CARBOHYDRATES OF THE BROWN SEAWEEDS

DESMARESTIA FIRMA AND DICTYOPTERIS PLAGIOGRAMMA,

AND OF THE DIATOM COSCINODISCUS NOBILIS

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Carbohydrates of the brown seaweeds Desmarestia firma and Dictyopteris plagiogramma, and of the diatom Coscinodiscus nobilis .

by Md. Anisur Rahman.

ABSTRACT

Dictyopteris plagiogramma: The carbohydrates of this weed have been chemically studied for the first time. Sequential extraction of the weed gave a mixture of laminaran and 'fucan' in the aqueous extract and a mixture of alginic acid and 'fucan' in the alkaline extracts.

A pure laminaran was separated on a DE-52 cellulose column and shown to comprise a (1→3)-linked β-glucan containing 1.4% mannitol. The 'fucan' contained fucose, glucuronic acid, xylose, galactose, mannose and half ester sulphate.

A variety of fractionation procedure gave 'fucans' with varying proportions of constituents and half ester sulphate.

The alginic acid from the sequential extraction was shown to be highly degraded, but an alkaline extract of a fresh sample of weed contained a less degraded alginate in which the mannuronic/guluronic acid ratio was ca. 60:40.

Methylation and periodate oxidation studies established the similarity of linkage pattern with other 'fucans'.

Alkaline desulphation of Dictyopteris 'fucan' and 3 'fucans' from other seaweeds revealed, after hydrolysis, the presence of a monosaccharide which was characterised as a 6-deoxyhexose indicating that $SO_4^=$ was attached to the fucose molecule at C-2 and/or C-3. Sodium methoxide treatment of the desulphated fucan followed by identification of 2-O-methyl fucose confirmed that $SO_4^=$ was attached to the interchain fucose residue at C-2.

Desmarestia firma: The sequential alkaline extract from this weed was also shown to comprise 16% of a highly degraded alginic acid and 1.8% of crude 'fucan'. Direct alkaline extraction yielded 19.8% of relatively higher viscosity alginate.

Coscinodiscus nobilis: After culturing the diatom under bacteria free conditions, 50 mg of extracellular polysaccharide and 30 mg of diatom were recovered. The former was shown to comprise a highly branched hetero-polysaccharide containing fucose, rhamnose, mannose, D-glucose, xylose, D-glucuronic acid, galactose (trace) and half ester sulphate. The positions of linkages between the monosaccharides have been established and evidence for the linkages between D-glucuronic acid and monosaccharides was obtained. The extracellular polysaccharides contained also a chrysolaminaran, but this may have been derived from dead cells. Fucose and mannose occur also in a separate polymer.

The diatom contained polysaccharide material consisting of glucose, mannose, fucose and uronic acid residues.

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PART-ONE

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Algae are a widely distributed class of plant life belonging to the lowest division (Thallophyta) which have attained about the same level of rudimentary development, and which possess chlorophyll(a); carry on photosynthesis, and are therefore independent to make their own food. They have a great diversity of form, some are single celled and may be motile or nonmotile; these unicellular forms again differ greatly in shape and usually are microscopic in size. In some the individual cells may become grouped into relatively loose aggregations known as colonies, which may be filamentous (threadlike), such as Spirogyra; spherical or platelike such as Chlamydomonas. In many of them however, the plant body is multicellular with the cells arranged end to end forming a branched filament, for example Cladophora. In certain groups numerous filaments are compactly organised to form a tissue which in cross-section resembles the parenchyma of higher plants as found in Macrocystis pyrifera and Lessonia flavicans.

1.2 Although algae may occur in variety of places, in the soil, in moist places such as rocks, cliffs, walls and trees, they occur mainly in salt water. As they are essentially photosynthetic organisms, their occurrence is therefore restricted to those parts of the sea where light is available. They are found either as a part of the free floating and free swimming organisms which make up the plankton or as benthic organisms lying on the bottom, whereas the larger algae are found attached to rocks.

1.3. Many of the algae are of considerable economic importance for providing food for fish, cattle and man. The larger species are used as soil improvers and fertilisers due to their gelatinous nature and to the presence of iodine, potassium and other minerals. Some are used as

sources of gelatinous substances such as agar, carrageenan and alginate. The two former as substrates for the culture of bacteria and all three as congealing agents for desserts and confections, as ingredients of cosmetics, dentifrices, shaving creams, shampoos and other industrial products.

1.4. Algae are commonly classified into several major groups on the basis of their colour. Although all the marine algae contain chlorophyll(a) the colour of the chlorophyll in some classes is masked by strongly coloured pigments. This has resulted in four main classes, namely brown (Phaeophyceae); green (Chlorophyceae); red (Rhodophyceae) and blue-green (Cyanophyceae) algae. In addition there are a number of other divisions including the Bacillariophyceae or diatoms.

The brown and red algae are found almost exclusively in marine habitats and are usually referred to as seaweeds. Nearly all species of Rhodophyceae are coloured bright red by biliproteins and the Phaeophyceae derive their brown colour from Xanthophyll, fucoxanthin.

1.5. The carbohydrates present in algae can be roughly divided into the following three groups.

A. Low molecular weight carbohydrates (soluble in 80% Ethanol).

B. Water soluble polysaccharides
   (a)Food reserve material
   (b)Other soluble polysaccharides.

C. Cell wall material or structural polysaccharides.

This thesis will deal only with marine algae belonging to the Phaeophyceae and Bacillariophyceae (diatoms). The different types of carbohydrates metabolised by these classes will be discussed in detail in Part I and Part II, respectively. A brief description of carbohydrates of the other two main classes of marine algae, namely Rhodophyceae and Chlorophyceae is being given here.

1.6. Carbohydrates of Rhodophyceae

1.6.1 Low molecular weight carbohydrates

A galactoside, named floridoside, which is a 2-O-α-D-galactopyranosyl glycerol is present in many species of Rhodophyceae,[2,3] and seems to be an end-product of photosynthesis and a reserve material in the red algae.[4] In some species 2-O-α-D-mannopyranosyl-D-glyceric acid seems to be more important sugar-derivative, e.g. in Polysiphonia.[5,6] In addition, O-α-D-mannopyranosyl-(1→3)-O-α-D-galactopyranosyl-(1→2)-glycerol and 1-O-α-D-galactopyranosyl glycerol (isofloridoside) have been isolated from several red algae[7] and various alditols and inositols have also been found in some species.

1.6.2 Water soluble polysaccharides

(a) Food reserve material

Floridean starch. This reserve polysaccharide is present in the shape of characteristic granules. It was first isolated from Furcellaria fastigiata by Kylin in 1913, and shown to be a glucan which is related structurally to amylopectin of land plants.[8] It is essentially an α-(1→4)-linked glucan with (1→6)-branch points and an average chain length of 9-15,[9] though a small proportion of α-(1→3)-linkages has been reported in some species.

(b) Other soluble polysaccharides

The water soluble polysaccharides from the red algae are mainly galactans. They are essentially linear polymers of alternating (1→3)- and (1→4)-linked galactose units. This simple pattern is often masked by other features, depending on the alga from which the polysaccharide has been isolated. The differences are due to the presence of both D and L-galactose, 3,6-anhydro-D and L-galactose, 6-O-methyl-D-galactose, and occasionally galactose substituted at O-4 and O-6 with pyruvic acid and various degrees of sulphation.[10] These units offer

a broad spectrum of polysaccharides comprising chains of alternate (1→3)- and (1→4)-linked galactose or modified galactose units.[11] The individual polymers differ in their finer details of structure possibly due to a particular environment.[12]

The galactans can be divided into three different groups of polysaccharides, namely agar, porphyran and carrageenan type, although polysaccharides related to more than one of these types have been found.[13,14]

Agar

Agar is synthesised by a wide variety of species of the red algae, such as Gelidium and Gracilaria species generally called agarophytes. It is the common name for a mixture of polysaccharides, which originally were thought to consist of two polysaccharides only, namely the neutral agarose and the sulphated agaropectin.[15] Comprehensive recent studies however, have indicated that it is composed of a complex series of related polysaccharides ranging from an almost neutral to a highly charged galactan. The extremes of structure can be defined as follows.

(A). Neutral agarose.

This consists of chains of alternating (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked-β-D-galactose.

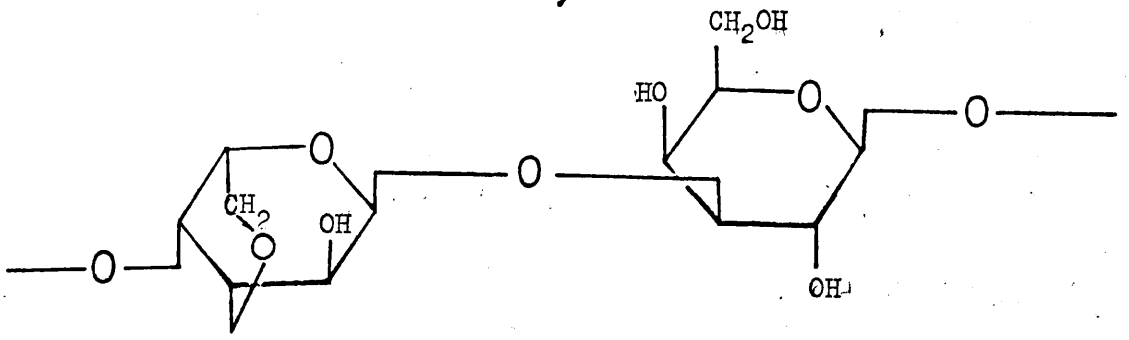


Fig. 1.1

(B). Pyruvylated agarose with a little sulphate

The molecules with substitution of pyruvic acid consist of the same alternating pattern with about one in twenty D-galactose residues being pyruvylated as 4,6-O-(1-carboxy-ethylidine)-D-galactose units[16] 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. 1.2.

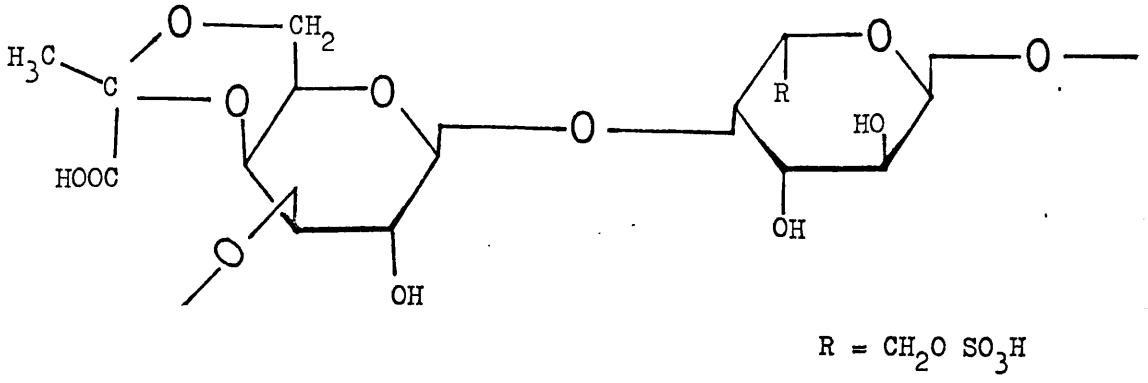


Fig.1.2

(C). A highly sulphated galactan

These molecules contain little or no 3,6-anhydrogalactose or pyruvic acid but consist of alternating (1→3)- and (1→4)-linked galactose residues mono- and disulphated.

The above classification represents only the extreme case, recent fractionation studies on DEAE-Sephadex[17] have indicated that agar is not made up of one neutral and one charged polysaccharide, but is composed of a complex series of related polysaccharides ranging from almost neutral to a highly charged galactan. Further evidence of heterogeneity was obtained by fractionation on Dowex 1 x 2 ion exchange columns.[18]

Carrageenan

Carrageenan is extracted from red algae such as Chondrus crispus, Gigartina and Eucheuma species. It differs from agar mainly in that 3,6-anhydro-D-galactose replaces the 3,6-anhydro-L-galactose of agar,[19,20] and has a higher content of mainly alkali stable ester sulphate.[21] Smith and Cook[22] separated the carrageenans by fractional precipitation

with potassium chloride into an insoluble fraction k-carrageenan, and a soluble fraction λ-carrageenan.

The repeating unit of k-carrageenan consists of chains of alternate (1→3)-linked-D-galactose-4 sulphate and (1→4)-linked-3,6-anhydro-D-galactose (see figure 1.3).

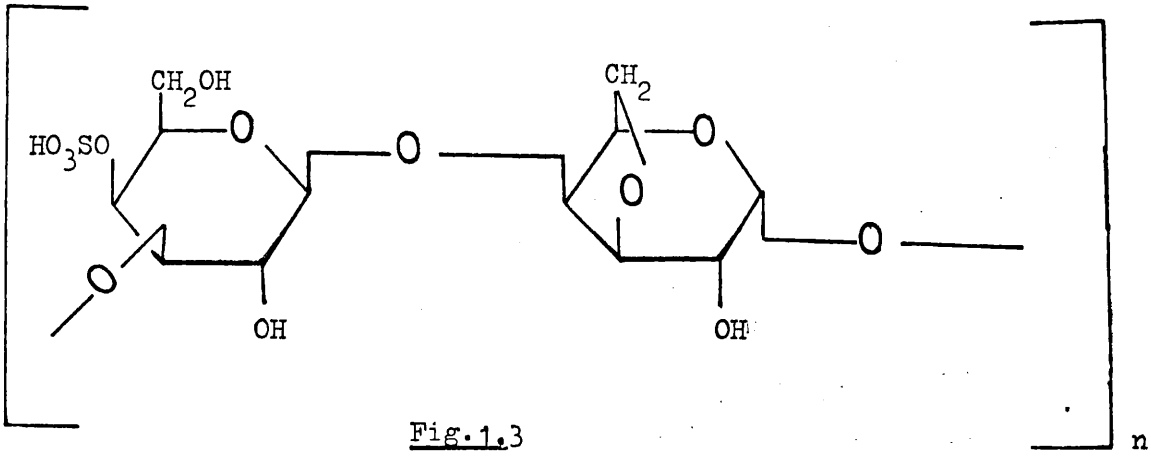


Fig.1.3

λ-Carrageenan, the soluble fraction obtained by potassium chloride fractionation, represents a very complex system. It is highly sulphated, contains mainly D-galactose, but also some 3,6-anhydro-D-galactose. It may be fractionated further by precipitation with ethanol, thus giving a polysaccharide containing only galactose,[23] this consists of highly sulphated alternating (1→3)- and (1→4)-linked galactose units. Some of the sulphate is alkali labile, yielding 3,6-anhydrogalactose indicating its presence on C-3 or C-6 of (1→4)-linked units.

Porphyran

This type of polysaccharide is mainly found in Porphyra and Laurentia species. It resembles agarose in containing 3,6-anhydro-L-galactose and 6-O-methyl-galactose and resembles carrageenan in containing galactose-6-sulphate.[24] Porphyran consists mainly of β-(1→3)-linked-D-galactose or 6-O-methyl-D-galactose residues alternating with

α-(1→4)-linked-L-galactose-6-sulphate or 3,6-anhydro-L-galactose,[25] the proportions of the anhydro sugar again varying with the season and environment.[26] The mucilage obtained from Anatheca dentata (a member of the Grateloupioceae) is one example of a polysaccharide not belonging to any of the three just mentioned groups. This polysaccharide has the "backbone" structure of a type (C) agar, but the high sulphate content (ca. 30%), 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.[27] A recent investigation of the extracellular mucilage produced by Rhodella maculata,[28] a microscopic unicellular red alga, showed that it comprised a sulphated heteropolysaccharide containing mainly xylose and glucuronic acid together with smaller proportions of 3-O-methyl-xylose, galactose and glucose. The xylose units are (1→3)- and (1→4)-linked, and glucuronic acid is (1→2)-linked. Glucuronic acid is also present as end groups together with (1→3) and (1→4)-linked galactose, while branch points are occupied by galactose linked through C-1, C-3 and C-4 as well as C-1, C-3 and C-6. Though water soluble xylans and mannans have been obtained from red seaweeds these will be discussed under the structural headings.

1.6.3. Structural polysaccharides

Cellulose: The cellulose content of the red algae is very small and it is present as the cell wall component.[29,30] The structure of the cellulose from Gelidium amansii was found to be similar to that of cotton except for its colloidal properties.[31]

Mannan: Mannan from Rhodophyceae is largely obtained by hot concentrated alkali extraction, although a small proportion may be extracted by cold

water and dilute alkali. The different extracts are structurally similar and differ only in their molecular size or shape. A β-(1→4)-linked mannan with an average chain length of about twelve has been extracted from the cell wall of Porphyra umbilicalis,[32] while a sulphated α-(1→3)-linked mannan was found in the aqueous extract of a Nemalion vermiculare Sur sample.[33,34]

Xylan: The xylans of Rhodophyceae are mainly of two types, either cell wall material or water soluble. The first type is essentially linear and consists of either β-(1→3)-linked[32] or β-(1→4)-linked[35] 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.[35]

1.7. Carbohydrates of Chlorophyceae

1.7.1. Low molecular weight carbohydrates

The main low molecular weight carbohydrate in green algae seems to be sucrose.[36] Sucrose, glucose and fructose have all been found in all the species investigated. In addition small quantities of other sugars, some alditols and myoinositol have been found in a few species.[37,38]

1.7.2. Water soluble carbohydrates

(a) Food reserve material. 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.[39] Aqueous extraction yields these glucans together with sulphated heteropolysaccharides. They can be separated as starch-iodine complexes or as glucans left in aqueous solution after complexing the sulphated

polysaccharides with cetyltrimethyl ammonium hydroxide. The structures of the glucans have been established through enzymic, periodate-oxidation, methylation and X-ray studies.[39] The main difference between the starches from the green seaweeds and those from the land plants seems to be that the former have a lower molecular weight and hydrolyse more easily than the latter.[40]

Fructans have also been found in various green algae and they are thought to be the food reserve material.[41] In Acetabularia crenulata[42] the fraction was shown to be of the inulin type, i.e. a linear (2→1)-linked polysaccharide with average molecular size varying from 33 to 62 units.

(b) Other soluble polysaccharides

According to Preston,[43] these polysaccharides are mainly constituents of the continuous amorphous phase of the cell walls and comprise 30-70% of the dry weight in different algae.[44] 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,[45] Chaetomorpha,[46] Caulerpa,[47] Codium[48] and Rhizoclonium.[49] The second group contains mainly rhamnose, xylose and glucuronic acid and has been obtained from Enteromorpha compressa,[50] Ulva lactuca,[51] Acrosiphonia arcta[52] and Urospora penicilliformis.[53] The polysaccharide from Acetabularia crenulata[54] 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 ($O.SO_3H$) 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 homopolysaccharides have been unsuccessful.

Cladophora rupestris[45] was the first alga in this group to be studied in detail. These studies established the major linkages, galactose linked through C-1 and C-3, as well as through C-1 and C-6, arabinose linked through C-1 and C-4, xylose linked through C-1 and C-4 as well as linked through C-1 (end group). 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.[46] 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).[55] 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[46] indicate very similar structures to that found in Cladophora, the major difference being a higher galactose content in the latter.

The polysaccharide from Codium fragile[48] 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.

The water soluble polysaccharides obtained from those 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[51] and Enteromorpha[50] and 8% in Acrosiphonia.[52]

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

The polysaccharide from Ulva lactuca has been most extensively studied. Methylation studies of the desulphated and carboxyl reduced polymer has indicated rhamnose linked through C-1 and C-4, as well as through C-1, C-3 and C-4, xylose linked through C-1 and C-4 as well as through C-1 and C-3. Tentative evidence for (1→3)-linked and (1→4)-linked glucose was also obtained.

It has been deduced from alkaline desulphation and periodate oxidation experiments that the position of sulphate ester is mainly at C-2 of rhamnose and to a lesser extent at C-2 of xylose.[57]

An acidic oligosaccharide 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.[56]

D-GlcAP(1→4)L-Rhap(1→4)D-GlcAp(1→3)D-Xylp(1→4)L-Rhap(1→3)
2S 2S 2S

D-Glucp(1→4)D-Xylp
6S

where D-GlcAp = D-glucopyranuornic acid.
L-Rhap = L-rhamnopyranose.
D-Xylp = D-xylopyranose.
D-Glucp = D-glucopyranose.
S = sulphate ester $S(=O)(=O)-O-$

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[42] 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 C-?-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.

1.7.3. Structural Polysaccharides

The main organic substances in cell walls are polysaccharides but it has been proved that proteins and lipids are also present.[58] The carbohydrate part of the cell wall in algae has normally a less complicated structure than the soluble polysaccharides. They can be divided into three groups, according to their constituent sugars, e.g. glucan (cellulose), xylan and mannan.

Glucan: X-ray diffraction studies[59] 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.

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.[60,61] 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.[62]

Mannan: Mannans have been shown to be present in the cell wall of certain green algae.[60] The mannans from Codium fragile[63] and Acetabularia crenulata[42] have been extensively studied and found to be β-(1→4)-linked mannans with indication of some degree of branching at C-2.

CHAPTER 2 GENERAL METHODS

2.1. Physical techniques:

(i) Evaporations were carried out under reduced pressure between 30 and 40°C with a 'Buchi' rotary evaporator.

(ii) Water. Deionised, distilled water was used in all experiments unless stated otherwise.

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

(iv) Dialysis was performed with Visking Cellophane tubing against distilled water with toluene added as a bacteriostat. The tubing was initially rendered free of glycerol by boiling with water.

(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 resins used were either Amberlite or Biodeminrolit.

(vii) Lyophilisation (Freeze-drying) was carried out on a 'Chem Lab' freeze-drier. 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.

(ix) Ultraviolet and visible absorbances were measured using a 'Pye Unicam' SP500 spectrophotometer.

(x) Molecular Sieves 3A and 4A molecular sieves (BDH) were activated by heating at 400° for 4 h.

(xi) Cotton wool bungs for stoppering of culture flasks were autoclaved at 30 p.s.i.

2.2. Purification of Common Solvents and Reagents

(i) Dimethyl sulphoxide was distilled from calciumhydride under reduced pressure so that the boiling point was about 80°. It was stored over activated 4A molecular sieve.

(ii) Chloroform was distilled from anhydrous sodium sulphate and stored, refrigerated, in brown glass bottles over anhydrous sodium sulphate.

(iii) Methanol ('Superdry') was prepared as described by Vogel,[67] distilled with the exclusion of moisture, and stored over activated 3A molecular sieve.

(iv) Methyl iodide was distilled from silver oxide and stored in the dark over silver oxide, activated by heating to 110° for 4h.

(v) Pyridine was distilled from sodium hydroxide pellets and stored over sodium hydroxide or potassium hydroxide pellets (BDH Analar).

2.3. Acid Hydrolysis

2.3.1. Formic acid method

The sample was dissolved in 90% formic acid and solid carbon dioxide was added to ensure an inert atmosphere. The tube was sealed and heated for 6 h at 100°C. The hydrolysate was diluted with water (5 vol.) and heated at 100°C for a further 2 h, in order to hydrolyse the formyl esters. The solution was evaporated to dryness at reduced pressure and residual formic acid removed by co-distillation with methanol.

2.3.2. Sulphuric acid method (Molar)

The sample was dissolved in M-sulphuric acid, and heated in a sealed tube for 4 h in boiling water bath. The hydrolysate was then neutralised either by adding solid barium carbonate or by shaking with 5% N-methyldioctylamine in chloroform.[68]

Neutralisation with barium carbonate was carried out as follows. The hydrolysate was cooled, and excess barium carbonate was added to it; the insoluble barium salts were then removed by centrifugation. The supernatant was deionised by shaking with IR 120 (H^+) resin followed by filtration and concentration at reduced pressure.

Neutralisation with N-methyldioctylamine was as follows. The hydrolysate was transferred to a separatory funnel and equal volume of 5% N-dioctylamine in chloroform was added to it. After vigorous shaking for a few minutes it was allowed to settle, and the organic layer was discarded. This operation was repeated. The aqueous layer was then again shaken with chloroform and the chloroform layer discarded. The aqueous layer was evaporated to dryness under reduced pressure.

2.3.3. 72% Sulphuric acid.[69]

Cold 72% sulphuric acid was added to the sample and the mixture left at room temperature for 1 h. Water (10 volumes) was added with cooling and the solution heated at 100°C for 4 h. After cooling the acid was neutralised with barium carbonate.

2.3.4. Trifluoroacetic acid method

The sample was dissolved in 2M-trifluoroacetic acid and the sealed tube was heated at 120°C in an oven for 2 h. The hydrolysate was then cooled, diluted with water and evaporated under reduced pressure; dilution with water and the subsequent evaporation was continued until the hydrolysate was neutral.

2.4. Chromatography

2.4.1. Paper chromatography

Paper chromatography was carried out by the descending technique using Whatman No.1 paper for general qualitative work and No.3 and No. 17 papers for preparative work (medium and large scale, respectively).

The maximum loadings for the two paper types were 100 mg per standard sheet for No.3 papers and 1000 mg per standard sheet for No. 17 papers.

No.17 papers were fitted with a wick of No.3 paper, and all preparative papers were washed before use with distilled water.

The method for the location of compounds on preparative papers was as follows.

After developing the papers in the chosen solvent system, three one-centimeter strips were cut from the air dried paper and these were sprayed with, or dipped in, the appropriate staining reagent. The position of the bands in these strips was then used to locate the material in the body of the paper.

The strips containing the separated material were cut from the chromatogram, stapled to paper wicks, and the compound was eluted from the paper with water.

The solvent systems used for paper chromatography were as follows.

(a) Ethylacetate: acetic acid: formic acid: water (18:3:1:4) by volume.[70]

(b) n-butanol: pyridine: water (6:4:3) by volume.[71]

(c) n-butanol: ethanol: water (40:11:19) by volume.[72]

(d) 2-butanone: acetic acid: water (9:1:1) by volume saturated with boric acid.[73]

2.4.2 Paper electrophoresis[134,135]

The Shandon high voltage electrophoresis apparatus L.24 was used with Whatman No.3 mm paper and the following electrolytes.

(a) Borate[74]

0.2M-sodium borate in water adjusted to pH 10 with sodium hydroxide. Electrophoresis was carried out for 2 h at 2.5 kV and 40 mamp. The non-migrating reference compound was 2,3,4,6-tetra-O-methyl-D-glucose.

(b) Pyridine/Acetic acid[75]

Pyridine (1-litre) adjusted to pH 6.8 with 5% acetic acid in water. The electrophoresis was carried out for 2 h at 3.0 kV. Glucose was the non-migrating marker.

(c) Borate with calcium ions.[76]

0.01M-sodium tetraborate (borax) in water containing 0.005M calcium chloride (pH 9.2). Electrophoresis was carried out for 2.0 h at 2 kV.

2.4.3. TGFP (Trimethylsilylated glass fibre paper) electrophoresis[77]

(a) Preparation of Trimethylsilylated glass fibre paper (TGFP)

Glass fibre paper (Whatman GF/C) was cut into strips and heated at 400°C for 2 h to burn out organic material, then immersed for 18 h in a shallow dish of CCl_4 containing 2% dimethyldichlorosilane, rinsed in toluene and dried. The TGFP strips were wetted by floating for 18 h in trays containing acetate buffer (pH 5) made of 0.1M-sodium acetate containing 0.02M-EDTA to which Tween-20 (0.2% by volume) had been added.

(b) Analytical electrophoresis

TGFP strips were blotted only lightly before being subjected to electrophoresis. Before application of the sample it was found advantageous to pre-equilibrate for 20 minutes at the running voltage.

The sample, usually 1-5 µg in ca. 2 µl of buffer, was applied at or near the midpoint of the strip. Separation conditions were, potential 1000 to 1200 volt, current 40-45 mamp, running time 20 to 40 minutes.

(c) Location and quantitation

When the separation was complete the strips were dried in air and the polysaccharides were located either by (i) dipping[78] first in a saturated toluidine blue solution in ethanol for 5 minutes, then in 40% aqueous ethanol for a further 5 minutes and finally washing in ethanol. Spots appear in pale blue background. Or (ii) by placing the strips[79] for 15 minutes in the fixing solution made of 20 ml 35% formaldehyde in 80 ml ethanol. After drying the strips were sprayed with a spray solution made of 0.04 g toluidine blue in 80 ml acetone and 20 ml water. The excess dye was first rinsed with a dip solution (5% acetic acid solution) and finally with water.

2.5. Staining reagents

The following staining reagents were employed for the location and identification of compounds on paper chromatograms and electropherograms.

2.5.1 Silver nitrate dip[80]

The developed paper was passed sequentially through solutions of saturated aqueous silver nitrate (2.5 ml) in water (10 ml) with acetone (500 ml); sodium hydroxide (20 g) in water (40 ml) and ethanol (960 ml); and finally in 10% aqueous sodium thiosulphate Air drying of the papers was allowed between dips.

2.5.2 (a) Aniline oxalate spray[81]

A saturated solution was prepared by adding aniline oxalate (25 g) in 50% aqueous ethanol (1 L) and stirring continuously at room temperature for 16 h.

(b) Aniline oxalate spray[82]

A saturated solution of aniline oxalate (25 g) in glacial acetic acid (1 L). The mixture was stirred overnight to obtain maximum dissolution.

2.5.3 Glucose oxidase[83]

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

2.5.4 Galactose Oxidase[83]

'Galactostat' kit (Worthington B ochemical Company) was made up as directed. A pink colour indicating the presence of D-galactose develops after 5 minutes at room temperature.

2.5.5 Periodate Permanganate spray[219]

The spray reagent was prepared freshly before use by mixing 4 parts of 2% aqueous sodium-metaperiodate and one part of 1% potassium permanganate in 2% aqueous sodium carbonate solution. This spray was used to locate the non-reducing sugars. If the paper was washed with water after the spots have finished appearing and before the c e llulose has discoloured the permanganate background, a permanent record of the chromatogram is obtained in the form of brown spots on an almost white background.

2.5.6 Urea hydrochloride[85]

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.

2.5.7 Ninhydrin

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

2.6. Gas liquid chromatography (g.l.c.)

2.6.1 Instrumentation

A Pye 104 gas chromatograph with nitrogen carrier gas and flame ionisation detector with glass columns (3 m x 5 mm) was used. [Column (i) for the TMS derivatives of the alditols, column (ii) for the alditol acetates and methylated alditol acetates].

2.6.2 The columns were packed with the following materials.

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

(ii) OV 225, 3% of cyanopropyl-methyl-phenyl-methyl silicone coated on 100-120 Gaschrom Q.

(iii) 3% of neopentylglycolsuccinate on Chromosorb W, 60-80 mesh.

2.6.3 Gas chromatography linked to Mass spectrometry[64,65,66]

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 20μA target current under a pressure of 10^{-6} torr. For CI spectra the 'ion source' was operated at about 150°C, 50 eV and 1000μA under a pressure of 0.4 - 0.5 torr with isobutane as the reactant gas.

2.7 Assays and Analyses

2.7.1 Carbohydrate content was assayed by the phenolsulphuric acid method.[84] 4% Phenol solution (1 ml) was added to water (1 ml) containing 10 - 100μg of sugar and concentrated sulphuric acid (5 ml) added rapidly. The colour developed was read at 487 nm on a Unicam SP 500 spectrophotometer. Standard graphs were prepared for different sugars and mixtures of sugars in the ratios corresponding to those of the particular polysaccharide.

2.7.2 Uronic acid determination was carried out by two different methods.

(a) Modified carbazole reaction[85]

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 recooled. After mixing the sample was heated for 20 minutes 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 minutes and then cooled in ice. Thereafter the carbazole reagent (200 μl) was added. The solution was mixed and heated for another 10 minutes 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 minutes at 55°C. The colours which developed were read at 530 nm on a Unicam SP 500.Standard graphs for different acids under each of the conditions were prepared.

(b) The metahydroxy diphenyl method[86]

To a sample solution (0.6 ml) containing from 1.5 to 60μg 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 minutes. After cooling in ice m-hydroxydiphenyl solution (50μl, 0.15% solution in 0.5% sodium hydroxide) was added. The solutions were shaken and the absorbance measured at 520 nm. on a Unicam SP 500. Standard graphs were prepared for the different acids.

2.7.3 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 (1 ml) and evaporated to dryness, the tube was then placed in an oven at 110°C for 2 h. The sample was then ready for sulphate determination, and the following modification of the Jones and Letham method[87] was used.

To the sulphate solutions (1 ml) containing 30 - 100μg of sulphate in microcentrifuge tubes the reagent 4-Chloro-4'-aminodiphenyl (1 ml of 0.19% in 0.1$\underline{\underline{M}}$-hydrochloric acid) and a trace of solid hexadecyltrimethyl-ammonium bromide were added. After mixing, the solutions, including a blank, were kept for 2 h and then centrifuged. Aliquots (0.2 ml) of the supernatants were removed and diluted to 25 ml with 0.1$\underline{\underline{M}}$-hydrochloric acid. The optical densities were read at 254 nm on a Unicam SP 500. From a standard graph the differences over the blank reading gave the sulphate content.

2.7.4 Nitrogen and Protein content

Nitrogen content was measured by A. Bernhardt (W. Germany) and the protein content calculated by multiplying by 6.25.[88]

2.7.5 Thiobarbituric acid test (TBA)

The TBA[99] test for the presence of 4,5-unsaturated acid was used with the following modifications.[100] The sample in 0.20 ml or less of solution was added to 0.025$\underline{M}$-HIO_4 in 0.125$\underline{M}$-sulphuric acid (0.25 ml). After 20 minutes at room temperature, 2% sodium-arsenite in 0.5$\underline{M}$-hydrochloric acid (0.5 ml) was added with shaking and the solution was permitted to stand for 2 minutes.

0.3% TBA (2 ml) (pH 2) was added and after stirring, the mixture was heated at 100°C for 10 minutes. When the mixture had cooled, its ultraviolet spectrum was measured on a Unicam SP 500 spectrophotometer to detect any absorption maximum at 548 nm.

2.7.6 Degree of polymerisation

This was determined according to the Timell[137] modification of the method of Peat et al. Three solutions were prepared as follows.

1. A blank containing water (0.5 ml) and 2% potassiumborohydride solution (0.5 ml).
2. An aqueous sugar solution (0.5 ml, containing 60 - 80 μg as carbohydrate) and 2% potassium borohydride (0.5 ml).
3. A 2$\underline{M}$ sulphuric acid solution (0.5 ml) containing the same amount of sugar as in solution (2) and 2% potassium borohydride solution (0.5 ml).

These mixtures were left at room temperature for 20 h. The optical density of each solution was then measured by phenolsulphuric acid method. The optical density of solution 1 was used as blank. The DP of the carbohydrate material is given by the relationship:

$$DP = \frac{(O.D)N}{(O.D)N - (O.D)R}, \quad \text{where}$$

(O.D)N = optical density of non-reduced solution 3.

(O.D)R = optical density of reduced solution 2.

This equation applies only for homopolysaccharides. Otherwise, the O.D. may be converted into the corresponding weight of sugar (read off the appropriate standard graph). Then

$$DP = \frac{\text{wt. of non-reduced carbohydrate}}{\text{wt. of non-reduced carbohydrate} - \text{wt. of reduced carbohydrate}}$$

2.8 General Reactions and preparations

2.8.1 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.

2.8.2 Preparation of dimethyl sulphinyl carbanion[89]

Sodium hydride (0.75 g) (i.e. 1.5 g of 50% coated with mineral oil) was weighed into a 250 ml three-necked flask, and was washed with n-pentane (five times 50 - 100 mls portions), the latter being decanted off after each washing. (The decantate may contain a small amount of sodium hydride and can be rendered safe by the slow addition of ethyl alcohol). The pentane was previously dried by storing over sodium wire usually for about 1 week.

After the final washing, the flask was fitted with a condenser and fine bleed. The apparatus was evacuated through the bleed while the sodium hydride was stirred over a magnetic stirrer (Teflon follower).

Dry nitrogen (through $CaCl_2$ tower) was flushed through the bleed into the flask which was then evacuated again. The process was repeated several times (6-7) so as to provide a nitrogen atmosphere.

Nitrogen was allowed to flush through continuously and dry DMSO (15 ml) was added by means of a syringe to the flask from the top of the condenser. A vigorous reaction took place with the evolution of hydrogen. The content of the flask was heated in an oil bath at 55°C until the 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 0.1 M HCl.

(iii) Esterification of uronic acid[90]

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

(iv) Reduction of sugar to alditol.[91]

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 120 H^+ 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.

(v) Trimethyl silyl derivatives.[92]

The material (10 - 15 mg), dried by co-distillation with methanol and dry benzene, was dissolved in dry pyridine (1 ml) and trimethylchlorosilane (0.1 ml) added followed by hexamethyl disilazane

(0.1 ml). After shaking for 5 minutes the precipitate of pyridinium chloride was removed by centrifugation and the sample evaporated to dryness. The residue was dissolved in dry hexane and analysed on g.l.c. [column (I)].

2.8.6 Alditol acetates.[93]

Samples of sugar alditols or of partially methylated alditols, dried in a desiccator, were dissolved in pyridine : acetic anhydride (1:1 v/v) mixture and heated for 30 minutes at 100°C. A small amount of toluene was added (to ease the evaporation) and the sample was evaporated to dryness under reduced pressure at 40°C. The residue was dissolved in chloroform and analysed by g.l.c. and g.l.c.-m.s. [column (II)].

2.8.7 (a) Methylation by Hakomori method

Methylation procedure

The Hakomori method[94] (modified by Bjorndal and Lindberg[95]) was used. The dry 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 then agitated in an ultrasonic bath for 1 h. (or shaken on a mechanical shaker for 8 h). The solution was then kept at room temperature for 6 h.

Methyl iodide (0.1 ml) (stored in the dark over silver oxide) was added dropwise with cooling by tap water and agitated in the ultrasonic bath for 20 minutes (6 h in shaker).

A further 1 ml of carbanion was added and again agitated in the ultrasonic bath for 1 h or for 6 h on a shaker. The solution was kept at room temperature for 6 h. Methyl iodide (1 ml) was added with cooling and agitated for 20 minutes (Ultrasonic bath). After the final period of agitation the solution was poured into water (25 ml) and dialysed for 3 days.

The aqueous solution from the dialysis sac was then extracted twice with chloroform, both the aqueous and organic fractions were evaporated to dryness and the methylated product was acid hydrolysed for analysis as the alditol acetates of the methylated sugars.

2.8.7 (b) Methylation with diazomethane

Preparation of diazomethane

The method for the preparation of diazomethane was similar to that of DeBoer and Backer.[96] A solution of potassium hydroxide (3 g) in water (5 ml) was mixed with methyldigol (18 ml) and dichloromethane (5 ml) and heated in an oil bath to 70 - 75°. A solution of toluene-p-sulphonylmethylnitrosoamide (10 g) in dichloromethane (50 ml) was added over a period of 30 minutes, with continuous stirring, effecting distillation of diazomethane together with dichloromethane. The distillate was collected in a cooled receiver, and comprised approximately 65% diazomethane. The deep yellow solution was dried over sodium hydroxide pellets and stored at 0°.

