shown a striking temperature influence in the case of Urospora wormskioldii[5]. In this species, unbranched monosiphonous filaments, up to 15 cm in length, microscopic dwarf plants and a unicellular codiolum stage, can be found depending on conditions of growth. The filamentous U. wormskioldii survives only at low temperatures near 5°C. Its four-flagellated zoospores, which are

elongated into a point at the posterior end, reproduce the mother plant at low temperature. They give rise to dwarf plants, however, in cultures at middle and higher temperatures (10° or 15°C). Dwarf plants also propagate abundantly by 4-ciliated zoospores, growing into dwarf plants again under the same conditions. However, the same zoospores develop into filamentous plants at 5°C. Additional biflagellate zoospores are produced only in the higher range of temperature; they lack sexuality and grow into small unicellular stalked plantlets. At 15°C this Codiolum stage does not become fertile, zoospores with 4 flagellae are produced only at middle or low temperatures respectively. Depending upon the conditions of the culture experiments, the zoospores of Codiolum develop into Urospora-filaments or into dwarf plants. These results permit interpretation of aspects of the seasonal rhythm of Urospora wormskioldii, i.e. the presence of filaments in March to May, and of fertile Codiolum in late summer to autumn.

The two common British Urospora species are U. bangioides and U. penicilliformis and they are both smaller than the arctic U. wormskioldii whose cells are normally up to 4 mm across and 15 cm in length[6]. U. penicilliformis has recently been investigated chemically; some of the results are described below.

A homologous series of maltooligosaccharides, sucrose, glucose, fructose, myoinositol and glyceric acid were found in the 80% ethanolic extract. In several other genera of the Chlorophyceae the presence of glucose, fructose and sucrose has been reported and myoinositol has been found in Acetabularia crenulata (p.8). In contrast the glyceric acid and the homologous series of maltosaccharides appears to be unique to this alga.

The following percentage proportions of sugars was found in the water soluble polysaccharide from U. penicilliformis, rhamnose 26, xylose 35, mannose and glucose 22, glucuronic acid 17, galactose trace. This water soluble polysaccharide was fractionated on a DE52-cellulose column by elution with water followed by graded elution with increasing concentration of KCl solutions. A glucan and a mannan were eluted in the aqueous fraction. The glucan was shown to be of the amylose type, giving a blue colour with iodine. Methylation, periodate oxidation and enzymic studies confirmed the presence of an α-1,4-linked linear glucan. The mannan was found to be an α-1,3-linked polymer with evidence of a small degree of branching at C-6. This is the first example of an α-1,3-linked mannan to be found either in algae or the higher plants.

A glucuronoxylorhamnan found in the 0.8M- and 1.0M-KCl fractions gave a molar ratio of rhamnose:xylose:glucuronic acid of about 5:7:2 with ester sulphate ranging from 17-22% in the different fractions. Ultracentrifugation and gel electrophoresis experiments indicated that the glucuronoxylorhamnan is a single polydisperse polysaccharide.

Desulphation, periodate oxidation and methylation studies indicated an essentially linear molecule with a small degree of branching at C-2 of xylose. The major structural feature was found to be 1,3-linked rhamnose usually sulphated at C-2 and sometimes also at C-4,1,4-linked and end group xylose and 1,4-linked and end group glucuronic acid.

Alkali extraction of the alga yielded a β-1,4-linked glucan, thought to be a degraded cellulose. The residue also contains a cellulose-type glucan.

The Urospora wormskioldii and Codiolum pusillum investigated in the present work were kindly collected by Mr R Hooper of the Memorial University of Newfoundland. The U. wormskioldii was harvested in April 1973. Two samples were supplied. A very pure sample, 1.5 g after air drying. A second sample slightly contaminated with diatoms and Monostroma, total weight after air drying 3.4 g. The Codiolum pusillum was harvested from Bulls Bay, south St. John's Newfoundland on 22nd August 1973. Total weight after air drying 4.75 g.

Urospora wormskioldii

I Extraction procedure

The alga was plunged into absolute ethanol immediately after collection and the resulting dark green extract was combined with the ethanol extracts from the present experiments. The residual weedwas air dried and weighed. This weight is later referred to as the dry weight of the weed, though slightly incorrect since a small amount of material had been extracted into the absolute ethanol.

The dry alga (872 mg of the pure sample) was ground to a fine powder under liquid nitrogen and the extraction procedure illustrated in flow chart I was followed.

Experiment 1 Ethanolic extraction

The green powder was extracted twice with 80% ethanol under the conditions given in the flow chart. After each extraction the solid was centrifuged off. The last extract was virtually colourless and found to contain negligible carbohydrate.

The combined dark green ethanolic solution was taken down to small volume and partitioned between toluene and water with about 5% n-butanol to break the emulsion. This procedure was repeated twice and this

Flow Chart I Extraction procedure of Urospora wormskioldii

Collection Kept in ethanol, air dried

Dry weight 872 mg

↓

Powdered Ground under liquid nitrogen

↓

Ethanolic extraction Extracted 2 x 300 ml 80% ethanol, 25°C, 8 h

3 x 300 ml 80% ethanol, boiling, 8 h

Combined extracts partitioned between toluene and water

From water layer 87.2 mg

↓

Aqueous extraction Extracted 2 x 300 ml water, 25°C, 8 h

3 x 300 ml water, 100°C, 8 h

Combined extracts

↓ Ethanol

→ Precipitate

↓ dialysed

Solution

→ freeze-dried 97.6 mg

↓

Combined weight 10.2 mg

↓

Acid extraction Extracted 5 x 300 ml dilute HCl pH = 2.0, 70°C, 8 h

Combined extracts dialysed

freeze-dried 29.2 mg

↓

Residue 405 mg

gave a virtually colourless ethanol/water layer and an intense dark green (Chlorophyll) toluene layer. The toluene layer was discarded and the ethanol/water layer was concentrated to dryness. The inorganic salts precipitated during this process were filtered off. After concentration a yellowish solid (87.8 mg) was obtained. This was combined with the alcohol soluble material (10.2 mg) present in the aqueous extract (Flow chart and expt. 5).

Experiment 2 Aqueous extraction

The air dried residual alga was extracted with water under the conditions detailed in the flow chart. After each extraction the solid was centrifuged off and the combined extracts were taken down to small volume and precipitated by 6 volumes of ethanol. The derived white solid was redissolved, dialysed and freeze-dried to a white powder (97.6 mg).

The dialysate and the ethanolic supernatant were combined and concentrated to a yellowish solid (10.2 mg). This solid was combined with the solid obtained from the ethanolic extraction.

Experiment 3 Acid extraction

The residue was thereafter extracted five times with dilute HCl (see flow chart). After each extraction the mixture was centrifuged and the last extract was found to contain little carbohydrate. The combined extracts were dialysed till chloride free and thereafter freeze-dried. A white powder (29.2 mg) was obtained. The residue after this extraction was not extracted further, but washed with cold water and freeze-dried as a suspension, yielding a brownish solid (405 mg).

II Investigation of the different extracts

The ethanolic extract

Experiment 4. Separate aliquots of the combined ethanol soluble solid were redissolved in water and treated with Biodeminrolit and IR 120H$^+$ resins respectively to remove inorganic material still present, and thereafter examined as follows.

Paper chromatograms run in different solvents [GM III (i),(a-c)] were developed with a number of staining reagents [GM IV (i, ii, iv and vi)].

Ionophoresis was carried out in pyridine/acetic acid buffer [GM III (ii) (b)].

A 3MM paper was run for 18 hours in solvent (GM III (i) (a)] and the different strips were eluted and the individual sugars derived therefrom were characterised by paper chromatography, ionophoresis and g.l.c. of the sugar TMS and alditol TMS derivatives [GM V A (ii), B (iii)]. Some of the substances with low chromatographic mobility were hydrolysed (GM II (i)] and the hydrolysates examined by paper chromatography, ionophoresis and g.l.c..

An aliquot was hydrolysed [GM II (i)] and the hydrolysate separated on 3MM paper in solvent (GM (i) (a)]. The different strips were eluted and the individual sugars were characterised by paper chromatography and g.l.c..

The aqueous extract

A. The ethanol soluble material

Experiment 5. Paper chromatograms of aliquots were run in solvents [GM III (i), (a and b)] and treated with locating agents [GM IV (i and ii)]. The chromatographic pattern was very similar to that of the ethanolic extract and it was therefore combined with the latter.

B. The ethanol insoluble material

Experiment 6 Characterisation of the constituent sugars

An aliquot of the white powder (8 mg) was hydrolysed [GMII(i)] and half the hydrolysate was analysed by paper chromatography in solvents [GM III (i), (a-c)] and sprays [GM IV (i, ii and iv)] and by ionophoresis [GM III (ii) (b and c)]. The other half was analysed by g.l.c. [GM V A (i)] as the TMS and alditol TMS derivatives [GM V B (iii)].

Experiment 7 Fractionation of the ethanol insoluble material

The white powder (56.7 mg carbohydrate) was dissolved in water (25 ml) and layered on to a DE-52 cellulose column [GM VII (xi)]. The column was eluted with about 600 ml of each of the following solutions: water, 0.3 MHCl, 0.5 MKCl, 0.8 MKCl and 1.3 MKCl. In later fractionations only water and 1.0 MKCl were used and material from the latter is hereafter called the acidic polysaccharide.

Experiment 8 Composition of the different fractions

The specific rotation of some of the fractions were measured [GM I (v)]. Furthermore the carbohydrate content [GM VI (i)], the sulphate content [GM VI (iii)] and the uronic acid content [GM VI (ii)(c)] of some of the fractions were determined. The sugars in the different fractions were characterised from hydrolysates, by paper chromatography in solvents [GM III (i) (a-c)] and locating agents [GM IV (i), (ii) and (iv)] and by g.l.c. of the sugar and alditol TMS derivatives [GM V A (ii), B (iii)].

The aqueous eluate was tested with KI/I_2 reagent.

Experiment 9 Methylation of the aqueous eluate

An aliquot (4.5 mg) was methylated once by the Hakomori method [GM VII (viii)]. The product was hydrolysed [GM II (i)], reduced [GM VII (v)], converted into the alditol acetates [GM VII (vii)] and analysed by g.l.c. and g.l.c.-m.s. [GM V A (ii), B (iv), C (ii)].

Experiment 10 Methylation of the 0.3M KCl fraction and the acidic polysaccharide

Aliquots (ca. 10 mg) of the acidic polysaccharide and of the 0.3M KCl fraction were methylated by the Hakomori method [GM VII (viii)] once and twice respectively. Half the methylated acidic polysaccharide and all of the methylated 0.3M KCl fraction were analysed as detailed in experiment 9. The other half ot the methylated acidic polysaccharide was converted into the methyl sugar glycosides [GM VII (iv)] after hydrolysis and analysed by g.l.c. [GM V A (i), B (i and ii)].

Experiment 11 Molecular weight determination of the methylated alditol acetates from the acidic polysaccharide

The molecular weights of the methylated alditol acetates from the methylated acidic polysaccharides obtained in experiment 10 were determined by the CI method on the mass spec. [GM V (ii)].

Experiment 12 Reduction of the glucuronic acid in the acidic polysaccharide and characterisation of the derived glucose

An aliquot of the acidic polysaccharide (38.2 mg) was reduced by the carbodiimide method [GM VII (x)]. After dialysing and freeze-drying (34 mg) the carbohydrate content [GM VI (i)] and the uronic acid content [GM VI (ii) (c)] were estimated. An aliquot (4 mg) of the recovered material was hydrolysed [GM II (i)] and the sugars characterised by paper chromatography in solvents [GM III (i) (a and b)] with locating reagents

[GM IV (i, ii and iv)] and by g.l.c. [GM V A (ii), B (iii)] as the sugar and alditol TMS derivatives.

Experiment 13 Methylation of the reduced acidic polysaccharide

An aliquot (6.3 mg) of the reduced acidic polysaccharide recovered from experiment 12 was methylated once by the Hakomori method [GM VII (viii)] and analysed (see expt. 9).

Experiment 14 Attempted removal of sulphate from the acidic polysaccharide with alkali[7]

An aliquot (13 mg) of the acidic polysaccharide was dissolved in water (5 ml) and sodium borohydride (about 25 mg) was added. The solution was set aside for 20 h after which sodium hydroxide solution (3M, 2.5 ml) and more sodium borohydride (about 50 mg) were added. The mixture was then heated at 80°C for 7 h after which it was cooled dialysed and freeze-dried. The recovered material was hydrolysed [GM II (i)] and half the hydrolysate was analysed by paper chromatography in solvents [GM III (i) (a and b)] and sprays [GM IV (i and ii)]. The other half was analysed by g.l.c. as the sugar and alditol TMS derivatives [GM V A (ii), B (iii)].

Experiment 15 Desulphation of the acidic polysaccharide by methanolic hydrogen chloride

An aliquot (40 mg) of the acidic polysaccharide was desulphated by methanolic hydrogen chloride [GM VII (xii)].

Experiment 16 Methylation of the desulphated acidic polysaccharide

An aliquot (12 mg) of the desulphated material obtained from the previous experiment was methylated once [GM VII (viii)] and analysed (see expt. 9).

Experiment 17 Alkali degradation of the methylated acidic polysaccharide

A methylated aliquot (40 mg) of the acidic polysaccharide (methylated once by the Hakomori method [GM VII (viii)] was dissolved in DMSO (3 ml) and 2.0M carbanion (2 ml) [GM VII (iii)] was added. The reaction mixture was set aside overnight and then 50% acetic acid (6 ml) was added to neutralise the carbanion. The mixture was extracted with chloroform and the carbohydrate contents of the chloroform solution and of the residual aqueous solution [GM VI (i)] were measured. The chloroform solution was taken to dryness and treated with 50% acetic acid (10 ml) for 1 h at 100°C. This solution was taken to dryness and the syrup redissolved in water. Sodium borodeuteride (50 mg) was added and the solution left overnight. The mixture was then treated with IR $120H^+$ resin and concentrated. Boric acid was removed by repeated codistillations with methanol. An aliquot (25%) of the recovered material was hydrolysed and analysed as outlined in experiment 9. The molecular weight of the peaks in the g.l.c. spectrum was determined by m.s. by the chemical ionisation method [GM V c (ii)].

Another aliquot of the recovered material from the chloroform solution was remethylated once using deuterated methyl iodide [GM VI (viii)]. The remethylated material was hydrolysed and analysed (see expt. 9).

The residual aqueous fraction of the methylated polysaccharide was taken to dryness, hydrolysed and analysed (see expt. 9).

The above procedure was repeated on a second aliquot (40 mg).

The acid extract

Experiment 18. The carbohydrate content [GM VI (ii)] of the white powder was estimated. An aliquot (5 mg) of the powder was hydrolysed [GM II (i)] and half the hydrolysate was analysed by paper chromatography in solvents [GM III (i) (a and b)] and locating agents [GM IV (i, ii and iv)], while the other half was analysed by g.l.c. as the sugar and alditol TMS derivatives [GM V A (ii), B (iii)].

An aliquot (2 mg) was heated with dilute KI/I_2 reagent.

Experiment 19. An aliquot (6 mg) of the powder was methylated by Hakomori's method [GM VII (viii)] and the methylated polysaccharide analysed (see expt. 9).

The residue

Experiment 20. A sample (41.2 mg) of the freeze-dried residue was hydrolysed [GM II (i)]. After removal of the residual solid the hydrolysate was analysed by paper chromatography in solvents [GM III (i) (a and b)] and by sprays [GM IV (i, ii and iv)].

The protein content of the freeze-dried residue was determined [GM VI (iv)]. The residue was tested for cellulose with Herzberg's reagent[8].

III Results and discussion

Both samples of U. wormskioldii were used in these investigations. Apart from some of the acidic polysaccharide experiments all the detailed investigations in the experimental section were on the original pure sample. The main differences in carbohydrates of the two samples were in the relative proportions of 1,2 and 1,3 linked rhamnose and of xylose derivatives in the acidic polysaccharide.

Low molecular weight carbohydrates

Paper chromatograms of the ethanolic extract and the alcohol soluble (and dialysable) fraction of the aqueous extract revealed their essential similarity. They were therefore combined to give 11.2% of the dry weight (54% carbohydrate) and examined as follows.

Paper chromatograms had spots with the mobilities of glucose, fructose and sucrose together with two faster [Rglc 2.14 and 1.62 in solvent (a)] and two slower [Rglc 0.45 and 0.20 in solvent (a)] moving spots. A faint "streak" with very low mobility was also observed. In order to remove a small amount of inorganic material two aliquots were separately treated with Biodeminrolit (neutral form) and with IR 120H^+ resins. The former resin removed not only the inorganic ions but also the fast moving spots. In contrast the latter resin removed the cations and left the fast moving spots. These facts indicated that the latter were due to charged molecules. An aliquot of the material was hydrolysed and it was found that the slow moving spots except one with the mobility of myo-inositol had disappeared and that the glucose spot was greatly enhanced.

Both the hydrolysate and the original material were separated on 3MM paper and the different substances were characterised as follows:

Glyceric acid.- From the fast moving charged material a non-reducing syrup was obtained. Ionophoresis and paper chromatography gave two spots with mobilities identical to those of glyceric acid run as control. It is well established that this acid occurs as the monomer and dimer and both monomer and dimer were obtained from the control and also from the sample under investigation.

Fructose.- A reducing syrup which on paper chromatograms and g.l.c. had the same retention times as those of fructose. The presence of a ketose group was confirmed by the blue colour given with urea hydrochloride.

Glucose.- A reducing syrup which had the same mobility as that of glucose on paper chromatograms and g.l.c. was obtained. The presence of D-glucose was confirmed by glucose oxidase.

Sucrose.- A non-reducing syrup was obtained which on paper chromatograms and g.l.c. had the mobility of sucrose. Locating reagents gave a positive reaction for ketose and after hydrolysis only glucose and fructose could be detected.

Myo-inositol.- A reducing syrup was obtained which under further investigation was shown to contain two substances, one with the mobility of maltose (reducing) and the other with the mobility of myo-inositol (non-reducing). Hydrolysis of an aliquot followed by paper chromatography and g.l.c. analysis showed glucose (which was confirmed as D-glucose by glucose oxidase) and myo-inositol to be present.

Glucose containing oligosaccharides.- All the reducing spots with Rglc less than 0.3 were eluted as a single fraction. After hydrolysis of an aliquot only glucose could be detected by paper

chromatography and g.l.c. analysis. This was confirmed as D-glucose by glucose oxidase. These results show the presence of a number of glucose containing oligosaccharides.

Glucose and fructose were the two major components in this extract, while the amount of sucrose was fairly small.

The aqueous extract

The ethanol insoluble material

A white powder (11.2% of the dry weight, 70% carbohydrate) was isolated. Analysis of hydrolysate showed the following sugars to be present:

Sugar	Approximate relative proportions
Glucose	1
Glucuronic acid	3.5
Mannose	trace
Xylose	6.5
Rhamnose	8

Table 1

Glucose, xylose, mannose and rhamnose were each characterised by their paper chromatographic mobilities in several solvents and by the measurements of the retention times of their sugar TMS, alditol TMS and alditol acetate derivatives on g.l.c.. Glucose was confirmed as the D-sugar with glucose oxidase. Because of the small amount of material available it was not possible to separate a sufficient amount of each of the other sugars to determine their D or L configuration.

However by analogy with similar polysaccharides separated from U. penicilliformis[3] it can be deduced that the present sugars are D-xylose, D-mannose and L-rhamnose. The presence of glucuronic acid was indicated by its paper chromatographic mobility. On ionophoresis in borate buffer containing calcium ions (expt. 6) a single spot with the mobility of glucuronic acid was obtained. This technique separates glucuronic, galacturonic, mannuronic and guluronic acids. The acid, after reduction (expt. 12) gave only glucose when examined by paper chromatography and by g.l.c. as the TMS derivative. The glucose was confirmed as the D-sugar with glucose oxidase proving the presence of D-glucuronic acid.

Fractionation of the polysaccharide.

The recovery, after fractionating part of the white powder (56.7 mg carbohydrate) on a cellulose column (expt. 7) are shown in the following table:

Fraction	Recovery in mg (as carbohydrate content)
aqueous	4.2
0.3M KCl	38.5
0.5M KCl	9.5
0.8M KCl	1
1.3M KCl	1
	54.2

Table 2

The overall recovery from the column was therefore about 94%. Because of the small amount of carbohydrate found in fractions other

than the 0.3M KCl, laterextracts were fractionated into an aqueous fraction and a 1.0M KCl fraction only, and the carbohydrate in this fraction is hereinafter called the acidic polysaccharide. In all these fractionation experiments the proportion of carbohydrate found in the aqueous fraction to that found in the 1.0M KCl fraction (or the combined KCl fractions) was about 1:12.

The aqueous fraction

This fraction, isolated as a white powder (carbohydrate content 83%), had $[\alpha]_D = +60^\circ$ (c = 0.6) and gave a deep blue colour with dilute KI/I_2 reagent indicating the presence of a starch-type glucan. However it was found to contain glucose, xylose and mannose in the approximate proportions of 5:2:trace.

To find the linkages between the sugars present in this polysaccharide(s) an aliquot of this fraction was methylated (expt. 9) and the following methylated sugars were characterised as their alditol acetates by g.l.c.-m.s..

Sugar	Relative amount
2,3,4-tri-O-methyl xylose	small
2,3-di-O-methyl xylose	large
3-O-methyl xylose	small
2,3,4,6-tetra-O-methyl glucose	large
2,4,6-tri-O-methyl glucose	large
2,3,6-tri-O-methyl glucose	large
4,6-di-O-methyl glucose	small
2,6-di-O-methyl glucose	small
2,3-di-O-methyl glucose	small

Table 3

The presence of end group-, 1,4-linked and 1,4,6-branched glucose residues confirms the presence of a starch-type polysaccharide including both amylose and amylopectin-type glucans. The $[\alpha]_D$ for starches separated from other green algae has been found to vary between $+158^{\circ}$ and $+171^{\circ}$ [9]. However the presence of other polysaccharides in this fraction could well explain the low rotation, ($[\alpha]_D = +60^{\circ}$), of this fraction.

Because of the positive starch test it can probably be assumed that most if not all of the 1,4-linked glucose residues are part of starch-type polysaccharides, since a polysaccharide with both 1,3- and 1,4-linked glucose units or a heteropolysaccharide with 1,4-linked glucose and xylose present would not be expected to give a positive starch test.

The peak areas of the 1,3- and 1,4-linked glucose residues (table 3) are of about the same size, with the latter slightly larger. The presence of 1,2,3- and 1,3,4-linked glucose residues suggests that the 1,3-linked glucose units are part of a highly branched glucan (with branches at C-4 and C-2) with fairly short chain lengths as does the large amount of end-group glucose residues present. The essentially linear β-1,3-linked glucans such as laminaran and callose[10] have low negative rotations and this glucan could therefore contribute to the low specific rotation of this fraction.