Methylation

In a typical experiment a solution of the mixture of partially acetylated monosaccharide (0.8 g) in absolute methylene chloride (10 ml) was treated with borontrifluoride etherate (0.04 ml). The solution was cooled to -5°C and diazomethane solution in methylene chloride was added until a permanent yellow colouration persisted. After 30 minutes the precipitate of the polymethylene was filtered off, the filtrate was washed quickly with 10% sodium carbonate solution and with water, it was then dried over magnesium sulphate. The methylene chloride was evaporated off under vacuum.

2.8.8 Periodate oxidation

Estimation of the extent of oxidation was measured by the spectrophotometric method of Aspinall and Ferrier.[97] The polysaccharides (15 - 20 mg) were oxidised with 0.015 M-sodium metaperiodate (20 ml) in the dark. Aliquots (1 ml) were withdrawn at intervals and diluted to 250 ml in a standard flask. The absorptions of these solutions were read in the Unicam spectrophotometer, SP500, in 1 cm cells at 223 nm Aliquots of 0.015 M-sodium metaperiodate (1 ml) and 0.015 M-sodiumiodate (1 ml) were diluted to 250 ml and their absorptions read under the same conditions. The periodate uptake of the polysaccharide was determined by comparison with the standard solution.

2.8.9 Preparation of a DE-52 ion exchange cellulose column

The cellulose (200 g, Diethylaminoethyl, D.E.A.E.; grade D.E.52 pre-swollen) was suspended in 0.5 M-HCl (2) and deaerated with magnetic stirring under vacuum for about 20 minutes. After allowing to stand for another 20 minutes the supernatant was decanted and the cellulose filtered on a Buchner funnel and washed with deionised water until the filtrate was neutral.

The wet material was then treated with 0.5M sodium hydroxide solution in the same way as the acid suspension. These two operations were repeated three times, and in the last alkali treatment instead of transferring into the Buckner funnel the alkaline slurry was transferred into a column (internal diameter 4.5 cm and length 30 cm). The material was washed with water until the eluate was neutral.

2.8.10 Preparation of DEAE-Sephadex molybdate column[73]

DEAE-Sephadex (A-50) (1.5 g) was heated at 98° for 1 h with three changes of 0.01 M sodium molybdate buffer (pH 5; 250 ml each change). The gel was then washed with freshly distilled water and packed as a slurry

in water into a column of internal diameter 2 cm and length 20 cm. This size of column can readily fractionate quantities of laminaran ranging from 10 - 200 mg. Larger columns can be used to fractionate gram quantities, but care has to be taken with insoluble laminarans to see that the total time for elution of the column does not exceed 5 - 6 h, otherwise, low yields are obtained due to precipitation. In a typical separation, laminaran (100 mg) dissolved in water (5 ml) was loaded on to the column. Fractions (5 ml) were collected; the column was eluted with water (100 ml) followed by 0.25 M-sodium chloride (100 ml).

2.8.11 Preparation of Sepharose 4B column

(a) Packing the column

The Sepharose 4B was obtained already in a swollen state, before packing the column it was first deaerated by means of a water pump until evolution of bubbles ceased. The column to be packed was first filled with eluant, until the dead space was taken up, and then the gel slurry was poured down the edge of the column until filled with gel. The column was then allowed to flow at a rate dictated by the working pressure. When very slow flow rates were required a peristaltic pump was used for packing the column. Equilibration was carried out by letting the column flow for at least two column volume.

(b) Determination of the void volume (Vo) and the total volume (Vt) of the column packed with gel filtration media.

Blue dextran (2 mg/ml, Pharmacia) and glucose (5 mg/ml) were dissolved in elution buffer and applied to the column. The column was eluted with buffer at the desired flow rate, and fractions collected. Glucose was determined by means of the phenol sulphuric acid method.

From the elution volumes (Ve) of the samples Vo and Vt could be found where:

Ve dextran blue = Vo (Interstitial volume between the gel granules)

Ve glucose = Vt (Total volume of the packed column).

2.8.12 Desulphation by alkali.[98]

The following is a typical experiment. An aliquot (4.7 g) of polysaccharide that had been pre-dried at 60° in vacuo over phosphorus pentoxide for 24 h was dissolved in water (300 ml), and potassium borohydride (1.0 g) was added. After 20 h at room temperature, 3 M-sodium hydroxide (200 ml) was added, with a further quantity of potassium borohydride (2.8 g), and the mixture was heated at 80° for 7 h. The solution was transferred to a dialysis sac and dialysed exhaustively against distilled water, concentrated and freeze dried (3.3 g).

PART ONE

CARBOHYDRATES OF THE BROWN SEAWEEDS

DESMARESTIA FIRMA AND *DICTYOPTERIS PLAGIOGRAMMA*

CHAPTER 3 CARBOHYDRATES OF PHAEOPHYCEAE

3.1 Introduction

The Phaeophyceae are characterised from a general chemical standpoint by their laminaran and alginic acid contents which replace amylopectin type polysaccharides and the galactan sulphates found in the Rhodophyceae. The polysaccharide sulphate esters are not excluded from the brown seaweeds, being represented in this case by 'fucans'.

The chemical composition of the brown marine algae has received intensive examination mainly because of the commercial importance of alginates, particularly during recent years. The various factors which affect the carbohydrate constituents have been studied, since harvesting the weed at optimum concentration of the alginic acid is a prerequisite for the industries based upon seaweed as a raw material. Thus, the effects of seasonal variations, depth of immersion and composition of sea water have been shown to affect the alginic acid, laminaran and 'fucan' contents of the seaweeds. It has also been shown that these polysaccharides are not evenly distributed throughout the frond or stipe of the plant.

Carbohydrates metabolised by the brown algae, the Phaeophyceae, can be divided into three major classes namely: Low molecular weight carbohydrates (soluble in 80% ethanol); Water soluble polysaccharides and Cell wall material or structural polysaccharides.

3.1.1 Low molecular weight carbohydrates

Among the low molecular weight carbohydrates of the Phaeophyceae, D-mannitol appears to be present universally, in some species and at some seasons in large quantities.[101] Mannitol is believed to be a storage material and also a substrate for respiration.[102] D-volemitol, a

seven carbon atom alcohol, has been found in the brown seaweed Pelvetia canaliculata,[103] and 1-O-β-D-glucopyranosyl D-mannitol and 1,6-di-(O-β-D-glucopyranosyl)-D-mannitol have been reported in several other brown algae.[104] Laminitol (3-O-β-D-glucopyranosyl-D-glucitol) has been found in Laminaria species and trace quantities of sucrose, galactose and mannose have been found in Cladostephus species.[105] In Desmarestia firma, the presence of a monouronic acid and lactone (with the same p.c. and ionophoretic mobilities as mannuronic acid) was also observed.[106]

3.1.2 Water soluble polysaccharides

This can again be subdivided into two classes (a) Food reserve material and (b) Other soluble polysaccharides.

(a) Food reserve material

Laminaran, a β-(1→3)-linked glucan is present in all brown algae.[107,108] Its proportion of the dry weight of the weed varies widely both with the species and with the season.

Laminaran yields glucose as the sole reducing sugar after complete acid hydrolysis, on one occasion a small proportion of mannose has been reported.[109] With better separation and monitoring techniques mannitol was found to be a constituent of the laminaran from some species.[110] From the proportions of mannitol present, it was calculated for laminaran from Laminaria hyperborea and L. digitata that 40% of the molecules have mannitol as the terminating carbohydrate at the potential reducing end of the molecule, linked at C-1, and the rest of the molecules are terminated by a reducing glucose unit linked at C-3. 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. Anderson et al calculated the M-chain and G-chain content of laminaran from Laminaria hyperborea by determining

the yield of formaldehyde liberated by periodate oxidation of laminaran before and after reduction.[111]

According to the solubility of laminaran in cold water it has been classified into two forms, 'soluble' and 'insoluble' 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 glucose units linked through C-1, C-3 and C-6 than the insoluble.[112] Laminarans are fairly low molecular weight polysaccharides (normally in the region of 4-5000); evidence for (1→6)-linked glucose within the chains has been reported.[112]

It is probable that laminaran is not a single molecular species but that the name covers a whole range of essentially β-(1→3)-linked glucans in some of which the reducing end is terminated by mannitol residues while other species are devoid of it. On the overall evidence Nisizawa[113] suggested that Eisenia laminaran is a linear β-glucan with a DP of about 20 containing both (1→3)-and (1→6)-inter-residue linkages in the approximate proportion of 2:1.

(b) Other soluble polysaccharides

Fucoidan or Fucan: One of the principal constituents of the brown seaweeds is fucoidan, more recently named 'fucan', a polysaccharide carrying half ester sulphate groups. Hydrolysis of 'fucan' yields fucose, but galactose, xylose and uronic acids (and sometimes mannose) are also generally present in the macromolecules. A 'fucan' containing only fucose units has so far never been isolated. In a highly purified sample from Himanthalia lorea,[114] the fucose content was found to be 57%, sulphate 38%, galactose 4%, xylose 1.5% and uronic acid ca. 3%. A 'fucan' from Fucus vesiculosus[115] with a fucose content of 38% and 33% sulphate, when treated with alkali lost 10% of the ester sulphate, indicating that 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 bulk of the L-fucopyranose unit cannot be (1→4)-linked as this would render all the sulphate groups alkali labile. Methylation of the 'fucan' revealed that the main structural feature of this polysaccharide is (1→2)-linked fucose units mainly monosulphated at C-4, evidence for fucose units disulphated at C-3 and C-4 was also found.

Structural studies of 'fucans' by other workers confirmed the above view. In addition it has been stated that the other sugar residues present might constitute an integral part of the 'fucan' macromolecule. 'Fucan' extracted from Fucus vesiculosus[116] and Ascophyllum nodosum[117] when subjected to free boundary electrophoresis gave rise to two bands in the former case and three in the latter, which is an indication of the heterogeneous nature of the polysaccharide.

Examination of the 'fucans' of Ascophyllum nodosum[117] and other brown algae revealed that the proportion of other constituent sugars vary not only from species to species but also from method of extraction or fractionation of the extract from the same species.

'Fucans' from alkali extract

Extraction of the brown seaweed with dilute sodium hydroxide, following the extraction of acid soluble carbohydrates, yields a mixture of alginic acid and 'fucans'. These latter polysaccharides contain varying proportions of fucose, xylose, fairly high amounts of glucuronic acid and half ester sulphate.[117] Alginic acid is precipitated by pH adjustments and the 'fucans' were recovered from the supernatant by fractional precipitation with ethanol. The major polysaccharide fraction from Ascophyllum nodosum, called ascophyllan contained approximately 25% fucose, 26% xylose, 19% uronic acid, 13% sulphate and 12% protein. Two other smaller fractions differ from ascophyllan

only in the relative proportion of the constituents. When examined by paper chromatography, the hydrolysates of all the three fractions exhibited similar chromatographic patterns, although variation in the intensity of the spots indicated variation in the amounts of the constituents.

Attempted separation of the polypeptide moiety from the carbohydrate in ascophyllan was unsuccessful, which suggests a chemical linkage between the two. When ascophyllan was subjected to mild hydrolysis with 0.5M-oxalic acid, cleavage occurred to peptide as well as glycosidic linkages of the polysaccharide resulting in dialysable mono and oligosaccharides and nondialysable degraded polysaccharide. The content of the dialysis sac when examined revealed the presence of almost all the uronic acid originally present in the polysaccharide and that it was almost devoid of fucose, xylose, and half ester sulphate. These results led the authors to conclude that ascophyllan consists of a glucuronan backbone with a relatively long side chain of sulphated fucose and xylose residues.

The residual Ascophyllum nodosum (after acid and alkali extraction) when extracted with ammonium oxalate/oxalic acid at pH 2.8, gave material consisting of a mixture of 17 to 20% alginic acid and a 'fucan', the latter comprised 49% fucose, 10% xylose, 12% uronic acid, 21% sulphate and 4% protein.[118] The alginic acid was removed from the polysaccharide mixture as the insoluble calcium salt, and attempted fractionation of the 'fucan' proved impossible. Autohydrolysis of the polysaccharide in a dialysis tube resulted in a degraded polysaccharide which 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 backbone. 3-O-(β-D-Glucopyranuronosyl) L-fucose, was characterised

as the major olgiosaccharide in a partial hydrolysate of this polysaccharide.

Studies on Himanthalia lorea, Bifurcuria bifurcata and Padina pavonia[119] in this laboratory indicate that the 'fucans' comprise a whole family of polysaccharides containing various proportions of fucose, xylose galactose, glucuronic acid, and sulphate. Fractionation of different extracts on DE-52 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 fractions with proportions of sugars and sulphate between these two extremes.

Sargassan

A sulphated heteropolysaccharide has been found in the acid extract of the brown alga Sargassum linifolium. Fractionation of this extract gave a neutral laminaran like glucan and a sulphated heteropolysaccharide (sargassan), which is composed of D-glucuronic acid, D-mannose, D-galactose, D-xylose, L-fucose and protein.[120] Sulphate groups have been detected on some of the D-galactose and L-fucose residues.[140] Evidence for the existence of a (1→4)-linked β-D-glucuronic acid and β-D-mannose backbone in sargassan was obtained from a study of the behaviour of the polysaccharide towards periodate before and after partial acid hydrolysis, alkali treatment, and methanolysis.[141] The heteropolymer also contains partially sulphated branches attached to the backbone, and these branches comprise various proportions of (1→4)-linked β-D-galactose, β-D-galactose 6-sulphate, and β-D-galactose 3,6-disulphate residues, (1→2)-linked α-L-fucose 4-sulphate residues, and (1→3)-linked β-D-xylose residues.

Alginic acid

Alginic acid is normally extracted with dilute sodium carbonate and purified by precipitation either with alcohol as the free acid or the calcium salt. It is a linear glycuronan composed of (1→4)-linked β-D-mannuronic acid and α-L-guluronic acid units. The proportion of the two components depend on species and on the region of the plant from which the polysaccharide is extracted. It also shows seasonal variations. The main location of alginic acid is in the middle lamella and in the primary cell wall of the seaweeds.[121,122] It was originally thought to be composed solely of D-mannuronic acid residues, but later studies[142] have shown that it also contains a considerable proportion of L-guluronic acid, a C-5-epimer of D-mannuronic acid. Periodate and bromine oxidation of alginic acid, followed by hydrolysis yielded both threaric and erythraric acid, showing that both uronic acids are (1→4)-linked.[126] (Figure 3.1).

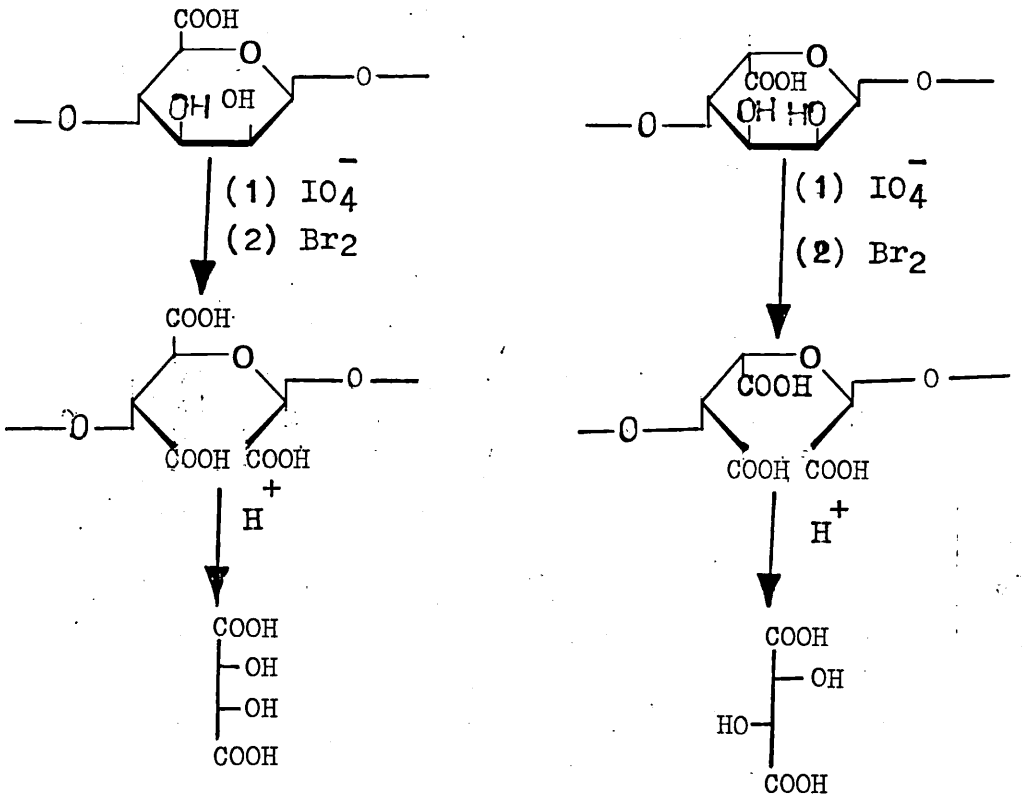


Figure 3.1

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 the disaccharide mannosylgulose from the hydrolysate.[127] Information about the sequence of uronic acid residues in the alginate molecule was obtained by heterogeneous hydrolysis. When a sample of sodium alginate[128] was subjected to hydrolysis with M-oxalic acid for 10 hours at 100°C, part of the polysaccharide was readily hydrolysed 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 acid resistant polysaccharide was separated into two fractions by adjusting the pH with hydrochloric acid or alkali 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 residue together with di- and tri-uronides. 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 were thought to be composed of dimannuronic and diguluronic residues.

In alginic acid the residues are arranged in blocks of either entirely mannuronic acid (M) or guluronic acid (G) units interspersed by regions of irregular alternation -M-M-M-M-M-M-G-M-G-G-M-G-G-G-G-G-G-M-M-G-

ca. 20

Fibres of alginic acid can be prepared and these can be used for X-ray examination. It has been shown from these studies[124] that the mannuronic acid regions form the spacing of 10.3 A^{o} and the guluronic 8.7 A^{o}.

The proportion of the various blocks in an alginate sample depends upon the source of the alginate and varies in different parts of the algal tissue.[148] The poly-guluronic blocks complex strongly with Ca^{2+}, Ba^{2+} and certain other divalent ions,[149,150] leading to alginates which gel readily with these ions, the other blocks complex only weakly and give alginate fractions, which form viscous solutions. In order to understand the relationship between gel formation and the block structure of alginates, it is necessary to know the chain lengths of the various blocks and how they are distributed in alginates from various sources. This has been done by enzymic degradation of alginates.[190]

Enzymes which depolymerise alginate have been obtained from various bacteria,[151,152] from brown algae,[153,154] and from marine molluscs.[155] In general, highly purified bacterial enzymes have shown a preference for cleaving the α-L-guluronic acid linkages, whereas purified enzymes from algae and molluscs have greater activity on the β-D-mannuronic acid linkages.[190] Enzymes having known specificity for only one type of linkage could advantageously be used to obtain information on the lengths of the blocks not attacked.

Recently a polarimetric procedure for determining the mannuronic/guluronic acid ratios in alginates has been developed.[191] Following the characterisation of brucine-L-guluronate,[192] it has been shown that the difference in $[\alpha]_D$ values of the brucine salts of D-mannuronic and L-guluronic acid is sufficient to provide the basis of a polarimetric method for determining the composition of a mixture.

However, the presence of dextrorotatory contaminant would tend to lower the mannuronic acid content and increase the guluronic acid content. Hence it is necessary that the samples of alginates for analysis be free of such contaminants as far as possible.

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.

3.1.3 Structural polysaccharides

Cellulose. A small proportion of cellulose seems to be a general structural polysaccharide in the brown algae. In most cases the X-ray diagram of cellulose I (the native cellulose of land plants) is shown after the microfibrillar material has been freed from encrusting materials of the cell wall.[129] Evidence for β-(1→4)-linked glucose units has been obtained by the isolation of cellobiose and periodate studies excluded the presence of (1→3)- and (1→6)-linkages.[130]

3.2 Investigation of the carbohydrates of brown seaweed Dictyopteris plagiogramma (Montague) Vickers.

Dictyopteris plagiogramma is a member of the brown algae Phaeophyceae and belongs to the order Dictyotales, family Dictyotaceae. It does not grow in the European waters, the present sample being collected from Brazil. Plants of Dictyopteris plagiogramma are erect and grow to a height of about 25 cm, are profusely branched, pale and translucent, the pattern of branching varies from alternate to somewhat irregular, at intervals of 1.0 - 2.5 cm.[131] The sinuses are narrow but rounded,

the segments are 3-7 mm broad, with a prominent midrib and pinnate venilets running obliquely to the margin. The membrane is in general one cell in thickness, the cells are not greatly elongated and sori of hairs are found in irregular rows beside the midribs, sporangia are irregularly scattered near the midribs and are between 80-120 μ in diameter.

Dictyopteris plagiogramma grows probably on rocks, old corals or shells dredged from the moderate depths of 9-18 m. to as much as 55 m. It is commonly found in the seawater of Bermuda, Florida, Mexico, Cuba, Jamaica, Puerto Rico, Brazil, Venezuela and the Island of Trinidad. The sample investigated in this work was collected from Marakazawa in May 1976, from six feet under water and sun dried after washing with water. The dried weed (150 g) was supplied to us by "Algimar Industrias Quimicas de Alginatos", Avenida Presidente Vargas, 417-A-180 Rio de Janeiro, Brazil. Marakazawa is about 40 kilometers from Natal, capital of the state of Rio Grande do Norte, Brazil.

3.3 EXPERIMENTAL - A

(Extraction of the carbohydrates)

Experiment 3.3.1 Examination of the weed before and after formaldehyde treatment

A portion (1 g) of the ground weed was hydrolysed for six hours first with 90% formic acid [GM 23.2] and for another six hours with M-sulphuric acid [GM 2.3.2]. Another portion (1 g) of the ground weed was immersed in formaldehyde (40%) solution and left overnight; it was then air dried and hydrolysed as above. Both the hydrolysates, after neutralisation, were analysed by paper chromatography, [solvents GM2.4.1 a, b and c] and sprays [GM 2.5.1, 2.5.2]. The hydrolysates were analysed by paper ionophoresis [GM 2.4.2] using buffer [GM 2.4.2 (b)]. (For results see page85).

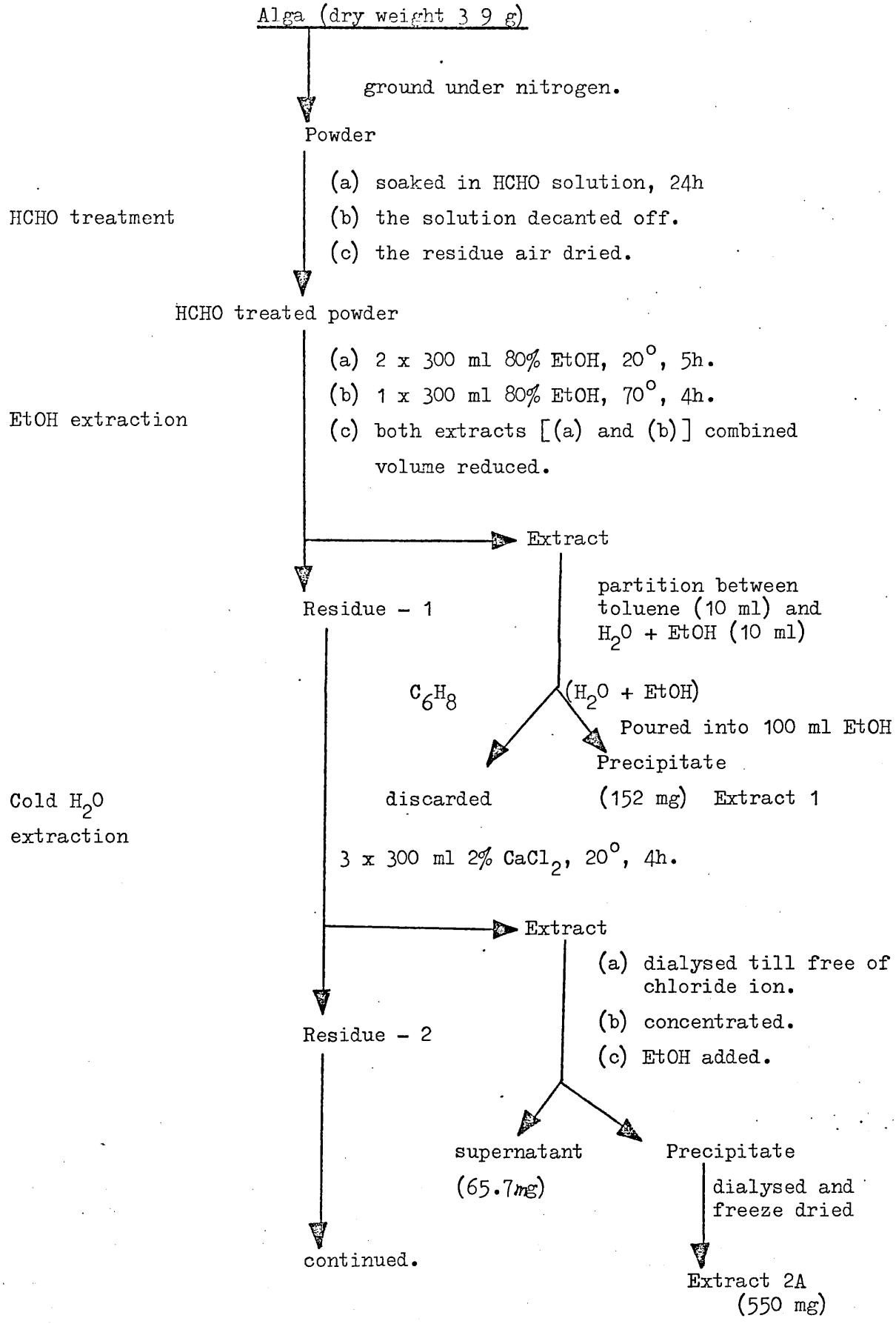
Sequential extraction

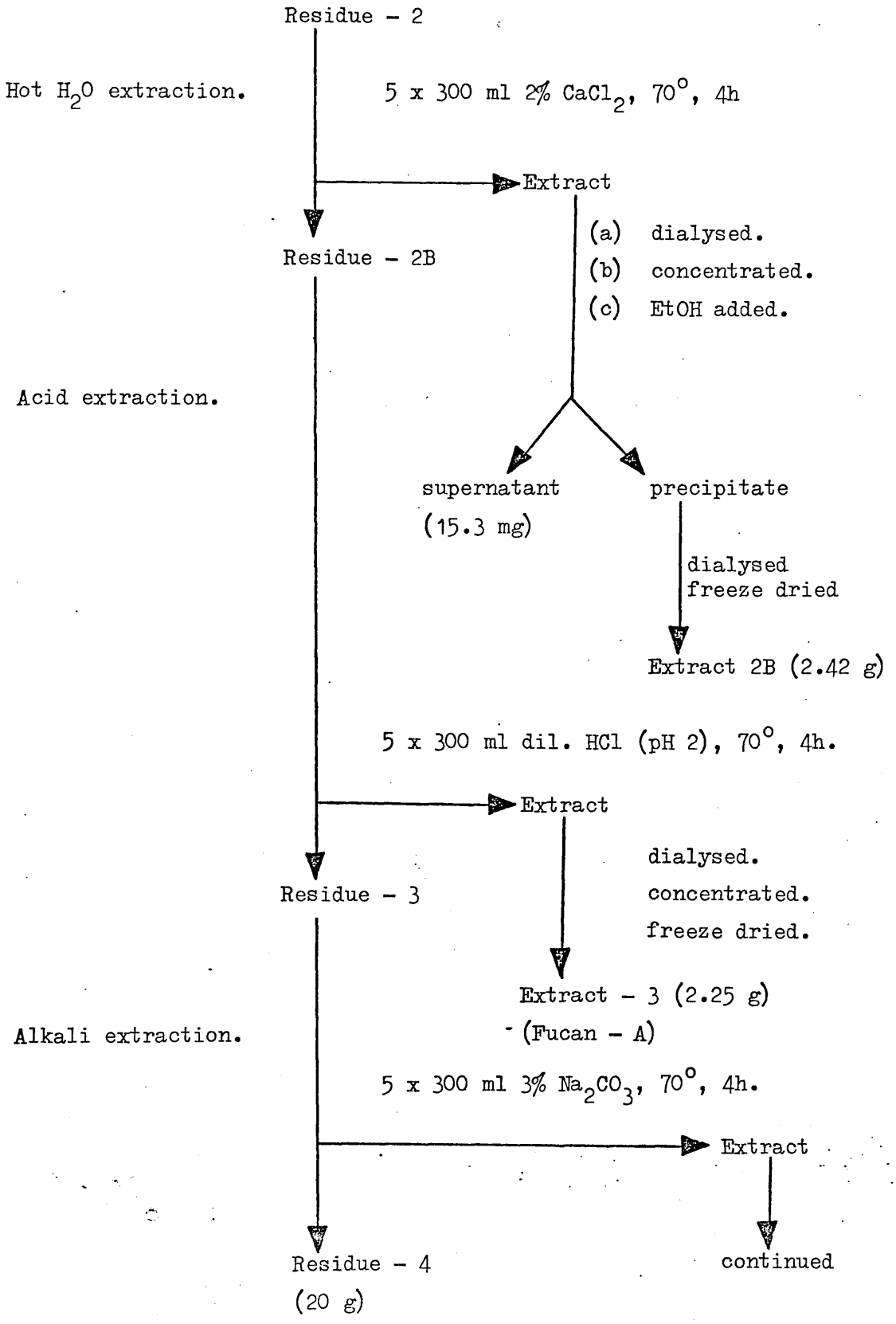
After the preliminary examination, the dry alga (39 g) was subjected to sequential extraction as outlined in flow chart 3.1.

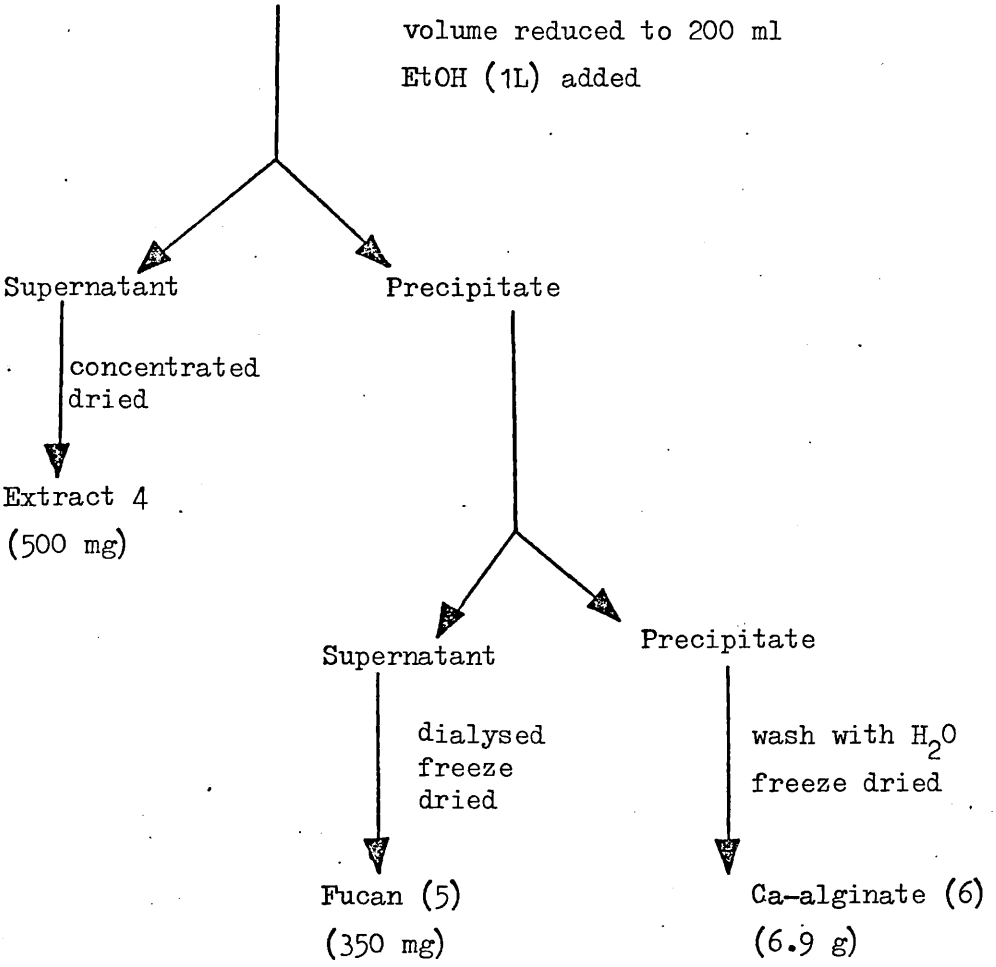
Experiment 3.3.2 Ethanolic extraction

The powdered weed was extracted twice with 80% aqueous ethanol under the conditions given in the flow chart. After each extraction the solid was centrifuged off. The last extract was virtually colourless. 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, a colourless ethanol/water layer and an intense dark green (Chlorophyll) toluene layer was obtained. The toluene layer was discarded and the aqueous ethanol solution (10 ml) poured into ethanol (100 ml), a white crystalline substance precipitated out. For subsequent treatment see experiment 3.4.1.

Flow chart 3.1




Residue - 2
Hot H_2O extraction.
5 x 300 ml 2% $CaCl_2$, 70°, 4h
Extract
(a) dialysed.
(b) concentrated.
(c) EtOH added.
Residue - 2B
Acid extraction.
supernatant
(15.3 mg)
precipitate
dialysed
freeze dried
Extract 2B (2.42 g)
5 x 300 ml dil. HCl (pH 2), 70°, 4h.
Extract
dialysed.
concentrated.
freeze dried.
Residue - 3
Extract - 3 (2.25 g)
(Fucan - A)
Alkali extraction.
5 x 300 ml 3% Na_2CO_3, 70°, 4h.
Extract
Residue - 4
(20 g)
continued


volume reduced to 200 ml
EtOH (1L) added
Supernatant
Precipitate
concentrated
dried
Extract 4
(500 mg)
Supernatant
Precipitate
dialysed
freeze
dried
wash with H_2O
freeze dried
Fucan (5)
(350 mg)
Ca-alginate (6)
(6.9 g)

Experiment 3.3.3 Aqueous extractions

After air drying the alga was extracted with aqueous calcium chloride (2%) solution 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 six volumes of ethanol. The derived white solid was redissolved in water, dialysed and freeze dried to a white amorphous powder. (Yield 550 mg from cold (2A) and 2.42 g from hot water (2B) extracts).

Experiment 3.3.4 Acid extraction

The residue was thereafter extracted five times with dilute hydrochloric acid (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 against deionised water till free from chloride ion. The extract was then freeze dried. A white solid (2.25 g) was obtained.

Experiment 3.3.5 Alkali extraction

The combined extracts were concentrated to 200 ml and poured into ethanol (1L) and after being left overnight the derived precipitate was centrifuged off. The centrifugate was concentrated, dialysed and freeze dried. A greenish solid (500 mg) was obtained (see page 89).

The ethanol precipitate was air dried, redissolved in water and dialysed for three days. The solution was then made up to a concentration of 1% alginic acid and a 2% aqueous calcium chloride solution was added slowly with stirring until precipitation of calcium alginate was complete. The gelatinous precipitate was centrifuged

off, washed with water and freeze dried to a fawn powder (6.9 g). The supernatant was dialysed till chloride free and freeze dried to a white solid (350 mg) hereinafter called the "fucan".

The residue (20 g) after alkali extraction was not examined further.

3.4 EXPERIMENTAL - B

(Investigation of the different extracts)

Experiment 3.4.1 The ethanolic extract (Extract - 1).

Both the crystalline material (152 mg), see flow chart 3.1) and the ethanolic supernatant from which the crystals had been removed were tested for carbohydrate [GM 2.5.5 2.7.1] both showed a negligible amount. Separate aliquots of the crystalline material were redissolved in water and treated with Biodeminrolit and Amberlite IR 120H$^+$ resins respectively, which virtually removed all the material indicating that the crystalline material is composed of inorganic matter. The ethanol soluble material of the aqueous extracts were also found to be devoid of any carbohydrate (for discussion of the results see page 85).

Experiment 3.4.2 The aqueous extracts (Extracts 2A and 2B)

The combined aqueous extracts (2A) and (2B), comprised 7.62% of the dried alga. The carbohydrate content [GM 2.7.1], sulphate content [GM 2.7.3] and uronic acid content [GM 2.7.2] of the polysaccharides obtained from both the cold and hot water extracts (hereinafter called 2A and 2B respectively) were determined. Aliquots of the polysaccharide were hydrolysed [GM 2.3.1, 2.3.2 and 2.3.4]. The hydrolysates were divided into two portions. One portion of the hydrolysates was analysed by paper chromatography in solvents [GM 2.4.1 (a) and (b)] and by sprays [GM 2.5.1, 2.5.2 and 2.5.3] and by paper ionophoresis

[GM 2.4.2 using buffer (2.4.2 (a)], while the other portions were analysed by g.l.c. after conversion into TMS alditol derivatives [GM 2.6.1 (i)]. The relative proportion of the individual monosaccharides were also determined. The results are shown in tables 3.1 and 3.2.

Experiment 3.4.3 The acid extract (Extract - 3)

The acid extract (extract 3) was investigated in a similar way as described in experiment 3.4.2. The results are discussed on page 88 and the relative proportions of the monosaccharide components of this extract is tabulated in table 3.3.

Alkali extract (Extract 4, 5 and 6)

Experiment 3.4.4 The "fucan" from alkali extract (extract 5)

A portion of the "fucan" (20 mg) (expt. 3.3.5) was hydrolysed, and the composition of the hydrolysate was examined as described in experiment 3.4.2. The sulphate content, uronic acid content and the carbohydrate content of the "fucan" were also determined in the usual way (for results see page89).

Experiment 3.4.5 Examination of the calcium alginate from the alkaline extract

Conversion of calcium alginate to sodium alginate

The calcium alginate (6.9 g) (expt. 3.3.5) was suspended in 0.5 $\underline{M}$ HCl (300 ml) in a large sintered funnel the base of which was filled with water. The suspension was occasionally stirred and after 3 h 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 0.1 M 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.

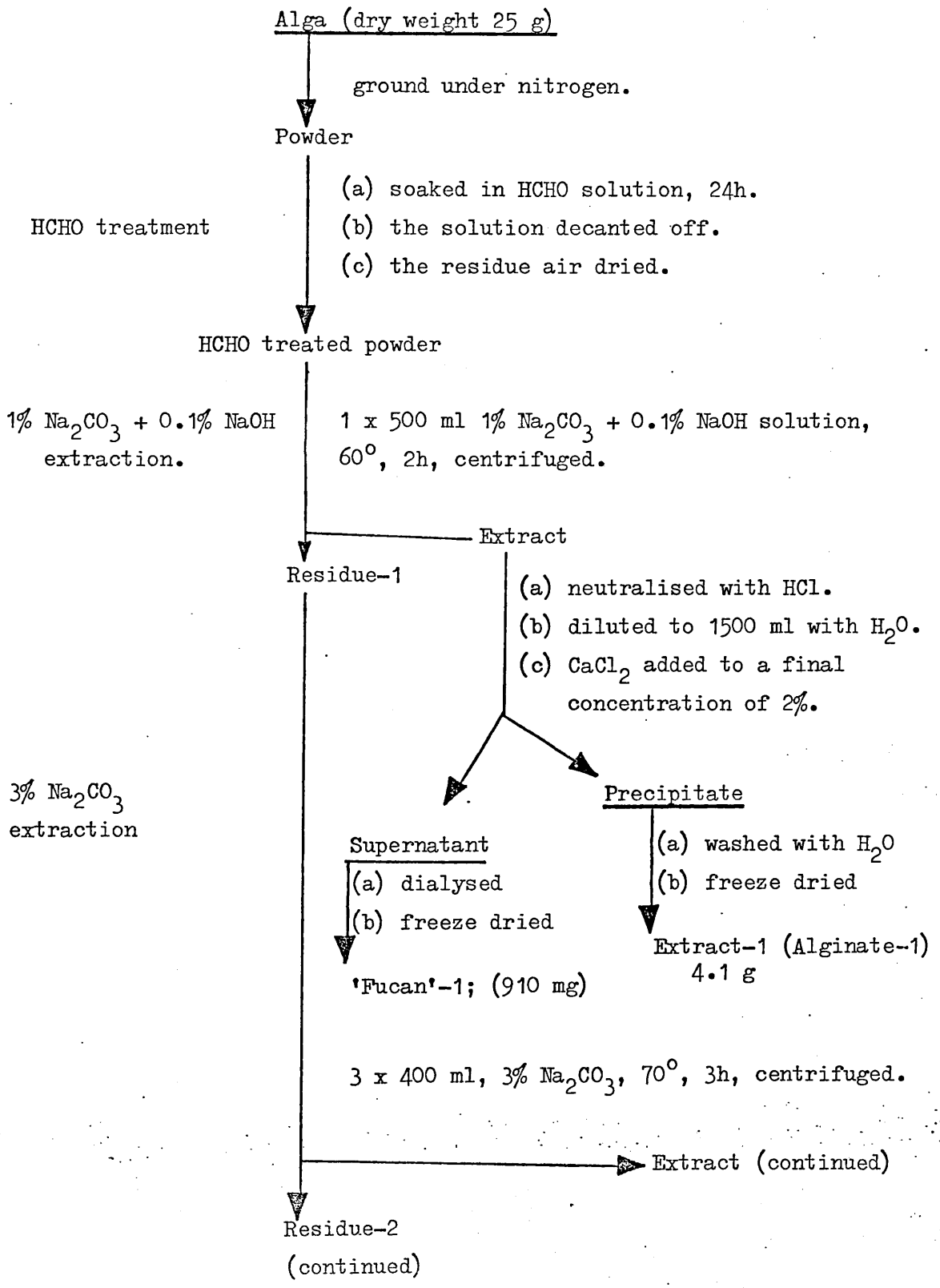
Experiment 3.4.6 Investigation of the sodium alginate

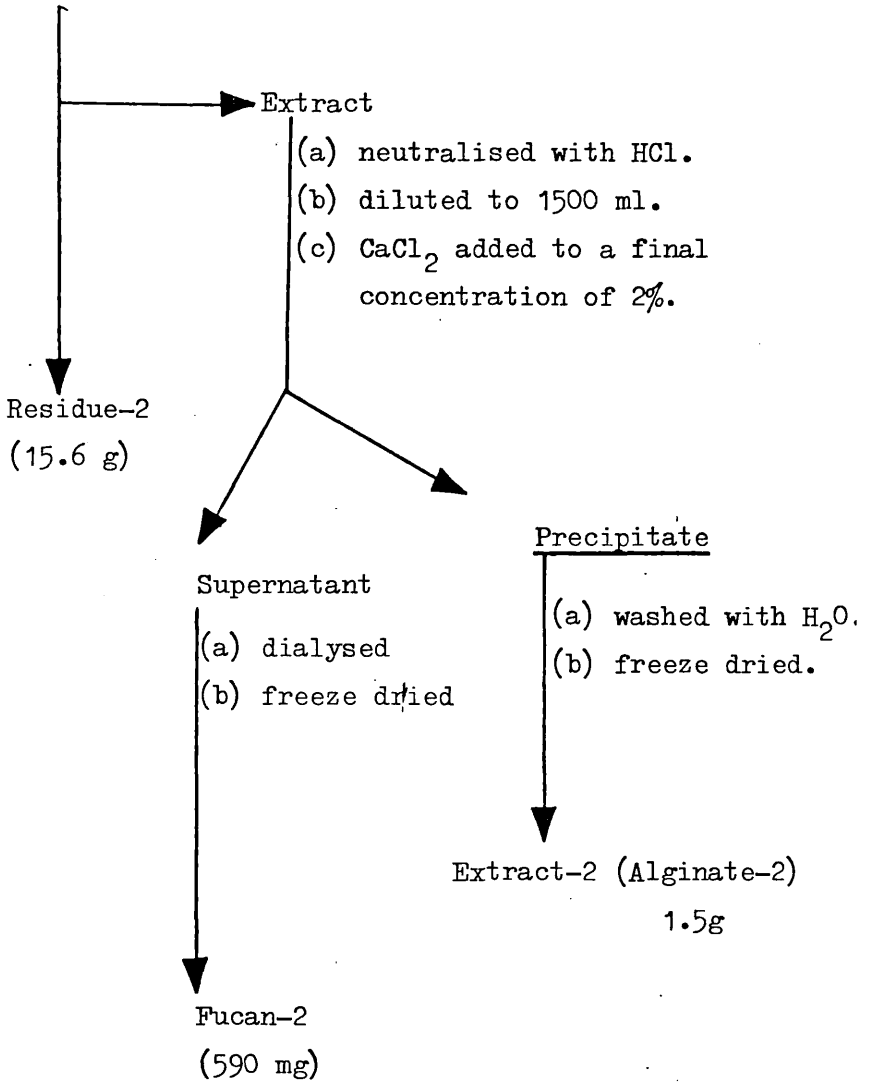
A portion (30 mg) of the sodium alginate was hydrolysed [GM 2.3.2] and investigated by paper chromatography [GM 2.4.1 a, b] and by ionophoresis [GM 2.4.2 (c)]. The viscosity of a 1% aqueous sodium alginate solution (25 ml) was measured at 25° in an Ostwald capillary viscometer (see page 90).

Experiment 3.4.7 Direct extraction of alginic acid (see flow chart 3.2)

A portion of the dry alga (25.0 g) was ground to powder under liquid nitrogen. A solution of 1.8% formalin (200 ml) was added to the alga and the mixture stirred for a minute and then left for thirty minutes. The supernatant was decanted off and water (500 ml) was added. To this mixture solid sodium carbonate (5 g) and a 10% sodium hydroxide solution (5 ml) were added and the mixture was kept at 60° for 2 h under stirring. The residue was filtered off and the solution neutralised (pH = 6.5) with dilute hydrochloric acid and diluted to 1500 ml. Calcium chloride solution (200 ml) was added under stirring so that the final concentration of calcium chloride in the solution becomes 2%. A gelatinous calcium alginate precipitated out which was centrifuged off and then washed with dilute calcium chloride solution. It was then suspended in water and freeze dried to an amorphous calcium alginate solid (Alginate - 1; 4.2 g). The supernatant was dialysed for 3 days and then freeze dried (fucan - 1; 910 mg).

Flow chart 3.2




Extract
(a) neutralised with HCl.
(b) diluted to 1500 ml.
(c) $CaCl_2$ added to a final concentration of 2%.
Residue-2
(15.6 g)
Supernatant
(a) dialysed
(b) freeze dried
Fucan-2
(590 mg)
Precipitate
(a) washed with H_2O.
(b) freeze dried.
Extract-2 (Alginate-2)
1.5g

The residue was extracted three times with a 3% sodium carbonate solution (400 ml) at 70°C for 3 h each time. The combined solutions were neutralised (pH = 6.5) with dilute hydrochloric acid and diluted to 1500 ml. This solution was treated as described above, and gave amorphous calcium alginate solid (Alginate - 2; 1.5 g) as well as a white solid (fucan - 2; 590 mg). The two calcium alginates were converted into the sodium salts as described in experiment 3.4.5. The weights of the sodium alginates 1 and 2 were 3.9 g and 1.4 g respectively. Their viscosity was determined as 1% solution at 25° with an Ostwald capillary viscometer (for results see page 90).

Experiment 3.4.8 Estimation of the guluronic to mannuronic acid ratio in the alginic acid[128]

Three aliquots (200 mg each) 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 0.8 M HCl was added to each flask and the mixtures were hydrolysed for 2 h at 100°C. The mixtures were centrifuged and the supernatants were used for analysis of the soluble components; the residues were washed with 0.4 M HCl. One residue (residue-1) was taken for analysis and it was further washed with 0.4 M HCl/acetone solution, suspended in water and freeze dried. The remaining residues (residue-2 and 3) were solubilised by the addition of 0.5 M NaOH. To these solutions 0.5 M HCl was added until the precipitation point was just reached. The mixtures were then made up to 10 ml with distilled water.

A solution of 0.8 M HCl (10 ml) was added to each solution of residues 2 and 3 and these were rehydrolysed for 2 h at 100°C.

The mixtures were centrifuged and the supernatants were kept; one 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 using a guluronic acid to mannuronolactone 1:1 graph.

The guluronic acid content of the superantants and the residues (the latter converted into soluble sodium alginate) were determined by the carbazole method measuring the ratio of chromophore produced in sulphuric acid with and without added borate at 55°C (for results see page92 table 3.5).

3.5 EXPERIMENTAL - C

(Fractionation experiments)

Experiment 3.5.1 Investigation of aqueous extracts (2A and 2B) by high pressure liquid chromatography (HPLC)

Portions of cold and hot aqueous extracts were dissolved in 0.1% acetic acid in deionised water (7.8 mg/ml and 7.2 mg/ml) respectively and filtered through millipore. These solutions (40 µl) were injected separately into a column of Lichrosphere Si 100. (spherical porous SiO_2 of 10 u diameter, the dimension of the column was 600 x 2 mm) at a flow rate of 0.1 - 0.2 ml per minute, and flow pressure of 1,500 p.s.i. The HPLC apparatus used was WATERS ALC 202 type with a fixed wavelength of U.V. 254 nm, differential refractrometer (twin cell), using visible wavelength and septum injector. The results are described on page 96 and figure 3.2).

Experiment 3.5.2 Fractionation of the aqueous extracts (2A) and (2B) on DE-52 cellulose column

The polysaccharides (2A, 206 mg and 2B, 507 mg) were dissolved separately in water (40 ml) and layered on to DE-52 cellulose columns [GM 2.8.9]. The columns were eluted with 600 ml of each of the following solutions: water, 0.1M KCl, 0.2M KCl, 0.3M KCl, 0.5M KCl, 0.8M KCl and 1.0M KCl. As the paper chromatography revealed similar composition in 0.1M, 0.2M and 0.3M KCl fractions, in subsequent fractionation experiments the columns were eluted with 0.3M KCl after eluting with water. The composition of the different fractions obtained from these fractionation studies is tabulated in tables 3.8, 3.9 and 3.11.

Experiment 3.5.3 Composition of the different fractions from Experiment 3.5.2

The carbohydrate content [GM 2.7.1], the sulphate content [GM 2.7.3] and the uronic acid content [GM 2.7.2] were determined for each of the fractions from Expt. 3.5.2. Aliquots of the aqueous and 0.3M KCl fractions of 2B were investigated by HPLC in the same way as described in experiment 3.5.1. After hydrolysis [GM 2.3.1] the monosaccharide components of the different fractions were characterised by paper chromatography in solvents [GM 2.4.1] (a) and (b) and locating reagents [GM 2.5.1 - 2.5.5] and by g.l.c. of TMS alditol derivatives. The specific rotation of the aqueous fractions was also determined.

Experiment 3.5.4 Fractionation of aqueous eluate[139] (glucan)

A portion (67.4 mg) of the aqueous eluate from extract 2B, expt. 3.5.2 was dissolved in water (2 ml) and layered onto a DEAE-Sephadex molybdate column [GM 2.8.10]. The column was eluted

first with water and then with 0.25M sodium chloride solution. Twenty fractions of 5 ml each were collected from each eluate and the absorbance of all the fractions were measured at 487 nm after treatment with phenol sulphuric acid (fig. 33). After dialysis of the combined sodium chloride fractions, both the combined aqueous and combined NaCl fractions were freeze dried and weighed (table 3.10).

Both the above fractions were hydrolysed [GM 2.3.1] and characterised by paper chromatography in solvent [GM 2.4.1 (d)] and locating reagent [GM 2.5.5]. The sodium chloride fraction was treated with glucose oxidase after hydrolysis and the oxidised mixture was analysed by paper chromatography.

Experiment 3.5.5 Fractionation of the hot aqueous extract (2B) with "Hyamine-1622" (Di-isobutyl-phenoxy-ethoxy-ethyl-dimethyl benzyl ammonium chloride).

The polysaccharide (1.0 g) was dissolved in water (100 ml) with constant stirring. An aqueous hyamine solution (1.5 g/10 ml) was then added dropwise while precipitation occurred. This was stirred for 20 h at room temperature. The precipitate was separated by filtering through glass fibre filter paper (filtrate I, +ve carbohydrate); and allowed to dry in air. The air dried precipitate was then transferred into a beaker, ethanol (250 ml) was added to it and the mixture stirred for 30 minutes, after which it was filtered again through glass fibre filter paper, (filtrate II). The precipitate II (754 mg) was washed several times with ethanol.

The precipitate (II) was not completely soluble in water; it was therefore again stirred with ethanol for 18 h, centrifuged and it was then freeze dried (584 mg). The freeze dried material was still insoluble in water; it was then extracted with chloroform after

dispersing in water. The cloudy aqueous fraction was filtered through glass fibre filter paper; both the residue and clear filtrate were freeze dried; these two fractions will be referred to as water insoluble complex WIP (495 mg) and water soluble polysaccharide WSP (41 mg) respectively.

Experiment 3.5.6 Composition of the different fractions from Experiment 3.5.5.

The carbohydrate content [GM 2.7.1], sulphate content [GM 2.7.3] and uronic acid content of all the fractions were determined. Aliquots were hydrolysed [GM 2.3.1] and the hydrolysates examined by paper chromatography [GM 2.4.1]. using solvents [a, b, c, d] and spray reagents [GM 2.5.1 - 2.5.5].

Aliquots of the hydrolysates of water insoluble and water soluble fractions were reduced and examined by g.l.c. [GM 2.6], using column II after converting into alditol acetates. The relative proportion of each sugar present in both the fractions was determined by measuring the peak areas of the derived alditol-acetates and also by phenol sulphuric acid method after separating the individual sugars from the hydrolysate on Whatman No.3 paper (for results and discussion see pages 104-110, Tables 3.12 and 3.13).

Experiment 3.5.7 Fractionation of acid extract (fucan-A) by 'Cetrimide' (cetyltrimethyl ammonium bromide) precipitation

The polysaccharide (fucan-A, 1.5 g) was dissolved in (0.02M) sodium sulphate solution (30 ml). 5% Centrimide (30 ml) was then added slowly with constant stirring; the polysaccharide formed a precipitate with the Cetrimide, this was then kept for 20 h at 4°C. The precipitate was separated by filtering through a nylon cloth.

The filtrate (S-A) was tested for carbohydrate [GM 2.7.1], it was then treated twice with Biodeminrolite, reduced to small volume and freeze dried (38.1 mg, off-white powder).

The precipitate, which is a cetrimide complex of the polysaccharide was decomposed in 4M potassium chloride solution (50 ml, 24 h, with constant stirring). The polysaccharide was recovered by precipitation with 200 mls of ethanol. The procedure was repeated 4 times to remove adhering 'Cetrimide' and the polysaccharide was finally recovered by dialysis and freeze drying (Product 1; 665 mg). The ethanolic supernatants were dialysed, concentrated and freeze dried (Product 2, 66 mg). All the three fractions were assayed for carbohydrate [GM 2.7.1], uronic acid [GM 2.7.2] and sulphate contents [GM 2.7.3]. Aliquots of each of the fractions were hydrolysed and examined on paper chromatography, and on g.l.c. after reduction and conversion into alditol acetates. The nitrogen content of each of the fractions were also determined. For results see pages 110-114 and table 3.14).

Experiment 3.5.8 Fractionation of the acid extract (Extract 3, fucan-A) by Trimethylsilylated glass fibre paper (TGFP) electrophoresis

The preparation of trimethylsilylated glass fibre paper (TGFP) and analytical procedure is already described in [GM 2.4.3 a, b, c]. Preparative method: Six strips of TGFP papers were used to fractionate 64.8 mg of the polysaccharide. After equilibrating each of the strips with acetate buffer for thirty minutes, the polysaccharide solution in acetate buffer was applied on a line of the paper and subjected to electrophoresis at the electric field strength of 22V/cm. for one hour. The paper was then air dried and the

moving and non-moving fractions were taken out by cutting the appropriate paper strips. Both the fractions were extracted separately with water at room temperature and freeze dried. The carbohydrate content [GM 2.7.1] and sulphate content [GM 2.7.3] of both the fractions were determined. Aliquots of each of the fractions were hydrolysed [GM 2.3.1] and analysed by paper chromatography [2.4.1] in solvents a, b and c and staining reagents [GM 2.5.1, 2.5.2].

Another aliquot of the hydrolysates was reduced and converted into acetate derivatives and analysed by g.l.c. [GM 2.6 column (ii)]. (See page 115 table 3.15).

3.6 EXPERIMENTAL - D

(Structural studies of glucan and fucan)

Experiment 3.6.1 Periodate oxidation of the aqueous fraction from Extract 2B (Expt. 3.3.3).

Determination of periodate reduced: A portion (295 mg) of the aqueous fraction obtained from Extract 2B, Expt. 3.3.3 and a laminaran (32.5 mg) of known composition from Bifurcaria bifurcata containing only G-chains were oxidised separately with 0.015 M sodium metaperiodate (20 ml) in the dark at 2°. Aliquots were withdrawn at intervals and diluted to 250 ml in a standard flask. The absorptions of these solutions were read in the Unicam spectrophotometer SP 500, in 1 cm cells against water at 223 nm. Aliquots of 0.015M sodium metaperiodate (1 ml) and 0.015M sodiumiodate (1 ml) were diluted to 250 ml and their absorptions read under the same conditions. The periodate reduced by the polysaccharides was determined by comparison with the standard solutions. The

periodate reduced by the polysaccharides at room temperature was also determined. The results are shown in table 3.6 and fig. 3.8 pages 119-123.

Experiment 3.6.2 Methylation of the 'fucan-A' by Hakomori method

A portion of 'fucan'-A (23.5 mg) was methylated once by the Hakomori method [GM 2.87 a]. An aliquot (ca. 1/3) of the methylated material was removed and after working up as detailed in [GM 2.87 a] this was hydrolysed [GM 2.3.1] and examined by paper chromatography. The hydrolysate was then reduced [GM 2.8.4], converted into the alditol acetates [GM 2.8.6] and analysed by g.l.c. and g.l.c. - m.s. [GM 2.6]. The remaining two thirds of the methylated polysaccharide was subjected to a further methylation procedure and hydrolysed as above. The hydrolysate was then analysed in a similar way as for the hydrolysate of (1 x Hakomori) methylated material (for results and discussion see pages 124-133 and table 3.18).

Experiment 3.6.3 Separation and characterisation of the slow moving component of methylated fucan-A hydrolysate

The hydrolysate of methylated fucan-A was placed on 3 mm paper and allowed to run in solvent system [GM 2.4.1 (c)] for 48 h. The fraction with slower chromatographic mobility was cut out and eluted with water. After evaporation of water at reduced pressure and at a temperature of 40°C, the sample was dried further over conc. H_2SO_4 in vacuo. The dried sample was then esterified, reduced [GM 2.8.3 and 2.8.4] and hydrolysed [GM 2.3.2]. The hydrolysate was then neutralised with N-methyldioctylamine. A portion of the neutralised hydrolysate was examined by paper chromatography. The remaining hydrolysate was reduced and acetylated. The derived alditol acetates were examined by g.l.c. and g.l.c.-m.s. [GM 2.6] using column (ii).

Experiment 3.6.4 Gel filtration studies of the methylated fucan-A

Portions of polysaccharide (ca. 2 mg) after one and after two Hakomori methylations were dissolved in water (0.5 ml each) and separately layered onto a Sepharose 4B column (16.5 cm x 1.3 cm). The column was eluted with M-KCl, 2 ml fractions being collected. Each of the fractions collected was analysed for its carbohydrate content [GM 2.7.1] and a graph of mls eluted against absorbance at 487 nm was plotted which shows the elution pattern of the methylated fucan-A (fig. 3.14)

The void and column volume of the column was determined with blue dextran and glucose respectively.

Experiment 3.6.5 Periodate oxidation studies of fucan-A

(a) The fucan-A (170 mg/170 ml H_2O) was oxidised with 0.03M-sodium metaperiodate (170 ml) in the dark at room temperature for 7 days; the reaction was monitored by the spectrophotometric method. When no more periodate uptake by the polysaccharide was observed, ethylene glycol (1 ml) was added to destroy excess periodate.

(b) Reduction of polyaldehyde to polyalcohols

The polyaldehyde solution (from 170 mg fucan) was reduced with sodium borohydride (800 mg). The solution was stirred for 2h at room temperature and then left at 2° for 20 h. It was dialysed (3-4 days) and freeze-dried to give the polyalcohol. An aliquot of the polyalcohol solution was tested for reducing sugar. (Recovery of the polyalcohol 143 mg i.e. 84%).

(c) Second periodate oxidation

The polyalcohol (100 mg/100 ml water) was treated with sodium metaperiodate [0.03M, 100 ml as (a)]. Aliquots (1 ml) of this solution were withdrawn at intervals of time and after dilution with

water (250 ml), spectrophotometric measurement was carried out at 223 nm to check if the polyalcohol reduced any periodate. No uptake of the periodate by the polyalcohol was observed. After 60 h the periodate was destroyed by the addition of ethylene glycol (1 ml) the whole dialysed against distilled water for three days and freeze dried (Recovery 95 mg).

(d) Mild hydrolysis of the polyalcohol with M-trifluoroacetic acid (TFA)

The polyalcohol (reduced, oxidised fucan) (118 mg) was submitted to mild hydrolysis in M-trifluoracetic acid, for periods ranging from 25 to 75 h at room temperature, under constant stirring. Aliquots of this solution were withdrawn after 25 h, 50 h and 75 h respectively and subjected to gel filtration studies on a Sepharose 4B column (for results see page135).

(e) Gel filtration studies

Portions of fucan A (3 mg/ml) and derived polyalcohols (2.6 mg/ml) after first and second periodate oxidation (2.3 mg/ml) before and after treatment with M-trifluoroacetic acid were layered on to a column (16.5 x 1.3 cm) of Sepharose 4B. Throughout packing and during elution of the column, a flow rate of 1 drop/15 seconds was maintained. The void volume (10 ml) was determined with blue dextran having molecular weight in excess of 5×10^6 which is the exclusion limit of Sepharose 4B. Glucose was eluted at 31 ml. The elution patterns of fucan-A, the polyalcohols and degraded polyalcohols are shown in (fig. 3.15).

(f) Formic acid hydrolysis of the polyalcohol from 'fucan-A'

A portion (15 mg) of the polyalcohol was hydrolysed with 90% formic acid [GM 2.3.1] and analysed by paper chromatography [solvents GM 2.4.1 (b)] and sprays [GM 2.5.1, 2.5.2].

Experiment 3.6.6 Desulphation of 'fucans' with alkali

Desulphation of fucan-A (acid extract of D. plagiogramma) along with two other 'fucans' previously isolated in this laboratory and a sample obtained from the Institute of Seaweed Research, Scotland, was carried out using sodium hydroxide [GM 2.8.12]. The carbohydrate content, uronic acid content and sulphate content of all the 4 samples were determined before and after alkaline desulphation (see page 140 table 3.20).

Experiment 3.6.7 Composition of the desulphated 'fucans'

The above four samples of 'fucans' after alkaline desulphation are designated as DSF-A (desulphated fucan from the acid extract of D. plagiogramma), DSF-B (desulphated 'fucan' from Bifurcaria bifurcata), DSF-H (desulphated 'fucan' from Himanthalia lorea), and DSF-X (desulphated 'fucan' from Institute of Seaweed research). Aliquots of DSF-A (45 mg), DSF-B (40.5 mg), DSF-H (35.8 mg) and DSF-X (38.4 mg) were hydrolysed [GM 2.4.1, a, b and c] and [GM 2.5.1, 2.5.2].

An aliquot of each of the hydrolysates, was reduced [GM 2.8.4.] and acetylated [GM 2.8.6]. The alditol acetates of the desulphated 'fucans' thus obtained were examined by g.l.c. using column II [GM 2.6]. The hydrolysates of the desulphated 'fucans' revealed the presence of a new monosaccharide in addition to the monosaccharide components which were originally present in the parent polysaccharides.

Experiment 3.6.8 Identification of the new sugar

The new sugar of the desulphated material was separated on 3MM paper [GM 2.4.1 (c)]. Better separation of the sugar was achieved by withdrawal of the paper at intervals of twelve hours, drying at

room temperature and again eluting. The separated sugar was converted into its alditol acetate [GM 2.8.6] and examined by g.l.c. [GM 2.6] and by g.l.c.-m.s. [GM 2.6.3 (i)] (see page 145 and fig.3.23)

Experiment 3.6.9 Gel filtration studies of 'fucans' before and after desulphation using Sepharose 4B column

A column was prepared (16.5 x 1.3 cm) which had a void volume of 11 ml. Glucose was eluted at 31 ml. The 'fucans' (2.5 mg/3 ml) were dissolved in the buffer and eluted with M KCl. Fractions (2 ml) were collected during elution. Each of the fractions collected was analysed for its carbohydrate content [GM 2.7.1] and a graph plotted of fractions eluted against absorbance at 487 nm (Figs.3.19a, 3.19b, 3.19c and 3.19d). The desulphated 'fucans' were also eluted and monitored for carbohydrate in similar way.

Experiment 3.6.10 Treatment of the desulphated 'fucan' with sodium methoxide[143]

The desulphated fucan-X (1.17 g) (dried over P_2O_5 at 60° in vacuo for one week) was soaked in dry methanol with occasional shaking for a further two days. The dried material, after rapid filtration, was added to a solution of lithium borohydride (0.2 g) and sodium (6 g) in dry methanol (250 ml) and the mixture was refluxed for 24 hours. The insoluble polysaccharide after filtration and washing with methanol was hydrolysed (M-sulphuric acid at 100° for 4 hours). Neutralisation with saturated $Ba(OH)_2$ soln. deionisation with Biodiminrolit resin and several additions of methanol with subsequent evaporations to remove the last trace of borate, gave a syrup (1.0 g). Separation of the syrup on Whatman 17 mm paper in solvent C [GM 2.4.1] gave 2-O methylfucose. It moved with the mobility of authentic 2-O-methylfucose. This was then converted into alditol acetate

[GM 2.8.6] and examined by g.l.c. and g.l.c.-m.s. (see page 147 figs. 3.24 and 3.25).

Experiment 3.6.11 Desulphation of 'fucan'-X by Pavlenko method[138]

The fucan (2.5 g) was subjected to mild hydrolysis and a mixture of monosaccharidesand their sulphates was obtained as ethanol soluble material after separating the ethanol insoluble polysaccharides (0.735 g). The resulting ethanol soluble material (1.76 g) was acetylated and the partially acetylated material (1.1 g) was desulphated with thionylchloride. The different steps involved are described below.

(i) Acid hydrolysis

The polysaccharide under investigation (2.5 g) was treated with 37 ml of water and then 37 ml of 2M-sulphuric acid was added. The resulting solution of the polysaccharide was heated at 95°C for 30 minutes. The precipitate was separated off by filtration, and the filtrate was neutralised with a saturated solution of $Ba(OH)_2$. The reaction mixture obtained was centrifuged, the supernatant was poured into ethanol (300 ml) and the mixture was left overnight, filtered, the filtrate was evaporated at 40°C and was dried over phosphorus-pentoxide. A mixture of monosaccharidesand their sulphates was obtained (1.76 g).

(ii) Acetylation of hydrolysate

A mixture of acetic anhydride (6 ml) and dry pyridine (7.5 ml) was cooled to 0°C, and added to the polysaccharide hydrolysate (1.76 g). The solution was kept at room temperature for 18 h and was then poured into a 50 ml separatory funnel 1/3 filled with ice. The aqueous solution was extracted with chloroform (4 x 6 ml) and the chloroform solution was evaporated to dryness. This gave a mixture of acetylated monosaccharide sulphates in a yield of (1.1 g).

(iii) Desulphation with thionylchloride

The mixture of acetylated monosaccharide sulphates (1.1 g) was dissolved in freshly distilled thionylchloride (6 ml) and was kept at room temperature for 6h. The solvent was then evaporated off in vacuo. The residue was dissolved in chloroform, and the solution was washed with 5% sodium bicarbonate solution and finally with water. The chloroform solution was then dried over calcined anhydrous sodium sulphate. The chloroform was evaporated off and the residue was dried over phosphorus pentoxide. The dried material was weighed (900 mg). (for results see page 158).

Experiment 3.6.12 Methylation of 'Pavlenko' desulphated 'fucan' with diazomethane

A portion (0.8 g) of the desulphated and partially acetylated monosaccharides was methylated with diazomethane [GM 2.8.7. b]. The methylated material was examined by paper chromatography and by g.l.c. using column (iii). This was then converted.into alditol acetates and examined further by g.l.c. and g.l.c.-m.s. using column (ii). (see table 3.22).

Experiment 3.6.13 Methylation of alkaline desulphated 'fucans' by Hakomori method

Portions of alkaline desulphated fucan-A (*D. Plagiogramma*) B (*Bifurcaria bifurcata*); H (*Himanthalia lorea*) and X were methylated once by Hakomori method. A portion (ca. 1/3) from each of the methylated material was taken out and after removal of the solvent these were hydrolysed and examined by paper chromatography. The hydrolysates were then converted into alditols and the derived alditols were acetylated. The alditol acetates of the partially methylated materials were then analysed by g.l.c. and g.l.c.-m.s.

The remaining portion (ca. 2/3) of the methylated polysaccharides were twice methylated by Hakomori method and analysed similarly as stated above after hydrolysis, reduction and acetylation (for results see pages 161-164 and tables 3.23 and 3.24).

3.7 RESULTS AND DISCUSSION

Preliminary studies of portions of the dried weed (with and without formaldehyde treatment) were carried out after acid hydrolysis. The hydrolysates contained glucose, xylose, fucose, galactose, mannose and uronic acids (p.c. and ionophoresis). Unlike other brown seaweeds, mannitol was not observed in this weed. Having this preliminary information in hand the weed was extracted sequentially according to the flow chart 3.1 (page 61).

3.7.1 The ethanolic extract (Extract-1)

The combined ethanolic extracts after removal of the chlorophyll (expt. 3.3.2) and concentration was poured into excess ethanol during which a crystalline substance (152 mg) precipitated out. The crystals were found to comprise almost entirely inorganic material. In spite of repeated crystallisation to remove this and resin treatment it proved impossible to detect any carbohydrate.

All previous studies on brown seaweeds showed the presence of D-mannitol ranging from 3 to 25% of the dry weight[133] of the weed. The fact that D-mannitol could not be identified in the ethanol extract of the present weed was rather surprising. By a further enquiry to 'Algimar Industrias Quimicas de Alginatos', the supplier of the weed, it was eventually learned that the dried weed was washed several times with water before it was supplied to us. In our opinion, this water treatment of the weed probably washed away the mannitol and other low molecular weight sugars which might have been present originally in the weed.

3.7.2 Aqueous extracts (Extracts 2A and 2B)

The combined aqueous extracts comprised 7.62% of the dried alga. Properties of the cold (2A) and hot (2B) extracts are given in table (3.1)

Table 3.1 Properties of the aqueous extracts

Extract (from 39 g dried alga)	Wt. of the freeze-dried extract in grammes	Carbohydrate content %	Uronic acid content %	Sulphate content %	Specific rotation $[\alpha]_D^{25^\circ}$
Cold water (2A)	0.55	63.3	20	5.7	-24.3 (c = 1.0)
Hot water (2B)	2.42	64	12	5.6	-26.5 (c = 1.0)

Paper chromatograms of the hydrolysates of (2A) and (2B) in different solvent systems and with different sprays and staining reagents revealed the monosaccharides shown in table 3.2. The results suggest that the polysaccharide in both the extracts were similar. The presence of D-glucose and D-galactose was confirmed by the appropriate oxidases, whereas colours and mobility corresponding to standard monosaccharides were used to identify the remaining monosaccharide components. G.l.c. of the TMS alditol derivative confirmed the monosaccharides tentatively identified by the paper chromatography.

The approximate relative proportion of the different monosaccharides present in the hydrolysate were determined from the peak area of the g.l.c. of each monosaccharide and by phenol sulphuric acid method after separation of the individual monosaccharide on a 3 MM paper. As the retention times of the TMS derivative of glucose and

galactose are very close to each other the phenol sulphuric acid method was adopted as the more reliable method in determining the relative proportions of the sugars of the aqueous extract. In doing so however, two different solvent systems were chosen. As the solvent system n-butanol : pyridine : water (6:4:3) separates the acidic sugars very well from the neutral ones, the oligouronic acid was first separated from the neutral components by developing in this solvent system; the resolution of the neutral saccharides is however, not very good in this solvent; these were therefore eluted together and again applied on paper and developed in the solvent system ethylacetate : acetic acid:formic acid:water (18:3:1:4). The amount of each monosaccharide was determined by phenolsulphuric acid method after eluting the individual strip (table 3.2).

Table 3.2 Approximate relative proportion of the constituent monosaccharides in the hot aqueous extract (2B) of D. Plagiogramma.

Monosaccharides	Approximate relative molar proportion
glucuronic acid	1.5
D-galactose	0.5
mannose	1.2
D-glucose	6.0
xylose	1.0
fucose	6.0

3.7.3 The acid extract (Extract-3)

After dialysis and freeze-drying a white solid (5.8% of the dry weight) was obtained. The amorphous solid was found to have a carbohydrate content of 66% (compared to a standard graph comprised of 4:1:0.5:1:1 = fucose:galactose:mannose:xylose:uronic acid), a uronic acid content of 19.7% and sulphate content of 3.9%. Acid hydrolysis of portions of this polysaccharide with three different acids, namely 90% formic acid, M-sulphuric acid and 2M-trifluoroacetic acid, revealed a similar chromatographic pattern in each case. The hydrolysates contained fucose, galactose, xylose, mannose, trace of glucose and glucuronic acid.

The g.l.c. of the TMS alditol derivative of the hydrolysate of this extract also supported the presence of the above saccharides. Separation of the individual monosaccharides on paper and determination of their approximate relative amount was carried out in two steps, as for the aqueous extract. Firstly the uronic acid was separated from the neutral monosaccharides and then the combined neutral monosaccharides were separated on a different paper. The approximate relative molar proportions of these saccharides were then determined with the following results (see table 3.3).

Table 3.3 Approximate relative proportion of the monosaccharide components of the acid extract (Extract-3)

Monosaccharide	Approximate relative molar proportion
fucose	4
galactose	1.5
mannose	0.8
xylose	1.0
glucose	0.3
glucuronic acid	2

An alternative method of separating the uronic acid from the neutral sugars was attempted by precipitating the uronic acid as barium salt, but complete separation of the uronic acid could not be achieved by this method as paper chromatograms revealed the presence of uronic acid in the filtrate obtained after treatment with barium hydroxide.

3.7.4 Alkali extract from sequential extraction (Extracts-4,5,6)

The ethanol soluble greenish solid [(500 mg), extract 4 (flow chart 3.1, expt. 3.35)] was found to contain 4,5-unsaturated acids,[100] [GM 2.7.5] thereby indicating the presence of degraded alginic acid. The carbohydrate content of this solid was very low (only 4%) and it was therefore discarded. The 'fucan' (350 mg) extract 5, separated from the alginic acid (expt. 3.44) was found to have a carbohydrate content of 68.5% (compared to a standard graph of an artificial mixture of 5:1:2 = fucose:xylose:glucuronic acid), a uronic acid content of 17% and sulphate content of 4%. It was found to contain fucose, galactose, mannose, xylose and glucuronic acid.

The total recovery of 'fucans' from aqueous, acid and alkali extract was 4g, i.e. 9.8% of the dry weight of the weed.

To obtain a less degraded alginic acid a fresh sample of the alga was extracted as outlined in expt. 3.4.7 and flow chart 3.2. Two 'fucans' namely 'fucan-1' and 'fucan-2' were isolated and found to contain the same monosaccharide components as the 'fucans' from sequential extracts of the weed, except that 'fucan-1' contained glucose.

Alginic acid.

Freeze-drying of the calcium alginate (Extract 6) obtained from the sequentially extracted weed gave a fawn powder [6.9 g calcium alginate, 14.6% of the dry weight (as alginic acid)].

The calcium alginate was converted into sodium alginate by way of free acid (expt. 3.45). Paper chromatograms of a hydrolysate of the sodium alginate showed the presence of uronic acid as well as lactones. Ionophoresis of the hydrolysate in a borate buffer containing calcium ions (pH 9.8), revealed the presence of spots which had M_G value similar to those of authentic mannuronic acid and guluronic acid and their lactones respectively.

The relative viscosity of a 1% aqueous solution of the sodium alginate (from sequential alkali extract, expt. 3.45) was found to be 1.6 at 25°C. This very low value indicates that the alginic acid is considerably degraded. In view of obtaining a less degraded alginic acid, it was decided to extract the weed directly with alkali to minimise degradation of the alginic acid. The details and yields of this extraction are given in experiment 3.4.7 and flow chart 3.2 (p.68). Two samples of calcium alginates (alginate-1 and alginate-2) were isolated.

A combined overall yield of 18.56% of alginic acid was obtained compared with 14.6% from the previous sequential extraction. Solutions of 1% sodium alginate of the two alginic acid (alginate-1 and alginate-2) samples were found to have visoosities at 25° as follows:-

Alginate from 0.1% NaOH and 1% $NaCO_3$ extract (alginate-1)

$$\eta_{rel} = 48$$

Alginate from 3% Na_2CO_3 extract (alginate-2)

$$\eta_{rel} = 21$$

A comparison of some of the yields and viscosities of alginic acid obtained by sequential and direct alkaline extraction of Dictyopteris plagiogramma, with those of the alginic acid obtained from other brown seaweeds and reported by earlier workers are given in the following table.

Table 3.4 Yield and viscosities of alginic acids isolated from different brown algae

Name of the brown algae	Percentage of yield of Alginic acid		Viscosity (η_{rel})
	direct alkaline extraction	Sequential extraction	
Dictyopteris plagiogramma	18.6	14.6	48 (1.6)
Desmarestia ligulata[106]	19	16	300 (1.7)
Desmarestia firma[106]	23	17	68 (1.5)
Desmarestia aculeata[160]		12[a] 16[b]	
Himanthalia lorea[119]		16.0	
Bifurcaria bifurcata[119]		16.0	
Padina pavonia[119]		13.0	

[a] August collected weed; [b] March collected weed.
Figures in parenthesis indicate the relative viscosity of the alginate obtained by sequential extraction.

The above table reveals that species of brown seaweed from different families and very different morphological form all synthesise a reasonably similar proportion of alginic acid. It also reveals that drastic extraction procedure leads to a considerable degradation of the alginic acid, resulting in low percentage of yield and low viscosity.