β-1,3-linked glucans have previously been reported in Cladophora rupestris[11] and in Caulerpa filiformis[12] but no extensive structural studies were carried out on these two glucans due to the small amount present in each weed. This is the first time however that a highly branched 1,3-linked glucan with branches at C-2 and C-4 has been reported from any alga.

The xylose was found to be 1,4-linked with a small amount of 1,2,4-linked branch points and end groups (table 3). No trace of 1,3-linked xylose residues could be found. If a pure xylan is present in this fraction it must be a branched 1,4-linked polysaccharide. Though the 3-O-methyl xylose peak is partly covered by the 2,3,6-tri-O-methyl glucose peak in the g.l.c. spectrum, an average chain length of about 26 is indicated.

Xylans separated from green algae have so far all been of the β-1,3-linked type. These xylans are structural polysaccharides and water insoluble. A water soluble xylan has been isolated from the red seaweed Rhodymenia palmata[13a] but this contained at least 20% of 1,3-linked xylose as well as 1,4-linked xylose. Other xylans from the Rhodophyceae comprise both β-1,4- and β-1,3-linked xylose units but homoxylans containing only 1,3-or 1,4-linked residues have also been separated[13b]. Hemicellulose β-1,4-linked xylans are often associated in plant cell walls with cellulose, but these xylans are partially acetylated. Structural studies[14] have shown that incorporation of large equatorial acetyl substituents at C_2 and/or C_3 makes the xylan adopt a two fold ribbon-like helix typical of cellulose. Unsubstituted β-1,4-linked xylans on the other hand adopt a three-fold helix which converts the ribbon chain into a cylinder and distributes the two remaining hydroxyl groups more evenly around the helix than they are in cellulose, and therefore increasesthe solubility. The presence of a water-soluble β-1,4-linked xylan with a few branch points does therefore not seem unreasonable.

The xylan in U. penicilliformis was not examined. It failed to precipitate with Fehling's solution which indicated that it is not a β-1,3-linked xylan as these are known[13] to precipitate as the copper

complex. It is therefore possible that a similar xylan is synthesised in both these Urospora species. Since the xylose units in what is termed the "acidic" polysaccharide are similarly linked (p. 74) it is possible that the xylan present in the aqueous fraction is a precursor of this more complex material.

The potassium chloride fractions

The 0.3M KCl fraction had $[\alpha]_D = -58^\circ$ (c = 7.2), carbohydrate content of 71% (calc. from the appropriate graph), uronic acid content of 17%, and 11% sulphate and contained polysaccharide(s) comprising mainly rhamnose, less xylose and glucuronic acid and traces of mannose.

The 0.5M KCl fraction contained polysaccharide(s) comprising the same sugars in roughly the same proportions and had a carbohydrate content of 75% and a uronic acid content of 13%. Because of the small amount of material (9.5 mg carbohydrate) this fraction was not investigated further.

The acidic polysaccharide (the 1.0M KCl fraction from later fractionations) was found to contain rhamnose, xylose, glucuronic acid and mannose in the following proportions 7.5 : 5.5:3:trace. It had $[\alpha]_D = -47^\circ$ (c = 5.6), a carbohydrate content of 72% (calc. from the appropriate graph) and a uronic acid and sulphate content of 17% and 12% respectively. This corresponds to a little less than every fifth unit being sulphated.

Methylation studies

An aliquot of the 0.3M KCl fraction was methylated twice (expt. 10) and the following methylated sugars were characterised as their alditol acetates.

(Tabulated in order of descending peak area).

Sugar
2,3-di-O-methyl xylose
2-O-methyl rhamnose
2,3,4-tri-O-methyl xylose
3-O-methyl xylose
2,3,4-tri-O-methyl rhamnose
3,4-di-O-methyl rhamnose

Table 4

The small amount of methylated rhamnose was rather surprising since rhamnose is the major sugar in this fraction. This could be due to alkali degradation after addition of carbanion during the second methylation and will be discussed further on page 74.

In order to avoid this degradation a sample of the acid polysaccharide (none of the 0.3M KCl fraction was available) was methylated once (expt. 10) and hydrolysed. Half of the hydrolysate was analysed on g.l.c. as the methylglycosides and the following methylated sugars were identified.

Methyl glycoside	Retention time R_{TMG}	
	column (i)	column (ii)
2,3,4-tri-O-methyl xylose	0.53, 0.66	0.40, 0.52
2,3-di-O-methyl xylose	1.23,1.53,1.70	0.66,0.70,0.80
2,4-di-O-methyl rhamnose	1.23	0.70
2-O-methyl rhamnose	3.4	1.1

Table 5

Because many of the methylated sugars have retention times in the same regions and the g.l.c. spectra contain a large number of overlapping peaks a complete interpretation was impossible. The other half of the hydrolysate was therefore converted into the alditol acetates and from g.l.c.-m.s. measurements of these acetates the following sugars were characterised (in order of descending peak area).

Sugar
Unmethylated rhamnose
Unmethylated xylose
2-O-methyl rhamnose
2,4-di-O-methyl rhamnose
2,3-di-O-methyl xylose
2,3,4-tri-O-methyl xylose
3-O-methyl rhamnose
3-O-methyl xylose

Table 6

To confirm these methylation results the alditol acetates were subjected to molecular weight measurement by the CI method on the mass spectrometer (expt. 11) (see p. 20).

In table 7 the retention times (relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol) of the peaks in the g.l.c. spectrum are tabulated as well as the expected and observed molecular weights of the assigned methylated alditol acetates.

Peak	Retention time	Molecular weight found M+1	Molecular weight expected M+1	Alditol acetate of
1	0.64	279	279	2,3,4-tri-O-methyl xylitol
2	0.98	321	321	2,4-di-O-methyl rhamnitol
3	1.23	307	307	2,3-di-O-methyl xylitol
4	1.43	349	349	2-O-methyl rhamnitol
5	1.68	349	349	3-O-methyl rhamnitol
6	1.86	377	377	Unmethylated rhamnitol
8	2.00	335	335	3-O-methyl xylitol
9	2.10	363		Not characterised
10	3.76	363	363	Unmethylated xylitol
11	4.28	303		Not characterised

Table 7

As can be seen from table 7 these results confirm those obtained from the earlier mass spectrometry experiments. As sulphated and uronic acid containing polysaccharides are known to be difficult to methylate completely it is reasonable to assume that the relatively high proportions of free rhamnose and xylose found in the hydrolysate from the methylated polysaccharide must, in part, be due to under-methylation, probably due to steric hindrance by those groups.

The previous methylation studies were all performed on the acidic polysaccharide from the pure sample, (p.56) while all the investigations on the following pages were performed on the slighly contaminated sample. In spite of the reported contamination no new methyl sugars were detected in the methylated acidic polysaccharide from the contaminated sample.

It can only be assumed either that the contamination was extremely small or that any new carbohydrate derived from the contaminants was present in other fractions. The only differences that could be detected in the methylated sugars was a difference in their relative proportions.

Characterisation of the linkages of the uronic acid residues

After reduction the acidic polysaccharide was recovered in 90% yield (expt. 12) and the glucuronic acid content had been reduced from 17% to 4.5%.

The relative proportions of the different methylated sugars present in the hydrolysate after methylation of this reduced polysaccharide (expt. 13) are given in table 8.

Sugar	Approximate relative amount (peak areas)
2,3,4-tri-O-methyl xylose	11
2,3-di-O-methyl xylose	31
3-O-methyl xylose	- *
unmethylated xylose	12
2,3,4-tri-O-methyl rhamnose	5
3,4-di-O-methyl rhamnose	8
2,4-di-O-methyl rhamnose	trace
2-O-methyl rhamnose	26
unmethylated rhamnose	34
2,3,4,6-tetra-O-methyl glucose	10
2,3,6-tri-O-methyl glucose	31

Table 8

* This peak is partly covered by the tri-methylated glucose peak and the peak area can not therefore be obtained, though it can be established that it is a fairly small peak.

The presence of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl glucose provides evidence that the majority of the glucuronic acid is 1,4-linked although a considerable proportion is also present as end group.

Although the relative amount of glucose is slightly higher in this methylated polysaccharide than the uronic acid content of the original polymer the relative proportion of rhamnose to xylose is essentially the same, and it is therefore considered that this methylated material has the same overall structure as the initial polysaccharide.

The relative amount of free xylose from the methylated reduced polysaccharide (table 8) is considerably less than that present in the methylated non-reduced polymer (see p.65) indicating that reduction had removed some of the steric hindrance to methylation and this free xylose disappears completely after desulphation and methylation of the desulphated polysaccharide(s) (p. 72). Since all attempts to detect sulphated xylose residues (p.69) were unsuccessful it can only be concluded that the undermethylation was due to steric hindrance.

Assuming that all the sulphate is located on rhamnose and that rhamnose also occurs as the main branching unit, there is still a high proportion of free hydroxyl groups in the methyl- and free rhamnoses which can only be attributed to undermethylation. That this assumption is reasonable is shown by the almost complete disappearance of free rhamnose in the hydrolysate of the methylated desulphated polysaccharide (p. 72).

The methylation results of the (pure) acidic polysaccharide (table 6) and of the (slightly contaminated) reduced acidic polysaccharide (table 8) indicates that the rhamnose units are mainly 1,3-linked with a high

proportion of either sulphate groups or branching at C-4, while smaller amounts are present as 1,2-linked units and end groups.

To try to obtain more information about the location of the sulphate groups the infrared spectrum of the acidic polysaccharide was recorded (see fig. 7).

The presence of half ester sulphate groups in this molecule was confirmed by absorptions at 1230 - 1270 cm^{-1} (S = O stretching vibration), at 850 cm^{-1} (axial sulphate groups) and at 835 cm^{-1} (equatorial sulphate groups). That both equatorial and axial sulphate groups are present suggests that in addition to sulphation at C-4 at least some of the rhamnose units are sulphated at C-2, as this is the only axial position available in the most energy favourable rhamnose conformation as shown in fig. 8.

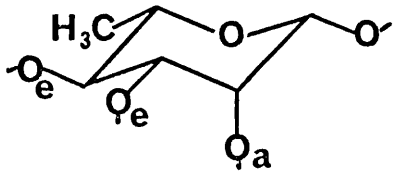


Figure 8

Attempted desulphation of the acidic polysaccharide with alkali

Though the 3-O-methyl xylose residue characterised in the acidic extract is present in very low abundance it was decided to try to establish if it is due to a sulphate group or a branch point. An aliquot of this fraction was treated with alkali (expt. 14). If the linkage at C-2 is due to sulphate then the following reactions could occur

Infra-red Spectrum of the acidic polysaccharide

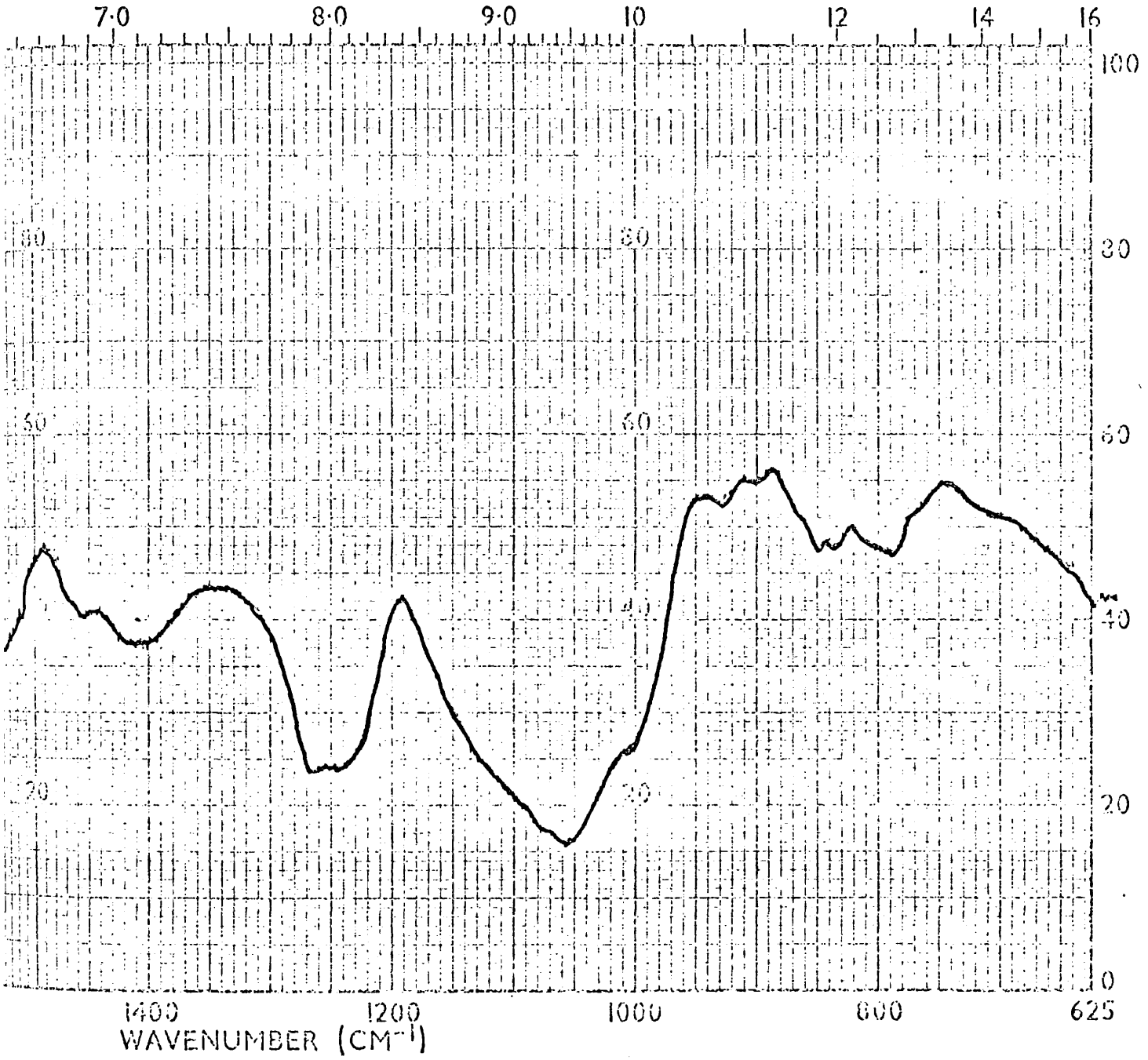


Figure 7

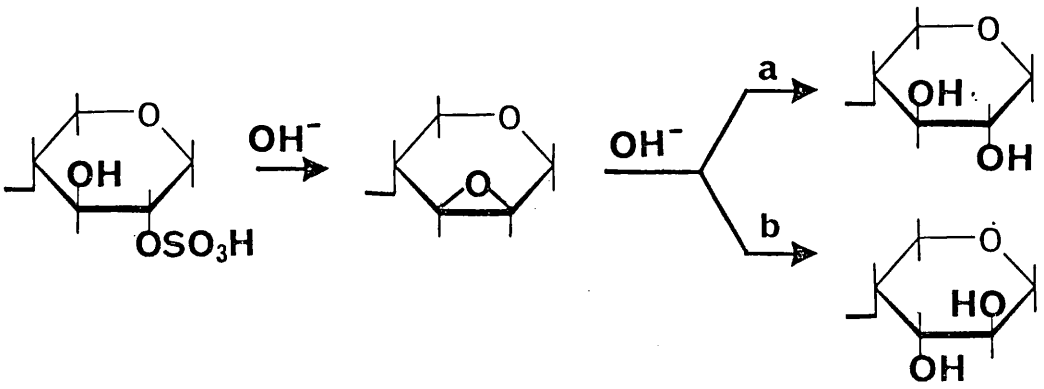


Figure 9

Pathway a) in figure 9 will give xylose while pathway b) will give arabinose which should be detectable even in the small quantities probably present. However neither arabinose nor any other new sugars could be detected after this experiment, which indicates that the 1,2,4-linked xylose residues are due to branching.

The fact that no new sugars could be detected in this experiment makes the presence of 1,2-linked rhamnose residues sulphated at C-3 or C-4 unlikely since these units could undergo the reaction outlined in figure 9.

Because of the small differences in sulphate content the polysaccharide would have on this desulphation no sulphate determination was performed after the alkali treatment.

Desulphation of the reduced acidic polysaccharide

An aliquot (45 mg) of the reduced acidic polysaccharide was desulphated with methanolic hydrogen chloride (expt. 15) and about 80% of the material was recovered. (Due to the small amount of desulphated material available the sulphate content was not determined).

The following sugars were characterised as their alditol acetates after methylation of the desulphated acidic material (expt. 16). (In order of descending peak areas).

Sugar
3,4-di-O-methyl rhamnose)
2,3-di-O-methyl rhamnose) *
2,4-di-O-methyl rhamnose)
2,3-di-O-methyl xylose
3-O-methyl rhamnose
2-O-methyl rhamnose
2,3,6-tri-O-methyl glucose
2,3,4,6-tetra-O-methyl glucose
2,3,4-tri-O-methyl xylose
3-O-methyl xylose
unmethylated rhamnose
2,3,4-tri-O-methyl rhamnose

Table 9

* This is by far the largest rhamnose peak. The three dimethylated rhamnose units have very similar retention times as their alditol acetates and this peak area represents all three. The m.s. studies suggests on on the other hand that the amount of 3,4-di-O-methyl rhamnose is fairly small compared to the other two.

After reduction and desulphation, methylation has resulted in greatly increased quantities of di-O-methyl rhamnoses and reduced amounts of free rhamnose compared with the results from the methylated sulphated polysaccharide (see tables 6, 8 and 9). The dimethyl sugars

could have arisen directly as a result of the removal of sulphate groups or indirectly from more complete methylation due to removal of steric hindrance.

The presence of only small amounts of 3,4-di-O-methyl rhamnose before as well as after desulphation further confirms the alkali desulphation result (p.69) that the 1,2-linked rhamnose units are not sulphated.

The presence of 2,3-di-O-methyl rhamnose in the methylated desulphated polysaccharide indicates for the first time 1,4-linked rhamnose units in the polysaccharide. This dimethyl sugar could have been derived from some of the units which previously gave rise to 2-O-methyl rhamnose units (see table 8) which themselves were derived from 1,4-linked rhamnose residues sulphated at C-3. The 3-O-methyl rhamnose, previously absent from the hydrolysate of the methylated sulphated polysaccharide (see table 8), could have been derived either from previously sterically hindered rhamnose units linked at C-2 and C-4 or units linked at C-2 and C-4 and sulphated at C-3.

Some of the dimethyl rhamnose residues in this spectrum might also be derived from disulphated rhamnose residues.

As IR spectroscopy indicated some sulphate at C-2 of the rhamnose (p.69) the 2-O-methyl rhamnose present in the hydrolysate of the methylated desulphated polysaccharide was derived either from sterically hindered rhamnose units linked at C-3 or C-4 and sulphated at C-2 or from 1,3,4-linked rhamnose units sulphated at C-2. A disulphated rhamnose unit would also fill this requirement.

A striking difference between the g.l.c. spectrum of the desulphated polysaccharide and those from the original material (p. 65) and also the carbodiimide reduced polysaccharide (p.67) is the total absence of free

xylose. At the same time the methylated xyloses from the desulphated polysaccharide are the same as those from the sulphated polysaccharide and this is further evidence that the xylose residues are devoid of ester sulphate and that the more complete methylation after desulphation is due to the removal of steric hindrance. The xylose residues are mainly 1,4-linked with smaller amounts present as either end groups or 1,2,4-linked branch points. It seems unlikely that they are present in a homoxylan since such a polysaccharide would be eluted with water from the DE-cellulose column.

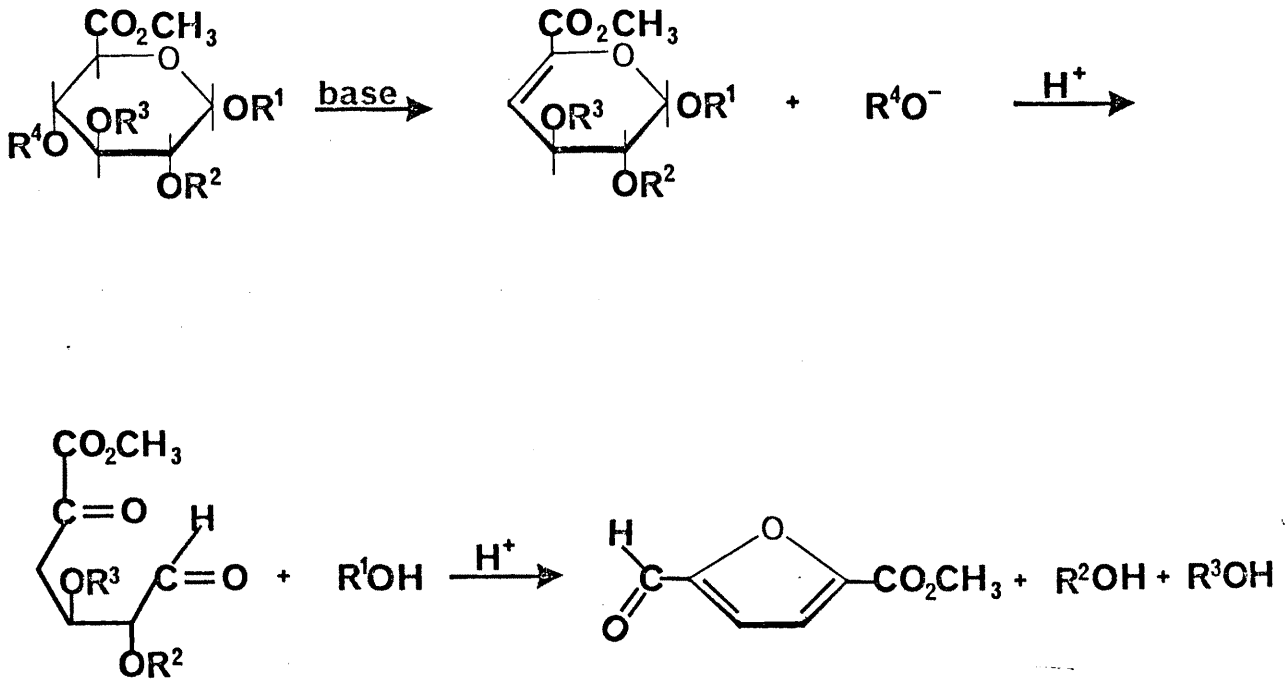
It seems very probable from the results that this acidic mucilage consists of a complex family of polydisperse highly branched polysaccharides, all built up on the same general plan but with considerable variation in size and in the extent of branching. There are 1,4-linked glucuronic acid and xylose units and all three sugars are present as end groups. The rhamnose and to lesser extent xylose are the only sugars to occur at branch points. Rhamnose appears to be linked in every possible way, although 1,3-linked units with sulphate on C-4 (and/or C-2) and 1,4-linked units with sulphate on C-2 and/or C-3 are dominant. At the same time 1,3- and 1,4-linked units could also be present as branch points since 3-O- and 2-O-methyl rhamnose are present in the hydrolysate of the methylated desulphated polysaccharide.