Previous workers have indicated that the viscosity of alginic acid depends to some extent on the proportion of mannuronic acid to guluronic acid residues in the alginate. In order to determine this proportion a method of sequential hydrolysis was used (expt. 3.4.8) and the amount of uronic acid present was determined by the carbazole method.

In all cases part of the material is solubilised and the insoluble residue can be fractionated into a mannuronic acid rich fraction soluble at pH 2.85 (M-blocks) and a guluronic acid rich fraction insoluble at that pH (G-blocks). The fractions soluble in 0.4M-hydrochloric acid contain roughly equal amounts of the two uronides and was at first thought to be built up of alternating mannuronic and guluronic units (MG-blocks). Later work[190,193] indicated a more random arrangement.

The recoveries and constituents of the uronic acids obtained from the different hydrolysates are shown in table (3.5).

Table 3.5 The D-mannuronic/L-guluronic (M/G) ratio of alginic acid obtained from Dictyopteris plagiogramma by direct alkaline extraction

Alginic acid	Flask	Number of hydrolysis	Soluble material %	Insoluble material %	Overall recovery %
Alginate-1	1	1	19.4(36)	80(45.7)	99.4
	2	1 2	20(47.5) 18(40)	58.5(40)	96.5
	3	1 2 3	19.1(44) 16.0(49.4) 9.6(39)	46.8(44)	91.5

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

The percentage of the soluble material is based on a (1:1) mannuronic: guluronic acid graph. (Phenol sulphuric). The insoluble material was weighed after freeze-drying.

It can be seen from the table th at slightly more soluble material is present in the first hydrolysates of all the samples. This difference is often considerably larger and indicates that in the present material a certain amount of degradation and loss has occurred during isolation, in agreement with the relatively low viscosity of the alginate.

Comparison of the properties of the fucans from the different extracts

The carbohydrate content, uronic acid content and sulphate content of the fucans from the aqueous, acid and alkaline extracts are given in table 3.6.

Table 3.6 Composition of the different extracts obtained by sequential extraction of D. plagiograma

Extract from 39 g	dry alga wt.	Carbohydrate content (%)	Uronic acid[a] content (%)	sulphate[a] content (%)	Specific rotation $[\alpha]_D^{25}$
Cold water	550 mg	63.3	20	5.7	-24.3 (c = 1.0)
Hot water	2.42 "	64	12	5.6	-26.5 (c = 1.0)
Acid	2.25 "	66	19.7	3.9	-37.9 (c = 1.0)
Alkali	350 "	68.5	17	4	-22.5 (c = 1.0)

[a] Calculated on the basis of carbohydrate content.

It appears from the above table that the 'fucans' obtained from the different extracts possess similar constituents as far as the carbohydrate, uronic acid and sulphate content is concerned.

A comparison of the monosaccharide components of the above extracts is given below in table 3.7.

Table 3.7 Monosaccharide components in the hydrolysate of different extracts

	EXTRACTS			
Monosaccharides	Cold water (2A)	Hot water (2B)	Dil. HCl	Alkali
glucose	xxxx	xxxx	trace	nil
fucose	xxxx	xxxx	xxxx	xxxx
galactose	xx	xx	xx	xx
mannose	xx	xx	xx	xx
glucuronic acid	xx	xx	xx	xx
xylose	xx	xx	xx	xx

Fractionation of extracts (2A), (2B) and 3.

Fractionation of the polysaccharides isolated from natural origin is an important prerequisite for their structural investigation, because the polysaccharides isolated from biological materials are very often found as mixtures of different types. Various methods are adopted for the fractionation; among the classical methods 'fractional' precipitation is still playing an important role. However, this method is not very efficient if the solubility properties of the components are only slightly different or if only small amounts are available. Even in cases in which larger differences in solubility occur, the yield of pure fractions is frequently small.

The precipitating agents for polysaccharides can be classified broadly into three categories namely (a) organic solvents (b) a series of complexing metals such as copper salts, barium hydroxide etc. and (c) detergent cations, such as cetyltrimethylammonium bromide (Ce trimide),

cetylpyridinium chloride (cpc) and hyamine.

The principles used for fractional precipitations has been applied in a column procedure, where columns are prepared from inert porus materials, such as cellulose powder.

One of the significant advances in the fractionation of polysaccharides is the introduction of cellulose ion exchanger for column chromatography, especially suited for the fractionation of water soluble high molecular weight substances. Cellulose ion exchanger such as DEAE (diethylaminoethyl)-cellulose can be used advantageously for the fractionation of acidic as well as neutral polysaccharides.[201] Acidic polysaccharides are readily adsorbed on the DEAE-cellulose columns at pH values nearer 6 and are eluted, depending on their content of acidic groups, (a) by increasing the buffer concentration at the same pH (only for weakly acidic polysaccharides), (b) by alkaline solution of increasing strength, (c) by acidic solutions of increasing strength. Neutral polysaccharides are usually not or only weakly retained on the column at pH 5-6; they are however adsorbed when the column is brought to a basic condition.

The adsorption of polysaccharides on DEAE-cellulose is strongly influenced by the structure of the polysaccharides to be fractionated, however the adsorption on DEAE-cellulose is generally enhanced with increasing amounts of acidic groups in the polysaccharide molecules. In a homologous series of linear polysaccharides, the lower molecular weight material is held less firmly than the higher molecular-weight material.

Among other fractionation methods gel filtration on Sephadex and Sepharose column,fractionation by ultrafiltration and by electrophoresis are also important.

Most recently high pressure liquid chromatography is also being used for the fractionation of polysaccharide mixtures. By this method polysaccharide mixtures can be separated according to their molecular weight and an idea about the molecular weight of each polysaccharide component present in the mixture can be obtained by correlating the retention time of the polysaccharides with the retention time of the polysaccharide mixtures of known molecular weights (standard). By use of a preparative HPLC, quantitative separation of the different polysaccharides present in the mixture is also possible.

3.7.5 Investigation of aqueous extracts by high pressure liquid chromatography

The extracts (2A and 2B) were subjected to high pressure liquid chromatographic (HPLC) investigation for the purpose of separating the individual polysaccharide components from the mixture (expt. 3.5.1). The chromatograms revealed that both the extracts 2A and 2B are identical, both being comprised of two different polysaccharide components. When compared with the peaks of polysaccharides of known molecular weight (standard) it was revealed that both the cold and hot aqueous extracts (2A and 2B) are built of polysaccharides of two different molecular weights, one having molecular weight in the range of 25,000 - 40,000 and the other between 3,500 and 4,000 (fig. 3.2).

3.7.6 Fractionation of the aqueous extracts on DE-52 cellulose column

Previous studies[106,119,161] on other brown seaweeds have shown that the aqueous extracts are a mixture of laminaran, a (1 → 3)-linked glucan and a family of polydisperse-heterofucans, and that fractionation can be achieved on columns of DE-52 cellulose by elution with water followed by increasing concentration of aqueous potassium chloride.

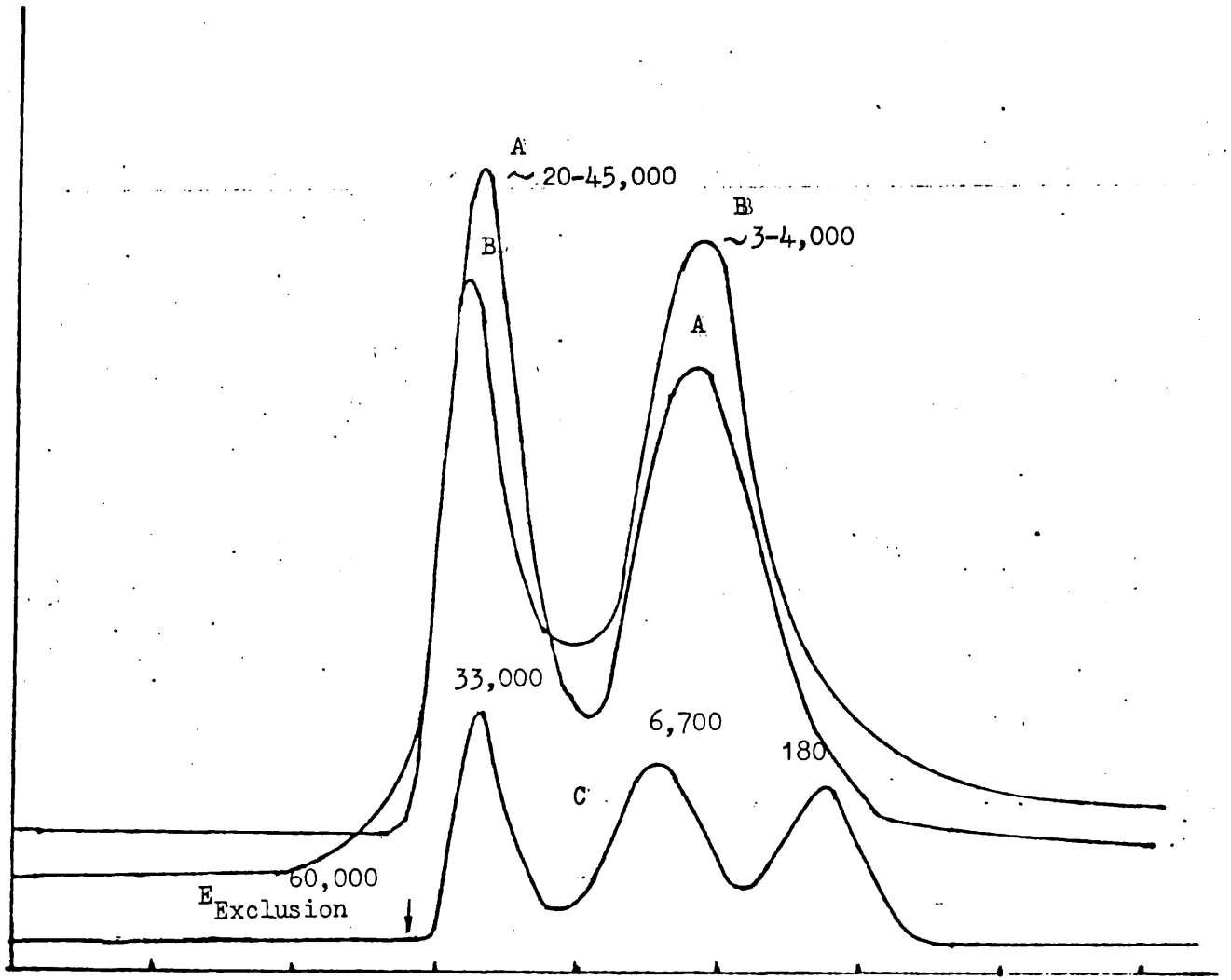


Figure 3.2 High pressure liquid chromatogram of the aqueous extracts from Dictyopteris plagiogramma. (A) cold aqueous extract (B) hot aqueous extract (C) polysaccharide mixture of known molecular weight (standard).

Samples of the aqueous extracts were therefore applied to DE-52 cellulose columns (expt. 3.5.2) and the following separations were achieved for the extracts (2A and 2B) (see tables 3.8 and 3.9)..

Table 3.8 Fractionation of (2A) on DE-52 column

Weight of the polysaccharide applied to the column = 206 mg

Fraction	Recovery (%)	Carbohydrate content (%)	Uronic acid content (%)	Sulphate content (%)
Water	28.5	87.4[a]	nil	nil
0.1M-KCl	4.8	60[b]	22.8	3.6
0.2M-KCl	21.65	62[b]	22	3.8
0.3M-KCl	21.45	67.2[b]	13.6	7.6
0.5M-KCl	4.2	52.4[b]	9.3	8.2
0.8M-KCl	2.9	43.3[b]	8.7	not determined
M-KCl	3.3	16.7	not determined	not determined

Total recovery, 86.8%; Specific rotation of the aqueous fraction $[\alpha]_D^{25^\circ} = -10.24^\circ$ (c = 0.41).

[a] carbohydrate content based on a standard glucose graph

[b] carbohydrate content based on a standard graph of an artificial mixture of fucose:galactose:mannose:xylose:glucuronic acid = (5:1:1:1:2) graph.

Table 3.9 Fractionation of (2B) on DE-52 column.

Weight of the polysaccharide applied to the column = 507 mg.

Fraction	Recovery (%)	Carbohydrate content (%)	Uronic acid content (%)	Sulphate content (%)
Water	36	84.5[a]	nil	nil
0.1M-KCl	2.1	55[b]	16.5	not determined
0.2M-KCl	15.4	66[b]	16.0	4.1
0.3M-KCl	20.9	73.1[b]	12	7.4
0.5M-KCl	7.3	44.5[b]	10	8.5
0.8M-KCl	3.5	46.2[b]	7.5	not determined
M-KCl	1.9	19.5[b]	not determined	not determined

Total recovery 87.1%, specific rotation of the aqueous fraction

$$[\alpha]_D^{25^\circ} = -11.3^\circ \qquad c = 0.23$$

[a] carbohydrate content based on a standard glucose graph

[b] carbohydrate content based on a standard graph of an artificial mixture of fucose:galactose:mannose:xylose:glucuronic acid = (5:1:1:1:2) graph.

The properties of the different fractions of (2A) and (2B) reveal a considerable similarity between the two extracts. The aqueous fractions of both the extracts were devoid of uronic acid and sulphate indicating that this is a neutral polysaccharide. For both the extracts 0.2M- and 0.3M-KCl eluted the major acidic polysaccharide fractions. The sulphate contents of these major fractions are also

very similar. In both the extracts, fractions with higher uronic acid content have lower sulphate content and vice versa. The sulphate content of 0.1M-, 0.8M- and M-KCl fractions were not determined due to the small percentage of recovery in these fractions.

From the above fractionation results it appears that the adsorption of the polysaccharide on DE-52 cellulose column is strongly influenced by the uronic acid and sulphate present in these polysaccharides. Although the adsorption on DE-52 cellulose is enhanced with increasing amounts of acidic groups in the polysaccharide molecules, this is not true in all cases. In the present fractionation studies, ideally one should have expected fractions with higher uronic acid and sulphate contents to be eluted with the eluant of higher concentration, but this was not the case. From the above tables it can be seen that, although increasing concentration of eluant eluted fractions with increasing percentages of sulphate content, the percentage of uronic acid in these fractions were not in increasing order. Other workers also reported similar results on the fractionation of fucans from other seaweeds.[161] This apparently anomalous behaviour of the fucans can be explained in two ways, either (1) the sulphate groups present in these molecules contribute more towards the acidity of the polysaccharides or (2) the uronic acid rich fraction of the polysaccharide eluted with eluant of low concentration possesses low molecular weight, because the adsorption on DE-52 column depends not only on the acidity but also on the size of the molecule. It has already been stated that in a homologous series of linear polysaccharides, the lower molecular weight materials is held less firmly than the higher molecular weight material.[205,206] Furthermore, it can be mentioned that the shape of the polysaccharide molecules also influences adsorption, for example, in fractionation of soluble starch and dextrans, the linear components were retained more strongly than the branched components.[201]

3.7.7 Fractionation of the aqueous eluate from the DE-52 column

Hydrolysis of the aqueous eluate and investigation of the hydrolysate by paper chromatography revealed the presence of glucose and a faint spot of a non-reducing saccharide, possibly due to mannitol. A portion of the hydrolysate was reduced and the product converted into TMS derivatives. G.l.c. analysis of the derived TMS-alditols revealed the presence of glucitol and mannitol, thereby confirming the conclusion made by paper chromatography. An aliquot of the hydrolysate was treated with glucose oxidase to convert the glucose into gluconic acid. Paper chromatogram of the derived solution was compared with the chromatogram of a mixture of glucose: mannitol (30:1) which had also been treated with glucose oxidase. The two mixtures revealed identical chromatograms confirming the presence of mannitol in the glucan. It has been reported[139] that the G- and M-chains of laminaran can be separated in an undegraded form by fractionation on a DEAE-Sephadex-molybdate column by eluting the former with water and the latter with 0.25M-sodium chloride solution. All attempts to separate the M-chains from G-chains of the present laminaran on a DEAE-Sephadex molybdate column proved unsuccessful.. Although two fractions were obtained (table 3.10 and fig. 3.3) they each proved to be mixtures of the two types of chains.

3.7.7 Fractionation of the aqueous eluate from the DE-52 column

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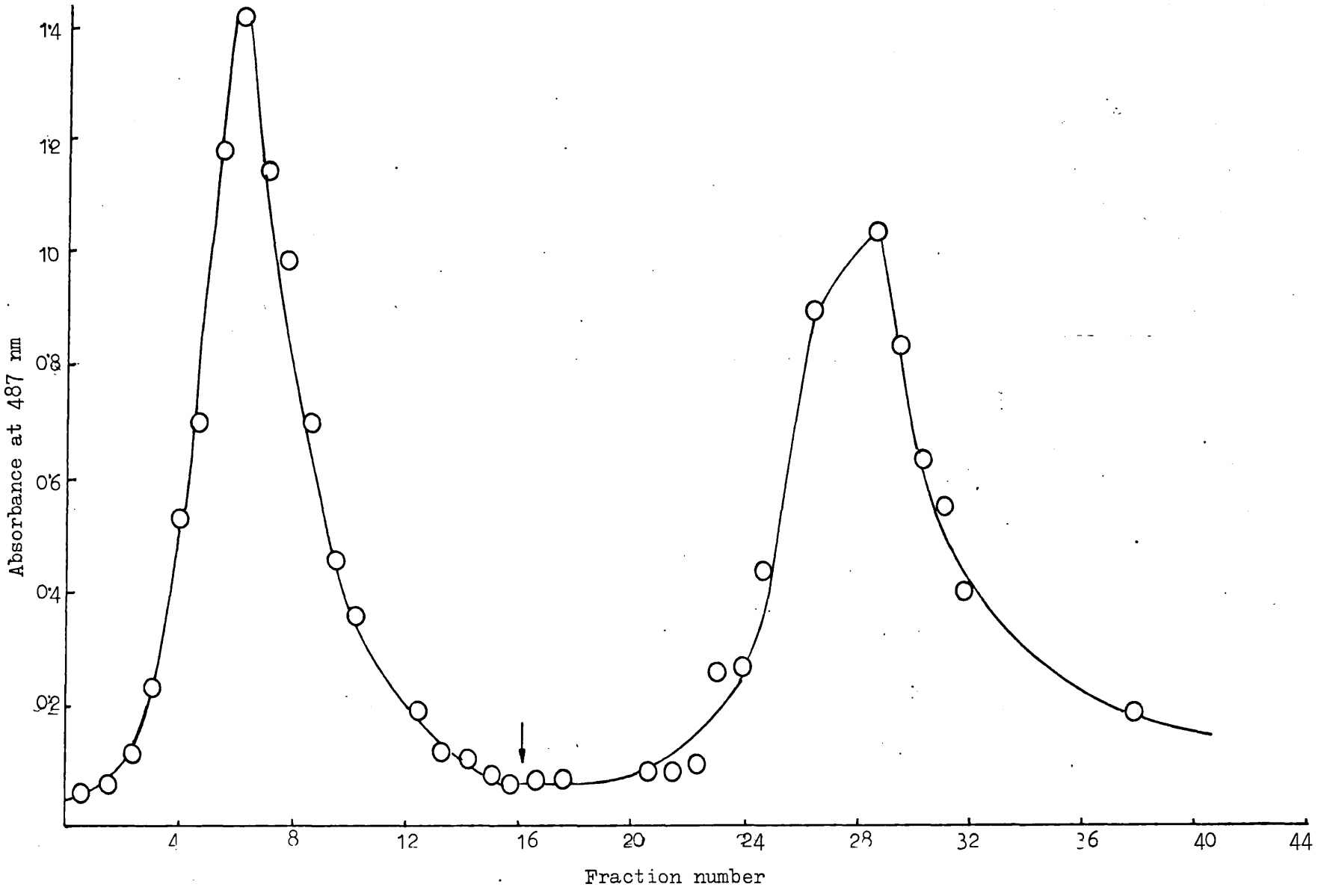


Figure 3.3 Fractionation of the aqueous eluate from the DE-52 cellulose column on DEAE-Sephadex-molybdate: —O— , phenol sulphuric acid reaction. The vertical arrow indicates the change from water to 0.25M sodium chloride.

Table 3.10 Fractionation of aqueous eluate on DEAE-Sephadex-molybdate column

Weight of the polysaccharide applied to the column = 75.6 mg

Recovery in fractions (mg		Total recovery (mg)	% recovery
Aqueous	0.25M-NaCl		
23.4	27.3	50.7	68

All the other fractions from DE-52 columns were also hydrolysed and examined by paper chromatography. The monosaccharide components of the different fractions of both the extracts (2A) and (2B) appeared to be identical. The monosaccharide components identified in the different fractions of both the extracts are shown in table 3.11.

Table 3.11 Monosaccharide components of the hydrolysates of extracts (2A and 2B)

Monosaccharide Components	Fractions						
	Water	M-KCl					
		0.1	0.2	0.3	0.5	0.8	1.0
Fucose	-	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
Glucose	xxxx	-	-	-	-	-	-
Galactose	-	xx	xx	xx	xx	xx	xx
Mannose	-	xx	xx	xx	xx	xx	xx
Xylose	-	x	x	x	x	x	x
Glucuronic acid	-	xx	xx	xx	xx	x	x
Mannitol	x						

xxxx indicates major component (by visual comparison)

x indicates minor component (by visual comparison).

The major fractions of both extracts were also investigated by g.l.c. after converting the components into TMS-alditols. The g.l.c. results confirmed the identification of the monosaccharides made by paper chromatography.

The aqueous and 0.3M-KCl fractions of (2A) and (2B) separated on the DE-52 column were investigated separately by HPLC. The aqueous fractions of both the extracts gave single peaks in the relatively low molecular weight range of 3,500 - 4,000 while the 0.3M-KCl fractions of both the extracts again gave single peaks with molecular weight ranging from 25,000 - 40,000 (fig.3·4a,3·4b) These results are in agreement with the HPLC results of these extracts before fractionation when two peaks were obtained.

Aliquots of the acid extract (Extract-3) were also fractionated on DE-52 cellulose column, which revealed a similar fractionation pattern, the only difference was that the aqueous fraction was devoid of any carbohydrate. The different KCl fractions, when examined by paper chromatography after hydrolysis revealed the presence of similar monosaccharide components as found in the KCl fractions of the aqueous extracts (2A) and (2B).

3.7.8 Attempted fractionation of the hot aqueous extract (2B) with hyamine-1622

Quaternary ammonium salts are effectively employed in the fractionation of polysaccharide mixtures. As the fractionation of the polysaccharides (Extract 2A, 2B and 3) on DE-52 cellulose column failed to produce any homogeneous fractions of fucans, except separation of a glucan from the acidic fucose containing polysaccharide, an attempt was undertaken to fractionate the above extract with hyamine-1622 (diisobutyl-phenoxy-ethoxy-ethyl-dimethylbenzylammonium chloride)

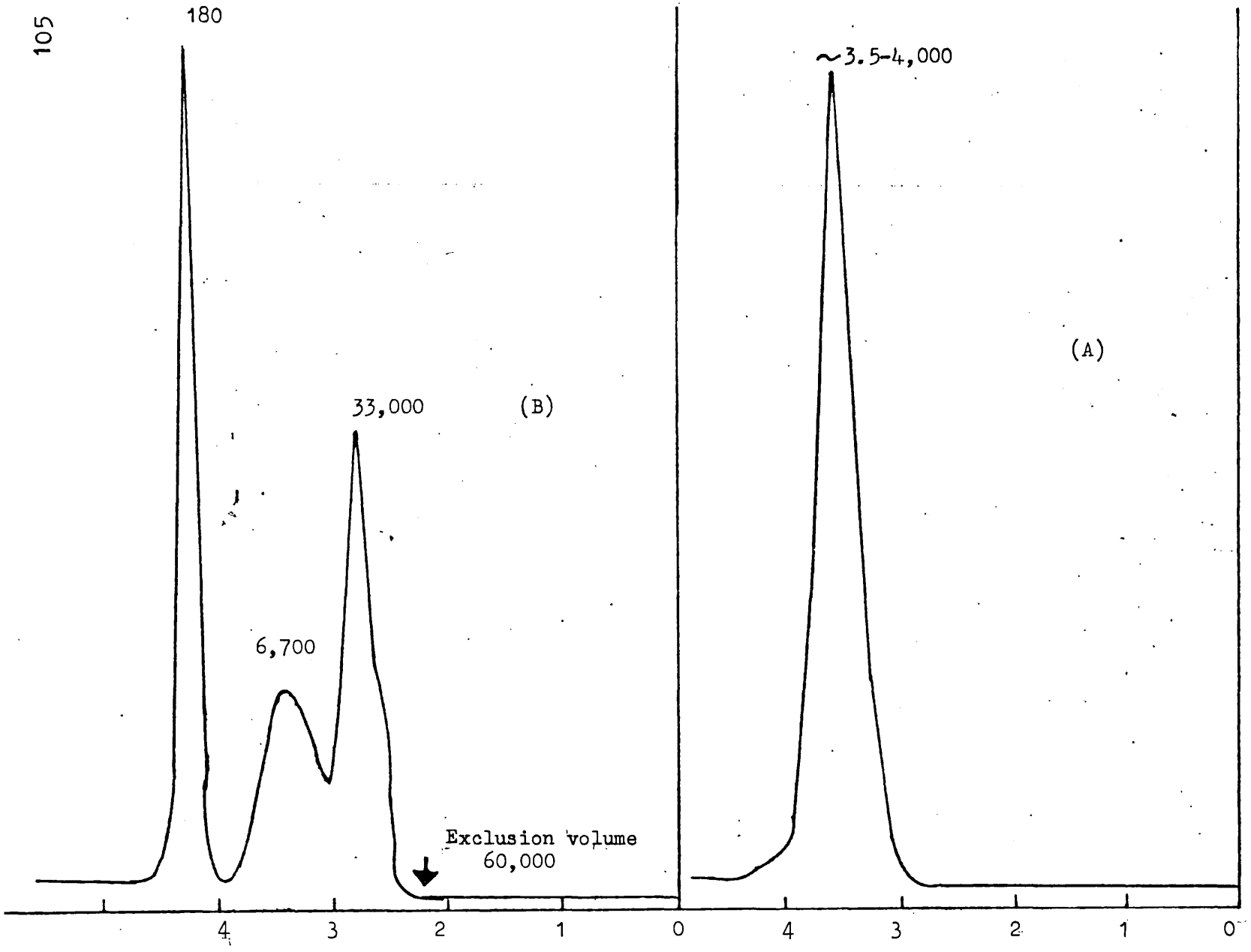


Figure 3.4a High pressure liquid chromatogram of the aqueous eluate obtained by fractionation of extract-2 on DE-52 cellulose column.(A): Aqueous eluate (B): polysaccharide mixture of known molecular weight.

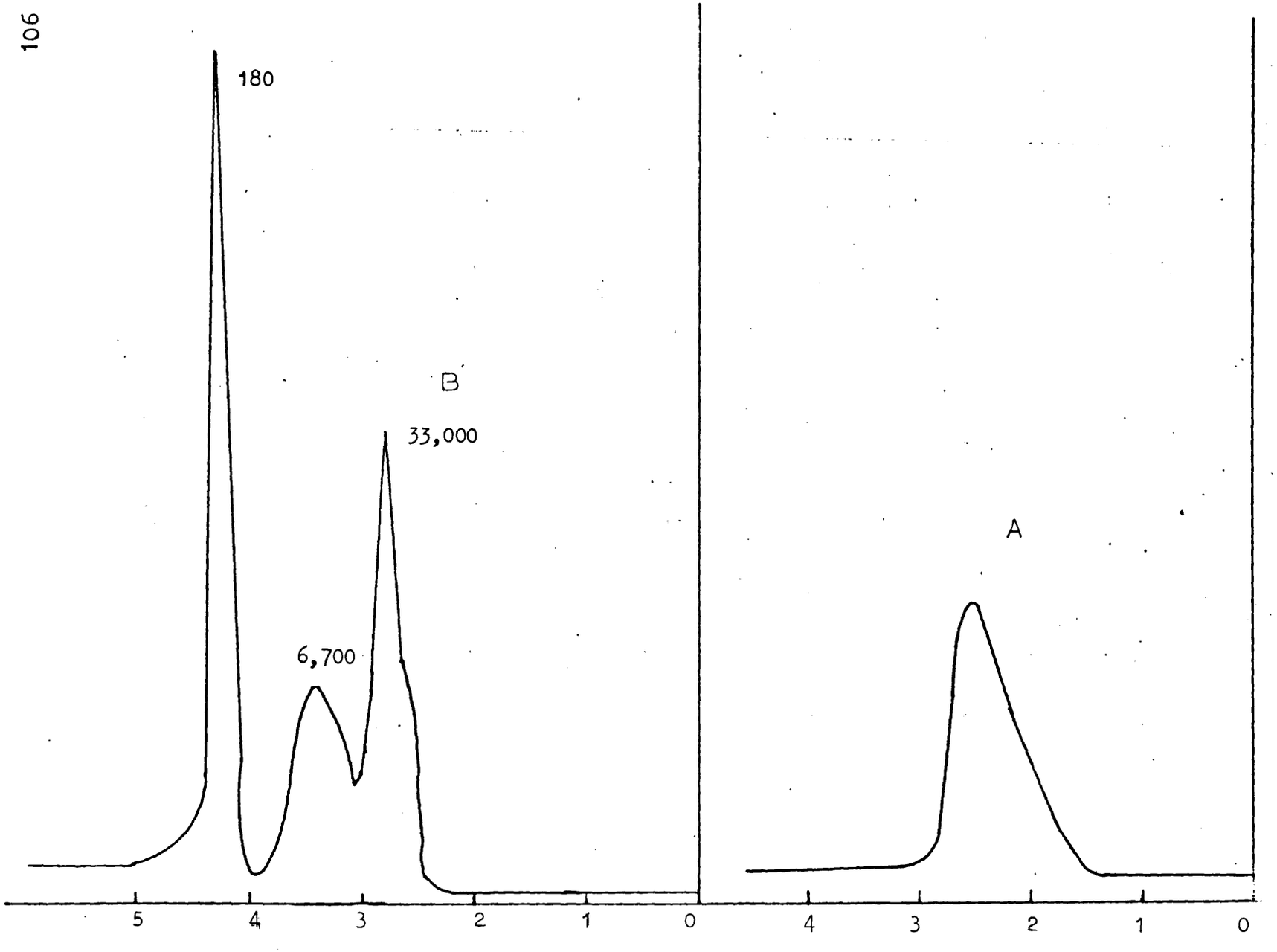
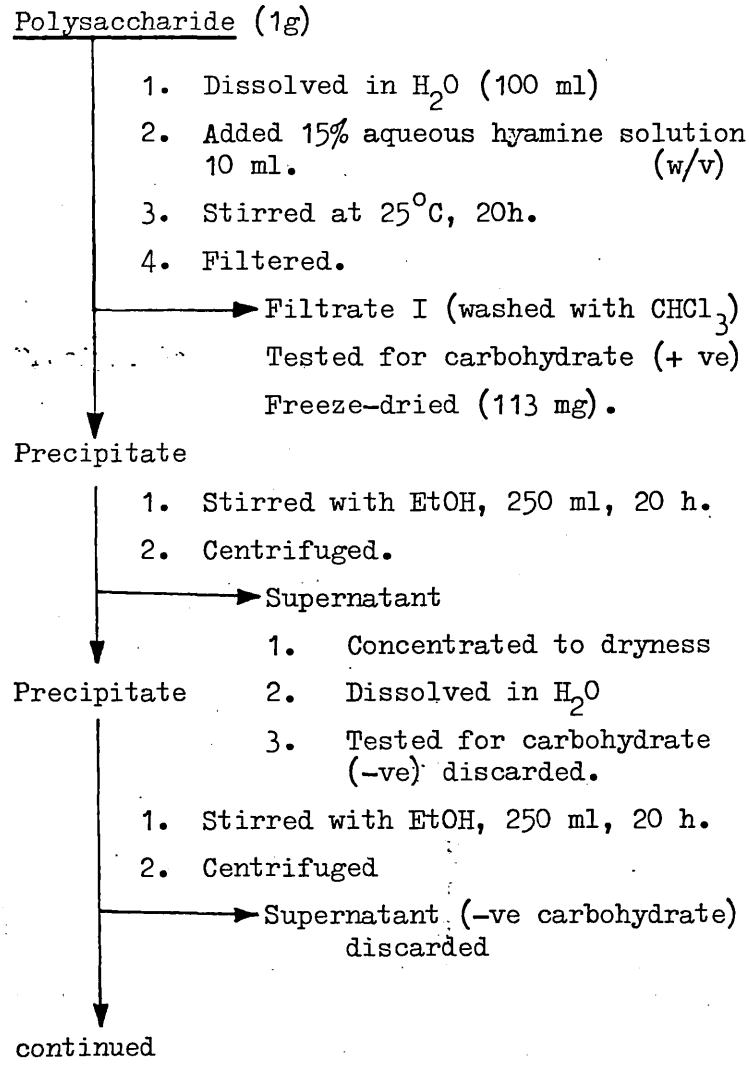
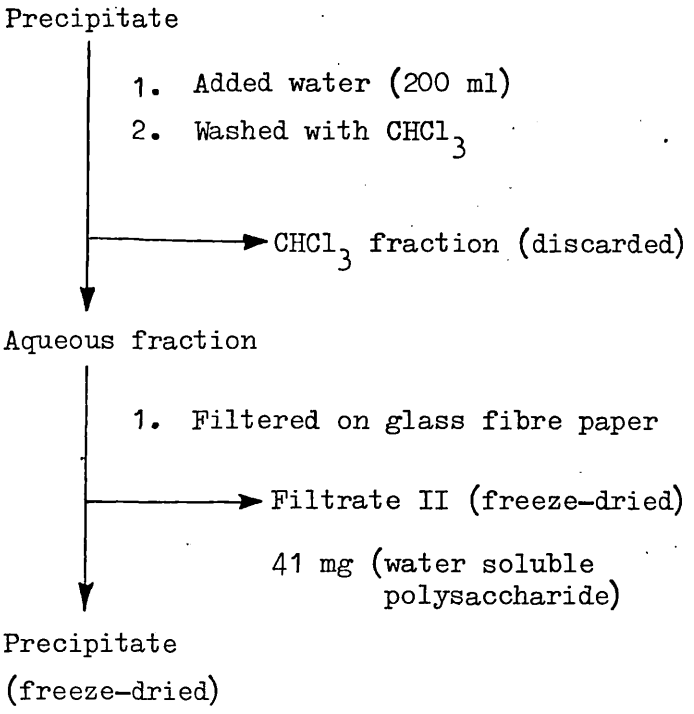


Figure 3.4b High pressure liquid chromatogram of the 0.3M KCl eluate obtained by fractionation of extract-2 on DE-52 cellulose column. (A) 0.3M-KCl eluate (B) polysaccharide mixture of known molecular weight.

in order to obtain a polysccharide containing only fucose as the monosaccharide unit. Although different quaternary ammonium ions are used for the fractionation of polysaccharide mixtures the use of hyamine for this purpose has not been reported before. It was hoped that the quaternary ammonia (QN^+) ion of hyamine will form an insoluble complex with the acidic polysaccharide of the extract thereby providing homogeneous polysaccharide fractions. The fractionation was carried out according to the following flow chart.

Flow chart 3.3





Examination of the different fractions

Filtrate-I The hyamine was removed from this fraction by extracting several times with chloroform. The aqueous layer was then reduced to smaller volume and freeze-dried, total yield 113 mg. Paper chromatographic examination of the hydrolysate of this fraction revealed glucose as the major monosaccharide along with a faint spot corresponding to mannitol. This corresponds therefore to the glucan separated and characterised from (2B) on DE-52 cellulose column (page 101).

Filtrate-II - and final precipitate

It appears from the flow chart 3.2 that several treatments of the precipitate with EtOH and $CHCl_3$ to remove the hyamine failed to produce a precipitate soluble in water, thereby indicating that complete removal of the hyamine from the polysaccharide complex was not achieved. As the carbohydrate content of the water insoluble fraction

(precipitate) could not be measured due to its insolubility - in water, this was determined after hydrolysis. The properties of the different fractions are given in table 3.12.

Table 3.12 Properties of the different fractions of hyamine fractionated polysaccharide (2B); ($SO_4^=$ 5.6%)

Fraction	Weight mg	Carbohydrate[a] content (%)	Uronic acid content (%)	Sulphate content (%)
Filtrate-I	113	90	not determined	not determined
Filtrate-II	41	78	9.8	3.3
Precipitate	495	60*	not determined	2.15

[a] by phenol sulphuric acid method.

[b] carbazole method

* carbohydrate content of the hydrolysate.

The constituent monosaccharides of the different fractions and their relative proportions are presented in table 3.13.

Table 3.13 Monosaccharide components of the different fractions and their approximate molar relative proportion

Fraction	Monosaccharide components	Approximate molar proportions
Filtrate-I	glucose:mannitol	70:1
Filtrate-II	fucose:xylose:galactose: mannose:glucuronic acid	4.2:1:1.2:0.9:2.2
Precipitate	fucose:xylose:galactose: glucuronic acid	8:1:5:2

In analogy with previous fractionation methods (DE-52 cellulose column, page 103) fractionation of the 'fucan' by hyamine precipitation also failed to produce a homopolymer, it can however be mentioned here that controversial opinion about the structure of 'fucan' still exists among the different groups working in this field. Although it has been known for 65 years that fucose-containing polysaccharides are present in many brown algae[119] and on the basis of detailed structural work[119] carried out on a sulphated fucan obtained from a single species (Fucus vesiculosus), it has become widely accepted that the fucose in other species are present as homopolymers of the same type and the residues of xylose uronic acid, galactose and mannose which have been detected in the fucan preparations were regarded as impurities. More recent studies[210] on the basis of examination of a wide range of brown algae have claimed that xylose and a number of other sugars besides fucose must be regarded as normal structural units of fucans.

In contrast to the fractionation on DE-52 cellulose the hyamine method separated all the mannose containing fucan in the filtrate II, although it is clear that a considerable proportion of the carbohydrate (33%) was lost in the fractionation.

3.7.9 Fractionation of Extract-3 (Fucan-A) by 'Cetrimide' precipitation

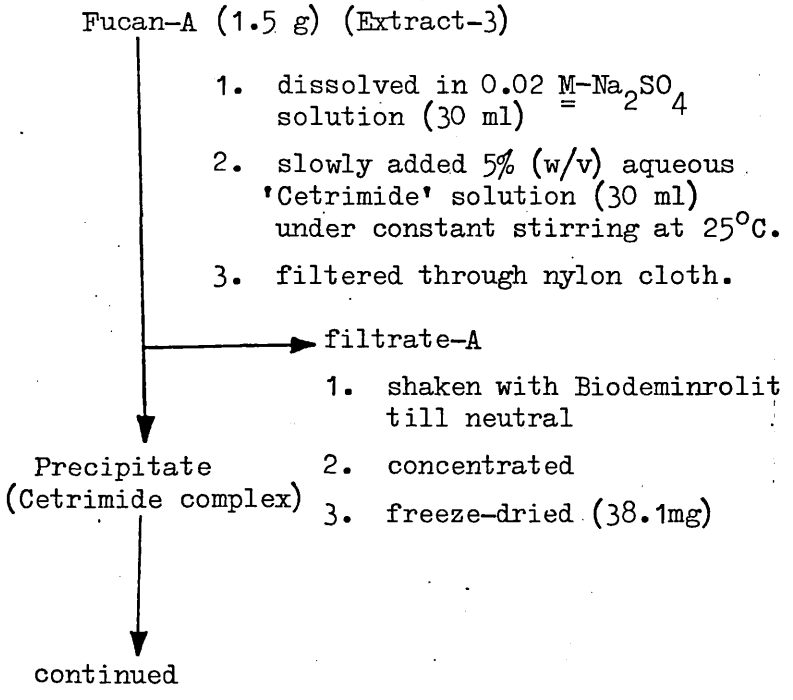
As the attempts to establish the homogeneity of 'fucan' by different methods, namely fractionation on DE-52 cellulose column and hyamine precipitation gave no well-defined fractions and a proportion of the material was lost during the fractionations, another attempt was undertaken to fractionate the 'fucan' into homopolymers by forming a complex with hexadecyltrimethyl ammonium bromide (Cetrimide).

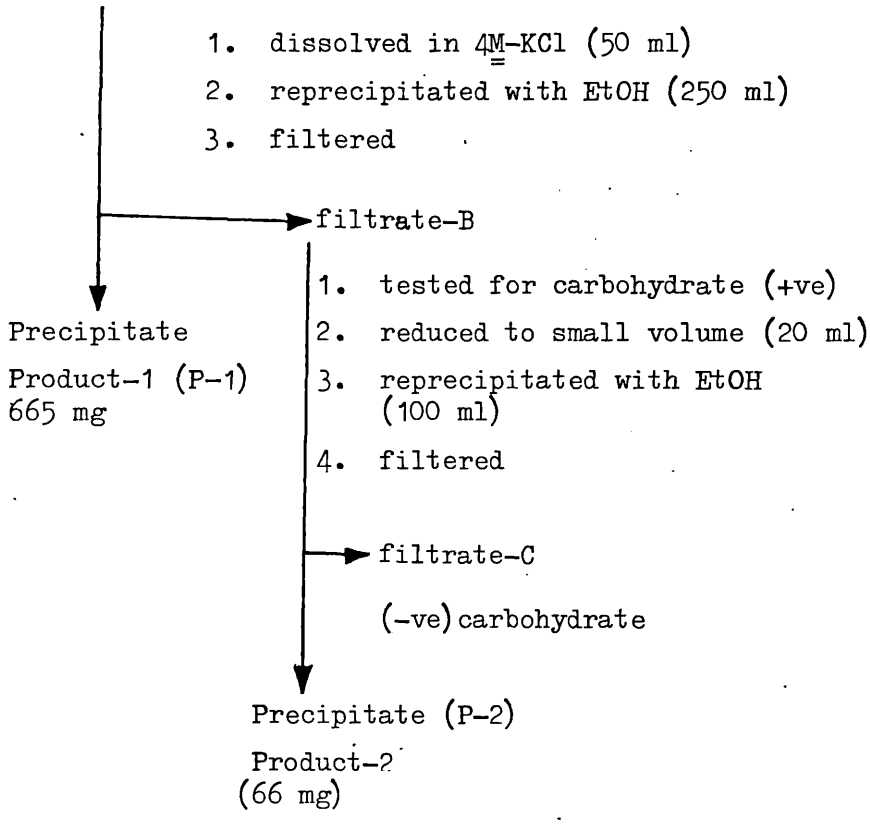
It is well known[202,203] that detergent cations, such as hexadecyltrimethyl ammonium, form salts with polyanions which are very insoluble in water. Neutral polysaccharides capable of forming complexes with borate ions can however react with 'Cetrimide' if present as borate complex. In the absence of the borate complex of neutral polysaccharides acidic polysaccharides are therefore easily separated from neutral polysaccharides by precipitating them directly with 'Cetrimide' in acidic, neutral or mildly alkaline solution.

A small concentration (0.02 $\underline{M}$ - or less) of sodium sulphate is added to the cetrimide solution in order to increase the rate of aggregation of the precipitate. Electrolytes other than the added sodium sulphate should be kept to the minimum to avoid the possibility of redissolving the precipitates. The precipitate, are soluble in some organic solvents and in salt solutions.[204]

The different fractions of the 'fucan' resulting from this fractionation are shown in the following flow chart.

Flow chart 3.4





Although three different fractions as shown in the flow chart were obtained, except the contaminating glucan (filtrate-A), each fraction was found to contain the same monosaccharide components. The carbohydrate content, uronic acid content, sulphate content and nitrogen content of all the fractions were determined. The properties of the different fractions are given in table 3.14.

From the table it is evident that a fractionation of the 'fucan-A' containing fucose as the only monosaccharide unit was not achieved. The material which complexed with 'Cetrimide' closely resembled the starting material. A small proportion which remained in solution was proved to be a glucan with mannitol as the minor constituent (p.c.). Further dissolution of the precipitate in potassium chloride (4M-) and reprecipitation with ethanol resulted in product-1 (P-1) and product-2 (P-2) (see flow chart 3.4). The product-1 and product-2 were found to contain similar monosaccharide units. One of the major differences between product-1 and product-2 is the relatively higher proportion of

Table 3.14 Properties of the different fractions obtained by 'Cetrimide' fractionation of the extract-3 (Fucan-A) of D. Plagiogramma

Fraction	Weight mg	Carbohydrate Content %	Uronic acid content %	Sulphate content %	Nitrogen content %	Monosaccharide components
Filtrate-A	389	85	-	-	1.4	glucose, mannitol
Product-1 (P-1)	665	62.5	2.2	3.5	0.83	fucose, xylose, galactose, mannose, glucuronic acid
Product-2 (P-2)	66	45	40	5.3	3.39	fucose, xylose, galactose, mannose, glucuronic acid.
Fucan-A (before fractionation)	1.5g	66	19.7	3.9	0.78	fucose, xylose, galactose, mannose, glucuronic acid, glucose.

Total recovery: 769.1 mg

Percentage of recovery: 51.3%

uronic acid content in the latter. This probably represents the extreme of this family of polydisperse molecules. The high nitrogen content of product-2 indicates that a considerable amount of Cetrimide is still present in this fraction despite repeated dissolution and precipitation to remove the Cetrimide. The loss of about 50% by weight of the starting material by this fractionation method is difficult to explain; however repeated dissolution and precipitation is necessary to remove all the cetrimide from the polysaccharide and this could account at least in part for the loss.

All these methods indicate a family of polydisperse heteropolysaccharides with a higher uronic acid at one extreme of the family. This is in keeping with the fractionation studies of fucans from other brown seaweeds.[106.119]

3.7.10 Fractionation of the extract-3 (Fucan-A) by trimethylsilylated glass fibre paper (TGFP) electrophoresis

It has been stated that many polysaccharides which were designated as homogeneous on the basis of different fractionation studies, can be separated into two or more components when examined by glass fibre paper electrophoresis.[211] The advantage of using the glass fibre paper rather than cellulose paper is that complexes between the polysaccharide and the fibre are avoided[212] and any polysaccharide can be detected for there is no restriction on the type or vigour of chemical test that has to be applied to locate the polysaccharides.

As a result of the failure of the different fractionation methods to produce a homogeneous fucose-containing polysaccharide, it was

decided to fractionate the 'fucan-A' on trimethylsilylated glass fibre paper by electrophoresis at pH 5 using acetate buffer. The purpose of treating the glass fibre paper with trimethylsilylating agent is to overcome the high rate of endosmotic flow in unmodified glass fibre paper.[145] Recently in an attempt to develop a set of techniques for separating plant cell wall polysaccharides easily at the required level of resolution, on the analytical as well as the preparative scale, Jarvis et al.[144] reported that the trimethylated silylated glass fibre paper almost eliminates the electroendosmosis at pH 5, and would float indefinitely on water, but could be wetted in buffer to which a surfactant had been added.

Application of this technique in the fractionation of 'fucan-A' (extract-3) (expt. 3.5.8) resulted in two different fractions, one remained at the starting line and the other fraction moved along the paper, the two fractions thus separated were examined by paper chromatography and g as liquid chromatography, which again revealed, in line with other fractionation results, that neither of the fractions obtained by this method is a homopolysaccharide, although the composition of the two fractions differed considerably. The composition of the two fractions as obtained from a typical experiment is shown in table 3.15.

Table 3.15 Composition of the stationary and moving fractions of the TGFP fractionated 'fucan-A'

Fraction	Amount of carbo-hydrate (mg)	Percentage of recovery	Percentage of sulphate	Monosaccharide components
Stationary	1.7	20	0	fucose, xylose, glucose galactose, mannose.
Moving	6.0	70	4.0	fucose, xylose galactose, mannose, glucuronic acid.
'fucan-A' (before fractionation)	8.6		3.9	fucose, xylose, galactose, mannose, glucuronic acid, glucose.

Total recovery 7.7 mg i.e. 90%

It is evident from the above results that although this method of fractionation failed to prove homogeneity of the 'fucan' molecule, it indicates the possibility of the existence of some blocks with non-sulphated fucose units; the moving fraction was shown to comprise similar monosaccharide components to the original 'fucan' and this is the major fraction. In analogy with other fractionation methods the present method also produced a small fraction which differed from the original material in that it is charged group free and may be the precursor of the fucans.

3.7.11 Periodate oxidation studies of glucan (aqueous eluate from DE-52 column).

Previous structural studies on glucan isolated from the aqueous extracts of other brown seaweeds confirmed its structure as β-(1→3)-linked glucan. In order to investigate whether the glucan obtained from the aqueous extracts of D. plagiogramma, has similar structural features as the structure of the glucans from other brown seaweeds and to determine the percentage of M-chainsin the glucan, periodate oxidation studies were carried out.

Periodate oxidation gives useful information in structural investigation. Thus sodium metaperiodate will cleave the C-C bond in 1,2-diols depending on pH of the solution and reaction time. If more than two contiguous hydroxyl groups are present formic acid is produced. Primary alcohol end groups give rise to formaldehyde. Thus α(1→3)-linked glucan will only be attacked by periodate at the reducing and non-reducing end with the liberation of one mole of formaldehyde per molecule. (fig. 3.5).

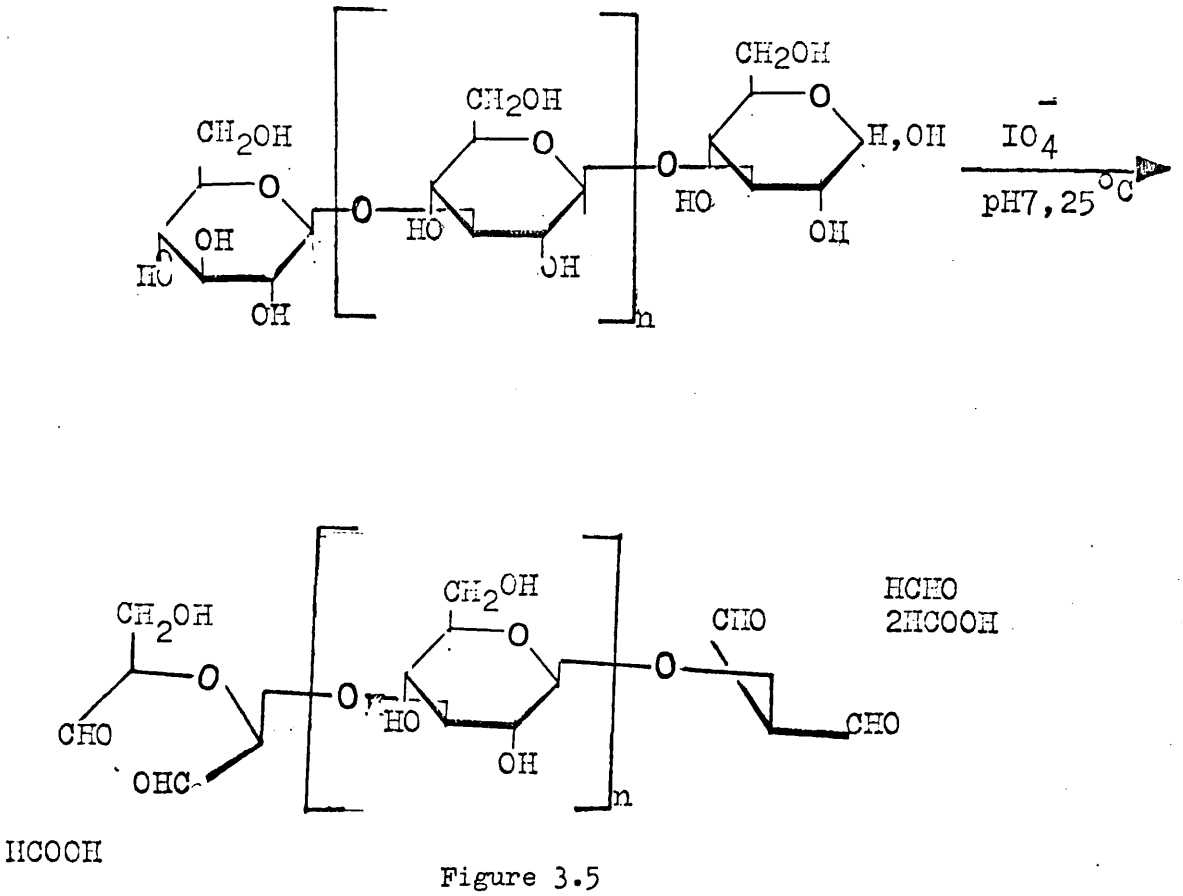


Figure 3.5

It is possible however, for a (1→3)-linked glucan to be "overoxidised" by periodate by a stepwise procedure from the reducing end, due to the formation of a malondialdehyde derivative which contains an activated hydrogen atom and is further subject to periodate oxidation. This "overoxidation" takes place slowly at room temperature but much more quickly at pH 8 and 35°C. This process proceeds with the liberation of 1 mole of formaldehyde from each monosaccharide residue. (fig. 3.6).

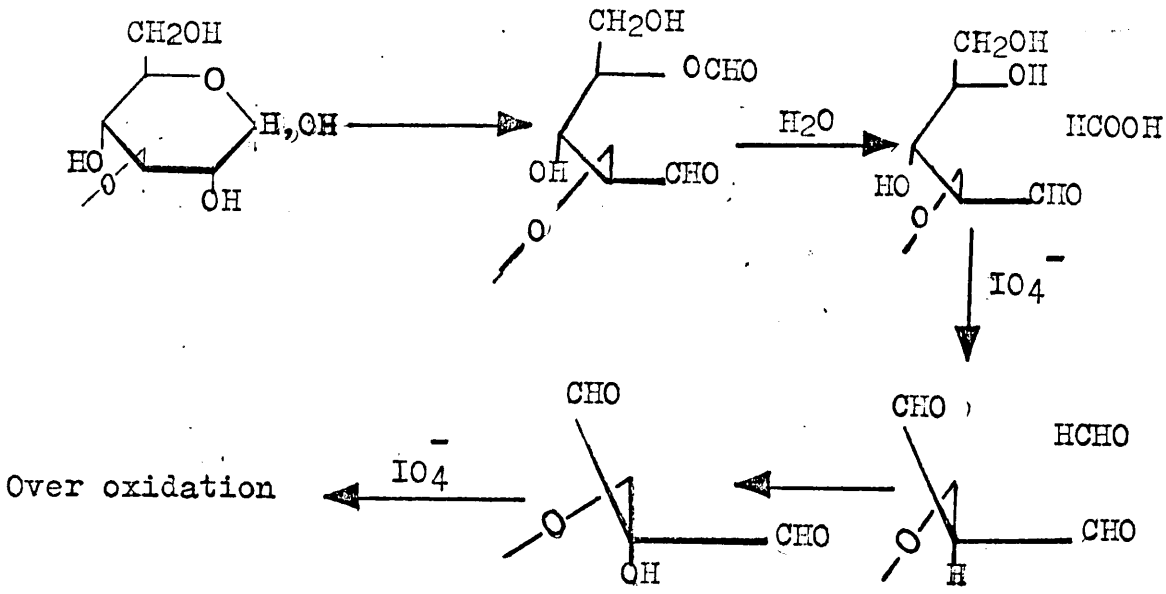


Figure 3.6

The number of glucose units degraded can be determined by estimating the amount of formaldehyde released.

Periodate oxidation reaction may be made more selective by lowering the temperature of reaction. Thus at 2° and at pH 7 the reducing end group of a polysaccharide is oxidised to form a formyl ester, which is fairly stable at this temperature and no formaldehyde is released from the primary alcohol group (fig. 3.7).

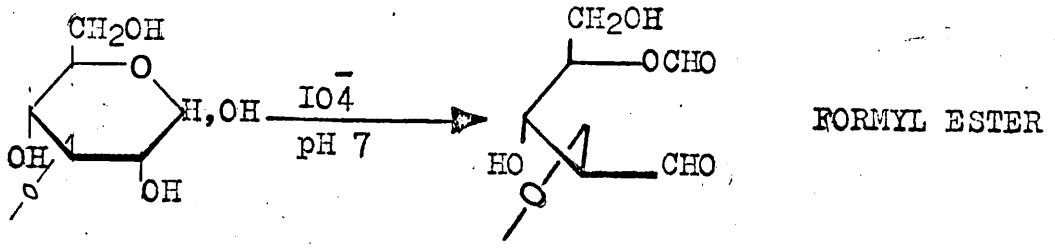


Figure 3.7

If a C-1 substituted hexitol is present in the molecule however, formaldehyde is produced from the primary alcohol group under these conditions. By calculation from the yield of formaldehyde, the percentage of hexitol in the molecule can be obtained. Provided the molecular weight of the polysaccharide is known, the percentage of hexitol in the molecule can also be determined by measuring the amount of periodate reduced under a given set of reaction conditions. Using the latter method the percentage of mannitol in the glucan from D. plagiogramma has been determined.

The amount of periodate reduced at different intervals of time at 2^{o} and at room temperature by the present glucan and by another sample of laminaran (Bifurcaria bifurcata) of known composition, which is devoid of the mannitol end group was determined (see table 3.16).

Table 3.16 Reduction of periodate

$NaIO_4$ reduced (moles/A.G.U.)

	Temperature					
	2^{o}	2^{o}	2^{o}	2^{o}	25^{o}	25^{o}
	Time in days					
	1	2	4	5	5	6
Laminaran (G-chain) 32.5 mg	0.193	0.193	0.193	0.193	0.354	0.354
Glucan (G + M-chain)	0.255	0.255	0.255	0.255	0.374	0.374

The small reduction of periodate by both the glucans indicates that both these samples are (1→3)-linked polysaccharides, thereby confirming that the glucan isolated from the aqueous extract of the D. plagiogramma is identical with the laminarans isolated from similar extracts of other brown seaweeds. At 2° the G-chain laminaran (B. bifurcata) reduced 0.193 moles and (G+M-chain) Dictyopteris laminaran reduced 0.255 moles of periodate for every anhydrohexose unit. Since B. bifurcata laminaran is comprised solely of G-chains this corresponds to an average chain length of 15. The degree of polymerisation of the Dictyopteris laminaran was 14 as determined by the Timmell[137] method. From the amount of periodate reduced by this laminaran, the percentage of M-chain laminaran was calculated and it was found that 19% of the laminaran possesses mannitol end group. At room temperature 0.354 and 0.374 moles of periodate are reduced for each anhydrohexose unit by G-chain and (G+M)-chain laminaran, respectively, indicating an average chain length of 18 for the former and the presence of 23% of M-chain laminaran in the latter polysaccharide. The percentage of M-chain laminaran in Dictyopteris plagiogramma, calculated from the amount of periodate reduced at 2° and at room temperature is in reasonable agreement with each other and it can be concluded that on average about 20% of Dictyopteris laminaran has mannitol end groups. The overall yield of laminaran in the present weed was 3% of the dry weight.

The percentages of yield of laminaran obtained from other brown seaweeds are given in the following table for comparison.

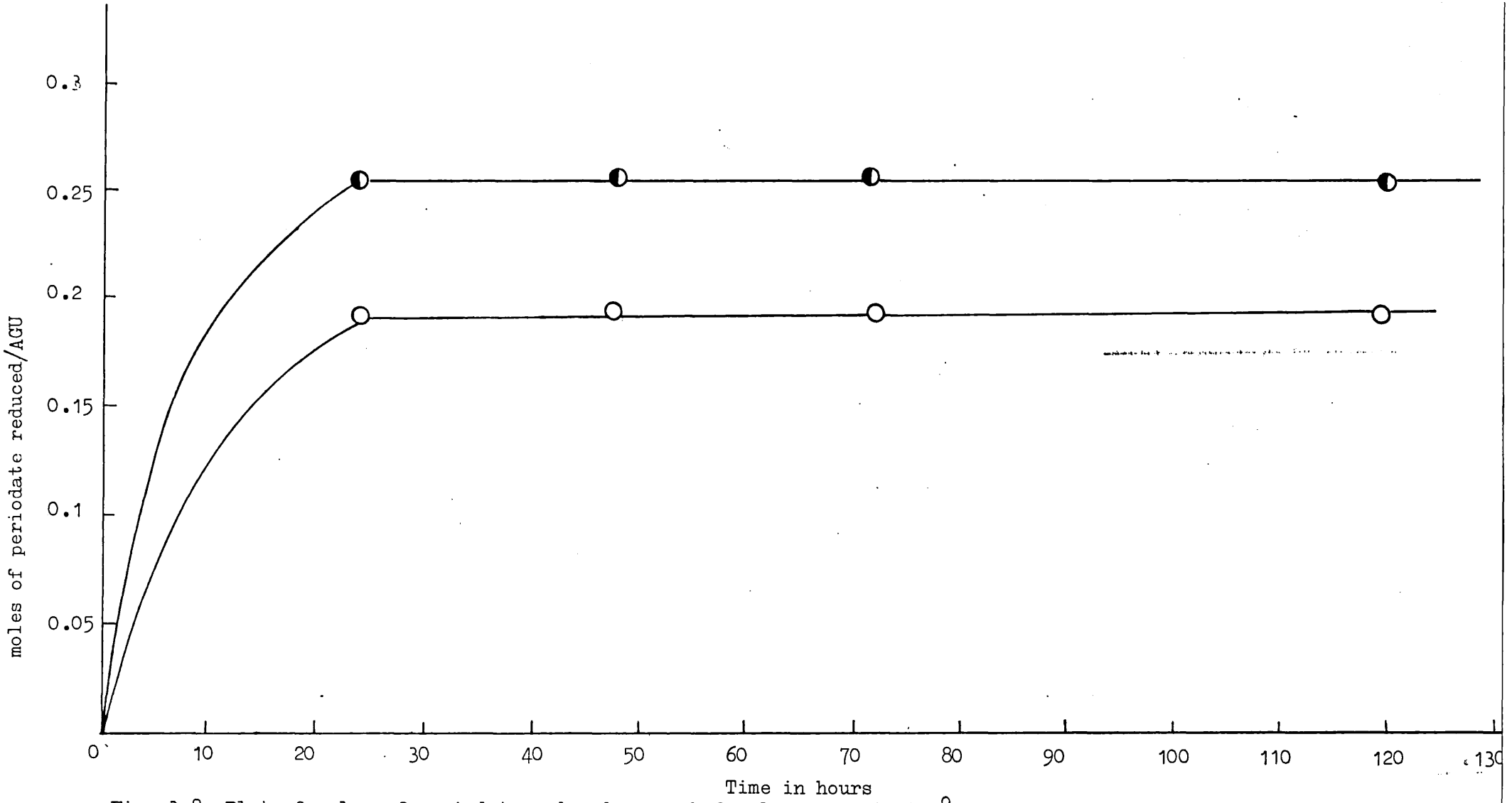


Fig. 3.8 Plot of moles of periodate reduced per anhydroglucose unit at 2°C

–O–, G–chain laminaran (Bifurcaria bifurcata)

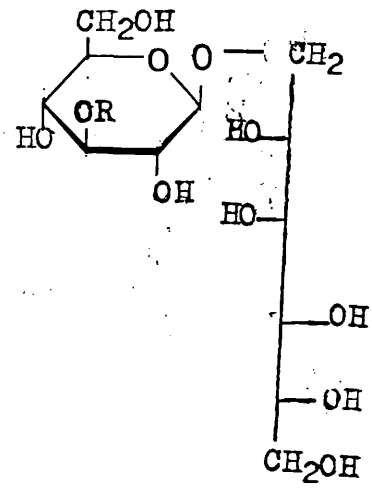
–◐–, (G + M)–chain laminaran (Dictyopteris plagiogramma)

Table 3.17 Percentage and composition of laminaran in different species of brown seaweed (based on dry weight of the weed)

Species	Percentage of laminaran	Monosaccharide components	Percentage of (G:M)-chain
Dictyopteris plagiogramma	3	glucose[a], mannitol[b]	80:20
Himanthalia lorea[119]	0.02	glucose	
Bifurcaria bifurcata[119]	0.20	glucose	
Padina pavonia[119]	0.07	glucose	
Desmarestia aculeata August collected	5.6	glucose[a], mannitol[b]	not determined
March collected	1.8	glucose[a], mannitol[b]	
Desmarestia ligulata[106]	0.08	glucose[a], mannitol[b]	not determined
Desmarestia firma[106]	1.8	glucose	

[a] major component ; [b] minor component.

From the above table it is evident that there are two types of laminaran present in the brown seaweeds. One type comprisesonly glucose monosaccharide unit and the other comprises glucose along with a minor component, mannitol. Although it has been accepted from methylation and periodate studies that all the laminarans are (1→3)-linked, it has been proved[199,200] that in laminaran of some speices the mannitol is linked through its C-1 to C-1 of the glucose residues at the reducing end of some of the glucan chains, giving non-reducing molecules (fig. 3.9).



R= Glycosyl residue

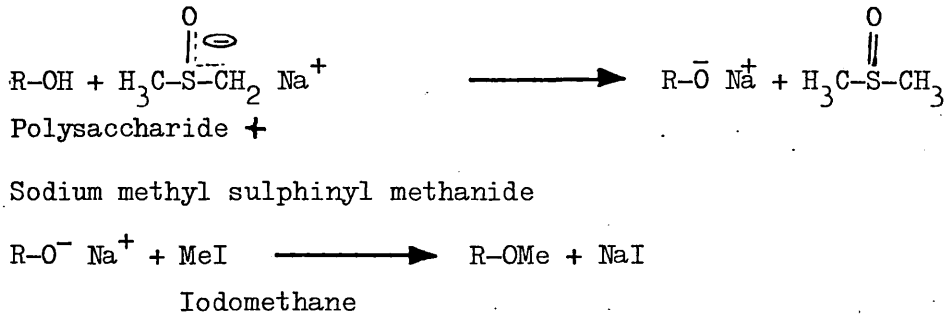
Fig.3.9

3.7.12 Methylation of fucans

Methylation analysis is an accurate method for determining the nature and proportions of the differently linked glycosyl residues in oligo- and polysaccharides or the carbohydrate moieties of glyco-conjugates. The method gives details of the types and percentages of the linkages present in the polymers, but gives no information on their sequence or the anomeric nature of the linkages. The methylation analysis of a polysaccharide can be considered as a three step operation, containing (1) the complete methylation of the polysaccharide in which all the hydroxyl groups are converted into methoxyl group, (2) the hydrolysis of all the glycosidic linkages in the O-methylated polysaccharide to yield the partially methylated monosaccharides and (3) analysis i.e. the characterisation and quantitative determination of the partially methylated monosaccharides.

Several methods are available for the methylation of poly-saccharides which have been reviewed by Lindberg et al.[212] Among these methods the most widely used in recent times is that developed by Hakomori[94] and involving the sodium methyl sulphinyl methanide and methyl iodide in dimethylsulphoxide (fig. 3.10).

Fig. 3.10 Function of sodium methyl sulphinyl methanide in the Hakomori methylation procedure



The substution pattern of O-methyl groups in the partially methylated monosaccharides reflects the substitution of the corresponding monomers in the native material. The hydroxyl → methoxyl substitution thus occurring as a result of methylation can often be analysed by paper chromatography. Since Lindberg and co-workers[212] reported that O-acetyl-O-methyl alditols can be characterised by electron impact (e.i.) mass spectrometry, the problems for the linkage analysis have been greatly simplified. The third step in Lindberg's method involves reduction of the partially methylated monosaccharides, acetylation and g.l.c.-mass spectrometry of the alditol derivatives thus obtained. The steps involved in methylation analysis by the Hakomori method of methylation is illustrated on the following page for laminaran, a β-(1→3)-linked glucan.

The transformation of sugars into acyclic derivatives removes the problem of multiple components due to the presence of anomers, but the conversion of aldoses into structurally symmetrical alditols may result in loss of stereochemical information.

Stephen and co-workers[229] have compared methylation analysis of partially methylated alditols as their trimethyl silyl ethers with that of the alditol acetates. The trimethyl silyl derivatives have the advantages of ease of preparation and ease of de-etherification when individual components are separated by preparative g.l.c.. The trimethyl silyl ether procedure is recommended for simple mixtures, but better resolution is obtained of complex mixtures of acetylated alditols.

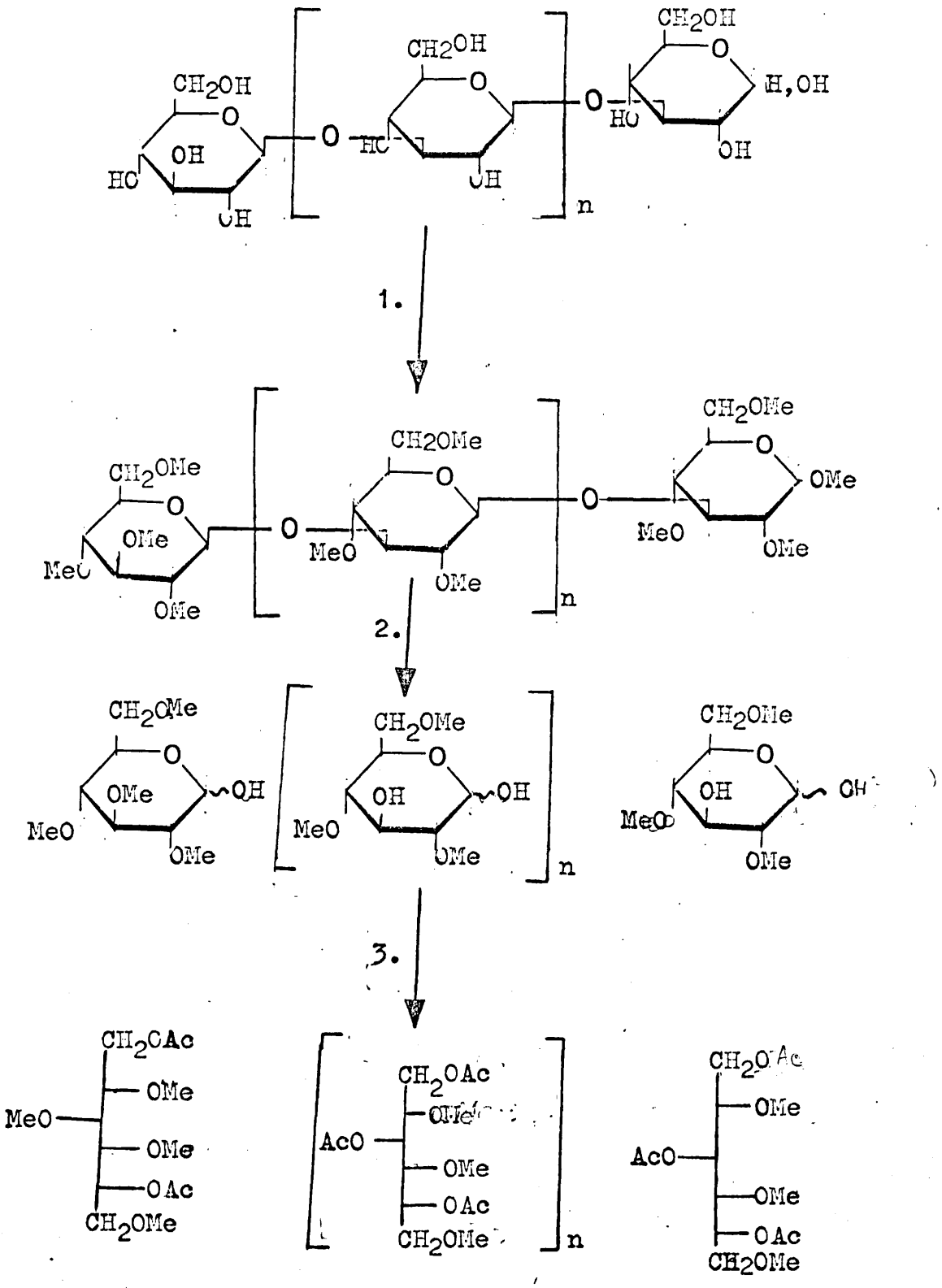


Fig.3.11

G.l.c.-m.s. of partially methylated alditol acetates[214]

The alditol derivatives having the same substitution pattern give similar mass spectra that are typical of that substitution pattern. Small differences in peak intensities may be observed for stereoisomers, but these differences are too small for identification purposes. The mode of fragmentation of the alditol derivatives have been extensively studied by Lindberg and co-workers.[212]

Primary fragments are formed by α-cleavage, resulting in fission between the carbon atoms in the alditol chain, the intensities of the primary fragments decrease with increasing molecular weight. Fission between two methoxylated carbons is more abundant than fission between an acetoxylated and a methoxylated carbon, which in turn is more abundant than fission between two acetoxylated carbon atoms. Thus fission (1) and (2) in fig. 3.12 are more important than fissions (3) and (4).

The methoxylated radical formed in (1) seems to be more stable than the acetoxylated radical formed in (2) and consequently the former fission is favoured.

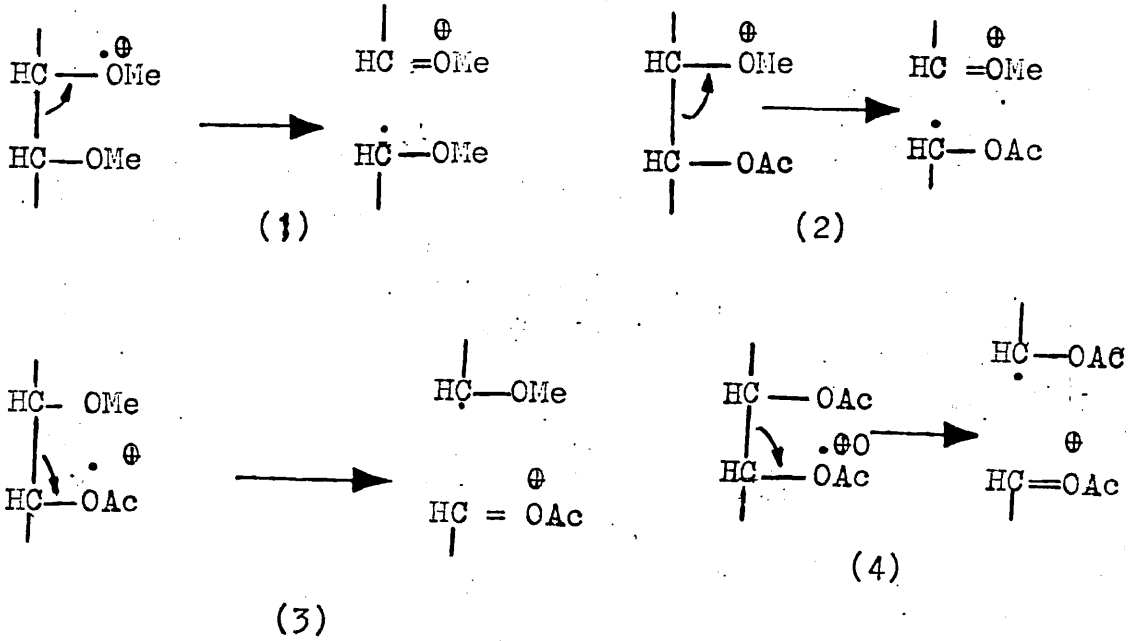


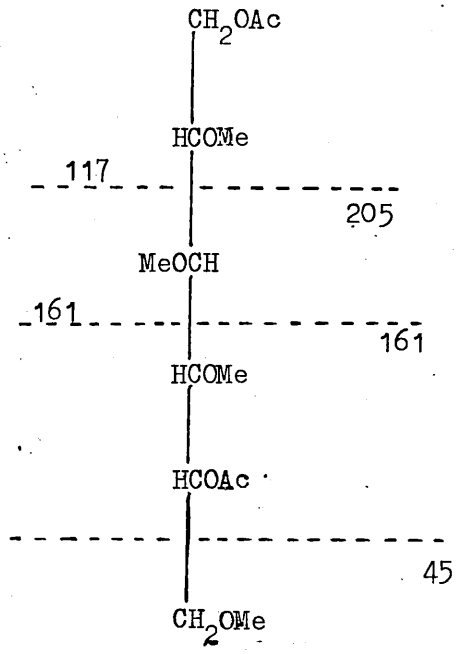
Fig. 3.12

In a deoxy-alditol, cleavage between the methylene carbon atom and a methoxylated carbon atom is significant, but less important than cleavage between a methoxylated and an acetoxylated carbon atom.

Secondary fragments are formed from the primary ones by single or consecutive eliminations of formaldehyde (30), methanol (32), ketene (42), acetic acid (60), methyl acetate (74), methoxy methylacetate (104), or acetoxy methyl acetate (132). By using the principles of primary and secondary fragmentation of partially methylated alditol acetates, most signals obtained in mass spectra of these compounds can be rationalised. As for example, most of the signals in the mass spectrum of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl D-glucitol can be explained (see fig. 313).

For materials having only one sugar of any one class (e.g. pentose, hexose, 6-deoxyhexose etc.), the identification of the components by m.s. is unambiguous. When several sugars of each class are present, the mass spectral evidence, together with the relative retention time in g.l.c. lead to an unequivocal identification of each component.