Alkaline degradation of the methylated acidic polysaccharide

In recent years a highly successful method in structure elucidation of polysaccharides containing uronic acid residues has been developed. It involves methylation of hydroxyl and carboxyl groups, base-catalysed elimination of the uronic acid followed by mild hydrolysis with acid[15].

The degraded product can then be etherified with trideuteriomethyl groups and rehydrolysed and converted into alditol acetates and analysed by g.l.c.-m.s.. Comparison of this analysis with the analysis of the original methylated polysaccharide gives information on the nature of the sugar residues on either side of the uronic acid residue.

When polysaccharides containing hexopyranosyluronic acid residues are methylated by the Hakomori method with sodium methyl sulphinyl and methyl iodide in methyl sulphoxide, complete methylation and esterification is usually obtained in one step. Degradation by β-elimination or reaction of the ester group with the methyl sulphinyl anion are insignificant, during this process probably because of the faster reaction between the anion and the methyl iodide which is used in excess. The methyl uronate residues in the methylated polymer carry a good leaving group at position 4, either a derivative of a sugar residue also present in the original polysaccharide or a methoxyl group. On treatment with base this group will be eliminated and an unsaturated uronate residue is formed. This gives an acid labile moiety and mild hydrolysis with acid, using conditions under which glycosidic linkages are stable yields a furan derivative as shown in figure 10.

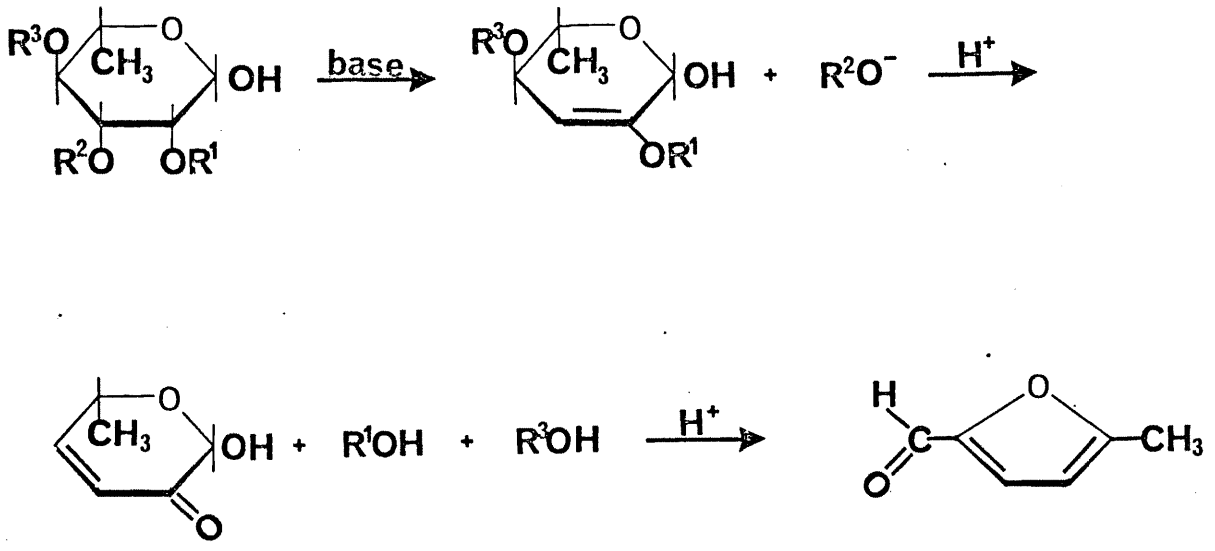


R^1 = sugar residue

R^2, R^3, R^4 = CH_3 or sugar residue

Figure 10

When a sugar, e.g. L-rhamnose, is linked to C-4 in the uronate residue which after methylation is eliminated by base, the resulting reducing sugar residue has a good leaving group at position 3 and will undergo a further β-elimination and ultimately give a furan as shown in figure 11.



R^1, R^2, R^3 = CH_3 or sugar residue

Figure 11

A chain of 1,3-linked rhamnose residues linked to C-4 of the uronate residue will therefore be completely degraded.

To help interpret the results from degradation experiments there are two ways of labelling the degraded molecule and thereby obtaining more information about the groups linked to uronic acid residues. The residue linked to position 1 of the uronic acid can be labelled as shown in figure 12.

When this rhamnose residue is analysed as its alditol acetate on g.l.c.-m.s. the presence and the siting of the deuteromethyl group will reveal that this rhamnose residue was linked through C-4 to the uronic acids potential reducing group.

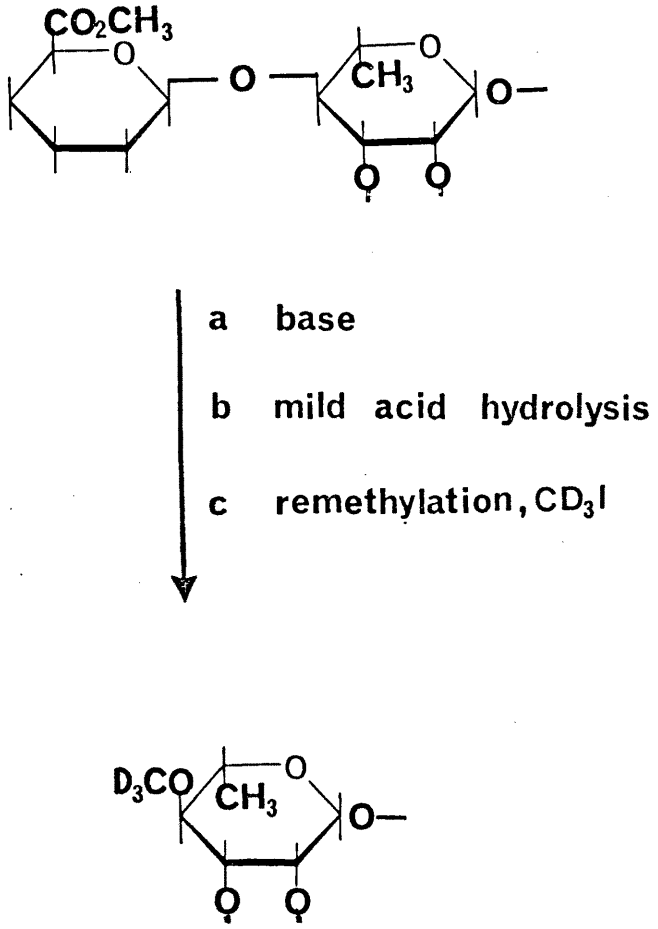
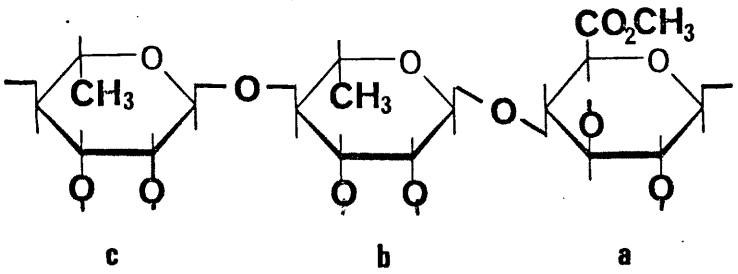


Figure 12

Since the residues linked to the uronic acids at C-4 are always degraded this residue cannot be labelled in any way but sometimes its neighbouring group can be as shown in figure 13.



1 degradation

2 mild hydrolysis

3 reduction, $NaBD_4$

4 acetylation

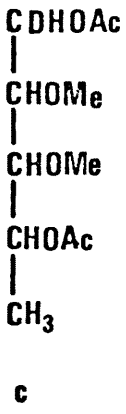


Figure 13

As can be seen from this figure the residue (c) is deuterated at C-1 and this is easily recognised during analysis and it can therefore be established that this particular residue is next but one to the uronic acid. By analysing the methylated polysaccharide before and

after degradation it can often be established which residue is missing in the degraded polysaccharide and the sequence a - b - c can be established. On the other hand if residue (b) was 1,3-linked all three units would be degraded and the next unit along the chain would be the one to be deuterated on reduction.

This method has been used successfully on a number of polysaccharides with a regular repeating structure, and the structures of polysaccharides with fairly large repeating units have been determined by combining the alkaline degradation method with periodate oxidation and other experiments[16,17].

To use this method on a family of polysaccharides, such as the present acidic polysaccharide(s) with no obvious repeating unit will probably give information that is both limited and difficult to interpret, as the following quotation from Professor B. Lindberg indicates[18]. "Some polysaccharides have regular structures, the biosynthesis of other polysaccharides seems to be governed by statistical principles. For the latter, there may not even be two identical molecules in the polysaccharide sample and concepts such as purity and structure may be difficult to define".

It was nevertheless decided to try this degradation procedure on the present acidic polysaccharide (expt. 17) one of the main reasons being that all the polysaccharides so far subjected to this method have been devoid of sulphate and it was of interest to observe the effect if any of the sulphate groups on this reaction.

In the first degradation experiment of the acidic polysaccharide no recognisable methylated carbohydrate residues were identified, neither in the water nor in the chloroform extracted fraction and the experiment was therefore repeated.

After extraction and mild acetic acid hydrolysis of this second degraded sample about equal amounts of carbohydrate (ca.12 mg in each) were found in the chloroform and in the water extract.

The chloroform extract was found to contain traces of 2,3,4-tri-O-methyl xylose and 2,3-di-O-methyl xylose as the only recognisable sugars. Mass spectra of the peaks from this g.l.c. trace contained fragments expected from methylated alditol acetates together with unidentified fragments. This indicates the presence of degraded sugar residues. To try to identify these degraded residues the molecular weights of the peaks in the g.l.c. trace were recorded by the CI method. Most of the molecular weights recorded were less than 300 which further confirms the presence of degraded sugar residues. Even with the molecular weights and their breakdown patterns none of these degraded sugar residues could be successfully characterised. Remethylation of an aliquot of the chloroform extract with deuterated methyl iodide was equally unsuccessful.

From the water extract the following sugars were characterised as their alditol acetates:

(In order of descending peak area)

2,3-di-O-methyl xylose

2-O-methyl rhamnose

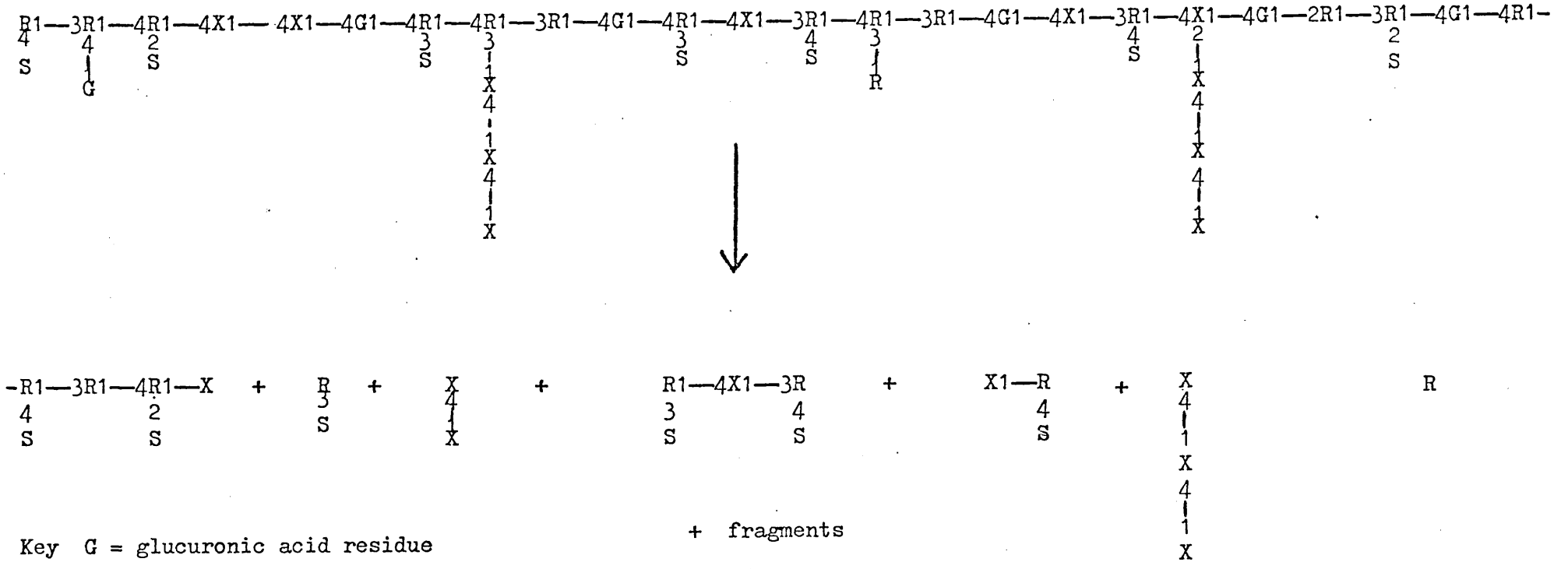
unmethylated rhamnose

unmethylated xylose.

Few peaks corresponding to degraded residues were found in this extract.

These results indicate that about 50% of the molecule has been degraded. It is possible to devise a structure (Fig. 14 p. 82) which

Alkaline degradation of the acidic polysaccharide



Key G = glucuronic acid residue
R = rhamnose residue
X = xylose residue
S = half ester sulphate group.

Fig. 14

explains these results and which is in keeping with all the previous results. In this formula the uronic acid end group will be degraded without affecting its neighbouring residues. The uronic acid units in the chain, on the other hand, will degrade their neighbouring residues and there is a possibility of a degradation chain reaction through successive 1,3-linked rhamnose units as shown in figure 14. It follows that such a molecule is broken into small fragments and oligosaccharides. However it must be emphasised that this is only one of a number of possible formulae which could be drawn which would fit the facts.

Nevertheless the results of the alkaline degradation do permit the conclusion that all three sugars are mutually linked, that the glucuronic acid is present in the inner part of the molecule glycosidically linked to rhamnose and possible to xylose and that some of the xylose is present in side chains.

It was not expected that 1,4-linked units sulphated on C-2 and on C-3 or 1,3-linked units sulphated on C-2 would affect this degradation and the results apparently confirms this expectation.

The acid extract

After dialysis and freeze-drying this extract a small amount of a white powder (3.3% of the dry weight, carbohydrate content 82%) was obtained. Paper chromatography and g.l.c. analysis of a hydrolysate revealed that this powder consisted almost entirely of glucose residues with traces of rhamnose, xylose, mannose and glucuronic acid units also present.

An aliquot of the powder was treated with KI/I_2 solution and a bluish/purple colour was obtained. This indicates the presence of

1,4-linked glucose residues and to confirm this a sample was methylated. 2,3,6-tri-O-methyl glucose was found to be the main peak with small amounts of 2,3,4,6-tetra-O-methyl glucose and 2,3-di-O-methyl glucose also present. These results indicate the presence of an amylopectin type glucan. Traces of methylated xylose and rhamnose residues were also found.

The presence of an amylopectin type polysaccharide in the acidic extract is at first glance somewhat surprising. However this was also found in U. penicilliformis[3] and microscopic evidence on the latter after aqueous extraction indicated that not all the cells were broken by the aqueous extraction and that the glucan was "locked up" inside the cell until it could be released by a sufficiently drastic extraction method. The same is probably true for the present alga.

The residue

The brownish solid obtained after the acid extraction was found to contain 33% protein. The solid gave the characteristic reddish/brown colour with Herzberg's stain[8], indicating the presence of cellulose.

Hydrolysis with 90% formic acid left 40% unhydrolysed and the hydrolysate was found to contain mainly glucose and small amounts of rhamnose, xylose and glucuronic acid. The main residue was therefore not extracted further.

The presence of rhamnose, xylose and glucuronic acid in the hydrolysate of this residue suggests that while the bulk of the glucuronoxylorhamnan is easily removed as the mucilage with hot water, some of it is deeply imbedded in the cellulose/protein matrix of the cell wall, and thus is difficult to extract.

Codiolum pusillum

I. Extraction procedure

The alga was plunged into absolute ethanol immediately after collection and the resulting dark green extract was combined with the ethanol extracts from the present experiments. As before the residual material was air dried and weighed. This weight is later referred to as the dry weight though slightly incorrect since some material had been extracted into the absolute ethanol.

The dry alga (4.75 g) was ground to a fine powder under liquid nitrogen and the same extraction procedure was used for this alga as for Urospora wormskioldii.

The extraction procedure is illustrated in flow chart II.

Experiment 1 Ethanolic extraction

After removing chlorophyll by water/toluene partition a yellow solid (352 mg) was obtained.

Experiment 2 Aqueous extraction

The combined extracts were concentrated to small volume and polysaccharide was precipitated by the addition of 4 volumes of ethanol. The derived white solid was redissolved, dialysed and freeze-dried to a white powder (550 mg). The ethanolic solution was taken down to dryness, the solid was redissolved in water and freeze-dried to a white powder (120 mg).

Experiment 3 Acid extraction

After dialysing and freeze-drying the combined extracts, a white solid was obtained (770 mg). The residue after this extraction was not extracted further, but washed with cold water and freeze-dried as a suspension, yielding a brownish solid.

Flow Chart II — Extraction procedure of Codiolum

Collection
Kept in ethanol, air dried
Dry weight 4.75 g

↓

Powdered
Ground under liquid nitrogen

↓

Ethanol extraction
Extracted 2 x 300 ml 80% ethanol, 25°C, 8 h
3 x 300 ml 80% ethanol, boiling, 8 h
Combined extracts partitioned between toluene and water. From water layer 352 mg

↓

Aqueous extraction
Extracted 2 x 300 ml water, 25°C, 8 h
3 x 300 ml water, 100°C, 8 h
Combined extracts

↓ ethanol

Solution 120 mg ← | → Precipitate 550 mg

↓

Acid extraction
Extracted 5 x 300 ml dilute HCl, pH = 2.0, 70°C, 8 h
Combined extracts dialysed and freeze-dried 770 mg.

↓

Residue

II. Investigation of the different extracts

The ethanolic extract

Experiment 4. Separate aliquots of the ethanol soluble solid was redissolved in water and treated with Biodeminrolit and IR 120H$^+$ resins respectively to remove inorganic material still present, and thereafter examined as follows:

Paper chromatograms run in different solvents [GM III (i), (a-c)] were developed with a number of locating reagents [GM IV (i, ii, iv and vi)].

Ionophoresis was carried out in pyridine/acetic acid buffer [GM III (ii) (b)].

A 3MM paper was run for 18 hours in solvent [GM III (i) (a)] and the different strips were eluted and the individual sugars were characterised by paper chromatography, ionophoresis and by g.l.c. of the sugar TMS and alditol TMS derivatives [GM V A (ii), V B (iii)]. Some of the eluates were hydrolysed [GM II (i)] and the hydrolysates examined by paper chromatography, ionophoresis and g.l.c.

An aliquot was hydrolysed [GM II (i)] and the hydrolysate separated on 3MM paper in solvent [GM III (i) (a)]. The different strips were eluted and the individual sugars were characterised by paper chromatography and by g.l.c.

The aqueous extract

The ethanol soluble material

Experiment 5. An aliquot (15 mg) was hydrolysed and the sugars present characterised by paper chromatography in solvents [GM III (i) (a-c)] and locating agents [GM IV (i, ii, iv and vii)] and by g.l.c. of the sugar TMS and alditol TMS derivatives [GM V A (ii), V B (iii)]. Aliquots

of the original material were also run in the same solvents.

Furthermore the carbohydrate content [GM VI (i)], the sulphate content [GM VI (iii)] and the protein content [GM VI (iv)] were all determined.

Experiment 6 Methylation of the ethanol soluble material

An aliquot (20 mg) was methylated once by the Hakomori method [GM VII (viii)]. The product was hydrolysed [GM II(i)], reduced [GM VII (v)], converted into the alditol acetates [GM VII (vii)] and analysed by g.l.c. and g.l.c.-m.s. [GM V A (ii), B (iv), C (ii)].

The ethanol insoluble material

Experiment 7 Characterisation of the constituent sugars and the uronic acid

An aliquot of the white solid (30.4 mg) was hydrolysed [GM II (i)] and a tenth of the hydrolysate was analysed by paper chromatography in solvents [GM III (i) (a-c)] and sprays [GM IV (i, ii and iv)] and by ionophoresis [GM III (ii) (b and c)] (Table 11). Another tenth of the hydrolysate was analysed by g.l.c. [GM V A (ii)] as the TMS, alditol TMS and alditol acetate derivatives [GM V B (iii and iv)]. The rest of the hydrolysate was separated on the 3MM paper in solvent [GM III (i) (a)], the different sugars and uronic acid were eluted and the specific rotations were measured [GM I (v)] for some of the sugars.

Experiment 8 Fractionation of the ethanol insoluble material

An aliquot of the white solid (408 mg) was dissolved in water (40 ml) and layered on to a DE-52 cellulose column [GM VII (xi)]. The column was eluted with about 600 ml of each of the following solutions: water, 0.1M KCl, 0.2M KCl, 0.3M KCl, 0.5M KCl and 1.0M KCl.

Experiment 9 Composition of the different fractions

The specific rotations of some of the fractions were measured [GM I (v)]. The carbohydrate content [GM VI (i)], the sulphate content [GM VI (iii)] and the uronic acid content [GM VI (ii) (c)] of some of the fractions were determined (Table 12). After hydrolysis the sugars in the different fractions were characterised by paper chromatography in solvents [GM III (i) (a-c)] and locating agents [GM IV (i, ii and iv)] and by g.l.c. of the sugar TMS, alditol TMS and alditol acetate derivatives [GM V A (ii) and GM V B (iii and iv)] (Table 13). The aqueous fraction was reacted with KI/I_2 reagents and the protein content [GMVI (iv)] was determined for the 0.1M KCl fraction.

Experiment 10 Methylation of the aqueous fraction

An aliquot (12.2 mg) was methylated once by the Hakomori method [GM VI (viii)] and analysed (see expt. 6).