Primary fragmentation:



Secondary fragmentation:

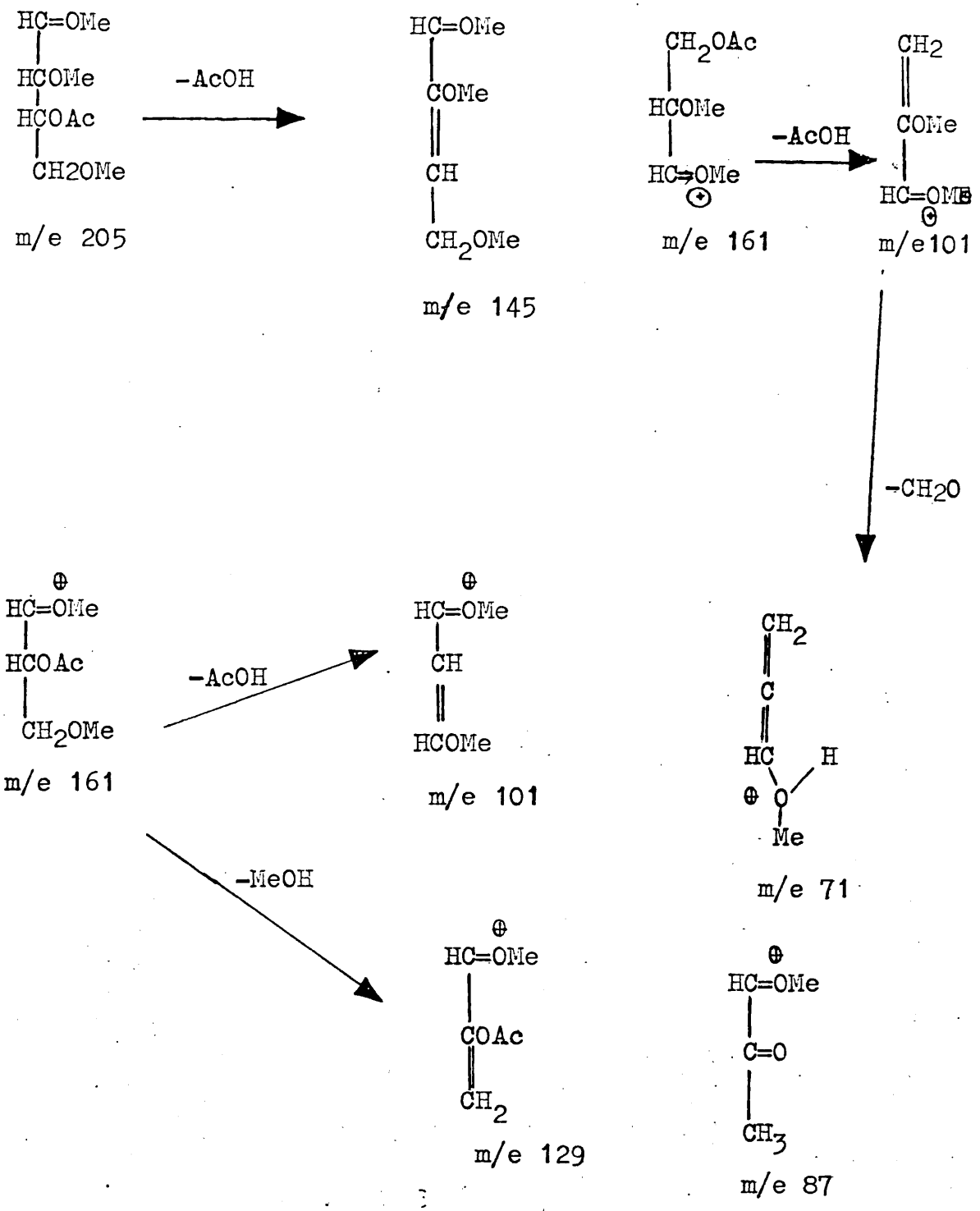


Fig.3.13

A prerequisite for Hakomori methylation reaction is that the substrate is soluble in DMSO. Solubilisation is often facilitated by ultrasonic treatment and/or warming to $\sim 70^{\circ}C$. Most 'undermethylations' are due to incomplete dissolution of the substrate. In these cases a portion of the material may be completely methylated while the remaining insoluble part is unmethylated. Presence of uronic acid and sulphate also hinders complete methylation.

The methylation experiment (3.6.2) was performed to determine the linkage pattern of the 'fucan'-A obtained from the Dictyopteris plagiogramma. The extent of degradation, if any, which might have occurred during the methylation was ascertained by gel filtration. The elution patterns of 'fucan'-A, and the once and twice methylated polysaccharides (fig. 3.14) show that indeed some degradation seems to have occurred. This will have to be borne in mind when the results of the methylation analysis are interpreted.

The partially methylated monosaccharides from the hydrolysate of methylated 'fucan'-A were tentatively characterised by paper chromatography and then confirmed by g.l.c. and g.l.c.-m.s. The results are shown in table 3.18.

Paper chromatography of the methylated 'fucan'-A hydrolysate revealed the presence of a slow moving component as well as the partially methylated monosaccharides. The slow moving component (oligouronic acid) was subsequently separated (expt. 3.6.3) from the rest of the partially methylated monosaccharides and the monosaccharide components involved in the formation of oligouronic acid were characterised (see table 3.19).

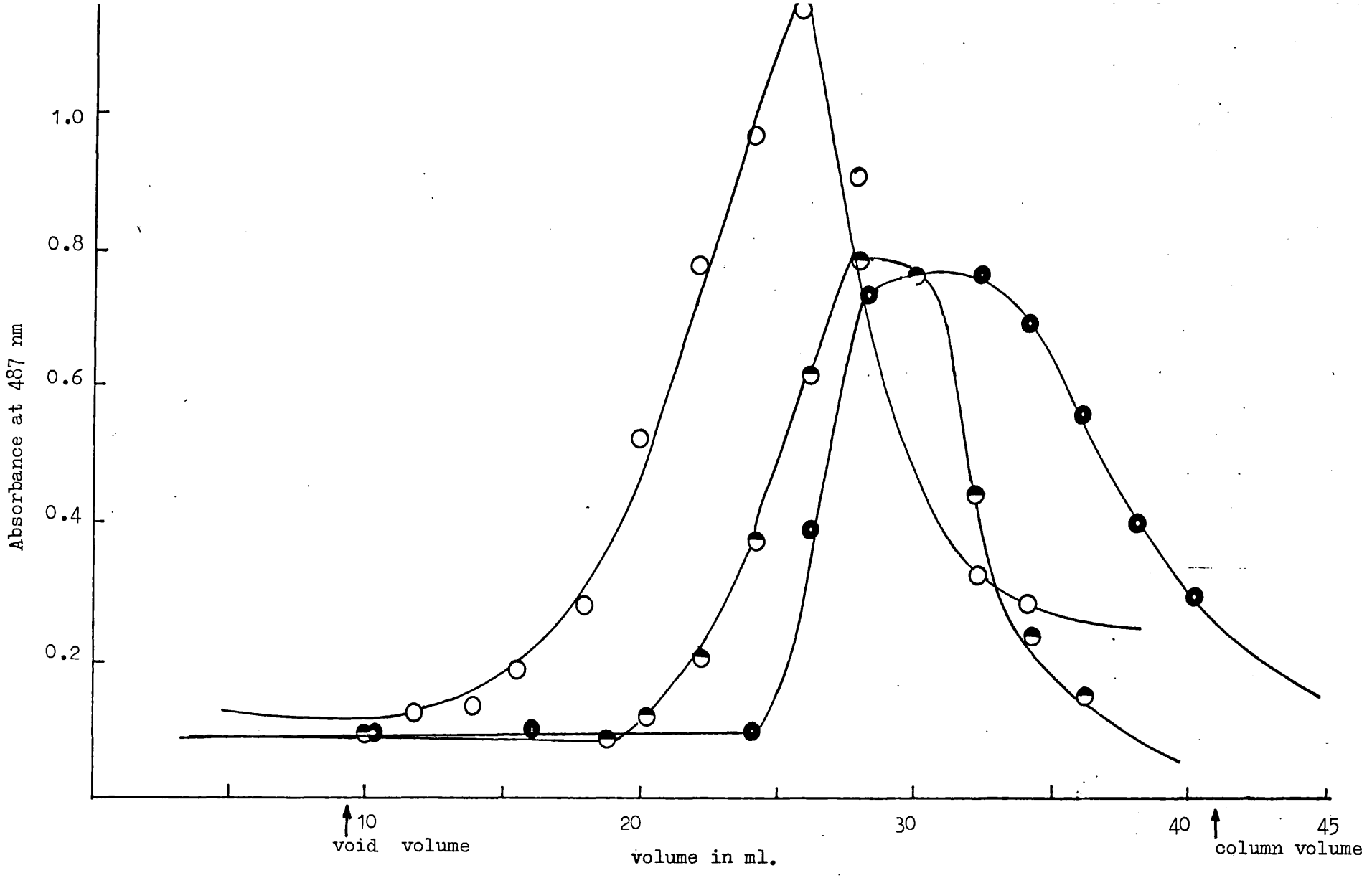


Fig. 3.14 Elution profile of 'fucan-A' on a Sepharose 4B column.
-O- , unmethylated; -⊖-, methylated once, -●-, methylated twice.

Table 3.18 Partially methylated monosaccharides characterised by g.l.c. and g.l.c.-m.s. from the hydrolysates of methylated 'fucan'-A; af ter conversion into alditol acetates

Retention time R_{TMG}[a]	Monosaccharides characterised by g.l.c. and g.l.c.-m.s.
	fucose
2.0	unmethylated (vl)
1.43	2-O-methyl fucose (l)
1.79	3-O-methyl fucose (m)
0.65	2,3,4-tri-O-methyl fucose (s)
0.91	3,4-di-O-methyl fucose (s)
	xylose
0.56	2,3,4-tri-O-methyl xylose (s)
1.02	2,3-di-O-methyl xylose (m)
3.40	unmethylated xylose (s)
	galactose
3.12	2,6-di-O-methylgalactose (l)
1.19	2,3,4,6-tetra-O-methylgalactose (s)
4.96	2,4-di-O-methylgalactose (s)
	mannose
4.58	2,4-di-O-methyl mannose (m)
2.27	2,3,4-tri-O-methyl mannose (s)

(a) Retention time relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

(vl) very large peak.

(l) represents large peaks, (m) medium and (s) small peaks.

The compositions of the mixtures of partially methylated monosaccharides obtained from 'fucan-A' by the application of one and two methylation procedures were virtually identical. In both products unmethylated fucose predominates. The presence of unmethylated fucose even after two methylations, is probably due to the fact that this 'fucan' in analogy with other 'fucans' from brown seaweeds,[119] is highly branched with all the hydroxyl groups in some fucose residues involved in glycosidic linkage and/or sulphation.

2-O-methyl and 3-O-methyl fucose were also major products along with small proportions of 3,4-di-O-methyl and 2,3,4-tri-O-methyl fucose in both of the methylated materials.

These results also indicate that all the monosaccharide components except mannose are present as end groups (although this may have resulted from cleavage of the molecules), as well as in the interior of the molecule linked in a variety of ways. It appears that xylose is present mainly linked through C-1 and C-4; and through C-1 (end group) indication of triply linked xylose units was also observed by the identification of free xylose in the mixture of partially methylated monosaccharides. Most of the galactose and mannose are present at branch points, linked through C-3 and C-4; and C-3 and C-6 respectively, although some of the galactose was present as end group. A small proportion of mannose is linked through C-1 and C-6.

In agreement with earlier methylation studies[119,161] these results confirm the presence of fucose linked through C-1 and C-2 as well as C-4; C-1 and C-3 as well as C-4; C-1 and C-2 as well as C-3 and C-4; through C-1 (end group) and through C-1 and C-2. Alternatively, the above secondary carbon atom may in some fucose residues be sulphated.

3.7.13 Characterisation of monosaccharides obtained from oligouronic acid

From paper chromatographic studies of the hydrolysates of methylated 'fucan'-A (page 130) it appears that any uronic acid residues which had not been degraded during the methylation had not been completely liberated by hydrolysis and are present as oligouronic acid. This oligouronic acid fraction of slower chromatographic mobility was therefore separated from the rest of the monosaccharide components and then esterified, reduced and hydrolysed. Paper chromatography of a portion of the hydrolysate revealed a complete conversion of the uronic acid to glucose. The glucose was confirmed as a D-sugar with glucose oxidase. The rest of the hydrolysate was reduced with sodiumborohydride and after conversion into O-acetyl alditols was analysed by g.l.c. and g.l.c.-m.s. The monosaccharide components identified by these methods are tabulated below (Table 3.19)

Table 3.19 Monosaccharides obtained from oligouronic acid

R_{TMG} [a]	Monosaccharide components
1.88	fucose [b]
2.1	2-O-methyl fucose
3.11	xylose [b]
4.59	2,3-di-O-methyl glucose [b]
6.29	glucose [b]
6.85	2-O-methyl glucose [b]
7.70	3-O-methyl glucose

[a] Retention time relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl D-glucitol.

[b] confirmed by m.s.

3.7.15 Periodate oxidation studies

The fucan-A' from Dictyopteris plagiogramma reduced 0.47 moles of periodate for every 'anhydrohexose' unit (expt. 3.6.5) and, after reduction, the polyalcohol was recovered in 84% yield. A repeated oxidation resulted in virtually no further reduction of periodate, indicating that, even if hemiacetal formation[147] had occurred during the first oxidation, the cleavage of the hemiacetal did not produce free vicinal hydroxyl groups possibly due to the presence of sulphate.

Gel filtration studies of the polysaccharide, the derived polyalcohol and the degraded polymer obtained after mild hydrolysis of the polyalcohol with M-trifluoroacetic acid was carried out on a Sepharose 4B column. This revealed that the polysaccharide and the polyalcohol were both very polydisperse, maximum elution occurring about midway between the void and exclusion volumes (fig. 3.15). The elution patterns of the polysaccharide and polyalcohol were very similar and it appears that there is not much difference in the molecular size of the two materials. The maximum elution of the degraded polymer, on the other hand, indicated a somewhat smaller molecular size, (fig. 3.15) indicating the fact that no cleavage had taken place in the interior of the molecule and that the cleaved units were on the periphery of the molecule.

The examination of the formic acid hydrolysate of the polyalcohol by paper chromatography (expt. 3.6.5f) revealed only a faint spot of glucuronic acid thereby indicating that most of the glucuronic acid present in the 'fucan' is linked through C-1 and C-4.

From these results it is clear that fucose and xylose are in close proximity to the uronic acid units in the polysaccharide and are probably mutually linked. The incomplete methylation of the monosaccharides in

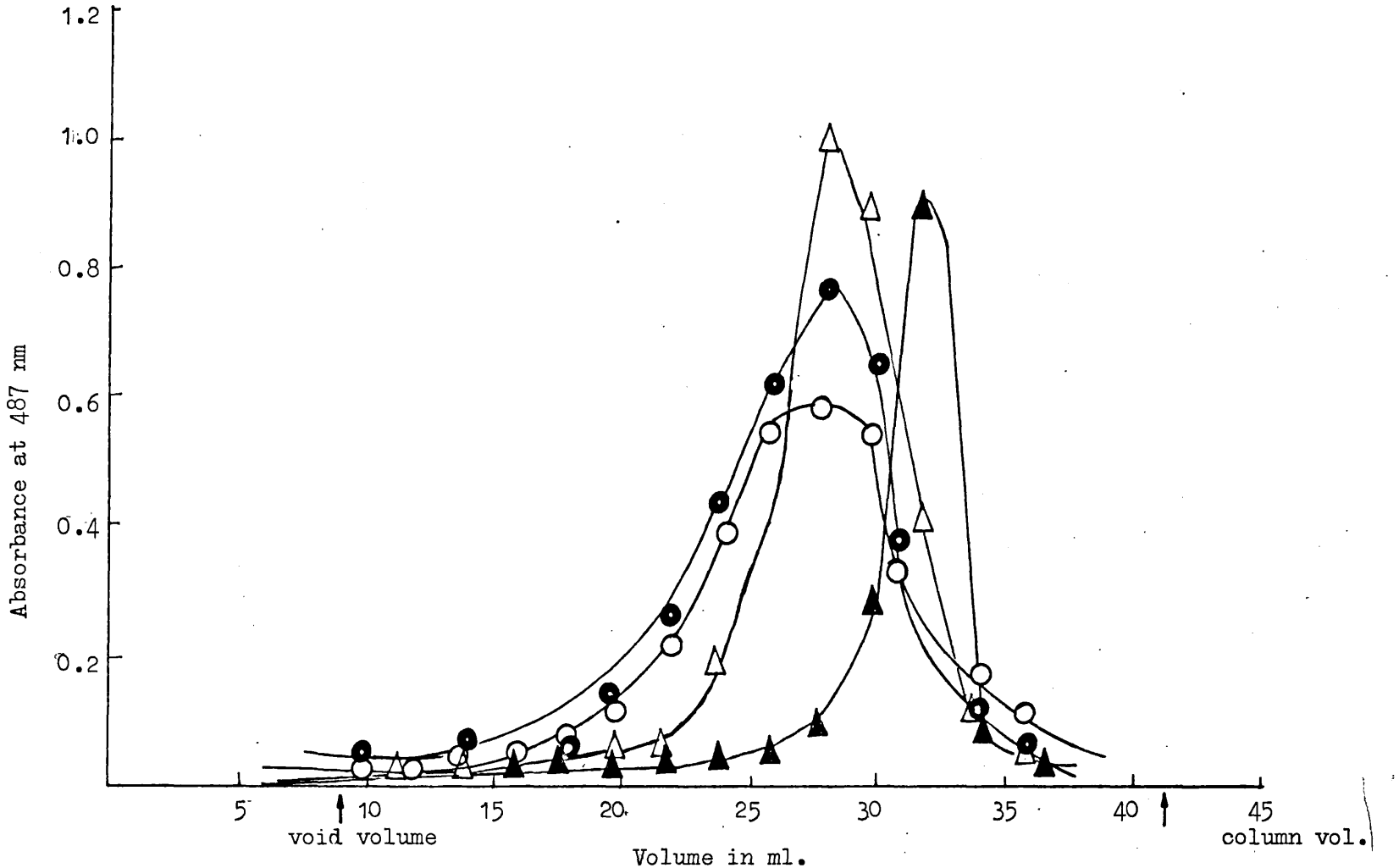


Fig. 3.15 Elution profile of fucan-A, polyalcohol and degraded polyalcohols on sepharose 4B column.

—●— fucan-A

—○— Polyalcohol from fucan-A

—△— Degraded polyalcohol at 25°C for 24 h.

—▲— Degraded polyalcohol at 25°C for 50h and 75h.

this material indicates a high degree of branching and is also partly due to the presence of sulphate.

The absence of any 4-O-methyl glucose indicates that the uronic acid is mainly linked through C-1 and C-4 and this is confirmed by the presence of a very small proportion of uncleaved uronic acid in the hydrolysate of the polyalcohol (page 134). The small proportion present indicates that the uronic acid may also occur at branch points. Although evidence of (1→4) linked glucuronic acid in other fucans have been given previously,[119,161] the presence of uronic acid in fucan at branch points is found for the first time.

In analogy with the previous investigations the methylation results indicate that the 'fucan-A' from Dictyopteris plagiogramma is a highly complex polydisperse heteropolysaccharide. However, these results indicate that all the different 'fucans' from different weeds are built up on the same general plan. Although these results give the major linkages of the individual sugars, they give little idea, apart from those which occur as end groups, of the overall structure of the macromolecules. Methylation of the alkaline desulphated 'fucan A' along with three other alkaline desulphated 'fucans' from Himanthalia lorea, Bifurcaria bifurcata and a 'fucan' obtained from the Institute of seaweed research, Scotland, was therefore carried out in the hope that further insight into the macromolecular structure would be obtained. (for detailed discussion of these methylated alkaline desulphated 'fucans' see page 161).

3.7.16 Desulphation of 'fucans'

One of the standard methods for determining the site of ester sulphate is the characterisation of methylated monosaccharides in the hydrolysates of the methylated polysaccharides before and after desulphation. Unfortunately complete methylation of these 'fucans' is not possible and furthermore complete desulphation by methanolic hydrochloric acid leads to complete degradation.

In view of the above fact desulphation of the fucans with alkali was attempted.

Desulphation of 'fucans' by alkali

It has been established[146] from experiments on model monosaccharide sulphates that the ester sulphate groups are cleaved by alkali only if they are linked to carbon atoms which are adjacent to carbon atoms carrying hydroxyl groups trans to the sulphate residues. The trans hydroxyl groups may be present as unsubstituted, acetylated, benzoylated or sulphated. The mechanism involves displacement of the sulphate group by rear side attack of the adjacent oxide anion to form an epoxide ring, accompanied by inversion of configuration at the carbon atom to which the sulphate group was originally attached. This can be represented as the intramolecular S_N2 process (see fig. 3.16).

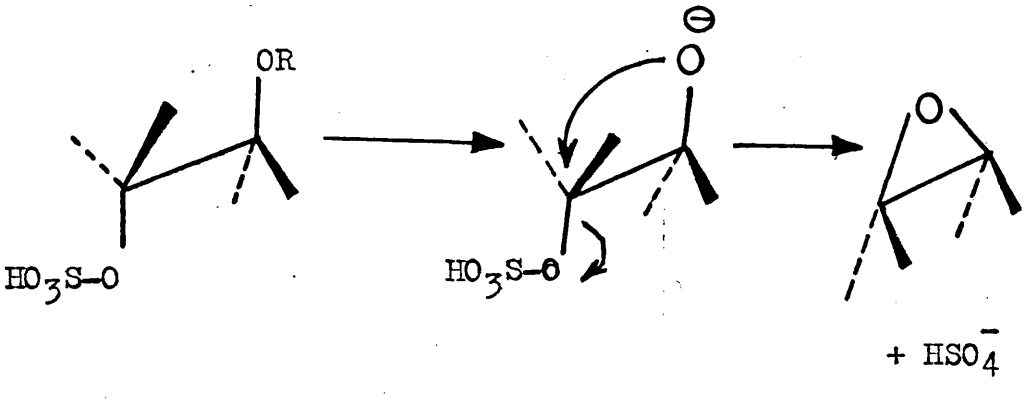


Fig. 3.16

The oxide anion is formed as a preliminary step from the adjacent ester group, by the base in which the ester groups undergo hydrolysis (alkali) or alcoholysis (alkoxide). The formation of oxide anion is reasonable for carboxylic esters, which are rapidly hydrolysed under the conditions employed, but is unusual for sulphate esters which are usually only slowly hydrolysed by the nucleophilic attack on sulphur required for this reaction.[213] With these disulphate esters the ease of hydrolysis has been explained by the inductive effect of the adjacent sulphate group.[214]

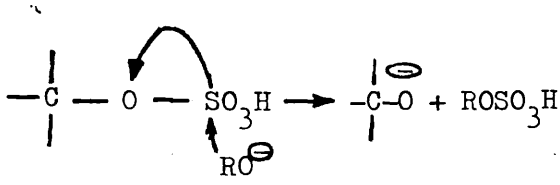
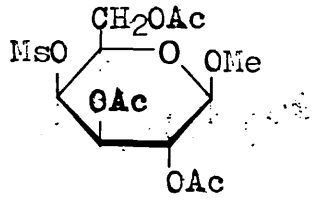


Figure 3.17

If the vicinal secondary group is cis, as in methyl 2,3,6-tri-O-acetyl-4-O-methanesulphonyl-β-D-galactoside (fig. 3.18), then only deacetylation occurs and the methanesulphonyl oxy- group is not displaced.



Ms = Methane sulphonyl)

Figure 3.18 2,3,6-tri-O-acetyl-4-O-methanesulphonyl-β-D-galactoside

3.7.17 The fucan-A along with three other fucans from three different brown seaweeds were subjected to alkaline desulphation. The properties of all the four 'fucans' are shown in table 3.20

Table 3.20 Properties of the different 'fucan' before and after alkaline desulphation

Fucan	Percentage of recovery by weight	Percentage of carbohydrate		Percentage of uronic acid		Percentage of sulphate	
		Original	Desulphated	Original	Desulphated	Original	Desulphated
fucan-A (*D-plagiogramma*)	72.3	66	72	19.7	15.3	3.9	2.6
fucan-B (*B. bifurcata*)	74	52	59.2	7.2	3.5	10.8	1.7
fucan-H (*H. lorea*)	86	50.5	57	7.9	6.7	21.7	15
fucan-X (supplied by Institute of Seaweed research)	63	50	56	6.8	3.4	26.5	13.5

It can be seen from the table that in case of 'fucan-A' the sulphate content has been reduced by 33.3% 'fucan-H' by about 30.9% 'fucan-B' by 84.3% and (fucan-X' by 49.1% indicating that at least some of the monosaccharide components of 'fucan' molecule are linked through C-1 and C-4 with sulphate group linked to C-2 or C-3, in each of these 'fucans'. This loss of sulphate however, does not necessarily mean that only fucose is sulphated. The loss in weight of the desulphated material can be mainly explained by the loss of sulphate and its accompanying metals. This was further confirmed by gel filtration studies of the fucans on Sepharose 4B column (see figs.3.19a,b,c,d) It can be seen from the elution pattern of the 'fucans' before and after desulphation that little change in the molecular size had occurred.

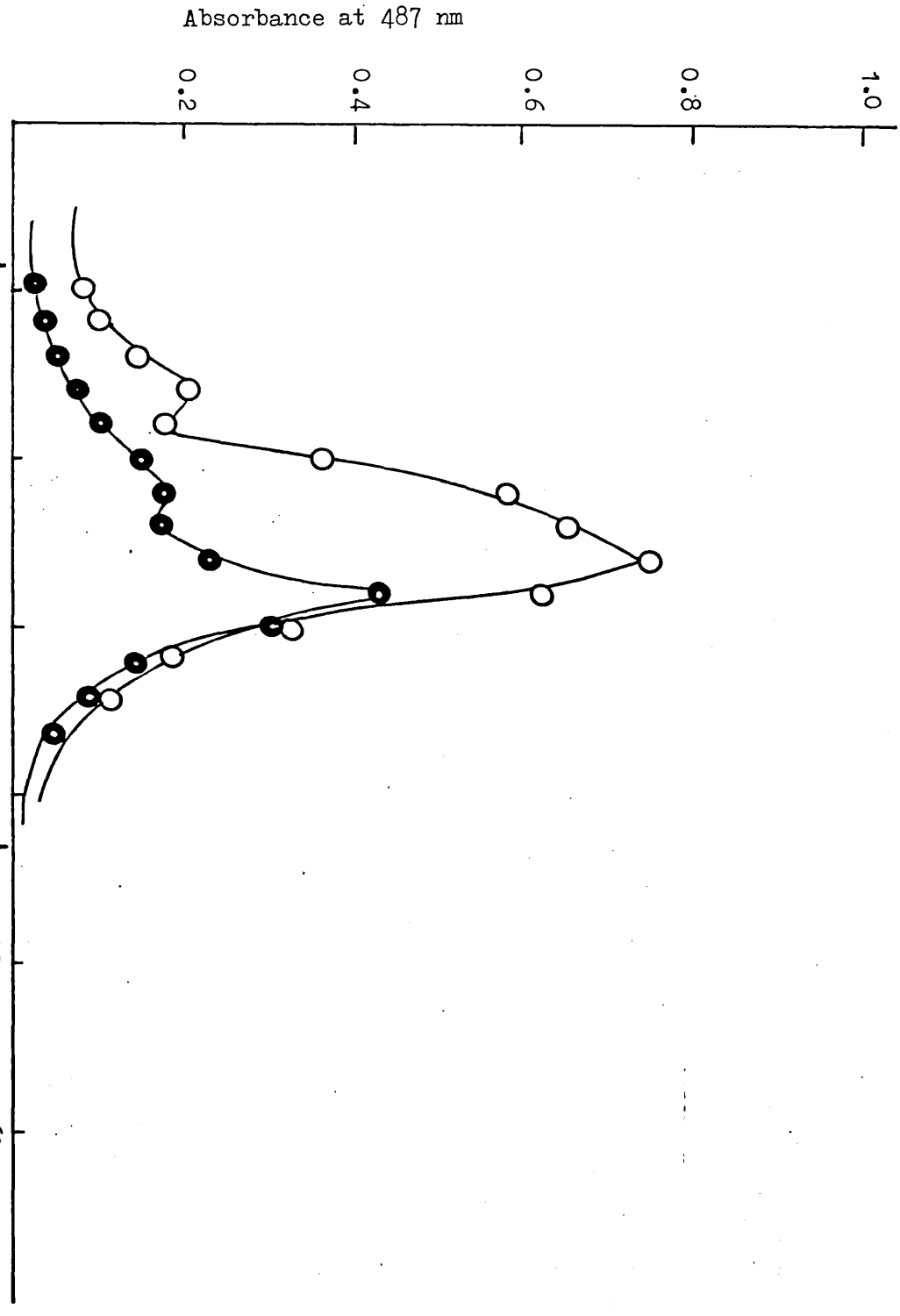


Fig.3.19a Elution profile of fucan-A on Sepharose 4B column.

-O- before desulphation

-●- after desulphation.

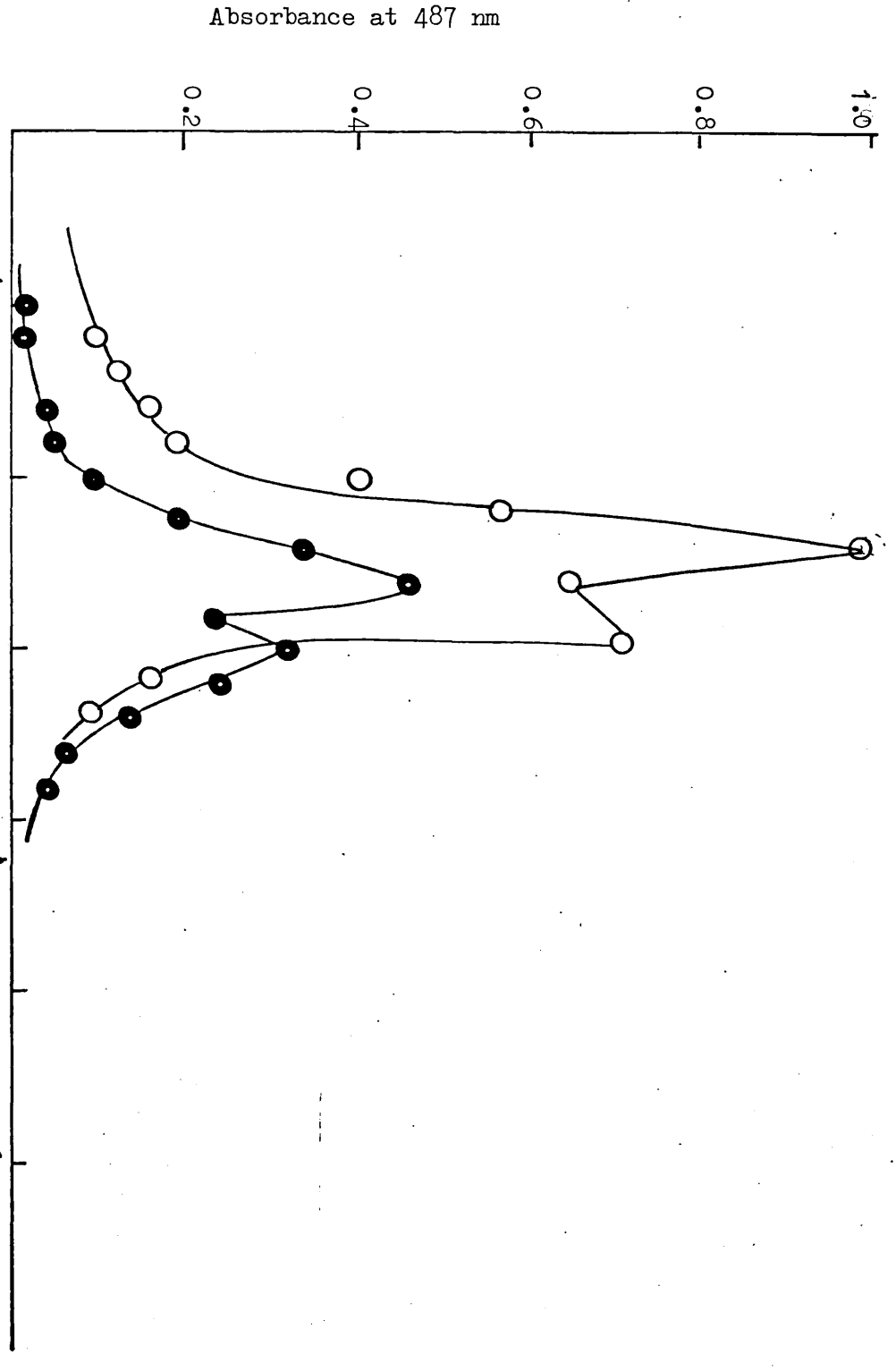


Fig. 3.19b Elution profile of fucan-B on Sepharose 4B column

-O- before alkaline desulphation

-●- after alkaline desulphation

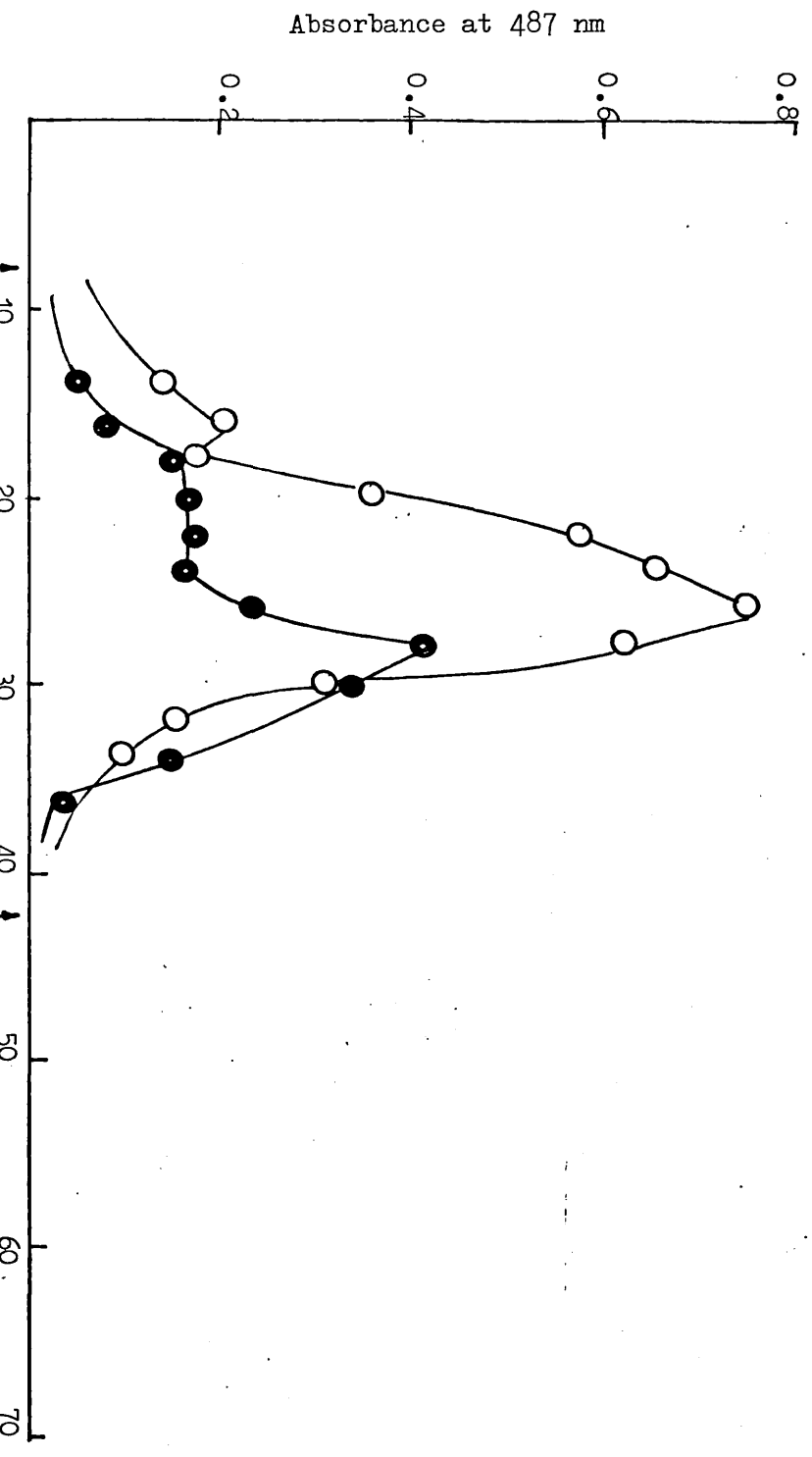


Fig. 3.19c Elution profile of fucan-H on Sepharode 4B column

-O- before alkaline desulphation

-●- after alkaline desulphation.

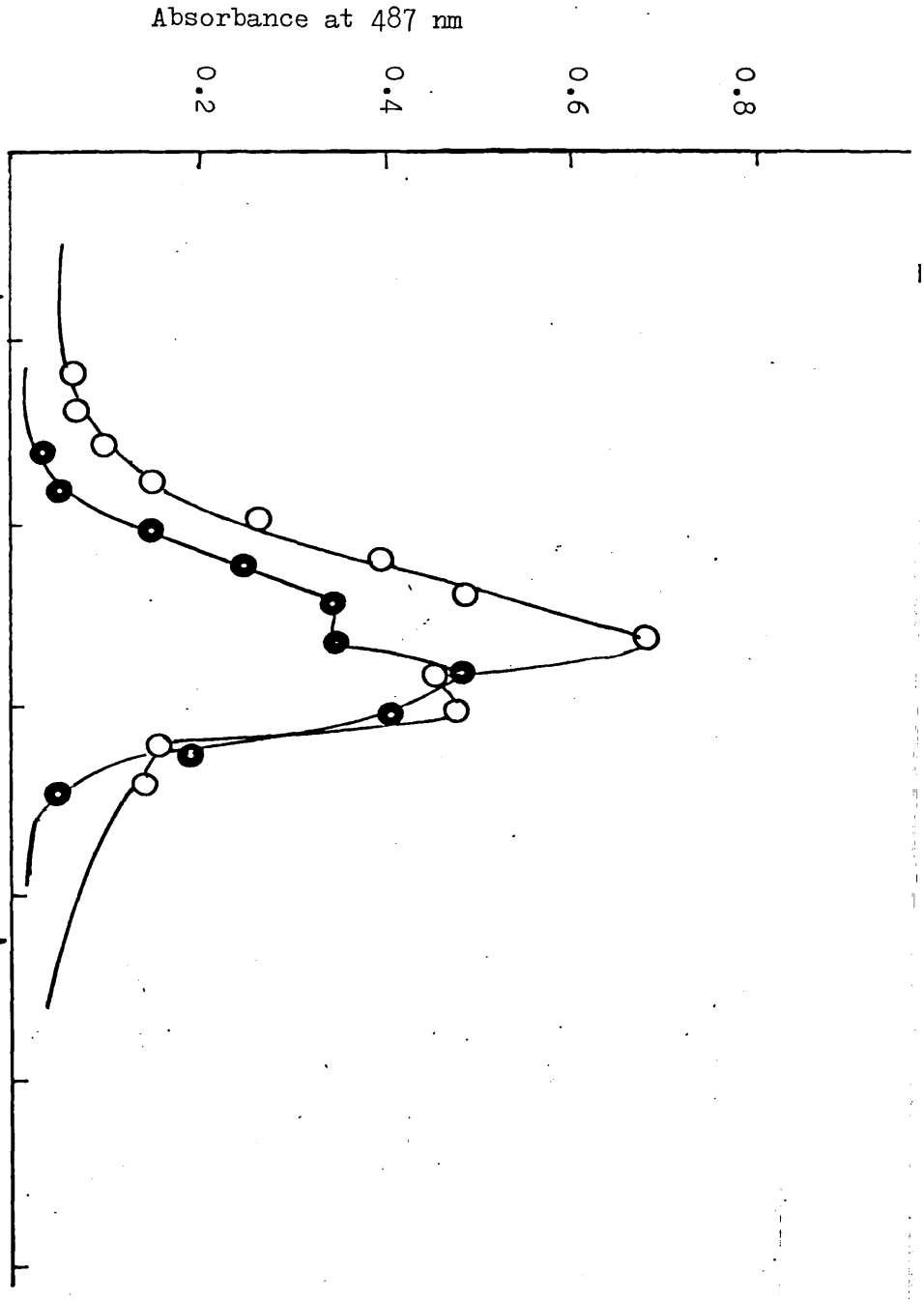


Fig. 3.19d Elution profile of fucan-X on Sepharose 4B column

-O- before alkaline desulphation

-●- after alkaline desulphation.