Experiment 11 Periodate oxidation of the 0.1M KCl fraction

An aliquot (27.3 mg) was dissolved in water (50 ml) and oxidised by a solution (50 ml) of 0.015M periodate [GM VII (ix)]. The reaction was monitored for 48 hours. An aliquot (6.0 mg) of the recovered polyalcohol was hydrolysed [GM II (i)] and analysed by paper chromatography [GM III (i) (a and b)] [GM IV (i and ii)].

Experiment 12 Methylation of the polyalcohol from experiment 11

The remainder of the recovered polyalcohol from the previous experiment was methylated once by the Hakomori method [GM VII (viii)] and analysed (see expt. 6).

Experiment 13 Methylation of the 0.2M KCl fraction

An aliquot (15.2 mg) of the 0.2M KCl fraction was methylated once by the Hakomori method [GM VII (viii)] and analysed (see expt. 6).

Experiment 14 Methylation of the combined 0.3M and 0.5M KCl fractions

An aliquot (8.2 mg) of the rhamnan (see p.97) was methylated once by the Hakomori method [GM VII (viii)] and analysed (see expt. 6).

Experiment 15 Periodate oxidation of the rhamnan

An aliquot (33.8 mg) of the rhamnan was dissolved in water (50 ml) and oxidised by a solution of 0.03M periodate (50 ml) [GM VII (ix)]. The reaction was monitored for 48 hours. An aliquot (6.2 mg) of the recovered polyalcohol was hydrolysed [GM II (i)] and analysed by paper chromatography [GM III (i) (a and b)] [GM IV (i and ii)].

Experiment 16 Reduction of the glucuronic acid in the rhamnan and characterisation of the derived glucose

An aliquot (28.0 mg) of the rhamnan was reduced by the carbodiimide method [GM VII (x)]. After dialysing and freeze-drying, the uronic acid content [GM VI (ii) (c)] of the reduced polysaccharide (25 mg) was determined. An aliquot of the recovered material was hydrolysed [GM II (i)] and the sugars characterised by paper chromatography in solvents [GM III (i) (a and b)] with locating reagents [GM IV (i, ii and iv)] and by g.l.c. [GM V A (ii), B (iii)] as the sugar and alditol TMS derivatives.

Experiment 17 Methylation of the reduced rhamnan

An aliquot (7.2 mg) of the reduced rhamnan recovered from experiment 16 was methylated once by the Hakomori method [GM VII (viii)] and analysed (see expt. 6).

The acid extract

Experiment 18. The carbohydrate content [GM VI (i)] and the sulphate content [GM VI (iii)] of the white powder obtained from the acid extract were determined as well as the specific rotation [GM I (v)]. An aliquot (5 mg) of the powder was hydrolysed [GM II (i)] and half the hydrolysate was analysed by paper chromatography in solvents [GM III (i) (a and b)]and by sprays [GM IV (i, ii and iv)], while the other half was analysed by g.l.c. as the sugar and alditol TMS derivatives [GM V A (ii), B (iii)]. An aliquot was treated with a dilute sample of KI/I_2 reagent.

Experiment 19 Ultracentrifugation of the acid extract

An aliquot of the solid (10 mg) was dissolved in water (1 ml) and analysed by a Beckman Model E ultracentrifuge at 59780 rpm using a synthetic boundary cell. The reaction was followed by photographs taken of the reaction mixture at time intervals.

Experiment 20 Fractionation of the acid extract by Fehling's solution[19]

Most of the white solid (755 mg) was dissolved in 4M NaOH (75 ml) and a solution of freshly prepared Fehling's solution was added under stirring till no more blue gelatinous copper complex was formed. The precipitate was filtered off and the slightly blue filtrate was dialysed for 3 days and freeze-dried and the resulting solid weighed. This constitutes the glucan . The precipitate was washed with water and then suspended in cooled water (100 ml). The copper complex was decomposed by the addition of 5M hydrochloric acid and the polysaccharide recovered by addition of 4 volumes of ethanol. After centrifugation the solid was dissolved in water, dialysed for 2 days and freeze-dried and

the solid weighed. This constitutes the mannan. The two fractions were investigated as outlined in experiment 18.

Experiment 21 Methylation of the glucan and mannan

An aliquot (6.0 mg) of the glucan was methylated once by the Hakomori method [GM VII (viii)], and analysed (see expt. 6).

Two aliquots (20.0 mg each) of the mannan were methylated once and twice respectively by the Hakomori method [GM VII (viii)]. Aliquots (5.0 mg each) of the two methylated mannans were analysed for sulphate [GM VI (iii)] and the rest of the methylated materials were analysed as for the glucan.

Experiment 22 Desulphation of the mannan by methanolic hydrogen chloride

An aliquot (100 mg) was partially desulphated by four treatments with HCl/MeOH [GM VII (xii)]. An aliquot (6 mg) of the recovered material was analysed for sulphate [GM VI (iii)].

Experiment 23 Methylation of the partially desulphated mannan

An aliquot (7.2 mg) of the partially desulphated mannan was methylated once by the Hakomori method [GM VIII (viii)] and analysed (see expt. 6).

The residue

Experiment 24. The protein content of the residue was determined [GM VI (iv)].

An aliquot (50 mg) of the residue was hydrolysed [GM II (i)] and after centrifuging off the solid [A] (5 mg), the centrifugate was analysed by paper chromatography in solvents [GM II (i) (a and b)] and by sprays [GM IV (i and ii)].

Both the residue and solid [A] were tested for cellulose with Herzberg's stain[8].

III Results and discussion

Low molecular weight carbohydrates

The yellowish solid (7.4% of the dry weight, 42% carbohydrate content) was examined by chromatographic methods (expt. 4) and the same results as for the ethanolic extract of U. wormskioldii (see p.56) were observed: Glucose and fructose were again the major components in this extract and smaller amounts of sucrose, myo-inositol, glyceric acid and glucose containing oligosaccharides (including a spot with the mobility of maltose) were all characterised as previously described (p. 56).

The aqueous extract

The ethanol soluble fraction

A white powder (2.5% of the dry weight) was obtained after freeze-drying. The carbohydrate content was 45% (rhamnose/glucose graph), the protein content was 30% and the sulphate content was 10%. An aliquot was hydrolysed and rhamnose and glucose in about equal amounts as well as traces of xylose were found. The presence of amino acids was shown by the ninhydrin spray. No monosaccharides could be detected on paper chromatograms of the unhydrolysed material, but the solubility in ethanol suggests fairly small molecules. The KI/I_2 test for starch was negative.

To obtain more information about the oligosaccharides present in this fraction an aliquot was methylated (expt. 6) and the following methylated sugars were characterised.

Sugar	Relative amount
2,3,4-tri-O-methyl rhamnose	X
3,4-di-O-methyl rhamnose	X
4-O-methyl rhamnose	XXX
2,4-di-O-methyl rhamnose 2,3,4,6-tetra-O-methyl glucose	XXXX
2,4,6-tri-O-methyl glucose	XX
4,6-di-O-methyl glucose	trace

Table 10

The 2,4-di-O-methyl rhamnose and the tetra-O-methyl glucose derivatives have very similar retention times as alditol acetates. The relative amounts of the two derivatives are therefore combined in the table.

These results indicate that the rhamnose residues are mainly 1,3-linked with a small amount of 1,2-linked residues and end groups also present. The 10% sulphate in this fraction and the small amount of end group rhamnose obtained suggests that the monomethylated rhamnose residues are due to sulphated rhamnose units rather than to branch points. Since the rhamnose residues are mainly 1,3-linked it is most probable that the sulphate is situated at C-2, although 4-O-methyl rhamnose could also be derived from 1,2-linked units sulphated at C-3.

The 1,3-linked glucose residues are probably part of a branched glucan similar to that found in the water extract of U. wormskioldii (p.60).

It is probable that the oligosaccharides found in this fraction are low molecular weight precursors of the rhamnans and glucans separated from water soluble alcohol insoluble fraction (p. 97).

The ethanol insoluble material from the aqueous extract

A white solid (11.6% of the dry weight, 80% carbohydrate measured on a glucose graph) was isolated. Analysis of a hydrolysed aliquot showed the following sugars to be present.

Sugar	Approximate relative amounts
Glucose	2
Glucuronic acid	1
Mannose	10
Xylose	5
Rhamnose	10
Galactose	trace

Table 11

Glucose, xylose, mannose, rhamnose and galactose were each characterised by their paper chromatographic mobilities in different solvents and by the measurements of the retention times of their sugar TMS, alditol TMS and alditol acetate derivatives on g.l.c. Glucose was confirmed as the D-sugar with glucose oxidase. Mannose was confirmed as the D-sugar by its optical rotation, $[\alpha]_D = +15.6^\circ$ (c = 0.41) [cf. D-mannose equil. $[\alpha]_D = +14.2]^{20}$. Rhamnose was confirmed as the L-sugar by its optical rotation, $[\alpha]_D^{24} = +7.8$ (c = 0.32) [cf. L-rhamnose equil. $[\alpha]_D^{20} = 8.2 — 8.9]^{20}$. Because of the small amount of material available it was not possible to separate sufficient of each of the other sugars to determine if they had the D or L configuration. However by analogy with similar polysaccharides separated from Urospora penicilliformis[3] it can be deduced that both galactose and xylose are present as the D-sugars.

The presence of glucuronic acid was indicated by its paper chromatographic mobility. On ionophoresis in borate buffer containing calcium ions a single spot with the mobility of glucuronic acid was obtained (see p.59). The separated acid, after reduction (expt. 16) gave only glucose when examined by paper chromatography and by g.l.c. as the TMS derivative. The glucose was confirmed as the D-sugar with glucose oxidase proving the presence of D-glucuronic acid.

Fractionation of the polysaccharide.- The recoveries after fractionating part of the white solid (228 mg carbohydrate) on the cellulose column (expt. 8) and the composition of the different fractions (expt. 9) are shown in the following table.

Fraction	Recovery (mg)	Carbohydrate Content %	Uronic acid Content %	Sulphate Content %
Aqueous	110	70	-	-
0.1M KCl	42	25	1	15
0.2M KCl	53	36	2	15
0.3M KCl	67	69	13	22
0.5M KCl	50	68	10	20
1.0M KCl	10	48	6	Not measured
	340			

Table 12

The overall recovery of carbohydrate (200 mg) from the column was about 90%.

The sugars present in each fraction are illustrated in Table 13.

Fraction / Sugar	Aqueous	M KCl				
		0.1	0.2	0.3	0.5	1.0
Glucose	XXX					
Mannose	X	XXXX	XXXX	small	trace	XXX
Rhamnose	X	trace	small	XXXX	XXXX	XXXX
Xylose	X	trace	trace	small	trace	trace
Galactose	trace	trace	trace	trace		
Glucuronic acid	trace*	trace*	X	X	X	X

* The presence of amino acids was also detected with ninhydrin spray.

Table 13

The results given in table 13 show that the fractionation has separated a glucan (aqueous fraction), a mannan (0.1 and 0.2M KCl fractions) and a glucuronoxylorhamnan (0.3 and 0.5M KCl fractions). The 1.0M KCl fraction (ca. 5 mg carbohydrate) does not seem to fit into this pattern and was not examined further. The 0.1 and 0.2M KCl fractions were investigated and gave structural details of a mannan while the polysaccharides from the 0.3M KCl and 0.5M KCl fractions were combined and the isolated polysaccharide(s) (117 mg, carbohydrate content 69%) is hereafter designated as the rhamnan.

The aqueous eluate. This eluate had an $[\alpha]_D = +80$ [c = 2,3]. After hydrolysis glucose was the main sugar present in the hydrolysate but appreciable amounts of xylose, rhamnose and mannose in about equal proportions were also found. Because of the small amount of material (112 mg) available it was decided not to attempt any further fractionation. The following methylated sugars were characterised as their alditol acetates after methylation.

(In order of descending peak areas)

Sugar
2,4,6-tri-O-methyl glucose
2,3,4-tri-O-methyl xylose
2,4-di-O-methyl rhamnose
2,3-di-O-methyl xylose
2,3,6-tri-O-methyl glucose
2,3,6-tri-O-methyl mannose
2,4,6-tri-O-methyl mannose
2,3,4,6-tetra-O-methyl hexose*
3,4-di-O-methyl rhamnose
2,4-di-O-methyl xylose
2,3,4-tri-O-methyl rhamnose
4,6-di-O-methyl glucose
2,6-di-O-methyl glucose
2,4-di-O-methyl mannose

Table 14

* as the tetramethylated mannose and glucose derivatives have very similar retention times this peak probably covers both of them

The large number of peaks obtained from this spectrum makes it very difficult to establish the identity of the polysaccharide(s) which might be present, but comparison with other Urospora species[3] (p. 60) permits the following conclusions.

This eluate gave a deep blue colour with KI/I_2 reagent, indicating the presence of an amylose-type glucan and this was confirmed by the 1,4-linked glucose residues observed. No trace of 1,4,6-linked

glucose units could be detected and this indicates the absence of an amylopectin type glucan in this extract.

Both in the ethanol soluble part of this aqueous extract and in the water eluate from U. wormskioldii 1,3-linked glucose residues have been found and furthermore in the latter a 1,3-linked glucan with 1,2,3- and 1,3,4-linked branch points were characterised. The methylation results indicate a similar glucan here.

The rhamnose residues are mainly 1,3-linked with smaller amounts of 1,2-linked residues and end groups also present. These are the same type of linkages as those found for the ethanol soluble rhamnan and the differences between the two polysaccharides are probably their molecular size and the presence of sulphate groups. No rhamnose containing polysaccharides were found in this fraction from either of the other two Urospora species.

The water soluble mannan in U. penicilliformis[3] was shown to consist of 1,3-linked mannose residues with a small amount of 1,3,6-branch points. As similar mannose peaks have been found in this spectrum it seems probable that a small amount of the same type of mannan is present in this alga. 1,4-linked mannose units are also present in this fraction but a mannan of this type is present in larger amounts in other fractions from this weed and will therefore be discussed later.

Various red seaweeds synthesise water soluble xylans consisting of both 1,4-linked (major) and 1,3-linked (minor) xylose residues[13]. The present findings indicate that this alga does the same. If this is so, this is the first green alga in which this type of xylan has been found. U. wormskioldii synthesises a 1,4-linked branched xylan, while the water soluble xylan present in small amounts in U. penicilliformis was not characterised.

The possibility of the presence of heteropolysaccharides in this fraction cannot be excluded. It is for instance possible that the xylose residues are part of the rhamnan but the small amount of material available makes partial hydrolysis studies and the characterisation of oligosaccharides containing the two sugars impossible.

The mannan

The 0.1M KCl fraction (10 mg carbohydrate) was found to have a protein content of 62.5%, and the 0.2M KCl fraction (19 mg carbohydrate) had $[\alpha]_D = -100^\circ$.

To obtain maximum information about the linkages in the small amount of mannan available, it was decided to periodate oxidise the 0.1M KCl fraction (ca. 7 mg carbohydrate remaining) and to methylate the 0.2M KCl fraction (ca. 18 mg carbohydrate remaining).

The low carbohydrate content of the 0.1M KCl fraction made accurate monitoring of the periodate reduction (expt. 11) difficult, but the polysaccharide was found to consume about 1 mole of periodate per anhydro sugar unit. On hydrolysing a portion of the resulting polyalcohol uncleaved mannose could still be detected by g.l.c. and paper chromatography. The remainder of the polyalcohol was methylated (expt. 12) and 3,6-di-O-methyl mannose and a trace of 2,3,6-tri-O-methyl mannose were the only methylated sugar residues which could be detected. This result indicates the presence of either 1,2,4-linked or sulphated mannose units, while the trace of 1,4-linked mannose units observed is probably due to underoxidation.

From the methylated 0.2M KCl fraction (expt. 13) the following mannose derivatives were characterised as their alditol acetates. (Trace quantities of methylated rhamnose and xylose were also detected).

Sugar	Relative amount
2,3,4,6-tetra-O-methyl mannose	large
2,3,6-tri-O-methyl mannose	large
3,6-di-O-methyl mannose	large
3-O-methyl mannose	trace
unmethylated mannose	small

Table 15

As can be seen from the table the tetra-, tri- and di-methyl mannose derivatives are the main peaks in the g.l.c. spectrum and the approximate proportions were found from peak area measurements to be 1:8:2 respectively. The low proportion of tetra-methylated to di-methylated residues suggests that some of the di-methylated residues result from the presence of sulphate groups.

These results provide strong evidence for the presence of a pure 1,4-linked mannan with a probable maximum chain length of 11 and the presence of sulpahte groups and possibly branch points at C-2 on every 5-6 mannose residues.

A polymer of this type should theoretically consume about 0.9 mole of periodate per anhydro sugar unit and the result obtained (about 1 mole periodate consumed per anhydro sugar unit) is in reasonable agreement with this.

The fact that the present mannan was eluted from the column with KCl and not with water supports the presence of charged groups in the polymer. The relatively high sulphate content (p.96) found in these two fractions again indicates that a large proportion of the di-methylated

mannose residues are derived from units carrying sulphate groups at C-2. If every 2 out of 11 mannose units were sulphated at C-2 (as would be possible from the methylation results) the polysaccharide should have a sulphate content of about 11%. That the actual sulphate content (15%) is higher is perhaps partly due to the presence of sulphated rhamnose, but more likely it is caused by partial desulphation on methylation (see p.112).

The trace of 3-O-methyl mannose is probably due either to undermethylation, due to steric hindrance or to di-sulphation at C-2 and C-6 or to a sulphate group at C-2 and a branch point at C-6 (or vice versa). The small amount of unmethylated mannose present is probably due either to undermethylation or to a mixture of sulphate groups and/or branch points. Although this mannan has a smaller average chain length and higher sulphate content (both of which facts would explain its higher sulubility), its essential similarity with the mannan extracted by acid from this weed is very clear (see p. 111).

The large negative rotation, $[\alpha]_D = -100^\circ$, found for the 0.2M KCl fraction was surprising as β-1,4-linked mannans previously isolated from other sources have recorded rotations of -22° to -50° [21]. The presence of small amounts of glucuronoxylorhamnan in this fraction probably enhances the negative rotation and the presence of protein might also have a similar effect. Little is known about the effect of sulphation on the rotation of mannans and this too might be the cause of the high negative rotation.

As the main bulk of the glucuronoxylorhamnan is found in the other potassium chloride fractions its structure will be discussed in the next section.

The Rhamnan

The rhamnan (p.97) was found to have an $[\alpha]_D = -89°$. This high negative rotation suggests that both the D-xylose and D-glucuronic acid are β-linked and that the L-rhamnose is α-linked. The approximate proportions of the sugars in this polysaccharide is rhamnose:glucuronic acid:xylose 8.3:1.2:0.3.

To obtain more information about the linkages present an aliquot was methylated (expt. 14) and the following methylated sugars were characterised.

(In order of descending peak area)

Sugar as alditol acetates
2,4-di-O-methyl rhamnose
3,4-di-O-methyl rhamnose
4-O-methyl rhamnose
2-O-methyl rhamnose
unmethylated rhamnose
2,3,4-tri-O-methyl rhamnose
2,3,6-tri-O-methyl mannose
2,3-di-O-methyl xylose
2,3,4-tri-O-methyl xylose

Table 16

The two dimethylated rhamnose derivatives have very similar retention times and the same is true for the two monomethylated derivatives and it is therefore difficult to establish the exact relative proportions. The g.l.c. spectrum indicates however that there are more 1,3-linked rhamnose residues present than 1,2-linked and that there are more 1,2,3-linked/sulphated rhamnose units present

than 1,3,4-linked/sulphated. The di- and mono-methylated rhamnose peaks are of about the same size and very much larger than any of the other peaks in the g.l.c. spectrum. The small amount of end groups present indicates that the polysaccharide is basically linear with few branch points. This confirms that the monomethylated and free rhamnose residues are derived from sulphated residues, rather than branch points. The relatively high proportion of sulphate (about 22%) found in this polysaccharide requires between every second and third unit in this polysaccharide to be sulphated.

The approximate composition of the rhamnan is calculated to be rhamnose 83%, uronic acid 12% and combined xylose and mannose 5%. Since half the rhamnose gives monomethyl rhamnose on methylation then probably about 42% of the rhamnose is sulphated and one in every two to three units of the polysaccharide is sulphated.

To get more information about the siting of the sulphate groups the IR spectrum of the rhamnan was recorded (see fig. 15).

The broad band at 1220-1270 cm^{-1} (S = O stretching vibrations) confirms the presence of half ester sulphate groups as does the band at 850 cm^{-1} (axial sulphate groups). That no band could be observed at 830 cm^{-1} indicates that very little sulphate is situated at equatorial positions and this implies that the majority of the sulphate groups are situated at C-2 of the rhamnose residues.

Characterisation of the uronic acid in the acidic polysaccharide

An aliquot of the rhamnan was reduced (expt. 16) and it was found that the uronic acid content was reduced from 12 to 2.5% and the overall recovery was about 90%. The uronic acid was proved chromatographically to be glucuronic acid (p.96). The reduced

Infra-red Spectrum of the rhamnan

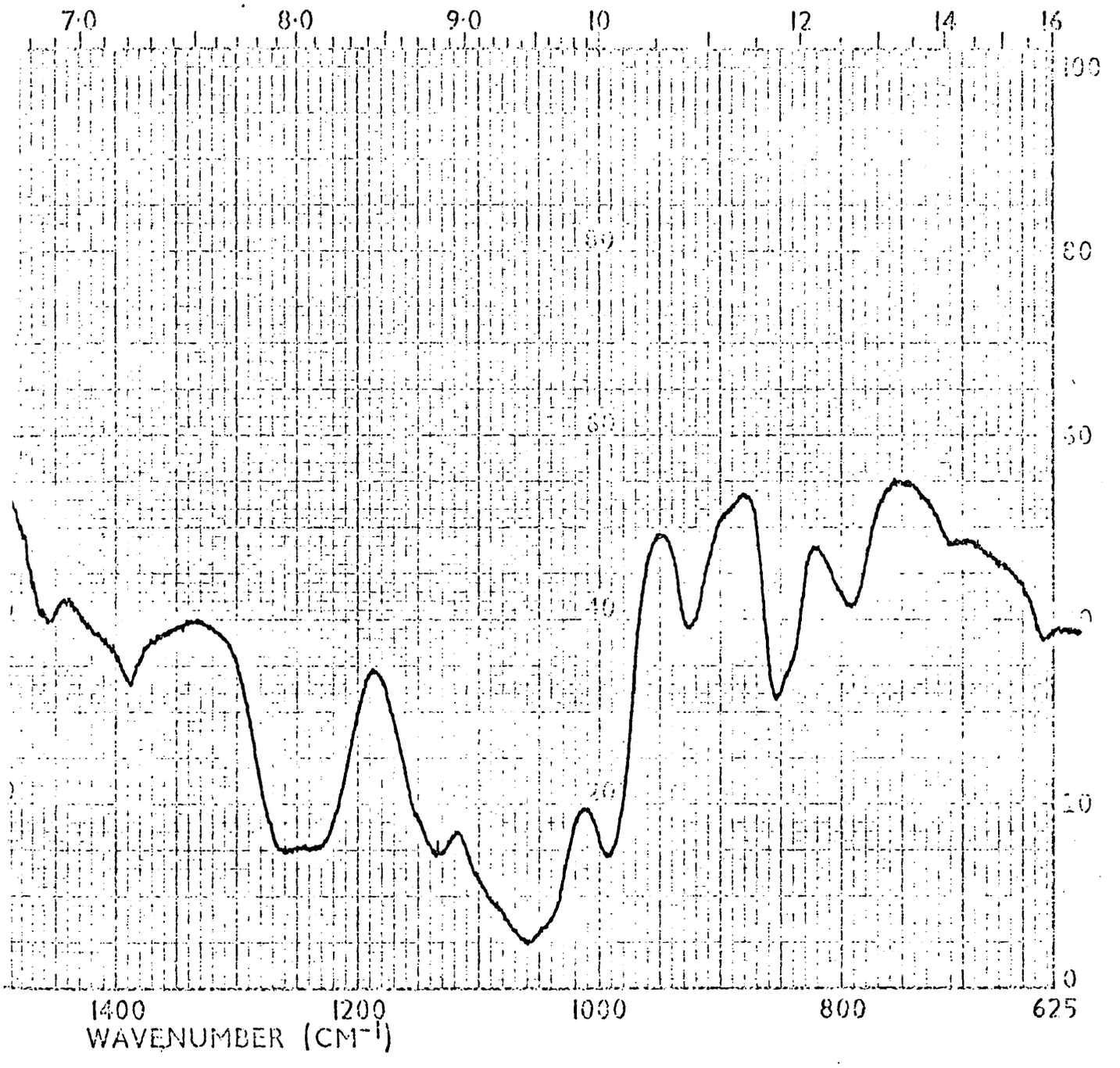


Figure 15

polysaccharide was methylated (expt. 17) and the following methylated sugars were characterised as their alditol acetates.

(In order of descending peak area)

Sugar
2,4-di-O-methyl rhamnose)
3,4-di-O-methyl rhamnose)
4-O-methyl rhamnose)
2-O-methyl rhamnose)
2,3,6-tri-O-methyl glucose
unmethylated rhamnose
2,3,4,6-tetra-O-methyl glucose)
2,4-di-O-methyl xylose)
2,3,4-tri-O-methyl rhamnose
2,3,6-tri-O-methyl mannose
2,3,4-tri-O-methyl xylose
2,3-di-O-methyl xylose

Table 17

The mono- and di- dimethylated rhamnose derivatives are again counted as only two peaks and these two peaks are very much larger than any of the others in the spectrum. Apart from the four largest peaks the others are all fairly small. As many of these derivatives have similar retention times the relative amounts present are difficult to estimate but the g.l.c.-m.s. analysis indicates the presence of more 1,3-linked rhamnose than 1,2-linked and more 1,2,3-linked/sulphated than 1,3,4-linked/sulphated. The amount of end group present seems fairly small, again indicating the presence of fairly long chains.

These results show that glucose (and therefore glucuronic acid) is present both as 1,4-linked (major) residues and end groups (minor).

The xylose residues were found to be 1,4- and 1,3-linked as well as end groups.

The majority of the rhamnose residues are 1,3-linked and to a large extent sulphated at C-2. Smaller amounts of rhamnose are also present as 1,2-linked residues and as end groups.

By analogy with U. wormskioldii (p.73) it is possible that some of the 2-O-methyl rhamnose residues and the free rhamnose are derived from 1,4-linked residues linked or sulphated at the other positions.

Periodate oxidation of the rhamnan

An aliquot of the rhamnan was oxidised by periodate (expt.15). The periodate reduction was found to be about 0.15 mole per anhydro unit. On hydrolyses of the recovered polyalcohol rhamnose with only traces of xylose and small amounts of glucuronic acid were observed. The uronic acid content had been reduced from 12 to 1.5% during the oxidation. This confirms that most of the xylose and glucuronic acid are either 1,4-linked or end groups and that most of the rhamnose residues are linked such that they are not cleaved by periodate.

Further discussion of the structure of this rhamnan and comparison with other rhamnose containing polysaccharides of Urospora sp. will be found at the end of the Codiolum section (p. 115).

The acid extract

A white powder (16% of the dry weight of the weed, 82% carbohydrate) was obtained from the acid extract after dialysis and freeze-drying. It had $[\alpha]_D = -5^\circ$ and a sulphate content of 5.5%. A hydrolysate was shown by paper chromatography and g.l.c. to contain mannose, glucose and

a trace of rhamnose. The proportion of mannose to glucose was found to be approximately 5:1.

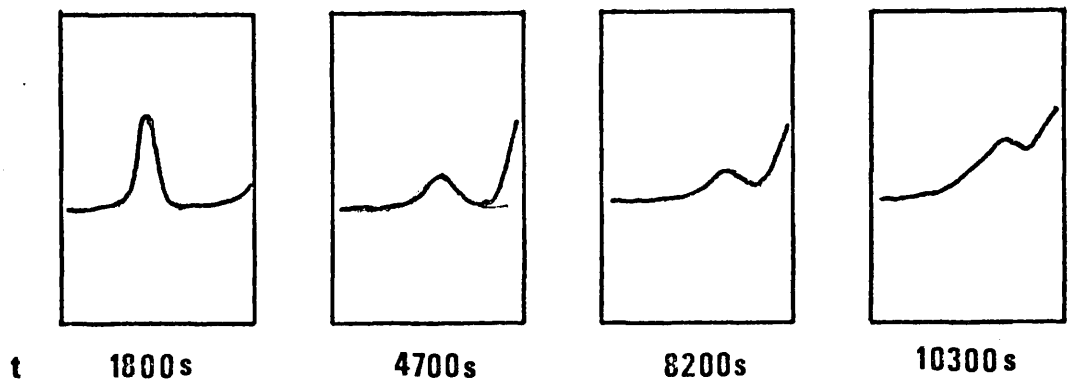
In an attempt to establish if there was more than one polysaccharide present an aliquot was ultracentrifugated(expt. 19). The changes observed with time as well as a plot of time t against $\log_e X$ whereX is the distance from the centre of the rotor is shown in fig. 16. From this plot the sedimentation coefficient was found to be $0.93 \times 10^{-13} S^{-1}$ for this polysaccharide(s).

The fact that only one peak could be observed throughout the experiment indicates that if more than one polysaccharide is present then they all have about the same molecular weight. Furthermore the slow speed with which the peak moved indicates that the molecular weight of the polysaccharide(s) in question is relatively small.

On treatment of an aliquot with a KI/I_2 solution a purple colour was obtained. This indicates the presence of an amylopectin-type glucan and bearing in mind the 1,4-linked mannan found in the water extract it seemed reasonable to assume the presence of a mannan and a glucan. As 1,4-linked mannans are known to complex with Cu^{++} [22] and 1,4-linked glucans do not,it was decided to try a possible separation with Fehling's solution.

Treatment of the extract with Fehling's solution (expt. 20) gave rise to a copper complex which consisted mainly of mannose units. From the solution a glucan containing a small proportion of mannose was recovered. To try to purify the two fractions further, both were given a second treatment with Fehling's solution but small amounts of mannose and glucose still persisted in the glucan and mannan fractions respectively.

Diagrams of sedimentation velocity of the combined mannan and glucan on the Ultracentrifuge



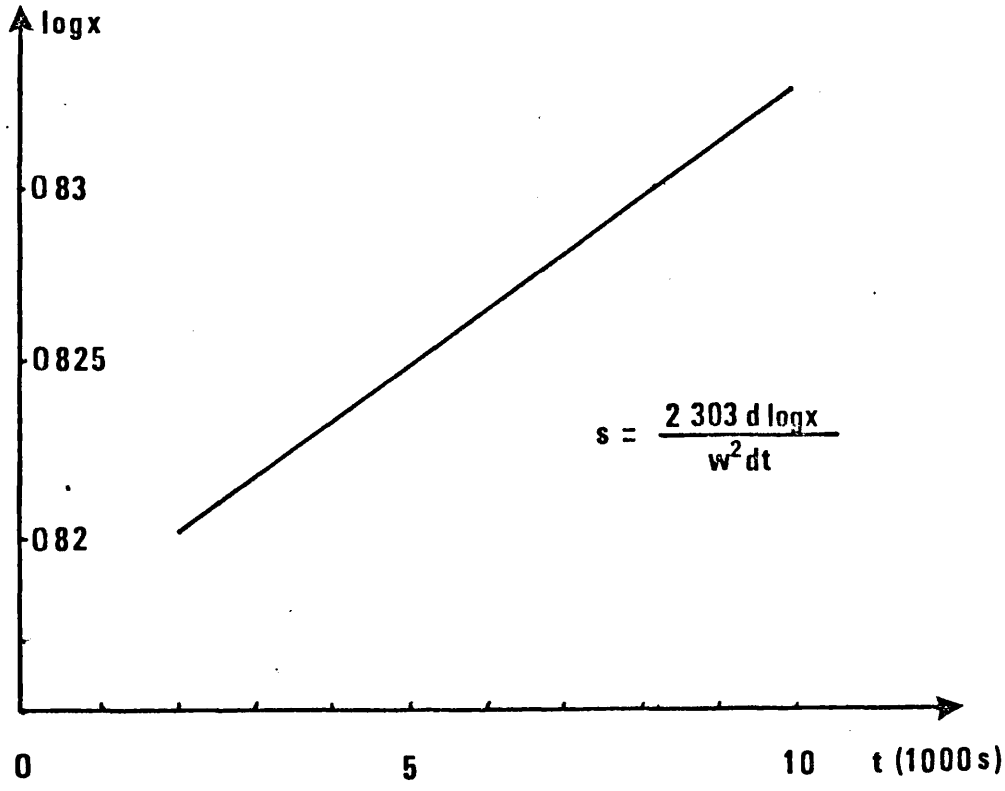


Figure 16

The yields and specific rotations of the two polysaccharides are summarised in the following table.

Fraction	Yield %	Carbohydrate Content %	Sulphate %	$[\alpha]_D$
Mannan	58	82	6.5	-25°
Glucan	11	87	-	$+131^\circ$

Table 18

The overall recovery from the Fehling's solution experiment was about 70%, the loss probably being due to degradation of the polysaccharides by the alkaline conditions.

The glucan

The high positive rotation and the purple colour with KI/I_2 suggests the presence of an amylopectin-type glucan. Such glucans extracted from different green algae have had specific rotations varying from $+154^\circ$ to $+205^\circ$. The presence of a small amount of mannose probably explains the lower value obtained for this material.

The glucan was tested for sulphate, but only neglible amounts were found and it can therefore be concluded that the glucan is sulphate free. To verify the amylopectin like structure the glucan was methylated (expt. 21) and the following methylated sugars were characterised.

2,3,4,6-tetra-O-methyl glucose

2,3,6-tri-O-methyl glucose

2,3-di-O-methyl glucose

Traces of methylated mannose were also found.

Peak area measurements gave the following approximate proportions of the methylated glucose units 1:21:1. This experiment shows the glucan to be 1,4-linked with 1,4,6-branch points and an average chain length of 23 units which is typical of a land plant amylopectin.

The mannan

The negative rotation ($[\alpha_D] = -25^\circ$) found for this mannan suggests that it might be of the β-1,4-linked type. However other mannans with this structure have been water insoluble and could only be extracted by alkali. On the other hand sulphate residues have not been reported in any of them and the presence of 6.5% sulphate in this mannan could explain its water solubility.

An aliquot was methylated and the following methylated sugars were characterised:

2,3,4,6-tetra-O-methyl mannose

2,3,6-tri-O-methyl mannose

3,6-di-O-methyl mannose

2,3-di-O-methyl mannose

Traces of methylated glucose residues could also be observed.

From peak area measurements the relative proportions were found to be 1:13:1.5:1. These results show that the mannose is 1,4-linked, sulphated and/or branched at C-2 and C-6 and has an average chain length of about 16.

If all the di-methylated mannose residues were due to sulphate groups a sulphate content of 8.3% would be expected. As the sulphate content is lower (6.5%) it can be concluded that both sulphate groups and branch points are present.

To try to establish the site(s) of the sulphate groups the mannan was partially desulphated (expt. 22). The overall recovery was 86% and the sulphate content had been reduced from 6.5 to 0.6%. An aliquot of the desulphated mannan was methylated (expt. 23) and g.l.c. analysis of the derived methylated sugars showed that the peak corresponding to 3,6-di-O-methyl mannose had almost completely disappeared while the proportion of the 2,3-di-O-methyl mannose peak was unchanged. The approximate relative proportions of the methylated mannose derivatives had changed from 1:13:1.5:1 (see above) to 1:13:0.2:1 after desulphation. The fact that the 3,6-di-O-methyl mannose peak disappeared with deminishing sulphate content while the 2,3-di-O-methyl mannose peak was unaltered indicates that this mannan is sulphated at C-2 and that it has branch points at C-6. This structure should give a sulphate content of 5.2%. As slightly more is actually found in the original material (6.5%) this might be due to desulphation during methylation. To try to establish if this is so two aliquots of sulphated material were methylated once and twice respectively and the sulphate content measured after each (expt. 21). It was found from this experiment that each methylation reduced the sulphate content by between 1 and 1.5%.

Part of the IR-spectra of the mannan before and after desulphation are shown in figure 17. The spectrum before desulphation has the characteristic broad band at 1210-1280 cm^{-1} assigned to sulphate groups together with a weak band at 850 cm^{-1} indicating axial positioned sulphate groups. No indication of equatorial positioned sulphate groups (band at 830 cm^{-1}) could be found. The spectrum after desulphation had lost both the broad band at 1210-1270 cm^{-1} and

Infra-red Spectra of the mannan
(a) before and (b) after desulphation

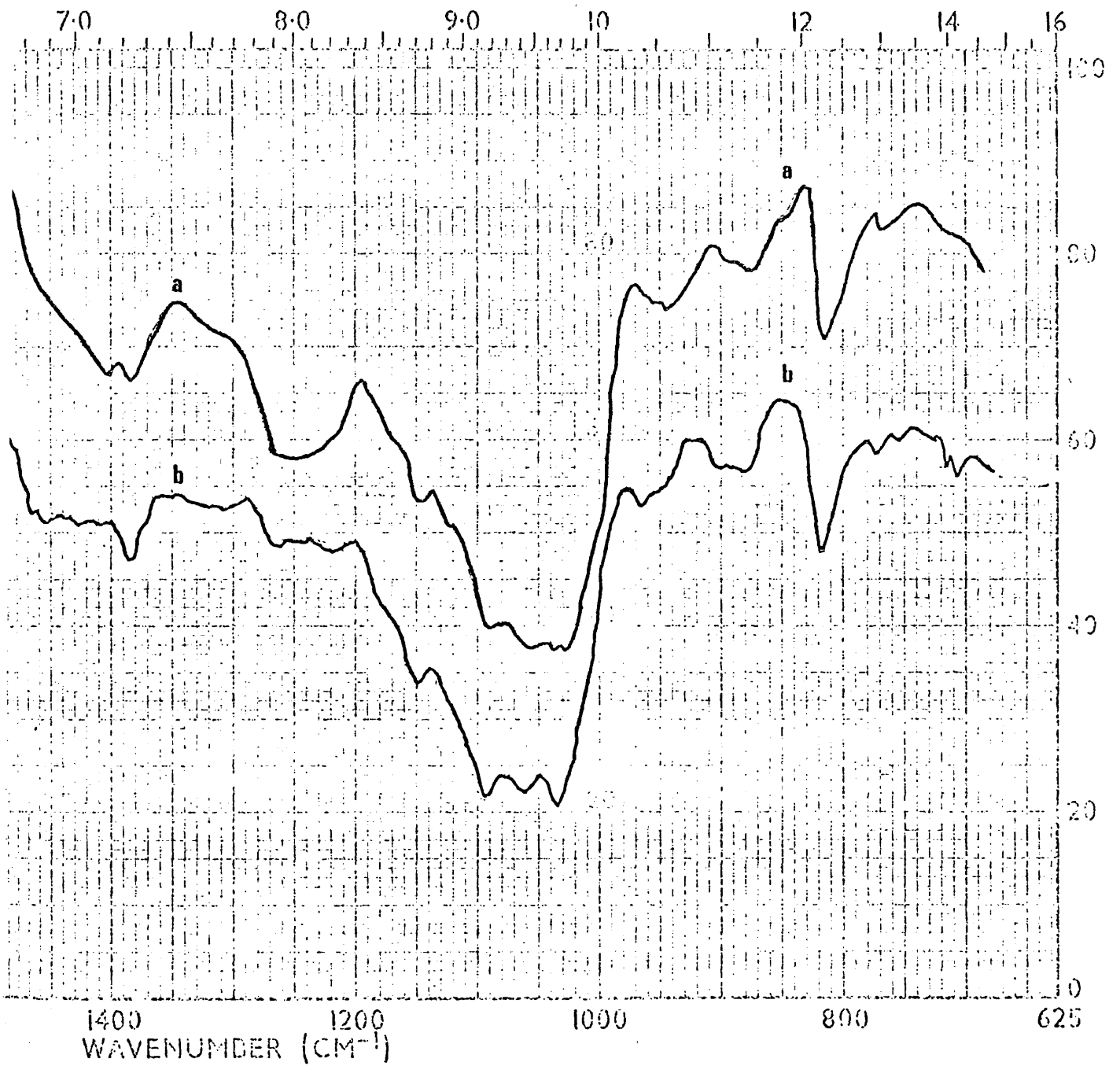


Figure 17

the weak band at 850 cm^{-1}, thus indicating that the sulphate groups in this mannan are in an axial position. In the most stable conformation of a β-linked mannose residue only the hydroxyl group at C-2 is in an axial position. The IR-spectroscopy therefore confirms the result obtained from the methylation studies.

All the present experiments indicate that this mannan is a β-1,4-linked mannan with an average chain length of about 16 and branch points at C-6 with every tenth residue sulphated at C-2.

The residue after acid extraction

The freeze-dried brownish solid was found to contain 51% protein. An aliquot of this solid was hydrolysed and only about 10% remained unhydrolysed (expt. 24). The hydrolysate was shown to contain mannose and glucose and traces of rhamnose. Both the solid and the residue (A) after hydrolysis were stained with Herzberg's stain and the resulting colours observed under a microscope. The solid showed both a yellowish green colour as well as smaller amounts of the reddish/brown colour normally found for cellulose. The residue(A) showed only the red/brown cellulose colour, thereby confirming the presence of cellulose in this alga.

The relatively large amount of mannose found in the hydrolysate of the residue suggests that while the bulk of the mannan is easily removed by water and dilute acid, some of it is deeply embedded in the cellulosic/protein matrix of the cell wall and thus is difficult to extract.

General conclusion and comparison with other members of the Chlorophyceae

It has been shown that the low molecular weight materials metabolised by U. wormskioldii and Codiolum are the same and consist mainly of glucose and fructose with lesser amounts of sucrose, myo-inositol, glyceric acid and a number of glucose containing oligosaccharides. Apart from the relative amounts these are the same as those obtained from U. penicilliformis[3]. In the latter alga it was shown that the oligomers consisted of a homologous series of maltooligosaccharides. Since a disaccharide with the mobility of maltose has been identified from the two present algae and because of the great similarity of the low molecular weight material in the three algae, it is reasonable to assume that at least some of the present oligomers are part of a similar maltooligosaccharide series. While glucose, fructose, sucrose and myo-inositol have been found before in green algae, the presence of glyceric acid and the glucose oligomers (maltosaccharides) seem to be limited to these three algae.

The aqueous extracts from the three Urospora species were found to contain carbohydrates with similarities as well as differences

Starches comprising amylose and amylopectin which are normally found in members of the Chlorophyceae[9], were characterised from both U. wormskioldii and Codiolum while only amylose appears to be synthesised by U. penicilliformis.

While essentially linear β-1,3-linked glucans have previously been reported[11,12] the highly branched 1,3-linked glucans found in U. wormskioldii and Codiolum are the first glucans of this type to be found in any alga.

Evidence for the presence of homo-xylans was found in both U. penicilliformis and U. wormskioldii. While the xylan in the former

was not characterised, the xylan in U. wormskioldii was found to be a 1,4-linked xylan branched at C-2, although no proof of a homo-xylan was found from Codiolum, it is possible that the xylose characterised in the hydrolysate of the aqueous fraction from the water extract is derived from a homo-xylan with both 1,4- and 1,3-linkages of the same type as those found in some red algae[13].

Although only traces of mannans were found in U. wormskioldii mannans were separated and characterised from both U. penicilliformis and Codiolum. The former mannan was found to be an α-1,3-linked polysaccharide while in the latter alga both this type of mannan in small amounts and large amounts of a β-1,4-linked mannan branched at C-6 and sulphated, at C-2, were found. The occurrence of β-1,4-linked mannans in algae has long been known[21] and recently a sulphated α-1,3-linked mannan has been isolated from a red seaweed[23]. Nevertheless this is the first time a sulphated and branched mannan of the type found in Codiolum has been reported. As both branched and unbranched sulphated mannose polymers were isolated from this Codiolum sample it is probable that this polysaccharide consists of a family of mannose polymers.

Since Codiolum is considered to be part of the life history of U. wormskioldii this difference in the polysaccharides is very interesting. The Codiolum is an erect form of the plant and it is possible that the mannan serves as a skeletal material to give mechanical rigidity to the alga, particularly as mannose is found together with glucose in the residue after aqueous and acid extractions.

As comparatively little sulphated rhamnan was isolated from the Codiolum it also seems possible that this sulphated mannan complements the rhamnan in the task of giving the alga a certain flexibility.

Mannose has previously been isolated and characterised from the hydrolysates of aqueous extracts of Acrosiphonia arcta[24] and of Ulva lactuca[25] but no mannans as such have been separated from either of these two algae.

The complex rhamnose containing polysaccharides (hereinafter called rhamnans) from all three species of Urospora and from species of Ulva, Enteromorpha, Acrosiphonia and possibly from Ulothrix[27] species have many similarities. They are all polydisperse hetero-polysaccharides which contain L-rhamnose, D-xylose and D-glucuronic acid and carry half ester sulphate and have fairly similar negative specific rotations. They probably all comprise a family of polysaccharides built up on the same general plan but with variable proportions of the individual sugars, extent of branching and sulphation.