3.7.18 Although the opening of epoxide rings does not always result in the production of a new monosaccharide, in the above partially desulphated 'fucan' hydrolysates all on visual examination on a paper chromatogram revealed the presence of a monosaccharide not found in the hydrolysate of the 'fucans' before desulphation.

As the percentage of sulphate originally present in 'fucan-X' before alkaline treatment was higher than the percentage of sulphate present in other'fucans' and as enough material was available, further investigation to identify the new monosaccharide component and thus to determine the site of the ester sulphate originally present in the 'fucan' was carried out with desulphated 'fucan-X' only.

The new component from 'fucan-X' was separated on a 3 MM paper, converted into the alditol acetates and examined by g.l.c. and g.l.c.-m.s. (fig.3.23) which revealed that the newly formed monosaccharide component is a 6-deoxyhexose. The formation of a new 6-deoxyhexose unit as a result of the alkali treatment of the 'fucan' can only have arisen from fucose, thereby confirming that at least some of the alkali labile sulphate group was situated on the fucose unit of the 'fucan'. The new 6-deoxyhexose can be formed from a fucan, the fucose residues of which are linked through C-1 and C-4 or from non-reducing end groups which are sulphated at C-2 and C-3. Either of these residues under the action of alkali yields epoxide derivatives which on hydrolysis of the polysaccharide gives 6-deoxy-L-galactose (L-fucose) and/or 6-deoxy-L-idose units (see fig. 3.20).

Fucopyranoside 2-sulphate

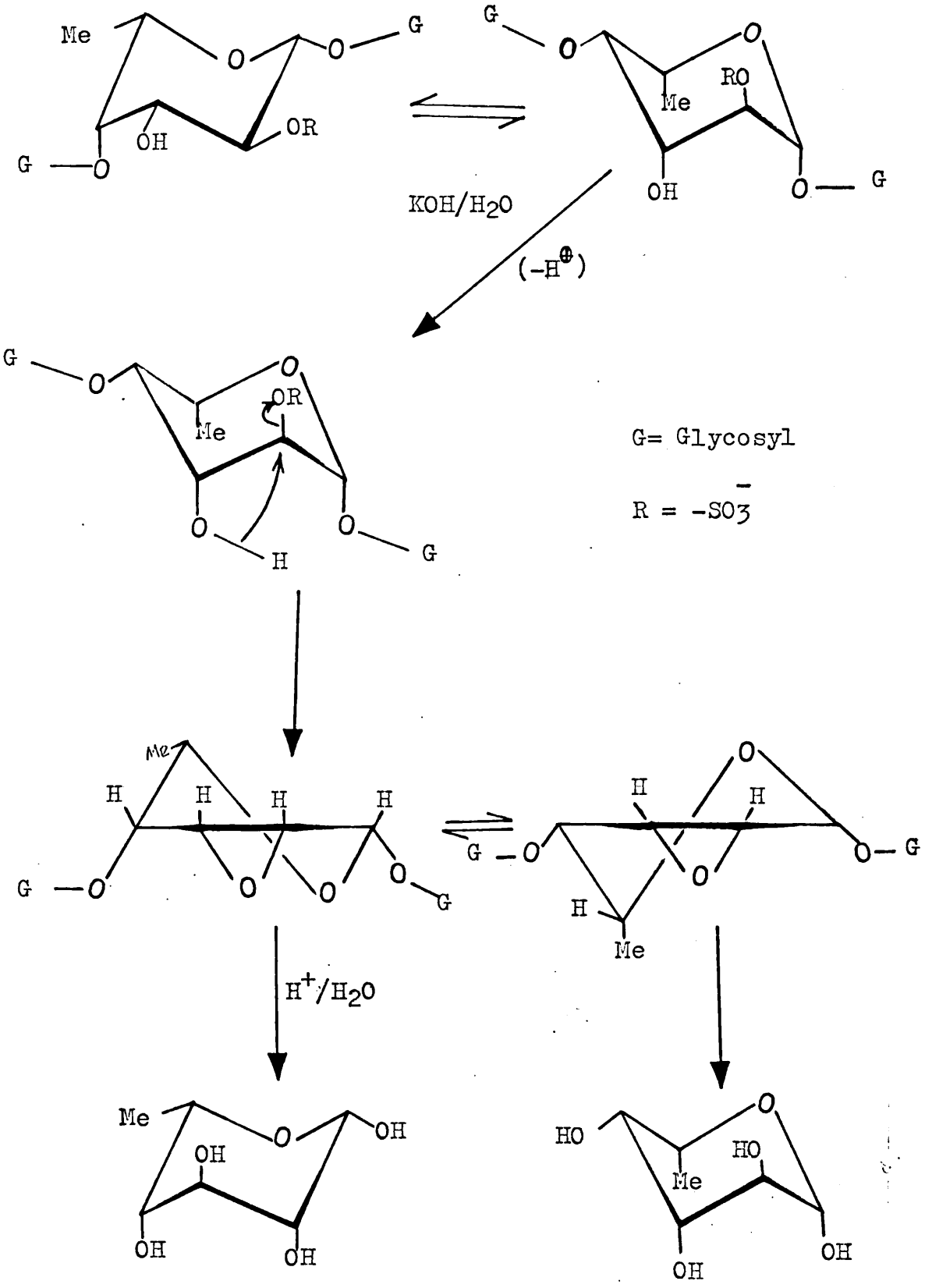


Fig.3.20

3.7.20 A similar reaction path is followed by fucopyranoside-3-sulphate, the final product of which will be the same as obtained from fucopyranoside-2-sulphate. The formation of 6-deoxy-L-idose therefore, does not distinguish between 2- and 3-sulphated fucose units. However, cleavage of the epoxide rings with sodium methoxide results in the formation of methyl derivatives, the methoxyl group entering the monosaccharide units at which the sulphate group was originally situated. Thus L-fucose-2-sulphate should yield 2-O-methyl-L-fucose and 3-O-methyl-6-deoxy-L-idose, whereas in the products from 3-sulphated fucose the position of O-methyl groups is reversed (see fig. 3.21, 3.22)

Fucopyranoside 2-sulphate

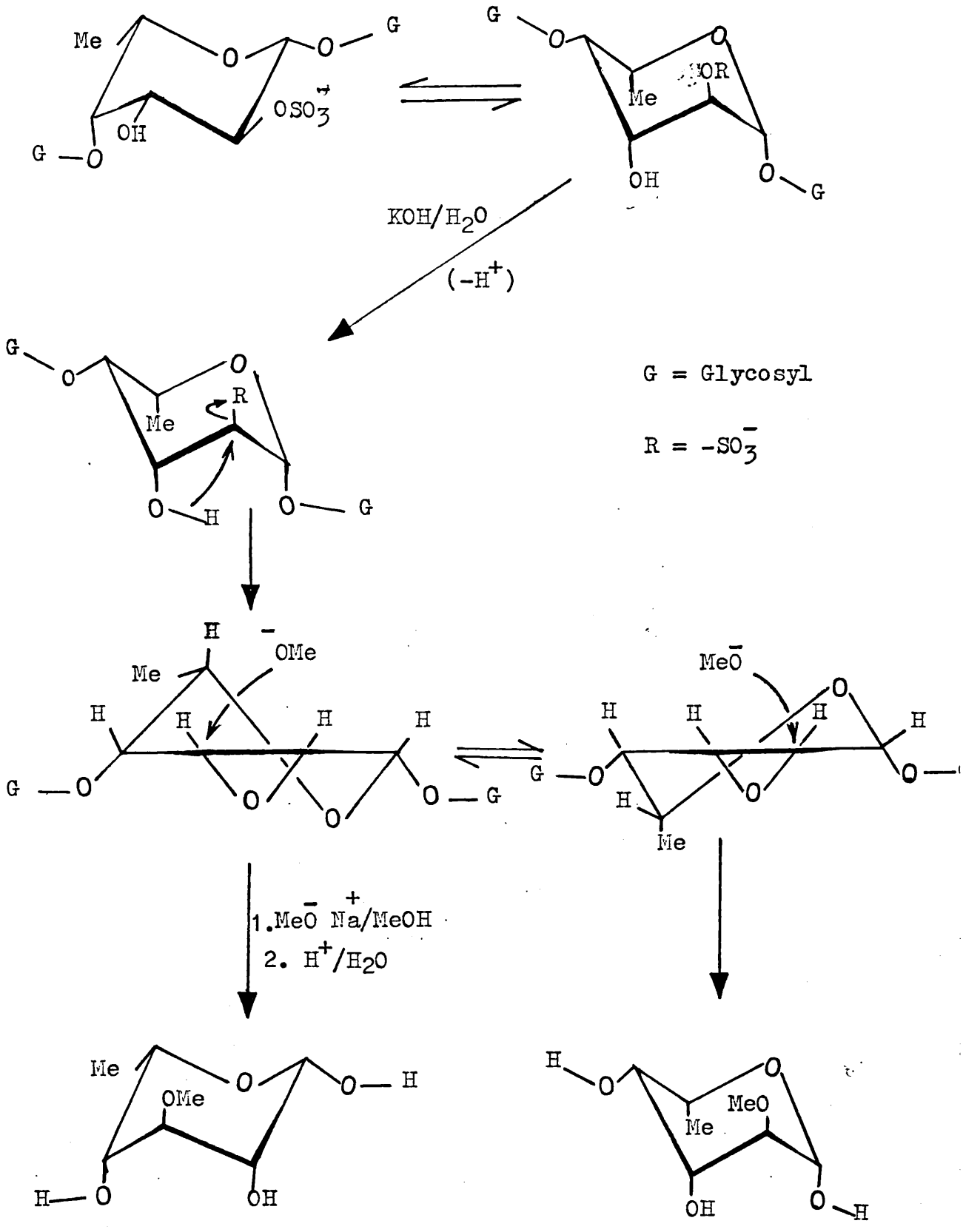
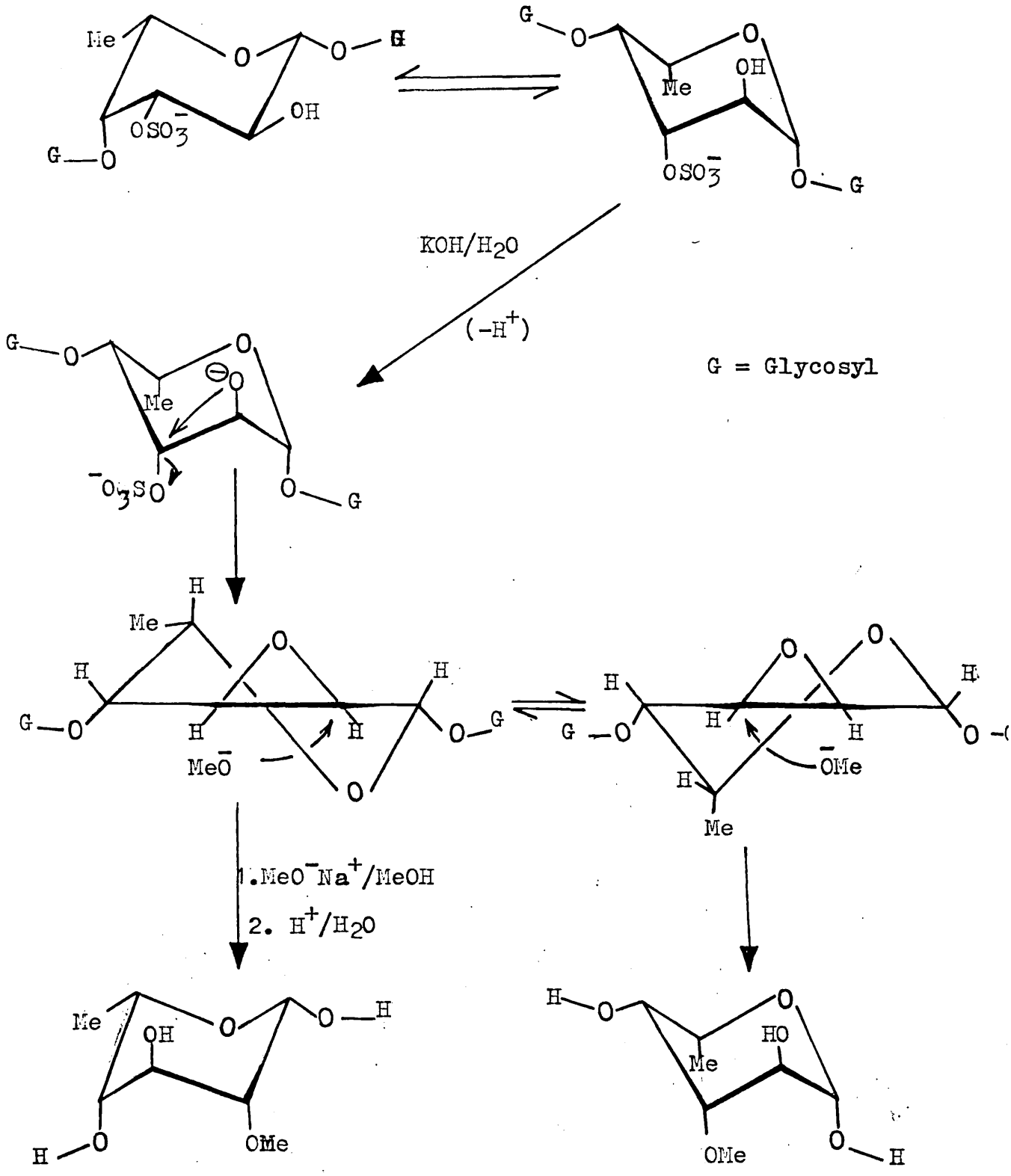


Fig.3.21

3-O-methyl 6-deoxy L-idose

2-O-methyl L-fucose

Fucopyranoside 3-sulphate



2-O-methyl 6-deoxy-L-idose

(V)

3-O-methyl-L-fucose

(VI)

Fig.3.22

In the present studies, the action of sodium methoxide on the alkaline desulphated 'fucan-X' followed by hydrolysis, led to the formation of two new components detectable by p.c. in addition to the monosaccharide components observed in the hydrolysate of the parent 'fucan'. The paper chromatographic mobility of these two new components were faster than the monosaccharide components of the original 'fucan' hydrolysate and the mobility of one of these components corresponded to the mobility of 2-O-methyl fucose. Both of these new components (hereinafter called 'Component-1 and Component-2) after separation on 3MM paper were examined by p.c., g.l.c. and g.l.c.-m.s.

The alditol acetate derivative of component-2 has the same retention time as that of 2-O-methyl fucitol acetate and its fragmentation pattern on e.i.-m.s. was similar to that of 2-O-methyl 6-deoxy hexitol acetate thereby indicating that component-2 is a 2-O-methyl-6-deoxy hexose. That this 2-O-methyl 6-deoxy hexose is indeed a 2-O-methyl fucose was confirmed by p.c. the R_F value of which corresponds with that of 2-O-methyl fucose and not with 2-O-methyl 6-deoxy idose or 3-O-methyl fucose thereby confirming that the site of ester sulphate group was on C-2 of the original 'fucan' and not on C-3 as in that case a 3-O-methyl fucose and/or 2-O-methyl 6-deoxy idose would have been the product.

Although it was thought at the first instance that the component-1 is a 3-O-methyl 6-deoxy hexose, repeated attempt to characterise this component as a carbohydrate derivative failed. It was therefore decided to investigate the acetate of the reduction product of 'component-1 by g.l.c. - c.i. m.s. The c.i. m.s. of this product was carried out using isobutane as the reactant gas.

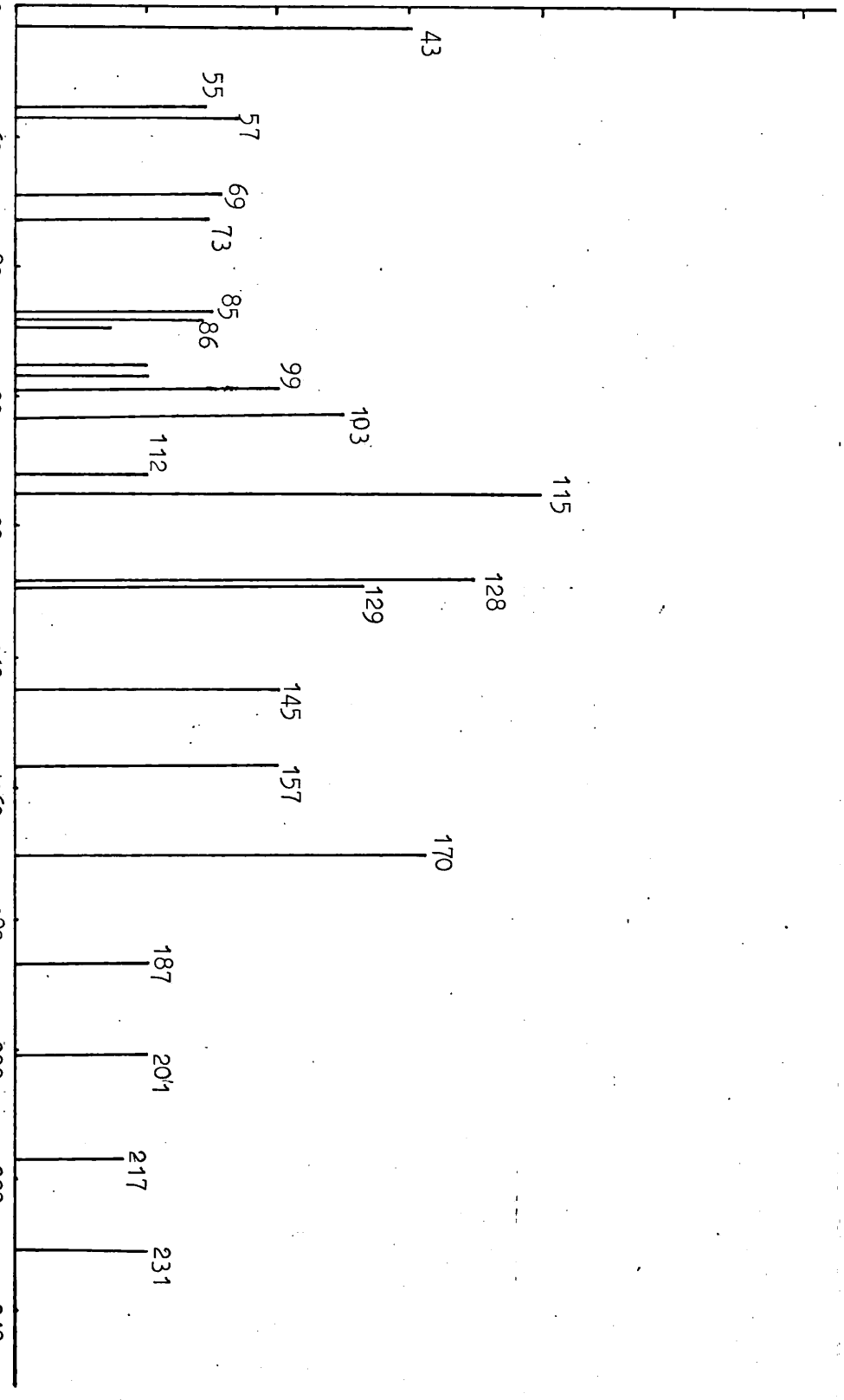


Fig. 3.23 Electron impact mass spectrum of 1,2,3,4,5 penta-O-acetyl-6-deoxy hexitol.

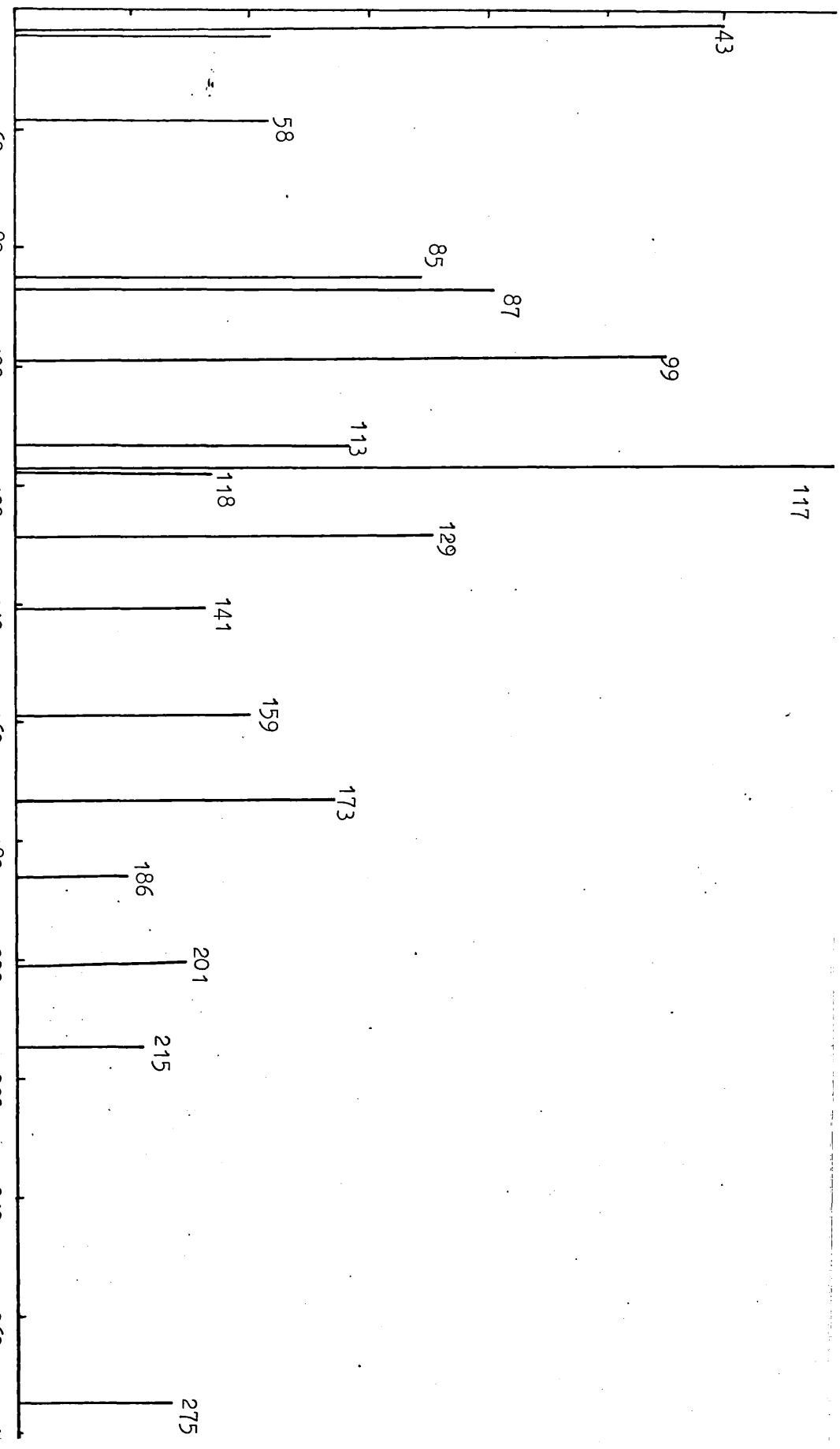


Fig. 3.24 Mass spectrum of 1,3,4,5-tetra-O-acetyl 2-O-methyl-6-deoxyhexitol.

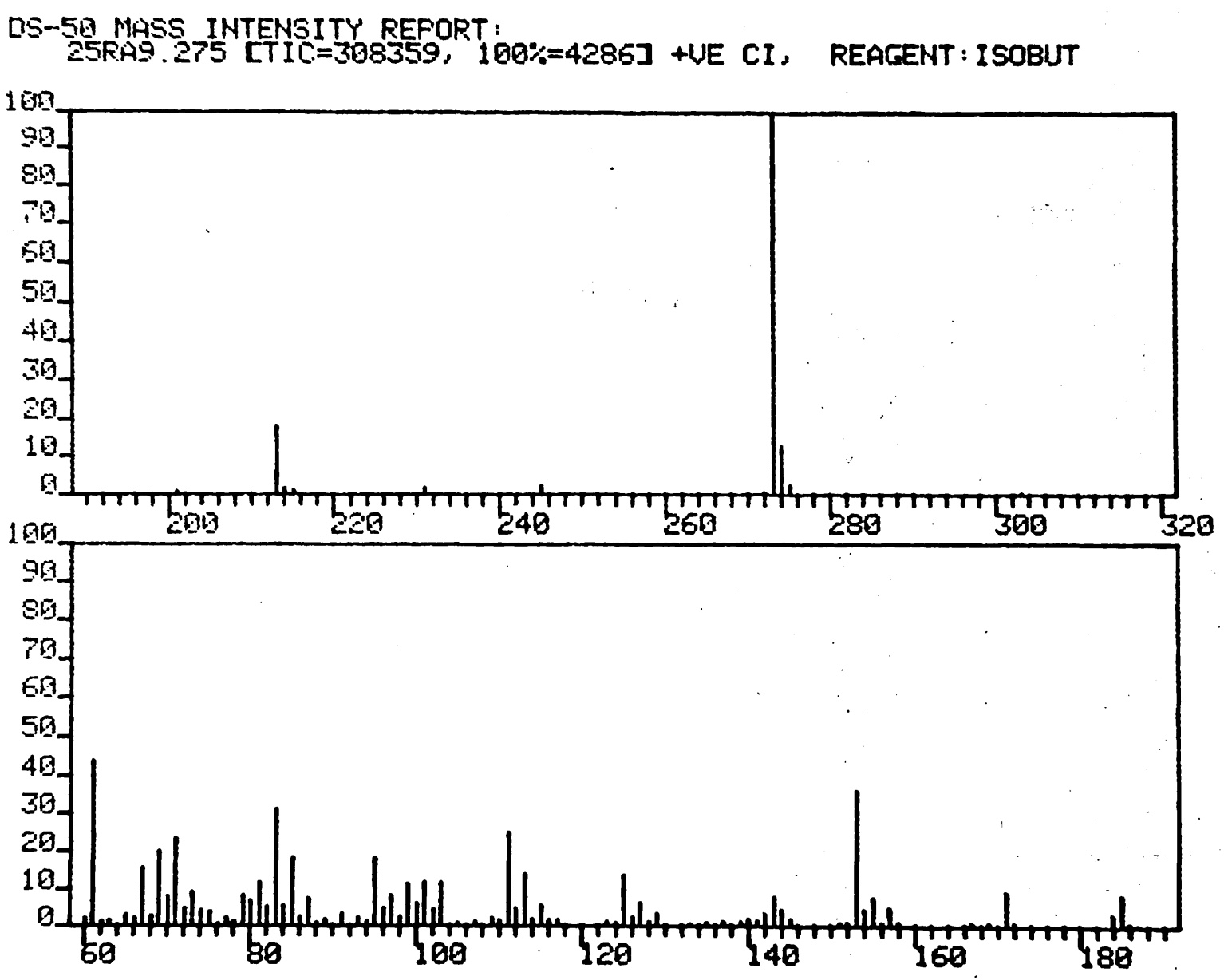
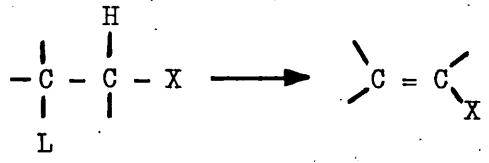


Fig.3.25 c.i-m.s of the alditol acetates of component 1

The spectra showed the protonated molecular ion $(M + 1)^+$ having m/e 273. As the alditol acetates of hexose, 6-deoxy hexose and pentose derivatives have molecular weight higher than 272; it was postulated that component-1 might have arisen from alkaline degradation of a C-4 substituted glucuronic acid which was originally present in the fucan. The possibility of formation of such a product by base catalysed β-elimination have been described by Lindberg[207] and Aspinall et al[208] and reviewed by Kiss.[218]

In general, polysaccharide and other sugar conjugates can be degraded in two ways; namely in a proton rich and in a proton-poor medium. Degradation in proton-rich media (e.g. acid hydrolysis) leads to oligosaccharide or monosaccharide fragments in which no important structural change in the sugar unit directly involved in this procedure. The position of the glycosidic linkage on the 4-hydroxyl group of the hexopyranuronate ring is of special nature, namely the β-position to the carboxyl group. Owing to this linkage, some special transformations have been observed in this class of sugars and sugar conjugates. As for example a new multiple bond is formed in the β-eliminative process by loss of two substitutents from adjacent atoms. The atoms involved in the β-elimination procedure in most of these reactions are carbon atoms; hence, the degradation results in a new carbon-carbon double bond (see fig.3.26).

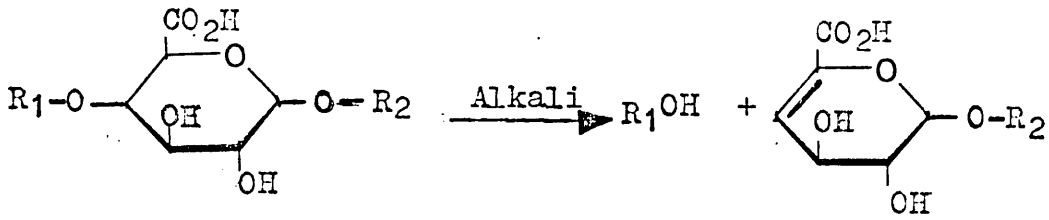


where L is the leaving group

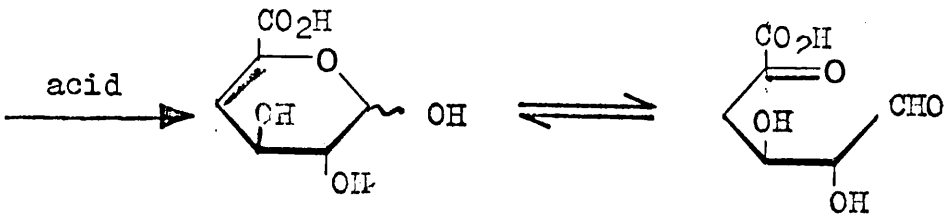
Figure 3.26

One of the two substitutents cleaved during the β-elimination is hydrogen activated, in the most commonly observed procedures, by an electron-withdrawing group X.

Formation of a product of molecular weight 272 from sodium methoxide treated 'fucan' containing glucuronic acid substituted at C-4 can be explained according to following reaction scheme.

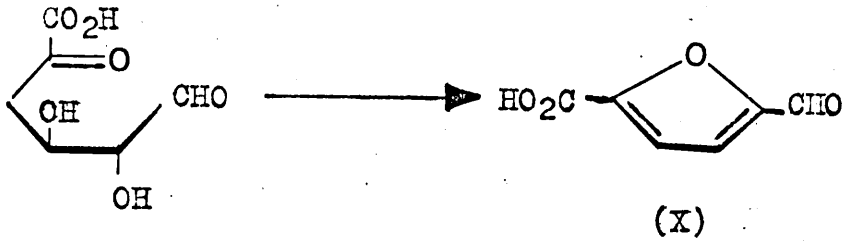


(VII) (VIII)



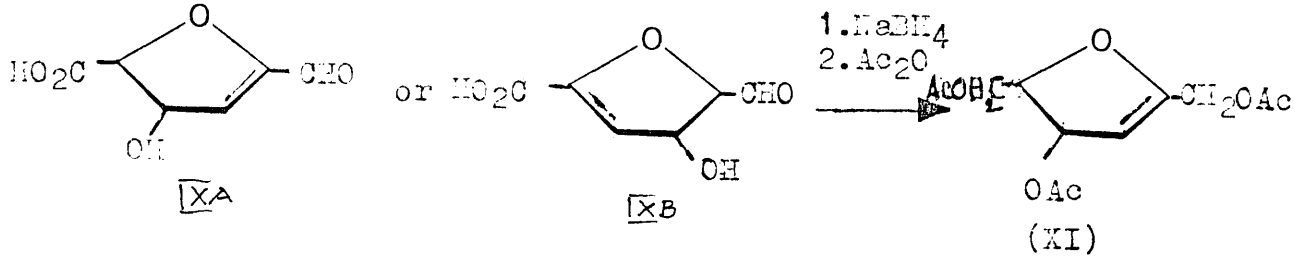
(IX)

Thus on treatment with alkali the uronate residue (VII) carrying a good leaving group at position C-4 will eliminate it and an unsaturated uronate residue (VIII) should be formed. The enol ether grouping in this moiety is acid labile, and mild hydrolysis with acid, should give a 4-deoxy-5-ulosuronate (IX) and the free aglycon. The product (IX) would probably react further, ultimately yielding a furan derivative (X)



(X)

It is likely that instead of yielding the final product (X) the intermediate (IXA) or (IXB) was formed which after reduction and acetylation resulted in the formation of product (XI).

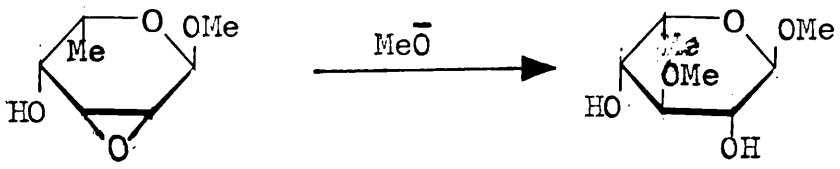


IXA IXB (XI)

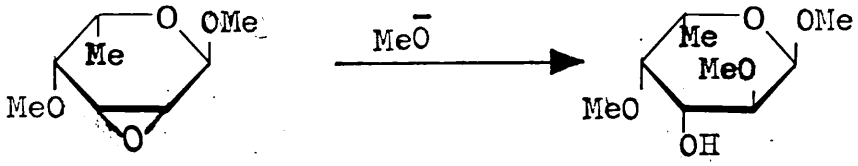
As shown in fig.3.21 an L-fucopyranose residue sulphated at C-2 might be expected to give two products, namely 3-O-methyl-6-deoxy-L-idose (III) and 2-O-methyl-L-fucose (IV). However, only one, the latter was formed.

It is profitable to compare this result with those obtained with methyl 2,3-anhydro-6-deoxy-α-L-talopyranoside (XII) and its 4-O-methyl derivative (XIII).[215,216,217] Treatment with sodium methoxide of the former gave the 3-O-methyl-L-idose derivative (XIV) as the only product, whereas that from the latter was the 2-O-methyl L-galactose derivative (XV). The products XIV and XV presumably arise from the conformations A and B, respectively. Although it is generally thought that the $^{0}HC_{5}$(B) conformation is more stable than $^{5}HC_{0}$ (A) conformation, it has been proposed that the latter is stabilised by hydrogen bond formation as shown.

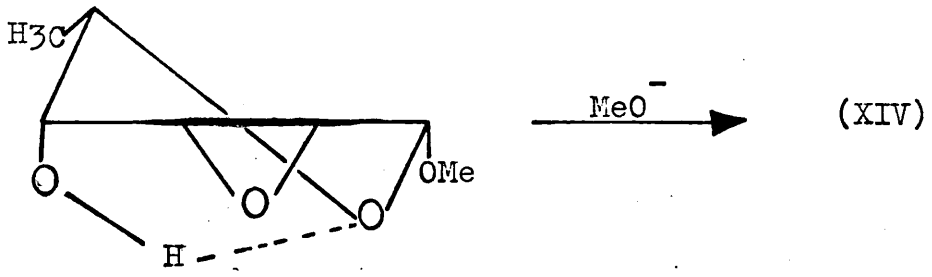
The results obtained with 'fucan-X' are therefore analogous to those obtained with methyl 2,3-anhydro-4-O-methyl-6-deoxy-α-L-talopyranoside. It is therefore likely that the 2-O-methyl-L-fucose obtained from 'fucan-X' arose from L-fucopyranose residues sulphated at C-2 which were indeed interchain residues (XVI)



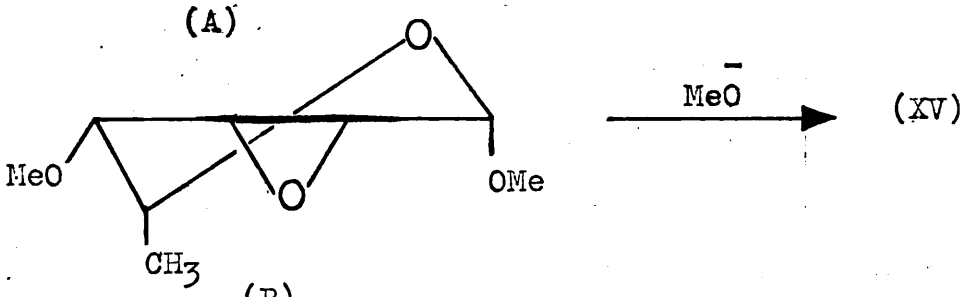
(XII) (XIV)



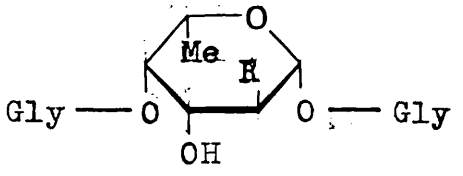
(XIII) (XV)



(A)



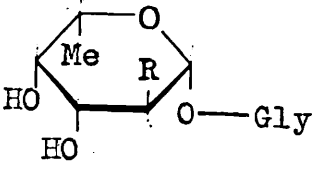
(B)



R = $-OSO_3H$

(XVI)

rather than non-reducing end groups (XVII)



R = $-OSO_3H$

(XVII)

3.7.21 Desulphation of 'fucan-X' by Pavlenko method

Pavlenko et al[138] claimed that partial acid hydrolysis of a sulphated polysaccharide followed by acetylation of the derived mixture of monosaccharide sulphates and desulphation with thionyl chloride results in complete removal of sulphate. Methylation of the sulphate free monosaccharide acetate by diazomethane and examination of the product by g.l.c. and g.l.c.-m.s. revealed the position of sulphate group in the original polysaccharide.

Application of Pavlenko method (Expt. 3.6.11) to fucan-X [(2.5 g), $SO_4^=$ 26.5%] resulted after mild hydrolysis in a water soluble (1.5 g) and water insoluble (0.735 g) fraction. The soluble material contained 11.5% sulphate and the insoluble material 32%. The acetate derivative of the soluble material

after thionyl chloride treatment contained 6% sulphate. It should however be realised that the sulphate contents were calculated from the weight of the monosaccharide acetates; based on a monosaccharide content these would be considerably higher.

3.7.22 Methylation of this material with diazomethane followed by examination of the methylated product by g.l.c. and g.l.c.-m.s. revealed the presence of 2-O-methyl, 3-O-methyl and 4-O-methylfucose along with large amounts of unmethylated fucose and galactose and some unmethylated xylose. The retention time of the alditol acetate derivatives of the different components are shown in table (3.21).

Table 3.21 Monosaccharide components identified as their alditol acetates by g.l.c. and g.l.c.-m.s. after desulphation with thionylchloride and methylation with diazomethane of the ethanol soluble fraction of fucan-X.

Retention time[a]	Monosaccharide components
1.85	free fucose (v.l.)
1.38	2-O-methyl fucose (l)
1.55	3-O-methyl fucose (l)
1.61	4-O-methyl fucose (l)
0.89	2,4-di-O-methyl fucose (l)
6.15	free xylose (m)
9.5	free galactose (v.l.)

[a] Retention times of (methylated) alditols, as their acetates, relative to 1,5 di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol (TMG)

v.l. = very large peaks, l = large peaks, m = medium peaks.