The rhamnose residues in the Urospora species are mainly 1,3-linked with smaller proportions of end group. Small amounts of 1,2-linked rhamnose units were found in Codiolum and U. wormskioldii and 1,4-linked rhamnose units have been characterised in both U. wormskioldii and U. penicilliformis.

The rhamnose residues in Ulva, Enteromorpha and Acrosiphonia species on the other hand, appears to be mainly 1,4-linked with smaller amounts of 1,3-linked rhamnose present.

The three Urospora species were found to be sulphated at C-2 of 1,3-linked (and to a smaller degree 1,4-linked) rhamnose units. Furthermore sulphate groups were also found to be attached to C-4 of 1,3-linked residues (and probably to C-3 of 1,4-linked residues in U. wormskioldii). Due to undermethylation of the polysaccharides, because of steric hindrance, no evidence of disulphation of the rhamnose

residues could be found but this possibility cannot be ruled out.

The xylose in the rhamnan of U. wormskioldii is mainly 1,4-linked with smaller amounts of 1,2,4-linked branch points and end groups. The xylose in the rhamnans of the other two Urospora species and of Ulva is again principally 1,4-linked though smaller amounts of 1,3-linked and end group xylose also occur. Furthermore some of the 1,4-linked xylose units in Ulva[26] were found to be monosulphated at C-2. No evidence for sulphation of the xylose in the Urospora species could be found.

The uronic acid in the rhamnans from Ulva and U. penicilliformis was found to be mainly 1,4-linked with smaller proportions of 1,3-linked and end group residues. Only 1,4-linked (major) and end group (minor) glucuronic acid were found from U. wormskioldii and Codiolum rhamnans.

The rhamnans metabolised by Codiolum and U. penicilliformis appear to be basically linear while those from U. wormskioldii, Ulva, Enteromorpha and Acrosiphonia all appear to be highly branched. The possible rhamnose branch points in Ulva[26] were found to be either 1,2,3- or 1,3,4-linked while those in U. wormskioldii were found to be either 1,3,4- or 1,2,4-linked

Evidence for the presence of cellulose have been found in U. wormskioldii and Codiolum as well as U. penicilliformis. The structural cell-wall polysaccharide in a number of other green algae has also been shown to consist of cellulose[28].

The present investigations have shown many similarities between the carbohydrates of Urospora penicilliformis, Urospora wormskioldii and (apart from the sulphated mannan) Codiolum pusillum, as would be expected from species belonging to the same genus.

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Chapter IV Carbohydrates metabolised by the brown seaweeds Desmarestia ligulata and Desmarestia firma

The brown seaweeds of the Desmarestia family belong to the order Desmarestiaceae. They are 30–180 cm in length and the fronds are filiformed or compressed sometimes with an obscure mid-riff. The branches are numerous, opposite or alternate, bearing fine filamentous hairs or short spine-like branchlets at various seasons. Unilocular sporangia develop from superficial cells of the cortical layer and contain a small number of zoospores which escape from the sporangium in one mass[1]. The Demarestia species are found growing on stones and rocks in the sea below low-water mark and therefore collection can only be achieved by divers. Desmarestia ligulata for instance is found on the rocky bottom of submarine tide-pools, near low-water mark and at greater depth[1]. Both D. aculeata and D. ligulata are found around the British Isles while the present sample of D. firma was found near Cape Town, South Africa.

Studies have revealed that there is a threshold light intensity below which reproduction in Desmarestia will not occur[2]. Furthermore there is evidence that the sexual reproduction of Desmarestia may be under photoperiodic control. The gametophytes of Demarestia were found to remain vegetative when grown in continuous light at 10°C, but one cycle of 10 h light, 14 h darkness, followed by a return to continuous light induced 13 of the branches to become fertile, and fertility was increased to 85% after 4 short-day cycles[3]. Interactions between photoperiod and temperature have also been investigated. In Demarestia gametophytic reproduction occurred in day lengths shorter than 10 hours at 18°, 12 hours at 14° and 14 hours at 10° and in all

day lengths the fertility was higher at the lower temperatures[3]. In this alga the critical daylength would seem to be increased at lower temperatures or the photoperiodic control of reproduction is relaxed at low temperatures.

It has been reported that Desmarestia species contain much sulphate as sulphuric acid in the vacuolar sap[4]. This is the main reason for the present interest in these Desmarestia species. One of the objects being to establish if the presence of free sulphuric acid in the alga causes the synthesis of different carbohydrates from those normally metabolised in brown algae.

Of the Desmarestia species only D. aculeata has been investigated chemically[5,6,7]. The results of these studies will be discussed at the end of this chapter.

Of the samples investigated in this work the D. ligulata sample (116g dry weight) was collected 17.7.73 from deep water at Port Erin, the Isle of Man by Dr Joanna Jones, University of Liverpool and the D. firma sample (105 g dry weight) was collected by Mr. R. Simon, The Dept. of Botany, University of Cape Town, again from below the low tide mark, (at 10 m depth) on 27th March.

Chapman[20] in a study of morphological variation in species of Desmarestia has reduced a number of varieties to D. ligulata var. ligulata and D. ligulata var. firma. These were found to be very polymorphic. Variety ligulata always has a branched thallus whereas firma is mainly unbranched, but some samples may have poorly developed branches. However these can be distinguished from var ligulata by their delicate papery texture after the end of the growing season. This contrasts with the much coarser, cartilagineous texture of D. ligulata var. ligulata.

Desmarestia ligulata

I. Extraction procedure

The alga was plunged into absolute ethanol immediately after collection and the resulting dark green extract (hereinafter called extract A) combined with the ethanol extracts from the present experiments. The residual material was air dried and weighed. This weight is later referred to as the dry weight though somewhat incorrect since a fair amount of material had been extracted into the absolute ethanol.

The dry alga (55.7 g) was extracted as outlined in flow chart III and explained in the following experiments.

Experiment 1 Ethanolic extraction

Solid barium carbonate was added to the 80% ethanol solution to keep the pH between 5 and 7. The last extract was found to be nearly colourless. The combined extracts (including extract A) were taken down to dryness, redissolved in water and extracted with ether (3 x 50 ml) to remove the chlorophyll and the yellowish aqueous solution was taken to dryness.

Experiment 2 Aqueous extraction

Solid barium carbonate was again added to the calcium chloride solution to keep the pH between 5 and 7. The residual alga was centrifuged off after each extraction and the final extract was found to contain very little carbohydate. The combined extracts were concentrated to small volume and freeze-dried to a white solid (1.62 g).

Experiment 3 Acid extraction

The pH was kept at 2.0 during these extractions by the addition of more dilute HCl acid when necessary. The combined extracts were dialysed for 3 days and after freeze-drying a white powder was obtained (1.50 g).

Flow Chart III Extraction procedure of Desmarestia ligulata

Alga plunged in ethanol after collection

↓

Dry weight 55.7 g

Extracted with 80% ethanol + solid $BaCO_3$, pH 5-7.

2 x 300 ml, 25°, 4h

3 x 300 ml, 70°, 4h

↓

Powdered under liquid N_2 and immersed in 40% formaldehyde overnight

supernatant discarded

↓

Extracted with a 2% $CaCl_2$ solution + solid $BaCO_3$, pH 5-7

1 x 300 ml, 25°, 4h

3 x 300 ml, 70°, 4h

↓

Combined extracts dialysed and freeze-dried 1.62g

↓

Extracted with dilute HCl, pH = 2.0 5 x 300 ml, 70°, 4h

↓

Dialysed and freeze-dried 1.50g

↓

Extracted with 3% Na_2CO_3 solution 5 x 300 ml, 50°, 4h

↓

Combined extracts

↓ ethanol

soluble → dialysed, freeze-dried 552 mg

insoluble → ↓ 2% $CaCl_2$

"fucan" 345 mg

calciumalginate 9.3g

↓

Residue

Experiment 4 Alkali extraction

The combined extracts were poured into ethanol (3 vol.) and after being left overnight the derived precipitate was centrifuged off. The centrifugate was concentrated, dialysed and freeze-dried. A greenish solid was obtained (552 mg). The dialysate was concentrated to small volume and tested for 4,5 unsaturated acids[8].

The ethanol precipitate was air dried and then redissolved in water and dialysed for three days. The solution was then made up to a concentration of about 1% alginic acid (compared to a mannuronolactone graph) and a 2% aqueous calcium chloride solution was added slowly with stirring until precipitation of calcium alginate was complete. This gelatinous precipitate was centrifuged off, washed with water and freeze-dried to a fawn powder (9.3 g). The supernatant was dialysed till chloride free and freeze-dried to a white solid (345 mg) hereinafter called the "fucan".

The residue after this extract was not examined further.

II Investigation of the different extracts

The ethanolic extract

Experiment 5. The solid from the ethanolic extracts was redissolved in water and treated with Biodeminrolit (neutral form) and the solution taken to small volume (ca. 3 ml) and the mannitol precipitated with six sequential additions of ethanol; the crystalline mannitol being removed by filtration after each addition. The residual syrup was investigated by paper chromatography [GM III (i) (a and b), IV (i, ii, iii, iv, v and vi)] and g.l.c. of the sugar and alditol TMS derivatives [GM V, A (ii), B (iii)]. The syrup was separated on 3MM paper in solvent (a) and the spots with $R_{mannitol}$ less than 1 were combined and the mixture hydrolysed. The hydrolysate was investigated

by paper chromatography [GM III (i) (a and b), IV (i, ii, iii, iv, v and vi)] and g.l.c. of the sugar and alditol TMS derivatives [GM V, A (ii), B (iii)].

The aqueous extract

Experiment 6. An aliquot (15 mg) was hydrolysed [GM II (i)] and the sugars present characterised by paper chromatography [GM III (i) (a and b), IV (i, ii, iv, v and vi)], by g.l.c. of the sugar and alditol TMS derivatives [GM V A (ii), V B (iii)] and by ionophoresis [GM III (ii) (b and c)].

The carbohydrate content [GM VI (i)], the sulphate content [GM VI (iii)] and the uronic acid content [GM VI (ii) (a)] were all determined.

Experiment 7 Characterisation of the mannose

An aliquot of the solid (100 mg) was hydrolysed [GM II (i)] and the hydrolysate subjected to ionophoresis at pH 6.8 [GM III (ii) (b)]. The material on the starting line of the ionophoretogram was eluted and spotted onto 3MM paper and the latter eluted in solvent (a) overnight. The "mannose" fraction was eluted and an aliquot was mixed with phenylhydrazine in ethanol[9]. An aliquot of this "mannose" was subjected to ionophoresis.

Another aliquot (100 mg) of the solid was treated as described above but the 3MM paper was developed in solvent (b) overnight. The "mannose" fraction was eluted and mixed with phenylhydrazine in ethanol[9]. After recrystallisation in ethanol the melting point of the derived hydrazone was measured as well as the mixed melting point with an authentic sample of mannose phenylhydrazone.

Experiment 8 Fractionation of the aqueous extract

An aliquot of the white powder (430 mg) was dissolved in water (40 ml) and layered on to a DE-52 cellulose column [GM VII (xi)]. The column was eluted with about 600 ml of each of the following solutions: water, 0.1M KCl, 0.2M KCl, 0.3M KCl, 0.5M KCl, 0.5M KCl and 1.0M KCl (Table 19).

Experiment 9 Composition of the different fractions

The carbohydrate content [GM VI (i)], the sulphate content [GM VI (iii)] and the uronic acid content [GM VI (ii) (a)] were determined for each of the fractions. After hydrolysis [GM II (i)] the sugars in the different fractions were characterised by paper chromatography in solvents [GM III (i) (a and b) and locating reagents [GM IV (i, ii, iv and v)] and by g.l.c. of the sugar and alditol TMS derivatives. The aqueous fraction was treated with glucose oxidase [GM IV (iv)] after hydrolysis and the oxidised mixture analysed by paper chromatography.

Aliquots of the 0.2M KCl and 0.5M KCl fractions [about 50 mg of each] were hydrolysed [GM II (i)] and the hydrolysates sequentially separated on 3MM paper in solvents (b) and (a). The appropriate strips were eluted and the quantity of the particular sugar determined by the phenol sulphuric acid method [GM VI (i)].

The acid extract

Experiment 10. An aliquot of the solid (15 mg) was hydrolysed and the hydrolysate and the composition of the solid were investigated as outlined in expt. 6.

Experiment 11 Fractionation of the acid extract

An aliquot of the white solid (205 mg) was dissolved in water (30 ml) and fractionated as detailed in expt. 8. A similar fractionation pattern (see table 19) was obtained.

The "fucan" from the alkali extract

Experiment 12. An aliquot of the "fucan" (16 mg) (expt. 4) was hydrolysed and the hydrolysate and the composition of the extract were investigated as described in expt. 6.

Experiment 13 Fractionation of the "fucan"

An aliquot of the fucan (220 mg) was dissolved in water and fractionated as described in expt. 8 and similar fractions (see table 19) were separated.

Experiment 14 Examination of the calcium alginate from the alkaline extract

Conversion of calcium alginate to sodium alginate

The calcium alginate (9.3 g) (expt. 4) was suspended in a 0.5M HCl (300 ml) solution in a large sintered funnel the base of which was filled with water. The suspension was occasionally stirred and after 3h the hydrochloric acid was filtered off. The filtrate was tested for calcium ions with an oxalate solution and the process repeated twice. No calcium was found in the last filtrate and the solid was washed with water till nearly neutral using tropolein as indicator. The solid was then suspended in water under vigorous stirring and titrated with 0.1M NaOH until pH = 7 was reached by which time all the alginic acid had dissolved. The solution was dialysed for two days and freeze-dried to a fawn solid.

Experiment 15 Investigation of the sodium alginate

An aliquot (20 mg) of the sodium alginate(S) was hydrolysed [GM 2 (i)] and investigated by paper chromatography [GM III (i) (a and b) and IV (i and ii)] and by ionophoresis [GM III (ii) (c)]. The viscosity of a 1% aqueous sodium alginate solution (25 ml) was measured at 25° in an Ubbelodhe suspended level viscometer.

Experiment 16 Extraction of alginic acid (see flow chart IV)

An aliquot of the dry alga (5.0 g) was suspended in a solution of saturated $CaCO_3$ (150 ml) under stirring and solid $CaCO_3$ was added to keep the pH between 5 and 7. The supernatant was decanted off and the solid washed with cold water. The supernatant was dialysed for 3 days and freeze-dried to give a white solid ("Fucan" A, 20 mg). A solution of 1.8% formalin (2 ml) was added to the alga and the mixture stirred for a minute and then left for 30 min. The supernatant was decanted off and water (150 ml) was added and the pH of the mixture was found to be 6.5. To this mixture solid Na_2CO_3 (1.5 g) and a 10% NaOH solution (1.5 ml) were added and the mixture was kept at 60°C for 2 h under stirring. The residue was filtered off and the solution neutralised (pH = 6.5) with dilute HCl and diluted to 800 ml. A 2% $CaCl_2$ solution (100 ml) was added under stirring and the gelatinous calcium alginate centrifuged off and then washed with dilute $CaCl_2$ solution, then suspended in water and freeze-dried to a brownish calcium alginate solid (B, 692 mg). The supernatant was dialysed for 3 days and then freeze-dried ("Fucan" B, 173 mg). The residue was extracted 3 times with a 3% Na_2CO_3 solution (150 ml) at 70°C for 3 h each time. The combined solutions were neutralised (pH = 6.5) with dilute HCl and diluted to 500 ml. This solution was treated as described above

and gave a brownish calcium alginate solid (C, 223 mg), as well as a white solid ("Fucan" C, 45 mg). The two calcium alginates were converted into the sodium salts as described in expt. 14 and their viscosity was determined for a 1% solution at 25° with an Ubbelodhe suspended level viscometer.

Flow Chart IV Extraction of alginic acid

Alga, dry weight 5.0 g

↓

Neutralising the free sulphuric acid. 150 ml saturated $CaCO_3$ solution + solid $CaCO_3$

Extract A 20 mg of "fucan"

↓

Formaldehyde treatment

↓

Extracted with 150 ml 1% Na_2CO_3 containing 0.1% NaOH, 60°C, 2h

Separation and purification

Extract B 692 mg calcium alginate and 173 mg of "fucan"

↓

Extracted with 150 ml 3% Na_2CO_3, 3x, 70°C, 3h

Separation and purification

Extract C 223 mg calcium alginate and 45 mg of "fucan"

↓

Residue

Experiment 17. The three "fucans" (A, B and C) obtained from experiment 16 were investigated as described in experiment 6 (see Table 20).

Experiment 18 Estimation of the guluronic to mannuronic acid ratio in the alginic acid

Three aliquots (about 200 mg) of sodium alginate were weighed accurately into three flasks. The alginates were dissolved in water (10 ml) and to obtain complete solubilisation the solutions were warmed. A solution (10 ml) of 0.8M HCl was added to each flask and the mixtures were hydrolysed for 2h at 100°C. The mixtures were centrifuged and the supernatants were taken for analysis of the soluble components and the residues were washed with 0.4M HCl. One residue (residue 1) was taken for analysis and it was further washed with 0.4M HCl/acetone solution, suspended in water and freeze-dried. The remaining residues were solubilised by the addition of 0.5M NaOH. The alginic acids were treated with 0.5M HCl until the precipitation point was just reached and the mixtures were then made up to 10 ml with distilled water. A solution of 0.8M HCl (10 ml) was added to each alginic acid solution and the combined solutions were rehydrolysed for 2 hours at 100°C. The mixtures were centrifuged and the supernatants were kept and one residue (residue 2) was taken for analysis and treated as described above. The last residue was resolubilised and hydrolysed as described above giving a supernatant and a residue (residue 3). The carbohydrate contents of the supernatants were estimated by the phenol sulphuric acid method [GM XI (i)] using a polyguluronic acid to mannuronolactone 1:1 graph.

The guluronic acid contents of the supernatants and the residues (the latter converted into soluble sodium alginate) were determined by the carbazole method [GM VI (ii) (b)] measuring the ratio of chromophore produced in sulphuric acid with and without

added borate at 55°C. Standard carbazole graphs were determined for polyguluronic acid and mannuronolactone.

Experiment 19 Estimation of the mannuronic to guluronic acid ratio in alginic acid (B) and (C) from expt. 14.

Three aliquots (about 200 mg each) of sodium alginate (B) were weighed into three preweighed flasks and the alginates were dissolved in water (10 ml each). The solutions were hydrolysed and the guluronic acid content in the different fractions was determined as outlined in expt. 18.

An aliquot (193 mg) of sodium alginate (C) was weighed into a preweighed flask and hydrolysed three times and analysed as described in expt. 18.

The residue

Experiment 20. The protein content of the residue after the extractions in flow chart IV was determined [GM V (iv)].

The residue was tested for cellulose with Herzberg's stain[10].

Two aliquots of the residue (about 50 mg each) were hydrolysed with 72% sulphuric acid (5 ml) [GM II (ii)] at 25°C and 100°C respectively for 1 hour. The uronic acid contents of the hydrolysates were determined [GM VI (ii) (a)] and the hydrolysates were investigated by paper chromatography [GM III (i) (a and b), IV (i and ii)].

Results and discussion

In the first instance the carbohydrates in half of the sample of alga were extracted according to the methods used for D. aculeata[7] namely extraction first with 80% ethanol and thereafter with an aqueous 2% $CaCl_2$ solution. The pH of this solution was found to be very low indicating that the alga's free sulphuric acid had been released into the solution. A large amount of solid $CaCO_3$ was therefore added to the mixture and the pH of the solution was kept between 5-7. When the pH of the 80% ethanol extract was measured this was also found to be very low.

Furthermore this alga had been plunged into boiling absolute ethanol immediately on collection and this ethanolic solution was again found to have an extremely low pH. The alga had thus been subjected to a hot acidic solution before the present extractions started. It is therefore probable that the polysaccharides extracted had been degraded to some extent. Nevertheless it was decided to proceed with these extractions as well as start a new extraction procedure based on flow chart III and to compare the two sets of results.

It was found that the two extraction procedures gave fairly similar results apart from the fact that the aqueous and acid extracts from the first half of the alga appeared to contain a fair amount of free uronic acid. Both these extracts had a uronic acid content of well above 40%. Since the rest of the results were found to be fairly similar only the results from the extraction procedure following flow chart III will be discussed.

To avoid degradation of the polysaccharides in algae containing free sulphuric acid it is obviously necessary to neutralise the sulphuric acid immediately the algae are collected. In the present experiments it was shown that though $BaCO_3$ was added to the 80% ethanolic solution there was still free sulphuric acid present in the alga after this extraction as proved by the low pH value found when the residue was subjected to aqueous extraction.

As ethanol does not extract any of the polysaccharides present in the alga it was decided to look for a compound that is soluble in ethanol and at the same time is a strong enough base to neutralise the acid quickly. Amines fit both these requirements and triethylamine and ethanolamine were both tested. After preliminary experiments in the laboratory with the present D. ligulata sample, two plastic containers each containing 80% ethanol and 1% of triethylamine and ethanolamine respectively, giving a final pH of about 10, were sent to the marine station Plymouth where Dr G. Boalch collected some D. ligulata. He placed a few strands of the weed in each container immediately on collection. The pH of these solutions remained about the same as before the addition of the alga.

Strands of alga were suspended in a small volume of water and the solutions were found to be neutral indicating that the amines had removed all the free sulphuric acid. 80% ethanol was used in these experiments because it was thought that the sulphuric acid would dissolve more easily into this solution than into absolute ethanol.

These results indicate that a method to deal with free sulphuric acid in these algae has been devised and it has been used in the extractions of D. firma discussed in a later part of this chapter.

After acidifying the solutions the sulphate present was immediately precipitated as barium sulphate dried and weighed.

The ethanolic extract

The ethanolic extract (A) and the 80% ethanolic extract were found to give similar chromatographic patterns and they were therefore combined. The mannitol was separated by repeated crystallisations with cold ethanol and this gave 1.9g (3.4% of dry weight) mannitol and a yellow syrup (50 mg, 10% carbohydrate) virtually mannitol free. This syrup was found to contain small amounts of xylose, mannose and galactose and six slow moving spots with $R_{mannitol}$ 0.8, 0.65, 0.5, 0.4, 0.26, 0.1. Because of the small amount of carbohydrate present in each spot they were combined and hydrolysed and from the hydrolysate xylose and galactose (major) as well as mannose and myo-inositol were characterised. None of the sprays (expt. 5) specific for glucose, ketoses and sedoheptuloses gave a positive reaction and it was therefore concluded that all these sugars were absent in this extract.

The aqueous extract

After dialysing and freeze-drying a white powder (2.7% of dry weight, 76% carbohydrate) was isolated. Analysis of a hydrolysed aliquot showed the following sugars to be present fucose (major), galactose, mannose, xylose, glucose and glucuronic acid.

Glucose, galactose, mannose, xylose and fucose were all characterised by their paper chromatographic mobilities in different solvents and by measurements of the retention times of their sugar and alditol TMS derivatives. Glucose and galactose were confirmed as the D-sugars by the appropriate oxidase spray. Fucose, xylose and galactose are normally found in fucans from brown seaweeds, but mannose is not and it was therefore decided to separate and characterise the mannose (expt. 7). However this proved more difficult than anticipated. A hydrolysate of the white powder was subjected to ionophoresis and the material remaining on the starting line was separated by paper chromatography and the mannose spot eluted. However this fraction failed to give a crystalline phenylhydrazone. When resubjected to ionophoresis the mannose fraction was found to be contaminated with uronic acid, which presumably was originally present as an equilibrium mixture of the free acid and lactone and the latter had remained on the starting line of the chromatogram with the neutral sugars during the initial ionophoresis. After separating the "apparently" pure mannose from contaminating acid by development of a paper in a basic solvent, the derived mannose gave a crystalline hydrazone with a melting point and mixed melting point with authentic D-mannose phenylhydrazone, of 188°. The pure mannose was also found to have the same mobility as a mannose control when subjected to ionophoresis in borate buffer.

The presence of glucuronic acid in this extract was confirmed by its paper chromatographic and ionophoretic mobility.

Indications of the presence of small amounts of mannuronic acid was also found in this extract.

Fractionation of the polysaccharides

The recoveries after fractionating part of the white solid (430 mg) from the aqueous extract on a cellulose column (expt. 8) and the composition of the different fractions (expt. 9) are shown in table 19.

Fraction	Recovery %	Carbohydrate Content %	Uronic acid Content %	Sulphate Content %
Aqueous	2.5	85[a]		
0.1M KCl	12.5	83[b]	14.8	3
0.2M KCl	41	70[b]	22.5	3
0.3M KCl	18	87[b]	17.1	7.5
0.5M KCl	11	71[c]	3.6	20
1.0M KCl	0			
	85			

Table 19

a) Compared to a glucose graph

b) Compared to a graph based on the carbohydrate composition of the 0.2M KCl fraction (see later)

c) Compared to a graph based on the carbohydrate composition of the 0.5M KCl fraction (see later).

The overall percentage recovery was found to be about 85% by weight and the carbohydrate recovery in this experiment was about 90% . Subsequent separations of this extract gave about 100% carbohydrate recovery.

Apart from the uronic acid content of the 0.1M KCl fraction it can be seen from this table that increasing concentration of potassium chloride gives fractions with less uronic acid and more sulphate. There is no obvious explanation for the fact that the 0.1M KCl fraction is found to have lower uronic acid content than the next two fractions.

The aqueous fraction

After dialysis and freeze-drying a small amount (10 mg) of a syrupy white solid was obtained. On hydrolysis glucose and a trace of a non-reducing sugar with the mobility of mannitol were found. This indicates the presence of laminaran and it also indicates the presence of mannitol terminated chains in the laminaran. It was difficult to detect with certainty the mannitol in the presence of the large excess of glucose. All the material was therefore hydrolysed and the hydrolysate was treated with glucose oxidase to convert the glucose into gluconic acid. Paper chromatograms of the derived solution were compared with the chromatograms from a mixture of glucose:mannitol 25:1 which had also been treated with glucose oxidase. The two mixtures gave identical chromatograms confirming the presence of mannitol end groups in the laminaran. The overall yield of laminaran found in this fraction corresponds to only 0.08% of the dry weight of the weed whereas the laminaran content in D. aculeata was found to be between 1.8 and 5.6% depending on the season of harvesting.

The potassium chloride fractions

As shown in table 19 the 0.2M KCl fraction had the highest uronic acid and lowest sulphate content while the opposite is true for the

0.5M KCl fraction. The determination of the proportions of the sugars present was therefore carried out on these two fractions by means of paper chromatography in different solvents (expt. 9). The appropriate strips of the chromatograms were eluted and the quantity of the particular sugar determined by the phenol sulphuric acid method. The following results were found for the two fractions

0.2M KCl Fraction

fucose:galactose:mannose:xylose:gluc.acid = 15:1.5:0.4:1:11

0.5M KCl Fraction

fucose:galactose:mannose:xylose:gluc.acid = 9.7:2.7:1.4:1:trace

The small amount of glucuronic acid present in the 0.5M KCl fraction was neglected when calculating the proportions. Glucuronic acid gives a weak colour reaction with the phenol sulphuric acid reagent thereby making this a somewhat less accurate method for uronic acid determination. This probably explains the high proportion of glucuronic acid of the 0.2M KCl fraction measured by phenol sulphuric acid compared with the value found by the carbazole method (table 19).

Numerous attempts were made to methylate the various fractions by the Hakomori method but without success. This may be due to difficulties in solubilising the polysaccharides in DMSO. Many attempts were also made to desulphate these polysaccharides by methanolic hydrogen chloride but as with other "fucans" it was found that this hydrolyse the glycosidic linkages faster than the sulphate groups in these polysaccharides. Unfortunately time was not available to reduce the uronic acid units as the carbodiimide complex to glucose, a process which might render the 0.2M KCl fraction more soluble in DMSO.

The acidic extract

After dialysis and freeze-drying a white solid (2.7% of the dry weight) was obtained. This solid was found to have a carbohydrate content of 72% (compared to the graph for the 0.2M KCl fraction in table 19), an uronic acid content of 18.2% and a sulphate content of 2%. A hydrolysate showed the presence of fucose, galactose, xylose, mannose and glucuronic acid together with small amounts of mannuronic acid.

On fractionating an aliquot on a cellulose column the same elution pattern was obtained as for the aqueous extract, except that the water eluate was devoid of carbohydrate.

The alkaline extract

The ethanol soluble greenish solid (1% of the dry weight) (expt. 4) was found to contain 4,5-unsaturated acids, thereby indicating the presence of degraded alginic acid. Its carbohydrate content was only 3% and it was therefore discarded. The fucan (0.6% of the dry weight) separated from the alginic acid (expt. 4) was found to have a carbohydrate content of 74% (compared to the graph for the 0.5M KCl fraction in table 19 and p.139), an uronic acid content of 10% and a sulphate content of 9%. It was found to contain fucose, mannose, galactose, xylose and glucuronic acid and gave the same elution pattern from the cellulose column as for the "fucans" from the aqueous and acid extracts (see table 19).

To obtain a least degraded alginic acid a fresh sample of the alga was extracted as outlined in experiment 16 and flow chart IV (see also the next section). Three "fucans" (A), (B) and (C) were separated and found to contain the same sugars as the above fucan and in addition "fucan" (A) contained glucose.

The carbohydrate, sulphate and uronic acid content of these three extracts are shown in table 20.

Extract	Carbohydrate Content % *	Uronic acid Content %	Sulphate Content %
A	36	4.8	13.5
B	65	7.1	11
C	54	4.2	9

* Compared to the graph for the 0.5M KCl fraction in table 19.

Table 20

It is clear from all these results that D. ligulata metabolises a family of polydisperse "fucans" with varying proportions of the different sugars, glucuronic acid and half ester sulphate.

Alginic acid

Freeze-drying of the calcium alginate obtained from this extract (expt. 4) gave a fawn powder [9.3g calcium alginate, 13.9% of the dry weight (as alginic acid)].

The calcium alginate was converted into sodium alginate by way of the free acid (expt. 14). Paper chromatograms of a hydrolysate of the sodium alginate showed the presence of uronic acids as well as lactones. Ionophoresis of the hydrolysate in a borate buffer containing calcium ions confirmed the presence of both mannuronic and guluronic acids (expt. 15). Ionophoresis of hydrolysates from other sodium alginate samples gave the same spots as those obtained for the present sample, but the guluronic acid spot seemed to be more predominant in the latter.

The viscosity of a 1% aqueous solution of the sodium alginate was found to be 1.7 at 25°C. This very low value indicates that the alginic acid is considerably degraded. The indication of a high guluronic acid content supports this since the mannuronic acid residues are more readily hydrolysed by acid. To confirm this it was decided to determine the mannuronic to guluronic (M/G) acid ratios.

To estimate this ratio a method of sequential hydrolysis was used[11] (expt. 18) and the amount of the uronic acids present were determined by the carbazole method though it is recognised that this method apparently overestimates the guluronic acid. An overestimation of the guluronic acid of between 8 and 15% has been reported for four alginic acid samples[12]. Considerabe difficulties were experienced in standardising the method and it was not until a sample of pure guluronic acid was received from Dr P. Brotherton (Alginate Industries) that satisfactory results were obtained.

The recoveries obtained from the different hydrolyses are shown in table 21.

Flask	Number of hydrolysis	Soluble material %	Insoluble material %	Overall recovery %
1	1	12.4	78.5(97)	90.9
2	1	12.2 (74.4)		
	2	6.1 (76.2)	70.7 (99)	89
3	1	12.1 (75.7)		
	2	5.2 (77.8)		
	3	4.6 (87.5)	65.1 (100)	87

Table 21

The numbers in the parenthesis are the percentages of guluronic acid found in the respective solutions or solids.

These results give a M/G ratio of 0.06 or a guluronic acid content of about 94% and even taking into account the overestimation of guluronic acid the amount of guluronic acid in this alginic acid is unusually high and supports the view from the other results that a large proportion of the mannuronic acid units have been hydrolysed when the alga was in contact with its own free sulphuric acid and with added acid during the extractions.

In an attempt to rectify this the alginic acid was extracted from the alga directly after neutralisation of the free sulphuric acid present in the weed. The details and yields of this extraction are given in experiment 16 and flow chart IV. Two samples of calcium alginate (B and C respectively) were isolated.

A combined overall yield of 16.4% of alginic acid was obtained compared with 13.9% from the previous extraction. 1% sodium alginate solutions of the two alginic acid (B and C) fractions were found to have the following viscosities at 25°

alginic acid from extract B (B) = 300

alginic acid from extract C (C) = 150

The mannuronic to guluronic acid ratio for alginic acids (B) and (C) were estimated as previously described (expt. 18) and the results are shown in table 22.

Alginic acid	Flask	Number of hydrolysis	Soluble material %	Insoluble material %	Overall recovery %
B	1	1	26.4 (69.5)	60.6 (67.1)	91
	2	1	26.5 (66.7)		
		2	9.0 (66.1)	54.5 (67.8)	90
	3	1	26.1 (70.3)		
		2	8.7 (68.1)		
		3	7.5 (44.3)	47.3 (71.4)	89.6
C	1	1	21.8 (68.8)		
		2	8.5 (68.2)		
		3	8.3 (57.7)	50.8 (76.1)	89.4

Table 22

The numbers in the parenthesis are the percentages of guluronic acid found in the respective solutions or solids.

These results give a M/G ratio of 0.47 for alginic acid (B) and a M/G ratio of 0.40 for alginic acid (C). This means a guluronic acid content of 67 and 71% respectively. These results show that this second method of extraction degrades the alginic acid to a considerably less extent than the first extraction procedure.

By comparing the results from tables 21 and 22 the following conclusions can be drawn.

The alginic acid with the lowest viscosity (table 21) is more resistant to acid hydrolysis than the other two acids thus supporting the earlier conclusions that the more easily hydrolysable parts of the molecule have already been removed during the exhaustive extractions. As would be expected the largest proportion of hydrolysed material is found in the first hydrolysate for all the samples of alginic acid. As the lowest guluronic acid content is found in the least degraded alginic acid (B) this supports the view that the mannuronic acid regions in the molecule are more easily hydrolysed than the guluronic acid regions. A reason for this might be the presence of the complexes formed between the guluronic acid blocks and calcium ions. It is difficult to say if the free sulphuric acid present in the alga degrades the alginic acid during the ethanol treatment.If it does this complex might again make the guluronic acid blocks more resistant to hydrolysis.

Even taking into account the probable overestimation of guluronic acid these results indicate a guluronic acid content of more than 50% in the alginic acid from this alga.

The residue

The residue after the extractions described in flow chart IV (the 5.0 g sample) was found to contain 27.5% protein. A portion of the residue was hydrolysed by 72% sulphuric acid (expt. 20) at room temperature. Paper chromatograms of the hydrolysate gave only spots with the mobilities of glucose and uronic acid. The presence of glucose was confirmed by glucose oxidase and the amount of uronic acid in the hydrolysate was estimated to be about 11%. Hydrolysis at higher temperature with acid reduced the amount of uronic acid to 1.3%.

It seems probable that even the room temperature hydrolysis of this residue degrades the uronic acid present and that the actual amount of uronic acid present is higher than 11%.

The residue was stained with Herzberg's stain and the resulting red/brown colour indicated the presence of cellulose as does the glucose found in the hydrolysate.

A comparison of these results with those from other brown algae will be made at the end of the results on Desmarestia firma.

Desmarestia Firma

The alga was freeze-dried immediately after removal from seawater. Due to lack of time a thorough investigation of this alga could not be performed and the present investigations can therefore only be regarded as preliminary ones.

The weighed alga (50.0 g) was extracted as described in flow chart V and in the following experiment.

Experiment 1 Extraction of the alga

It was necessary to add a few drops of triethylamine to keep the pH at about 7.0 during the extraction. The carbohydrate content of the combined ethanol extracts was determined before the extract was taken down to small volume and the mannitol precipitated by six successive additions of ethanol.

The subsequent extracts were separated from the residue by centrifugation and tested for carbohydrate.

Investigation of the extracts

Experiment 2 Estimation of free sulphuric acid in the alga

The ethanol solution containing the neutralised sulphuric acid was acidified slightly with 1M HNO_3 and the sulphate precipitated as barium sulphate by addition of a barium chloride solution.

The solid precipitating during the original neutralisation was dissolved in water, the solution made slightly acid by 1M HNO_3 and the sulphate precipitated as barium sulphate by addition of a barium chloride solution.

Flow Chart V Extraction procedure for Desmarestia firma

Dry weight of alga 50g

↓

Neutralising the sulphuric acid with 500 ml 80% ethanol including 4% triethylamine at 25°, pH 7.0

↓

Powdered under liquid nitrogen

↓

Extracted with 300 ml 80% ethanol 4x, 70°, 4h

Combined ethanol extracts carbohydrate content 0.9g

Mannitol 2.7g

↓

Immersed overnight in ca. 35% formaldehyde, dried 43g

↓

Extracted with 300 ml aqueous 2% $CaCl_2$, 2x, 25°, 4h, pH 5.0

3x, 70°, 4h, pH 5.0

Dialysed and freeze-dried to a white solid 1.60g

↓

Extracted with 300 ml dilute HCl, pH = 2.0 4x, 70°, 4h

Dialysed and freeze-dried 0.70g

↓

Residue

The combined barium sulphate solid was washed with water and the weight of barium sulphate determined (7.0g) after heating the solid in an iron crucible.

Experiment 3 Investigation of the ethanol extract

The residual syrup after removal of mannitol was investigated by paper chromatography [GM III (i) (a and b), IV (i, ii, iii, iv and vi)] and separated by 3MM paper developed in solvent (a). The different strips were eluted and the slowest moving broad band hydrolysed [GM II (i)] and investigated by paper chromatography [GM III (i) (a and b), IV (i, ii, iv and vi)], ionophoresis [GM III (ii) (b and c)] and by g.l.c. as the sugar alditol acetates [GM V A (ii), B (iv)].

The aqueous extract

Experiment 4. An aliquot (15 mg) of the white solid obtained from the aqueous extract was hydrolysed [GM II (i)] and the hydrolysate analysed by paper chromatography [GM III (i) (a and b), IV (i, ii, iv, v and vi)] and electrophoresis [GM III (ii) (b)]. The carbohydrate [GM VI (i)], uronic acid [GM VI (ii) (c)] and sulphate contents [GM VI (iii)] of the solid were estimated.

Experiment 5 Fractionation of the aqueous extract

Part of the solid (480 mg) was dissolved in water (40 ml) and layered on top of a cellulose column [GM VII (xi)]. The column was sequentially eluted with about 600 ml of water, 0.3MKCl, 0.5MKCl and 1.0M KCl and dialysed and freeze dried (see table 23).

Experiment 6 Investigations of the different fractions from the column

The carbohydrate [GM VI (i)], uronic acid [GM VI (ii) (c)] and sulphate contents [GM VI (iii)] of the different fractions were estimated.

An aliquot of each fraction (ca. 10 mg) was hydrolysed [GM II (i)] and half of the hydrolysate analysed by paper chromatography [GM III (i) (a and b), IV (i, ii, iv and v)]. The other half of the hydrolysate was reduced [GM VII (v)], converted into the alditol acetates [GM VII (vii)] and analysed by g.l.c. [GM V A (ii), B (v)].

Experiment 7 Carbodiimide reduction of the uronic acid in the 0.3M KCl fraction

A sample of this solid (75 mg) was dissolved in water (100 ml) and the acid reduced [GM VII (x)]. The uronic acid content of the recovered material (91%) was estimated [GM VI (ii) (c)].

Experiment 8 Methylation of the reduced 0.3M KCl sample

An aliquot (21 mg) of the reduced 0.3M KCl sample (expt. 7) was methylated once by the Hakomori method [GM VII (viii)]. The product was hydrolysed [GM II (i)], reduced [GM VII (v)], converted into the alditol acetates [GM VII (vii)] and analysed by g.l.c. and g.l.c. - m.s. [GM V A (ii), B (iv), C (ii)].

The acid extract

Experiment 9. The acid extract was analysed as described in expt. 4.

Experiment 10. Fractionation of the acid extract

Part of the solid (555 mg) was dissolved in water (40 ml) and fractioned as described in expt. 5.

Experiment 11 Investigations of the different fractions from the column

The different fractions from the column were investigated as described in expt. 6.

Experiment 12 Fractionation of the 0.3M KCl fraction by Fehling's solution

An aliquot (75 mg) of the 0.3M KCl fraction was dissolved in 5% NaOH (50 ml) and treated with Fehling's solution as described in expt. 20 on page 91.

Results and discussion

The ethanol extract

From the combined ethanol extracts 2.9 g free sulphuric acid (5.8% dry weight) and 2.7 g (5.4% of the dry weight) of crystalline mannitol was separated. Separation of the remaining syrupy liquid by paper chromatography showed the presence of mannitol, D-glucose (as confirmed by glucose oxidase), monouronic acid and lactone (with the same p.c. and ionophoretic mobilities as mannuronic acid) and a broad band with $R_{Mannitol}$ between 0.16 and 0.46. On hydrolysis this band was found to contain mannitol and xylose (major) as well as glucose and galactose (minor). The broad band gave a weak yellowish/brown colour with aniline oxalate spray thereby indicating hexose reducing end groups. By comparison with D. ligulata (page135) it is reasonable to assume that some of the xylose units are linked to galactose end groups and the large amount of xylose present indicates more than one xylose unit to each galactose residue.

Furthermore it is probable that the glucose is linked to mannitol either as 1-O-D-mannitol-β-D-glucopyranoside or as

1,6-O-D-mannitol di-(β-D-glucopyranoside) as these compounds have been found in a number of brown algae[5]. The presence of xylose linked to mannitol can not be ruled out and further studies of this extract are required in order to draw any certain conclusions about the sugars and linkages of the oligosaccharides present.

Sedoheptulose and ketose sprays both gave negative results thereby indicating the absence of these types of compounds in this extract.

The aqueous extract

The white solid (3.2% of the dry weight) was found to have a carbohydrate content of 88% (glucose graph), a uronic acid content of 9% and a sulphate content of 2%.

Investigations of a hydrolysate by paper chromatography and ionophoresis indicate the presence of glucose (major), galactose, fucose, xylose, glucuronic acid and small amounts of alginic acid in this extract.

Fractionation of the solid on a cellulose column (expt. 5) gave the recoveries shown in table 23. The composition of the different fractions is also given in this table.

Fraction	Recovery mg	Carbohydrate[a] Content %	Sulphate Content%	Uronic acid[b] Content %
Aqueous	120	92	-	1
0.3M KCl	292	83	1.5	9
0.5M KCl	34	69	11	13
1.0M KCl	-			
	446			

[a] compared to glucose graph

[b] compared to glucuronic acid graph

Table 23

The overall carbohydrate recovery was found to be 91%.

The aqueous fraction

From a hydrolysate of this fraction only glucose with traces of alginic acid could be detected, thereby indicating the presence of laminaran in this alga. No trace of mannitol could be found, therefore it appears unlikely that this laminaran contains mannitol end groups. In an attempt to make sure that no mannitol end groups were present a sample of the laminaran was analysed by a DEAE-cellulose-molybdate column[13]. This column separates molecules with mannitol end groups from those with glucose end groups and as no separation was obtained it can be deduced that no mannitol is present. Laminaran is also found in the 0.3M KCl fraction and will be discussed further there.

The 0.3M KCl fraction

Analysis of a hydrolysate of this fraction gave glucose as the

main sugar though fucose, galactose, xylose, glucuronic acid and alginic acid residues were also observed. The presence of alginic acid was indicated by paper chromatography and verified by g.l.c. When the sugars in the hydrolysate are reduced and converted to alditol acetates at least some of the mannuronic acid present is converted into mannose and detected as such, as was shown with a sample of authentic mannuronolactone.

From the g.l.c. results it can be seen that more than 50% of the carbohydrate in this fraction consists of glucose and the total amount of laminaran in the aqueous extract is therefore at least 55% and probably more.

Some of the uronic acid in this fraction was reduced as carbodiimide complex (expt. 7) and the uronic acid content went down from 9 to 7%. This shows that the uronic acid in this fraction is difficult to reduce which again indicates the presence of alginic acid (see appendix II page 174).

An aliquot of this uronic acid reduced sample was methylated (expt. 8) and the methylated sugars, characterised as alditol acetates, are listed in table 24 in order of descending peak areas.

Sugar
2,4,6-tri-O-methyl glucose
unmethylated mannose
unmethylated glucose
2,4-di-O-methyl glucose
2,3,4,6-tetra-O-methyl glucose
2,3,6-tri-O-methyl hexose
unmethylated fucose
unmethylated galactose

Table 27

The presence of 2,4,6-trimethylated glucose residues shows that the glucose in this fraction is part of a laminaran. The methylation results indicates furthermore that some of the glucose residues are either branched or sulphated at C-6. The small amount of sulphate found in this fraction and the fact that a "fucan" is present provides strong evidence of branching at C-6.

The 2,3,6-trimethyl hexose residues probably originate from reduced alginic acid.

The fact that no trace of either methylated fucose, galactose or xylose could be found indicates that the present "fucan" is as difficult to methylate by the Hakomori method as the "fucan" in D. ligulata.

The 0.5M KCl fraction

Analysis of a hydrolysate of this fraction shows the presence of fucose (major), galactose, xylose and glucuronic acid.

Methylation by the Hakomori method was again found to be unsuccessful even though DMF was tried as solvent as well as DMSO.

The acid extract

The white solid (1.4% of the dry weight) was found to have a carbohydrate content of 55% (glucose graph) and a sulphate content of about 1%.

Investigations of a hydrolysate by paper chromatography indicates that this extract consists to a large extent of alginic acid since most of the carbohydrate did not move in a basic solvent. Apart from the acid, glucose, fucose, galactose and xylose were all found. The solid was found to have a uronic acid content of about 15%

(mannuronolactone graph) but this is probably too low since this does not allow for the presence of acid as well as lactone.

Fractionation of the solid on a cellulose column (expt. 10) gave the recoveries shown in table 25. The composition of the different fractions is also given in this table.

Fraction	Recovery mg	Carbohydrate[a] Content %	Sulphate Content%	Uronic acid Content %
Aqueous	11	83	-	-
0.3M KCl	466	53	1	17[b]
0.5M KCl	22	78	8	7[c]
1.0M KCl	-			
	499			

[a] glucose graph

[b] mannuronolactone graph

[c] glucuronic acid graph

Table 25

The overall carbohydrate recovery was found to be 89.9%.

The aqueous fraction

On hydrolysis only glucose was found in this fraction thereby indicating that a small amount of laminaran was left in the residue after water extraction.

The 0.3M KCl fraction

Analysis of a hydrolysate by paper chromatography and g.l.c. indicated the presence of large amounts of charged materials and ionophoresis in borate buffer (expt. 9) further indicates that most

of the material is mannuronic acid. As small amounts of fucose was also observed in the hydrolysate a sample (100 mg) was methylated by the Haworth method[14] but again no methylated sugars were observed.

In an attempt to separate the "fucan" from the alginic acid, a sample of the extract was treated with Fehling's solution (expt. 12) and two fractions were obtained. On hydrolysis it was found that the non-complexed fraction consisted of fucose, galactose, xylose and glucuronic acid while the complexed fraction contained the alginic acid as well as some of the "fucan". Even though amounts of "fucan" was found in the complexed fraction this may be a method to remove the alginic acid in this and other fractions.

The 0.5M KCl fraction

Analysis of a hydrolysate by paper chromatography showed the presence of fucose, galactose, xylose and glucuronic acid. The chromatograms showed the same pattern as that found for the "fucan" in the aqueous extract, thereby confirming the expected similarities.

Conclusions and comparison with other brown seaweeds

As lack of time permitted only preliminary studies of *D. firma* the conclusions will mainly concern the results from *D. ligulata*.

Both the present *Desmarestia* samples were found to contain mannitol as the main ethanol-soluble material, as previously found for *D. aculeata*[7] and other brown seaweeds.

The galactose and xylose containing oligosaccharides found in the ethanolic extract of *D. firma* and the galactose, xylose and mannose containing oligosaccharides found in the ethanol extract of *D. ligulata*

appear to be unique to these two algae. They are possibly precursors of the "fucans" in these weeds and since neither fucose nor glucuronic acid were found in this extract it is possible that these polysaccharides are side chains to be linked on to a fucose/glucuronic acid backbone.

The ethanolic extract of D. aculeata[7] was found to contain mannitol, sucrose, laminitol, rhamnose, sedoheptulose, glucose, fructose, 2-O-methyl- and 3-O-methyl fucose. Apart from glucose (in D. firma) and mannitol none of these compounds were found in the ethanolic extracts from the two present Desmarestia species.

The laminaran obtained from D. aculeata and D. ligulata were both shown to contain mannitol end groups while the laminaran from D. firma and Himanthalia lorea[15], Bifucaria bifurcata[15] and Padina pavonia[15] previously investigated in this laboratory were all found to be devoid of mannitol.

The laminaran from D. firma and D. aculeata was found in both the acidic as well as the aqueous extract and these two algae were also found to have a much higher laminaran content than the other four weeds mentioned above (see table 26). Methylation studies of these two laminarans indicates that they both contain a proportion of 1,3,6-linked branch points.

While the "fucans" from H. lorea, B. bifurcata and P. pavonia were found to contain fucose, xylose, glucuronic acid and only traces of galactose, the "fucans" from the three Desmarestia species were found to contain the same sugars but larger proportions of galactose. Furthermore the "fucan" from D. ligulata was found to contain a fairly high proportion of mannose. "Fucans" containing fucose, mannose,

galactose, xylose and glucuronic acid have been isolated from *Cystoseira barbata*[16], *Sargassum linifolium*[17], *Sargassum pallidum*[18] and *Pelvetia wrightii*[19]. Evidence has been obtained to indicate that the three latter algae have a glucuronomannan backbone. Furthermore they have all been shown to be highly branched and sulphated polysaccharides.

The "fucans" from three *Desmarestia* species were found to have basically the same elution pattern from a cellulose column. The results obtained indicate that they are polydisperse and comprise a family of macromolecules all built up on the same general plan but containing different proportions of uronic acid and half ester sulphate. The linkages of "fucan" polysaccharides will be discussed in the next section.

The alginic acid in *D. aculeata*[6] has been found to contain about 46% mannuronic acid while the highest mannuronic acid content found in the alginic acid from *D. ligulata* was 33%. Exhaustive extraction of the alginic acid from *D. ligulata* gave an alginic acid with low viscosity as well as low mannuronic acid content, thereby indicating that the mannuronic acid regions of this alginic acid are more easily degraded than the guluronic acid regions. Preliminary studies of the alginic acid from *D. firma* indicate that the same is true for this alga. The mannuronic acid found in the acid extract from this alga is a further explanation of the low mannuronic acid content in the isolated alginic acid. The presence of alginic acid in the acid extract of both *D. firma* and *D. ligulata* (traces) suggests that the free sulphuric acid present in these algae have degraded/hydrolysed the alginic acids at least to some extent. The presence of amine during the neutralisation of the sulphuric acid in *D. firma* might

also be responsible for some degradation/hydrolysis of the alginic acid in this alga.

D. ligulata was, as expected for brown seaweeds, found to contain cellulose.

In the following table the relative yields of the different polysaccharides of some brown seaweeds are given.

Species	Laminaran	"Fucan"	Alginic acid	"Fucan" (Na_2CO_3)
Himanthalia lorea	0.02	18	16	1.8
Padina pavonia	0.07	5.0	13	2.0
Demarestia aculeata (August)	5.6	2.2	12	12.5
(March)	1.8	4.8	16	2.2
Desmarestia ligulata	0.08	5.6	13.9	0.6
Desmaresita firma	1.8	2.8*	-	-

* Alginic acid as well as "fucan"

Table 26

It can be seen from this table that the yields of the different polysaccharides from D. ligulata does not show any significant difference from those from the other algae in this table, thereby implying that the presence of the free sulphuric acid in this alga does not change the metabolic pathways from those of other brown seaweeds. No trace of free sulphuric acid was found in D. aculeata[7].

The preliminary studies of D. firma on the other hand indicates a very low content of "fucan" compared to the other weeds.

It has not been possible to find any explanation for the high free sulphuric acid content (about 6% in D. firma).

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Chapter V Investigations of "fucans" from Himanthalia lorea and Padina pavonia

As the investigations of the rhamnans (page 74) had shown that sulphated rhamnans can undergo the alkali degradation reaction it was decided to try this reaction on some of the "fucans" previously investigated in this laboratory. All the linkage analysis previously carried out on these "fucans" has been by g.l.c. of the methylated derivatives, and as many of the different methylated derivatives have similar retention times on g.l.c. it was decided to analyse the methylated sugars as their alditol acetates by g.l.c.-m.s.

"Fucan" samples from Himanthalia lorea and Padina pavonia (hereinafter called H. "fucans" and P. "fucans" respectively) were used since these "fucans" both contain only fucose, xylose and glucuronic acid and the results should therefore be easier to interpretate than similar results from "fucans" containing additional sugars.

Investigations of the different "fucans"

Two samples of 0.3M KCl H. and P. "fucans" (fractionated previously on a cellulose column from acid extracts[1]), a sample of M KCl P. "fucan" (separated during this work by a cellulose column from an acid extract as previously described[1]) and a sample of a highly purified H. "fucan" isolated[2] in 1948 were investigated.

Experiment 1 Characterisation of the 0.3M KCl H. and P. "fucans"

The uronic acid [GM VI (ii) (c)], carbohydrate [GM VI (i)] and sulphate [GM VI (iii)] content of the two "fucans" were estimated. Two aliquots (about 100 mg each) were hydrolysed [GM II (i)] and separated

on 3MM paper in solvent (a). The appropriate strips were eluted and the proportions of the different sugars estimated by the phenol sulphuric method [GM VI (i)].

Experiment 2 Reduction of the uronic acid in the 0.3M KCl *H.* and *P.* "fucans"

An aliquot (about 50 mg) of each "fucan" was dissolved in water (about 100 ml) and reduced *via* the carbodiimide complex [GM VII (x)]. The uronic acid contents of the two reduced "fucans" were estimated [GM VI (ii) (c)].

Experiment 3 Methylation of the reduced and unreduced 0.3M KCl *H.* and *P.* "fucans"

An aliquot (about 100 mg) of each of the unreduced "fucans" was methylated by the Hakomori method [GM VII (viii)]. An aliquot (about 10 mg) of each methylated "fucan" was hydrolysed [GM II (i)], converted into the alditol acetate [GM VII (vii)] and analysed by g.l.c. - m.s. [GMV A (ii), B (iv), C (ii)].

An aliquot (about 50 mg) of each of the reduced "fucans" was methylated by the Hakomori method. After methylation and dialysing the content of the dialysis sac was extracted with chloroform and the two extracts taken down to the dryness and after their carbohydrate contents were estimated they were analysed as described above for the unreduced "fucans".

Experiment 4 Alkaline degradation of the two methylated unreduced *H.* and *P.* "fucans"

The methylated fucan (50 mg) and toluene-p-sulphonic acid (trace) was dissolved in a mixture of DMSO and 2,2-dimethoxypropane (19:1) (4 ml)

and carbanion [GM VII (iii)] (2 ml) was added. The solution was stirred overnight after which an excess of 50% aqueous acetic acid was added. The mixture was poured into water (50 ml) and extracted with chloroform. The $CHCl_3$ and aqueous solutions were taken to dryness, hydrolysed [GM II (i)] and analysed as described in expt.3.

Experiment 5 Methylation of the MKCl P. "fucan"

An aliquot (101 mg) of the MKCl P. "fucan" was methylated and analysed as described in expt. 3.

Experiment 6 Alkaline degradation of the methylated MKCl P. "fucan"

The methylated M KCl P. "fucan" (50 mg) was subjected to the alkaline degradation procedure as described in expt. 4.

Experiment 7 Methylation of the purified H. "fucan"

An aliquot (70 mg) of the purified H. "fucan"[3] was methylated as described in expt. 3.

Experiment 8 Alkaline degradation of the methylated purified H. "fucan"

The methylated purified H. "fucan" (40 mg) was subjected to the alkaline degradation procedure as described in expt. 4 with the following modifications. The reaction mixture was dialysed for 3 days after the degradation and the carbohydrate of the dialysate estimated. The content of the dialysis sac was taken to dryness and given a mild hyrolysis with 50% acetic acid at 100° for 1 hour. The solution was taken to dryness again, the reaction mixture was reduced with sodium borodeuteride and hydrolysed and analysed as described in expt. 4.

Results and discussion

In the first experiment the 0.3M KCl fractionsfrom the acid extracts of the H., P. "fucans" were examined. The H. "fucan" was found to consist of about 67% glucuronic acid, 20% fucose, 10% xylose and 2.5% sulphate, while the proportions of the P. "fucan" was found to be about 45% glucuronic acid, 35% fucose, 15% xylose and 2.5% sulphate. To obtain information about the linkages of the glucuronic acids in the two "fucans" an aliquot of each was subjected to reduction by the carbodiimide method (expt. 2) before methylation. The uronic acid content of the H. "fucan" was reduced from 67% to about 30% and of the P. "fucan" from 45% to about 20%. The results from the methylation experiments (expt. 3) are tabulated in table 27. The reduced "fucans" were extracted with chloroform after methylation and this table also indicates from which extract the different sugar derivatives were characterised. (About 1/5 of the recovered materials were found in the aqueous fractions).

Only small amounts of methylated sugars could be charaterised from the unreduced P. "fucan" and none at all could be characterised from the unreduced H. "fucan" thereby confirming the difficulties of methylating a polysaccharide with a high uronic acid content.

As can be seen from table 27 the same methylated sugars were found in these two "fucans". The xylose and glucuronic acid residues are mainly 1,4-linked with smaller amounts of end groups. The fucose residues are mainly 1,3-linked with smaller amounts of 1,4-linked residues and end groups as well as traces of 1,2-linked residues in the P. "fucan". The possible branched/sulphate residues are 1,2,3,4- 1,2,3- or 1,3,4-linked and only traces of 1,2,4-linked residues could be found. The large amount of unmethylated fucose present is probably

Alditol acetates	H. "fucan"		P. "fucan"		
	Reduced		Non reduced	Reduced	
	Water	Chloro-		Water	Chloro-
2,3,4-tri-O-methyl fucose	X	X	X	-	X
2,4-di-O-methyl fucose	X	X	X	XX	X
3,4-di-O-methyl fucose				trace	
2,3-di-O-methyl fucose			X	X	X
2-O-methyl fucose	X		X	X	X
3-O-methyl fucose		trace			trace
4-O-methyl fucose			X	XX	X
Unmethylated fucose	XX		XX	XX	
2,3,4-tri-O-methyl xylose				X	
2,3-di-O-methyl xylose	X	X		X	X
2,3,4,6-tetra-O-methyl glucose	X	X		X	X
2,3,6-tri-O-methyl glucose	X	XX	X	X	XX

XX = major constituent

X = minor constituent

Table 27

due mainly to undermethylation because of steric hindrance but it is also probable that the "fucans" are highly branched with all the hydroxyl groups in some residues involved in linkage and/or sulphation.

The alkaline degradation technique (expt. 4) was attempted on these two "fucans" but due to the high glucuronic acid content and

probably the presence of 1,3-linked fucose residues they were both completely degraded.

No other fractions of this H. "fucan" were available and this "fucan" was therefore not investigated further. A sample of the acid extract from P. pavonia was available and this sample was fractionated on a cellulose column and the M KCl fraction separated. This fraction has been shown to contain about 40% carbohydrate, 5% uronic acid and 17% sulphate. On methylation (expt. 5) the following methylated sugars were characterised as their alditol acetates (in order of descending peak area).

Before degradation	Sugars characterised	After degradation
X	Unmethylated fucose	X
X	4-O-methyl fucose	X
X	2,4-di-O-methyl fucose	X
X	2-O-methyl fucose	X
X	2,3-di-O-methyl fucose	X
X	2,3,4-tri-O-methyl fucose	
X	2,3-di-O-methyl xylose*	
X	2,3,4-tri-O-methyl xylose*	

* The two methylated xylose derivatives were only found in small quantities.

Table 28

This methylated P. "fucan" was subjected to the alkaline degradation procedure (expt. 6) and only fragments were found in the

chloroform extract, while the methylated sugars characterised as their alditol acetates from the remaining water extract are tabulated in table 28.

The main differences between the g.l.c. spectra of the methylated alditol acetates before and after degradation are that the spectrum after degradation does not contain any end group fucose or any xylose derivatives and that the unmethylated fucose peak is relatively much larger in the latter spectrum.

The relative higher proportion of unmethylated fucose after the degradation indicates that the molecule has a highly branched fucose backbone that is not affected by the uronic acid degradation. This suggests that the uronic acid molecules are mainly situated at the outside of the molecule and that they are, to some extent, linked to fucose molecules through C-4. Since neither xylose nor end group fucose derivatives could be found after the degradation they must either be linked directly to C-4 of uronic acid groups or be part of chains that are degraded by the reaction. This indicates that the xylose as well as the glucuronic acid are situated at the outside of the molecule.

To verify the sugar linkages and to try to establish the neighbouring groups of the uronic acid (linked through C-4) a sample of a highly purified "fucan" from H. lorea[2] was investigated. This "fucan" contains 56.7% fucose, 4.1% galactose, 1.5% xylose, 3.3% glucuronic acid and 38.3% sulphate.

Part of the "fucan" was methylated and given the alkaline degradation treatment (expt. 8). The methylated sugars characterised after the degradation as their alditol acetates are shown in table 29 (in order of descending peak areas).

Sugar
2-O-methyl fucose
2,3-di-O-methyl fucose
2,4-di-O-methyl fucose
4-O-methyl fucose
unmethylated fucose
2,3,4-tri-O-methyl fucose
2,3-di-O-methyl xylose
2,3,6-tri-O-methyl galactose

Table 29

These results show that the fucose residues are mainly 1,3- and 1,4-linked with smaller proportions of end groups. The possible branched/sulphated fucose residues are mainly 1,3,4-linked with smaller amounts of 1,2,3- and 1,2,3,4-linked residues, while the galactose and xylose are both found to be 1,4-linked.

The high proportion of sulphate in this "fucan" and the relative amount of end groups and tri- and tetra-linked fucose residues characterised makes it highly probable that these multiply linked residues are sulphated rather than branched. This indicates that this "fucan" is a basically linear polysaccharide and by comparison with the other H. and P. "fucans" investigated in this section it is possible that they all have a linear fucose backbone.

The reaction mixture was dialysed after the degradation experiment and only neglible amounts of carbohydrate were found in the dialysate thereby confirming that little degradation had taken

place. This again indicates that the small amount of uronic acid residues are situated at the outside of the molecule. The relatively small amount of galactose characterised after degradation indicates that much of the galactose residues are situated fairly close to the uronic acid and are therefore degraded.

After the mild hydrolysis the reaction mixture was reduced with sodium borodeuteride in an attempt to label some of the residues at the end of the degraded chains formally linked through C-4 of the uronic acid (see page 79). Unfortunately large amounts of deuterated residues were found in the peaks of all the methylated fucose derivatives and taking into account the small uronic acid content of this "fucan" it seems probable that the 50% acetic acid hydrolysis has hydrolysed quite a number of the glycosidic linkages in the molecule, thereby making it impossible to obtain any information from this experiment. In future alkaline degradation experiments with "fucans" it will therefore be necessary to partially hydrolyse with a weaker acid.

The investigations of the H. and P. "fucans" indicates that the fucose residues in these molecules are mainly 1,3- and 1,4-linked with smaller proportions of end groups. The branched/sulphated fucose residues are either 1,2,3,4-, 1,2,3- or 1,3,4-linked, and the xylose, glucuronic acid and galactose residues were found to be 1,4-linked and end groups.

In the previous investigations of the "fucans"[1] mass spectrometry was not available and the linkages were determined by g.l.c. analysis of methylated sugar derivatives. The mass spectrometry analysis of these methylated derivatives agrees with

those obtained earlier but there are also some important differences.

The linkages of the xylose, glucuronic acid and galactose residues were found to be the same in both investigations. Unmethylated, end group and 1,3,4-linked fucose were also found in both samples, while the 1,2-linked and 1,2,4-linked fucose residues previously reported[1] could not be detected in the present "fucans". This is probably due to the similar retention times of the three dimethylated fucose derivatives and the similar retention times of the 3- and 4-O-methyl fucose derivatives whereas m.s. analysis enables complete identification of these derivatives.

1,4-Linked fucose residues have previously been reported in the "fucan" from Fucus vesiculosus[3]. The main methylated fucose derivative from this "fucan" was found to be 3-O-methyl fucose, but taking the similar retention times into consideration some of it might have been the 4-O-methyl fucose derivative by comparison with the present "fucans".

During the present investigations indications have been found for the presence of a linear fucose backbone in these H. and P. "fucans".

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Appendix I Linkage analysis of an *Ulva lactuca* rhamnan

A sample of a desulphated and uronic acid reduced rhamnan from *Ulva lactuca*[1] was methylated by the Hakomori method and the following methylated sugars characterised as their alditol acetates (in order of descending peak areas).

Sugar
2,3-di-O-methyl xylose
2,3-di-O-methyl rhamnose) **
2,4-di-O-methyl rhamnose)
2,3,6-tri-O-methyl glucose
2,3,4,6-tetra-O-methyl glucose
Unmethylated rhamnose
2,3,4-tri-O-methyl rhamnose
2-O-methyl rhamnose*
3-O-methyl rhamnose*
4-O-methyl rhamnose*

* Trace quantities

** The two dimethylated rhamnose derivatives have similar retention times and are therefore taken as one peak, though m.s. analysis shows that only small amounts of 2,4-di-O-methyl rhamnose is present.

Table 30

These results show that the rhamnose residues are mainly 1,4-linked with a small proportion of 1,3-linked and end group residues. Trace quantities of rhamnose derivativesmultiply linked through every hydroxyl group in every possible way have also been found. The xylose residues were found to be 1,4-linked and the glucuronic acid residues 1,4-linked and end groups.

These results correspond in the main to those obtained from previous methylation studies[1,2], though the previously reported 1,3-linked glucuronic acid and 1,3- and triply linked xylose could not be found in this experiment.

The small amount of end group and multiply linked residues found in this rhamnan indicates that it is basically a linear polysaccharide which is in contradiction with the earlier reported highly branched structure[1].

References

1. Haq, Q.N., Percival E., Proc. 5th int. Seaweed Symp. (Young E.G., McLachlan J.L., eds. Pergamon press 1965) 261.

2. Brading J., Georg-Plant M.M.T., Hardy J., J. Chem. Soc. (1954) 319.

Appendix II Reduction of alginic acids

It has been found during the present work that reduction of uronic acids with sodium borohydride *via* the carbodiimide complex have been very satisfactory. It was therefore decided to try this method in the hope of developing a new method for determining the guluronic to mannuronic ratio by determining the ratio of the reduced sugars.

A number of different sodium alginates supplied by Alginate Industries were tested and they were reduced once and twice. The recoveries and the uronic acid content from three typical samples are shown below in table 31.

Sample	Reduced once		Reduced twice	
	Recovery %	Uronic acid Content %	Recovery %	Uronic acid Content %
1	53	42	49	18
2	51	38	46	16
3.	48	40	45	21

Table 31

As can be seen from this table the yields from these experiments are too low to be certain that the products have the same proportions of the constituent sugars as the original materials and they are therefore of little value.

These experiments were therefore not taken further.

Methylation of the reduced polymers by the Hakomori method showed as expected only small amounts of end group hexose and large amounts of 1,4-linked hexose characterised as the methyl-glycosides and alditol acetates.