These results reveal that the fucose units of a portion of the fucan macromolecules re sulphated, the ester sulphate group being situated at C-2, C-3 and at C-4. A large amount of fucose units are also unsubstituted by sulphate groups as evident from the presence of a large peak of unmethylated fucose although these could have been removed during the mild hydrolysis. The galactose and xylose units of this portion of the 'fucan' also appear to be free of sulphate groups. These results agree well with previous findings, because evidence for the presence of sulphate groups on monosaccharide units other than fucose has not been reported so far in the majority of the fucans although in the case of sargassan which has a very high galactose content, some sulphate was reported to be on the galactose units.[140]

Examination of the insoluble material (precipitate 735 mg) recovered from the mild hydrolysis revealed the presence of similar monosaccharide components to those observed in the original fucan-X, however the insoluble material was found to contain more sulphate and uronic acid than the parent fucan. The properties of the different fractions are shown in table 3.22

Table 3.22 Properties of the different fractions of 'Pavlenko' treated fucan-X.

Name of the fraction	% carbohydrate content	% sulphate content	% uronic acid content
Original material (fucan-X)	50	26.5	6.8
Ethanol insoluble material (precipitate)	50	32	9.1
Ethanol soluble material (filtrate)	54.3	11.6	not determined

It appears from the above results that this method of desulphation and methylation is not suitable as a tool for structural studies of the 'fucans'; because it excludes ca. 30% of the polysaccharide (precipitate) which might have played an important role in the structure of the original polysaccharide. The original workers completely ignored this fraction nor did they determine the sulphate content of the soluble material at different stages of the experiment. Complete desulphation of the present 'fucan' by this method was not achieved as 6% sulphate was present in the final product and consequently this makes accurate conclusions of the results impossible. It appears that the results reported by Pavlenko et al should be viewed with considerable caution.

3.7.23 Methylation of alkaline desulphated 'fucans' by Hakomori method

In order to discover the position of alkali labile sulphate group in the fucan molecule and to determine the structural relationship of the Dictyopteris 'fucan' with other 'fucans' from different brown seaweeds methylation was carried out by Hakomori method (expt. 3.6.13) Following tables show the partially methylated sugars from alkaline desulphated fucans. They were characterised as their alditol acetates by g.l.c. and g.l.c.-m.s.

Table 3.23 Partially methylated monosaccharide components in the hydrolysates of methylated alkaline desulphated fucan-A (DSF-A) (one methylation)

Retention time R_{TMG}[a]	Monosaccharides characterised
	fucose
1.43	2-O-methyl fucose (vl)
1.97	Unmethylated fucose (l)
1.10	2,3-di-O-methyl fucose (m)
0.63	2,3,4-tri-O-methyl fucose (s)
1.76	3-O-methyl fucose (s)
0.71	3,4-di-O-methyl fucose (s)
	xylose
0.57	2,3,4-tri-O-methyl xylose
1.15	2,3-di-O-methyl xylose
3.57	Unmethylated xylose
	galactose
3.2	2,6-di-O-methyl galactose (l)
1.20	2,3,4,6-tetra-O-methyl galactose(s)
5.0	2,4-di-O-methyl galactose (s)
	mannose
4.26	2,4-di-O-methyl mannose (m)
2.12	2,3,4-tri-O-methyl mannose (s)

[a] Retention time relative to 1,5 di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol

vl = very large.

l = large, m = medium, s = small peaks.

The above partially methylated monosaccharides were also identified from the twice methylated samples. From a comparison of the methylated results of the sulphated and alkaline desulphated fucans it can be concluded that the alkali labile sulphate group of the fucan is present at C-2 of the fucose molecule, as the proportion of 2-O-methyl fucose

has been increased in the desulphated methylated product. The appearance of 2,3-di-O-methyl fucose in the desulphated fucan also supports this conclusion. These results also indicate that sulphate is not present in other monosaccharide units of the fucan molecule as the partially methylated xylose, galactose and mannose were identical in both the sulphated and desulphated fucan.

The methylation results confirm the conclusion made from the results of the alkaline desulphation of the 'fucan' in that the alkali labile sulphate group is present on C-2 of the fucose unit of the fucan.

To ascertain whether the above conclusion also applies to the other fucans investigated previously in this laboratory, alkaline desulphated fucans from Himanthalia lorea and Bifurcaria bifurcata were also methylated by the Hakomori method. The partially methylated monosaccharide identified in the product of once and twice methylated alkaline desulphated fucan hydrolysates from Himanthalia lorea (DSF-H) and Bifurcaria bifurcata (DSF-B) were very similar and are tabulated below.

Table 3.24 Partially methylated monosaccharide components in the hydrolysates of methylated DSF-H and DSF-B characterised by g.l.c. and g.l.c.-m.s. as their alditol acetates

Retention times R_{TMG}[a]	Monosaccharide components
	fucose
1.43	2-O-methyl fucose (l)
1.92	unmethylated fucose (l)
1.10	2,3-di-O-methyl fucose (m)
1.68	3-O-methyl fucose (s)
0.63	2,3,4-tri-O-methyl fucose (s)
1.22	3,4-di-O-methylfucose (s)
	xylose
0.56	2,3,4-tri-O-methyl xylose
1.18	2,3-di-O-methyl xylose
3.32	unmethylated xylose

[a] Retention time relative to 1,5 di-O-acetyl 2,3,4,6-tetra-O-methyl glucitol
l = large, m = medium, s = small peaks.

Comparison of the results of the methylated alkaline desulphated fucans from Himanthalia lorea and Bifurcaria bifurcata with those of the sulphated ones[119] indicate that 2,3-di-O-methyl fucose is present in the methylation products of alkaline desulphated fucans only. It also shows that the peak size of 3-O-methyl fucose decreases considerably relative to that of the sulphated fucan. It is therefore concluded that the 2,3-di-O-methyl fucose resulted from the fucose units linked through C-1 and C-4 and sulphated at C-2, thereby confirming the similarity of the 'fucan' from Dictyopteris plagiogramma with the fucans from other brown seaweeds.

3.8 Conclusion

The overall results from these studies reveal that species of brown seaweed from different families and very different morphological form all synthesise the same polysaccharides, namely laminaran, fucose containing polysaccharide and alginic acid and the only discernable difference is in the proportion of these materials in the different species. The fucose containing polysaccharides from each of the species examined so far comprise a broad spectrum of polysaccharides, all based on a similar structural pattern, but with different proportions of ester sulphate, uronic acid, xylose, galactose and in some cases mannose.

CHAPTER 4 INVESTIGATION OF THE RESIDUAL MATERIAL AFTER AQUEOUS AND ACID EXTRACTION OF *Desmarestia firma* AND OF THE ALGINATE FROM *D. ligulata* and *D. firma* BY DIRECT ALKALI EXTRACTION OF THESE WEEDS.*

4.1 The genus *Desmarestia* belongs to a small family Desmarestiaceae. All the species of *Desmarestia* are conspicuous plants, often growing to several feet in length. The species of *Desmarestia* are found attached to 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. Two of the four British species, *Desmarestia aculeata* and *Desmarestia ligulata*, though seldom seen growing, are frequently washed up and can be collected from floating drift weed. *Desmarestia viridis* is less common and the fourth species *Desmarestia firma* is not a British seaweed, it was harvested below low tide on the coast to the east of Capetown, South Africa.

A curious feature of this group is that some of its members decompose with remarkable rapidity when brought into contact with the air, changing to a bright verdigris colour, and quickly rotting other seaweeds carried in the same container. It has been reported that some *Desmarestia* species contain much sulphuric acid in the vacuolar sap.[157] (Among the species investigated it was shown that *Desmarestia aculeata* does not contain sulphuric acid[158,160]). The presence of free sulphuric acid in some species of *Desmarestia* is the main reason for the interest in the present work. 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.

* This work was a part of a publication which appeared in Phytochemistry 17 (1978) 1289-1292.

Among the species of the genus Desmarestia only D. aculeata[158,159,160] and D. ligulata [161] have been previously investigated chemically. A preliminary investigation on Desmarestia firma has also been carried out.[161]

The D. firma sample investigated in this work was collected by Mr R Simon, The Department of Botany, University of Capetown from below the low water tide mark (at 10 m depth) on 27th March, 1975. The weed was freeze dried immediately after collection and stored in a deep freeze for about a year before chemical investigation was undertaken.

Earlier work from this laboratory[161] had investigated the carbohydrates of the brown seaweed Desmarestia ligulata by sequential extraction with ethanol, water, dilute acid and alkali and also the 'fucan' and alginic acid obtained by direct alkali extraction. Desmarestia firma had also been extracted with ethanol, water and acid. The approximate percentage yields (of the dry weight of weed) of the different polysaccharides are given in the table 4.1.

Table 4.1

Approximate percentages of the dry weight of the polysaccharides isolated from two species of Desmarestia

Species	Laminaran	Fucan	Alginic acid
D. ligulata	0.08	5.5	19 (16)*
D. firma	1.8	1.9+	23 (17)

* Figures in the parenthesis indicate the percentage of alginic acid from the sequential alkali extract.

\+ 'Fucan' derived from aqueous and acid extracts only.

In view of the very low yield of 'fucan' from D. firma it was decided to investigate the residual material remaining after aqueous and acid extraction and also to extract this weed directly with alkali. Direct alkali extraction of D. ligulata was also carried out after neutralisation of the free sulphuric acid by a new method with triethylamine. Parallel experiments by neutralisation with saturated aqueous calcium carbonate were also performed.

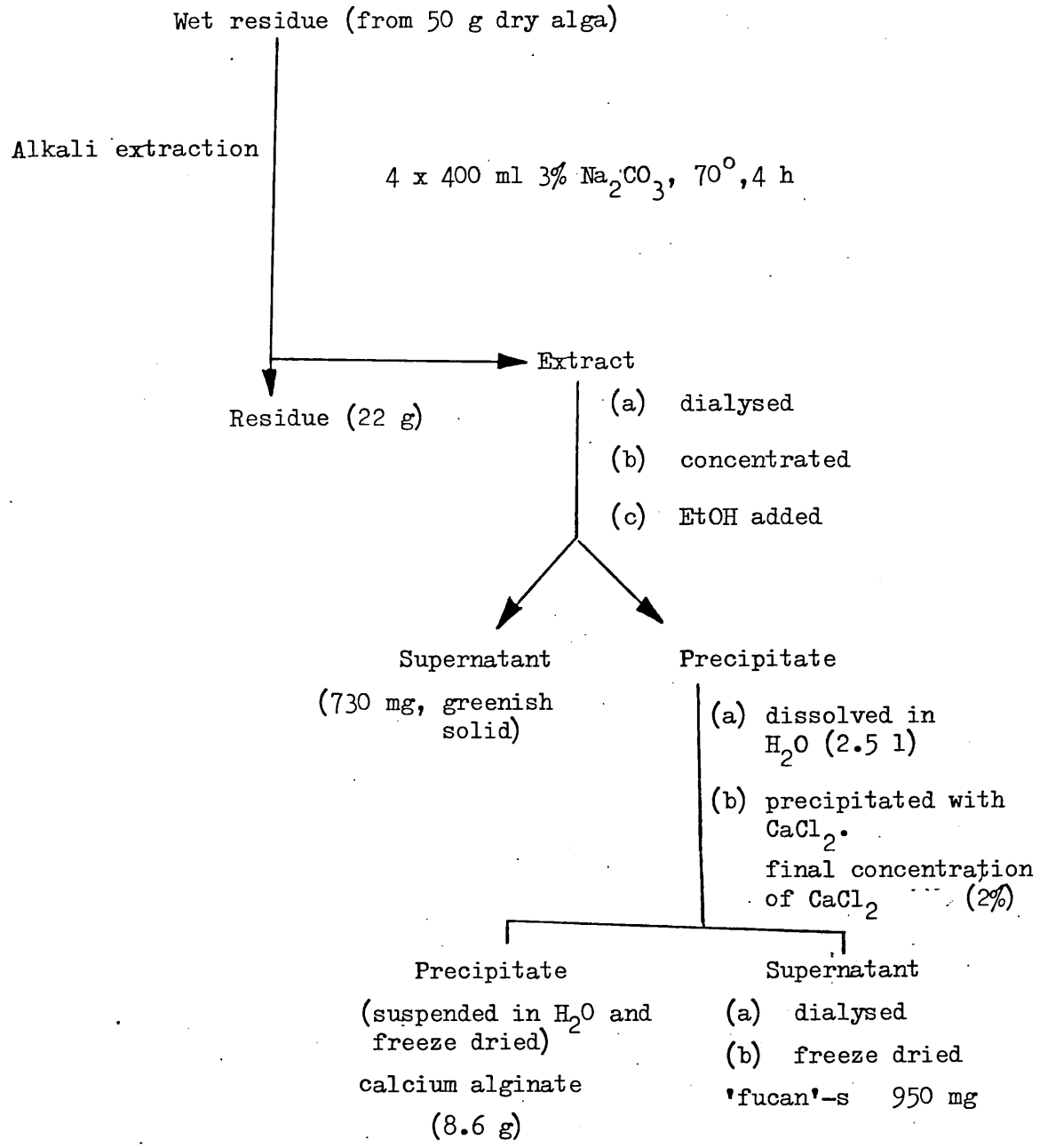
4.2 The extraction of the residual material remaining after sequential extraction of the dry weed with ethanol, aqueous calcium chloride and dilute hydrochloric acid was carried out with dilute alkali according to the flow chart 4.1.

Experiment 4.2.1 Alkali extraction

The wet residue (from 50g dry weed) after ethanol, aqueous and acidic extraction was extracted with 3% Na_2CO_3 according to the flow chart 4.1. The combined extracts, after concentration (200 ml), were poured into ethanol (1 L) and after being left overnight the derived precipitate was centrifuged off. The supernatant was concentrated, dialysed and freeze dried. A greenish solid was obtained (730 mg). Carbohydrate content 4%. This was tested for 4,5-unsaturated acid [GM 2.7.5].

The ethanol precipitate was air dried, redissolved in water and dialysed for 3 days. The solution was then made up to a concentration of about 1% alginic acid based on the weight of the air dried material. Calcium chloride was added slowly with stirring to a final concentration of 2% $CaCl_2$, during which precipitation of calcium alginate was complete. This gelatinous precipitate was centrifuged off, washed with dilute

Flow chart 4.1 Extraction procedure of the residual material from *Desmarestia firma*.



calcium chloride and water, and freeze dried to a fawn powder (8.6 g). The supernatant was dialysed till chloride free and freeze dried to a white solid (950 mg) hereinafter called the 'fucan'-S'

The residue (22 g) after this extraction was not examined further.

Investigation of 'fucan'-s

Experiment 4.2.2 Composition of 'fucan'-s

A portion of the 'fucan'-s (25 mg) was hydrolysed [GM 2.3.1]; and the hydrolysate was examined for the component monosaccharides by paper chromatography [GM 2.4.1 a,b,c] and [GM 2.5.1-2.5.4]. An aliquot of the hydrolysate was examined by paper ionophoresis [GM 2.4.2 in buffer b and c] (for results see page 174)

Experiment 4.2.3 Attempted fractionation of the 'fucan'-s by polyacrylamide gel electrophoresis

The Shandon SAE-2734 analytical polyacrylamide electrophoresis apparatus was used. The 'fucan'-s (5 mg) in water (1 ml) was shaken vigorously to achieve complete dissolution. An aliquot (100 μl) of this solution was applied to each tube of polyacrylamide gel (8 tubes in all). Stacking was done by applying 5mA per gel tube for 20 minutes. The run was continued for 50 min at 40 mA. Staining was with either toluidine blue, periodate-fuschin stain or coomasie blue. Shandon general instructions were followed for the preparation of the gel column (see page 175 and figure 4.1).

Experiment 4.2.4 Analytical ultracentrifugation of the 'fucans'

The 'fucan'-s along with three other samples of 'fucans' namely those from *Himanthalia lorea*, direct alkaline extracted 'fucan' of *D. firma* and of *D. ligulata*, were subjected to ultracentrifugation as a 1% solution in 0.1 M-KCl. The analysis was carried out on a Spinco

Model-E analytical ultracentrifuge, using analytical-D (An-D) Rotor at a centrifugal force of 259,700 g. The reaction was followed by photographs taken of the reaction mixture at time intervals (see page 177)

Experiment 4.25 Determination of the 'fucose content' of fucan

The fucose content of 'fucans' from the alkali extract of Desmarestia firma along with two other 'fucans' obtained from direct alkaline extraction of D. firma and D. ligulata as well as 'fucan' of known fucose content from Himanthalia lorea was determined by the method of Nicolet and Shinn[194] with a modification of Cameron, Ross and Percival.[195] As reproducible results were not achieved with a sample of 'fucan' of known fucose content a further modification of the method was made considering the following facts.

I. Sulphuric acid (2.5%), 5 ml used in the original work for hydrolysis might not be strong enough to hydrolyse the 'fucan' completely.

II. The amount of periodic acid added might not be sufficient to oxidise all the fucose obtained from the hydrolysis of 'fucan'.

III. A considerable amount of fucose might be degraded during hydrolysis.

Keeping these facts in mind later experiments were carried out by hydrolysing the 'fucan' with $\underline{\underline{M}}$-$H_2SO_4$ and the amount of periodic acid was doubled. An experiment was also carried out to investigate the degradation of fucose (if any) by acid hydrolysis, it was observed that no considerable degradation occurs under the conditions of hydrolysis used. The correct figure for the 'fucan' of known fucose content was obtained. So these modifications were used for the 'fucans' for which the fucose content was not known. The fucose content of the

powdered weed of D. ligulata and D. firma was also determined by this method. (Results are shown in tables 4.2 and 4.3).

Experiment 4.26 Conversion of calcium alginate into sodium alginate

The calcium alginate (8.6 g) (Expt 4.24) was converted into the sodium salt following the experimental procedure detailed in experiment 3.4.5 page 66.

Experiment 4.27. Investigation of the sodium alginate from sequential alkali extract

An aliquot of the sodium alginate (25 mg) was hydrolysed and investigated by paper chromatography [GM 2.4.1; a and b and 2.5.1, 2.5.2] and by ionophoresis [GM 2.4.2 c]. The viscosity of a 1% aqueous sodium alginate solution (25 ml) was measured at 25°C in an Ostwald capillary viscometer (for results see page 181).

Experiment 4.28 Direct alkaline extraction of alginic acid from Desmarestia firma and Desmarestia ligulata

Two parallel extractions were carried out for each of the above algae. In one of the extractions free sulphuric acid present in the alga was neutralised with triethylamine and in the other neutralisation was carried out with saturated calcium carbonate.

(a) Neutralisation with triethylamine

The dried weed (10 g) was soaked with 80% ethanol, stirring continuously. Triethylamine (1 ml) was added to keep the pH range of the solution neutral. Stirring was continued for 2 h and the aqueous ethanol centrifuged off; the residual weed was again extracted for 1 h with 80% ethanol, adding a few drops of triethylamine to keep the solution neutral, the supernatant was decanted off.

(b) Neutralisation with saturated calcium carbonate

The dry weed (5.5 g) was taken in a beaker and solid calcium carbonate powder was sprinkled on it. The weed was then covered with

water and stirred vigorously; addition of calcium carbonate was continued till the mixture became permanently neutral (1 h).

After neutralising the weed in the above ways, extraction of the alginic acid was carried out as follows.

Extraction of triethylamine treated weed (D. firma)

To the triethylamine treated and powdered weed (10.2 g) a solution of 1.8% formalin was added, stirred for a minute and then left for 30 minutes. The supernatant was decanted off and water (300 ml) was added to it, the pH of the mixture was found to be 6.8. To this mixture solid Na_2CO_3 (3.0 g) and a 10% NaOH solution (3 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 6.8) with dilute HCl and diluted to 800 ml. Calcium chloride was added under stirring to a final concentration of 2% during which precipitation of calcium alginate was complete. The calcium alginate was centrifuged off and washed with dilute calcium chloride solution. The calcium alginate was suspended in water and freeze dried (Alginate-1, 2.1 g). The supernatant was dialysed for 3 days and then freeze dried fucan-1 0.240 g). The residual weed was extracted three times with a 3% Na_2CO_3 solution (300 ml) at 70° for 3 h each time. The combined solutions were neutralised (pH = 6.8) with dilute HCl. This solution was treated as described above and gave calcium alginate (Alginate-2, 423 mg and fucan-2, 102 mg).

Extraction of calcium carbonate treated weed (D. firma).

The calcium carbonate treated weed was also extracted similarly as described above, the yield from 5 g of the weed in different extracts was as follows:

Alginate-1 = 1.13 g	Fucan-1 = 118 mg
Alginate-2 = 89 mg	Fucan-2 = 3 mg.

Extraction of triethylamine and calcium carbonate treated D. ligulata

Portions (10 g each) of D. ligulata were treated separately with triethylamine and with saturated calcium carbonate and extracted similarly as for D. firma. The yields of the different extracts from triethylamine treated and calcium carbonate treated weed are as follows:

Triethylamine treated	Saturated calcium carbonate treated
Alginate-1 = 1.16 g,	Alginate-1 = 1.19 g
Fucan-1 = 114 mg	Fucan-1 = 110 mg
Alginate-2 = 780 mg	Alginate-2 = 741 mg
Fucan-2 = 74 mg	Fucan-2 = 83 mg

Experiment 4.2.9 Examination of the alginates

The different samples of alginates obtained by the above extraction methods were purified and converted into sodium salts following the method adopted in expt. 3.4.5 chapter 3 (p.66) and all the alginates were hydrolysed and the hydrolysates analysed by paper chromatography and paper ionophoresis as described in experiment 7. The viscosity of 1% aqueous sodium alginate solutions were measured at 25°C. (see page 182, table 4.4).

Experiment 4.2.10 Composition of 'fucans' from direct alkaline extraction

The fucans (fucan-1 and fucan-2) obtained from each weed by the direct alkaline extraction method (expt. 8) were investigated after hydrolysis by paper chromatography [GM 2.4.1 (a and b), 2.5.1-2.5.4] and by g.l.c. after converting into alditol TMS derivatives [GM 2.6.1, 2.6.2(ii)] and by ionophoresis [GM 2.4.2 (b and c)]. The carbohydrate content [GM 2.7.1] the sulphate content [GM 2.7.3] and the uronic acid content were all determined.

4.3 Results and discussion

As already mentioned only the alkaline extract of the Desmarestia firma has been investigated in detail in the present work, chemical investigation of the carbohydrates of other extracts namely 80% ethanol, cold and hot water and dilute acid extracts was carried out previously and has been reported elsewhere.[106]

4.3.1 The sequential alkaline extract

The ethanol soluble greenish solid (1.4% of the dry weed) (expt 4.21) was thought possibly to contain some degraded alginic acid. This was tested as for 4,5-unsaturated acid[100] which gave a positive result, thereby indicating the presence of degraded alginic acid. Its carbohydrate content was only 4% and it was therefore, not investigated any further. The 'fucan' (ca. 2% of the dry weight of the weed) separated from the alginic acid was subjected to further investigation.

4.3.2 Composition of the 'fucan'-s from sequential alkali extract

The chromatographic investigation (expt 42.2) of the 'fucan'-s hydrolysate revealed the presence of a component of slow chromatographic mobility, along with galactose, xylose and fucose. In comparison with the slow moving spot the fucose spot was very faint.

In view of the small proportion of fucose, it was necessary to determine if the uronic acid (slow moving spot) was derived from the 'fucan' or from contaminating low molecular weight alginic acid. The ionophoretic studies of the 'fucan' hydrolysate using borate buffer in the presence of calcium ion (pH 9.2) revealed the presence of mannuronic acid as the major component of the hydrolysate, thereby confirming the contamination with alginic acid.

4.3.3 Attempted fractionation of the 'fucan'-s by polyacrylamide gel electrophoresis

In order to separate the 'fucan'-s from contaminating low molecular weight alginic acid an attempt was made to fractionate the crude material (see expt4.2.3). A fast moving sharp band was observed on 7.5% cross-linked gels which stained with periodate fuschin stain; this is an indication that the polysaccharide is of low molecular weight as well as anionic at pH 8.5. No other bands were visible (fig. 4.1) from which it may be concluded that the so called 'fucan'-s from the alkali extract comprise mainly low molecular weight alginic acid, and the other components of the polysaccharide are negligible compared to this low molecular weight alginic acid. The 'fucan' present in this material might be either so negligible as to escape detection or it was diffused throughout the gel column.

4.3.4 Ultra centrifugal studies of the different 'fucans'

The 'fucan'-s along with other fucans (see expt4.2.4)were subjected to ultracentrifugal studies. Only a single peak was observed for the 'fucan'-s from the sequential alkaline extract of D. firma indicating the presence of one major component, the s value was 0.98 Svedbergs, which suggests a low molecular weight for the sample. In three other runs 'fucans' from Himanthalia lorea, fucan-2 from D. firma obtained by direct alkaline extraction (expt4.2.8) and 'fucan'-1 from D. ligulata (expt4.2.8) were subjected to investigation. The Himanthalia 'fucan' exhibited a double peak corresponding to at least two components of low and moderate molecular weight.

'Fucan'-2 exhibited fast diffusing double peak, which suggests that at least two components of low molecular weight are present. Fucan-1 (D. ligulata) exhibited an unsymmetrical peak, indciating three or more fractions, major component had S = 2.2. The sample contains at least two low molecular weight components along with some higher molecular weight material.

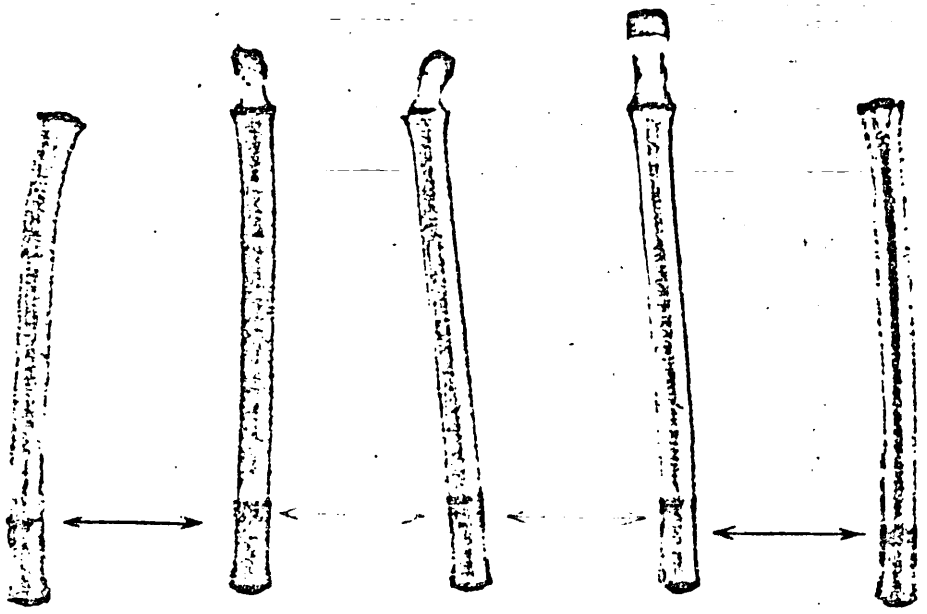
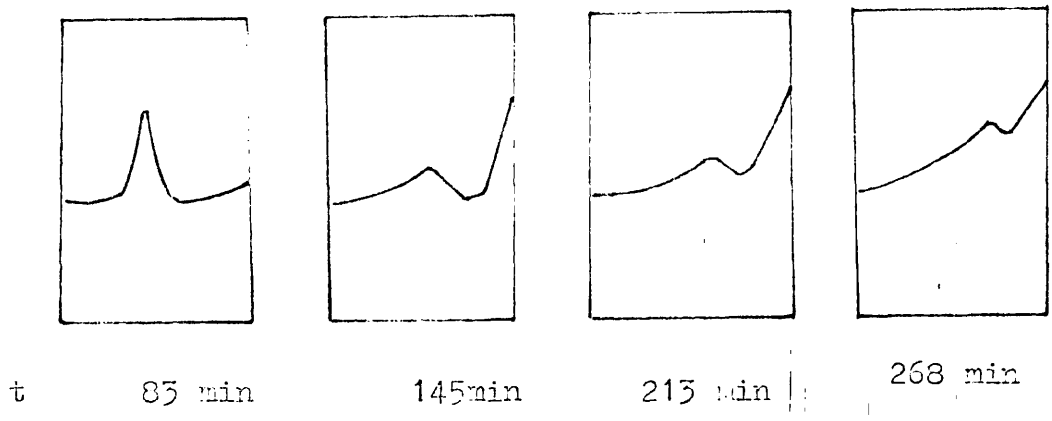
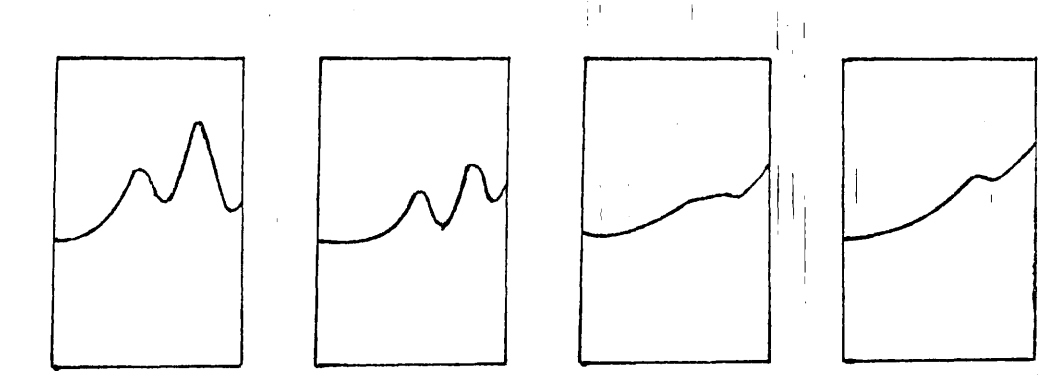


Figure 4.1 Fractionation of 'fucan-S' from Desmarestia firma by polyacrylamide gel electrophoresis on 7.5% cross linked gels at pH 8.5.



(A)



(B)

Fig. 4.2 Diagrams of sedimentation velocity of
(A) fucan-s (B) fucan-II

4.3.5 Fucose content

In view of the foregoing results it was found necessary to determine the fucose content of the 'fucan' (expt 42.5). The method of Nicolet and Shinn[194] modified by Cameron, Ross and Percival[195] was used. The underlying principle of this method involves the oxidation of the L-fucose with periodic acid whereby the terminal-$CHOH.CH_3$ group is converted into acetaldehyde, which forms an addition compound with sodium bisulphite, the amount of this compound being estimated by titration with iodine (see equation).

$$C_6H_{12}O_5 \xrightarrow{HIO_4} CH_3CHO$$

$$CH_3CHO + NaHSO_3 \longrightarrow CH_3CHO.NaHSO_3$$

$$NaHSO_3 + I_2 + H_2O \longrightarrow NaHSO_4 + 2HI$$

Hence, $C_6H_{12}O_5$ (164.16) = 2 litres N-iodine

= 200,000 ml N/100 iodine.

$$\therefore \ \% \text{ fucose} = \frac{\text{ml N/100 iodine} \times 164.16 \times 100}{200{,}000 \times \text{weight of sample.}}$$

As reproducible results could not be obtained by this method (table 4.2) M-sulphuric acid and twice the amount of periodate was used and these gave results of reasonable reproducibility (see table 4.3).

Table 4.2

'Fucose content' determination by modified Nicolet and Shinn method

Name of sample	Weight of sample mg	Vol. of 0.1M Na-Arsenite ml	Vol. of 0.5M Periodate ml	% Fucose
Fucose	11.3	14	3.5	72
Fucan (H. lorea) (S 97 Dr Percival)	51.0	14	3.5	22.6
Fucan (H. lorea) (S 98 Dr Percival)	29	14	3.5	24.63

Table 4.3

The 'fucose content' determination of the different fucans and powdered seaweeds by modified Ross and Percival method.

Name of Sample	Weight of sample mg	Vol. of 0.1M Na-Arsenite ml	Vol. of 0.5M Periodate ml	% Fucose
Fucose	6.5	14	7	100.9
Fucan (H. lorea)	13.5	14	7	60.2
Fucan-s (D. firma)	47.3	14	7	9.8
Fucan (D. ligulata)	42	14	7	5.85
Powdered weed (D. firma)	650	14	7	0.35
Powdered weed (D. ligulata)	664	14	7	0.32

The low percentage of fucose in the present 'fucan' gives further evidence that the material investigated (fucans from D. firma) is not a pure fucan; 60.2% of fucose in the 'fucan' from Himanthalia lorea agrees well with the previous recorded fucose content for this 'fucan'[196]; thereby confirming the reasonable accuracy of the modified method. The fucose content of pure fucose also shows that this method is reasonably accurate, only a slight over-estimation was obtained for the pure sample. Although, the fucose content of fucans vary to a great extent from species to species and the exact figure is still in doubt an average of 44% fucose in the fucans has been assumed by Cameron, Ross and Percival.[195]

That the present method gives an over-estimation of the 'fucose content' has also been shown by Black et al.[197] they obtained a lower percentage of fucose by estimating the liberated acetaldehyde colorimetrically, than the percentage obtained by titrimetric method. These authors, therefore, concluded that an aldehyde or ketone other than acetaldehyde or formaldehyde (the latter is retained by the alanine) is liberated on the treatment of the seaweed hydrolysates by periodate.

Due to the low percentage of 'fucan' in Desmarestia firma and Desmarestia ligulata it was thought that the presence of free sulphuric acid in these weeds might be responsible for the degradation of some of the 'fucan' after the death of the algae. With a view to prove this the fucose content of the whole weed was also determined. The percentage of fucose in D. ligulata was 0.32 which corresponds to 5.3% based on the 'fucan' present in this weed, this figure agrees reasonably well with the fucose content (5.85%) determined from the 'fucan' (6% of the dry weed).

Similarly the fucose content of D. firma weed was found to be 0.35% and this corresponds to 9.1% based on the 'fucan' (3.8% of the dry weed). Fucose content determined directly from this 'fucan' is 9.8% (see table 4.3).

All these observations indicate that sulphuric acid present in these algae did not degrade the fucan to any extent. If this were so the percentage of fucose content of the weed would have been larger, furthermore fragments of this fucan would have been found in the ethanolic extracts, but this was not the case.[106]

These facts, namely low fucose content, relatively high proportion of mannuronic acid, as well as the ultracentrifugal and gel electrophoresis results, indicate that the material studied, might contain low molecular weight alginic acid, (which did not precipitate out with calcium chloride) along with a small amount of 'fucan'-S. On these grounds further structural investigation of this material was abandoned.

4.3.6 Alginic acid

Freeze drying of the calcium alginate obtained from the sequential alkali extract of Desmarestia firma (expt 2.1) gave a fawn powder (8.6 g calcium alginate). The calcium alginate was converted into sodium alginate by way of the free acid (expt 3.45 chapter 3) (8 g Na-alginate 16% of the dry weed).

Paper chromatograms of a hydrolysate of the sodium alginate showed the presence of uronic acids as well as lactones. As the paper chromatography of uronic acids in solvents most commonly used for chromatography of carbohydrates does not give satisfactory separation of the individual uronic acids and for quantitative work it is necessary beforehand to transform all lactones to uronic acids;

the use of paper ionophoresis for the separation of uronic acids was made. Paper ionophoresis of a hydrolysate of the sodium alginate in a borate buffer containing calcium ions confirmed the presence of both mannuronic and guluronic acids.

The viscosity of the sodium alginate obtained by this method was very low (η_{rel} = 1.5 for 1% solution at 25°C). This very low value indicates that the alginic acid is considerably degraded. The presence of a relatively high proportion of mannuronic acid in the so-called 'fucan'-s from the alkaline extract and also in the alcoholic and acid extracts of D. firma supports this[106] since the mannuronic acid residues are more readily hydrolysed than guluronic. Carlberg and Percival[161] obtained similar results in the alkaline extract of D. ligulata, and confirmed it by determining the mannuronic to guluronic acid (M/G) ratio. Their results gave a M/G ratio of 0.06 or a guluronic acid content of about 94% in the alkaline extract of the sequentially extracted weed. Based on these results they concluded that a large proportion of the mannuronic acid units had been hydrolysed when the alga was in contact with its own free sulphuric acid and with added acid during the extraction. As D. firma also contains free sulphuric acid and the presence of mannuronic acid in the alcoholic and acidic extract was established we were also convinced that the low viscosity of the alginate is due to the loss of a substantial amount of mannuronic acid during the process of extraction.

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 4.2.8 (a and b). Two samples of calcium alginate-1 and 2 were isolated.

A combined overall yield of 19.8% of alginic acid was obtained compared with 16% from the previous sequential extraction. A parallel study with a sample of D. ligulata was also carried out, where an overall yield of 19.5% of alginic acid was obtained compared with 14% from sequential extraction. Viscosity measurements of a 1% solution of sodium alginate obtained from D. firma and from D. ligulata under identical conditions were carried out and the values obtained are tabulated below.

Table 4.4

Viscosities of 1% solutions of sodium alginates at 25°C

Extraction method	Name of alga	Alginate	Relative viscosity $\eta_{rel.}$
Sequential extraction	D. firma	Alginate from 3% NO_2CO_3 extract	1.5
	D. ligulata	Alginate from 3% Na_2CO_3 extract	(1.7)*
Direct extraction	D. firma	Alginate-1	68
		Alginate-2	56
Direct extraction	D. ligulata	Alginate-1	81 (300)
		Alginate-2	61 (150)

* Figures in parenthesis are previously recorded values.

It is evident from table 4.4 that the viscosity of the two samples from D. ligulata weed obtained by direct extraction has been decreased to a considerable extent. It can therefore be concluded that not only the drastic method of extraction is responsible for the low viscosity of the

alginates, prolonged storage of the weed also affects the viscosity to a considerable extent. The fact that D. firma had been stored for about a year before chemical investigation was undertaken might be one of the reasons for the low viscosity of the alginate.

4.3.7 'Fucans' from direct alkaline extracts

The fucan-1 and fucan-2 separated from the alginic acid of direct alkaline extracts exhibited similar composition to that of the 'fucan' from sequential extraction except that fucan-1 was found to contain glucose in addition to other components. The presence of glucose in fucan-1 is due to contaminating laminaran.

4.4 Conclusion

From the foregoing results it can be seen that these weeds have a low yield of 'fucans' and this low yield cannot be explained by the hydrolytic effect of the sulphuric acid present in these weeds. It can only be concluded that D. ligulata and D. firma synthesise less 'fucan' than most other brown seaweeds.[119] However, Durvillea species[198] also appear to be virtually devoid of 'fucans'.

It can also be speculated that the alginate content in the growing weed of the species under investigation is probably considerably higher than the estimated value (table 4.1) since the results clearly show that some degradation does occur during storage and extraction.

Finally it can be concluded that the presence of sulphuric acid in Desmarestia firma has no effect on their carbohydrate metabolism, this is equally true in the case of D. ligulata.

PART TWO

CARBOHYDRATES OF THE EXTRACELLULAR MUCILAGE OF THE MARINE DIATOM (*Coscinodiscus nobilis*)*

* This work has been accepted for publication in Phytochemistry.

CHAPTER 5

5.1 INTRODUCTION

Diatoms belong to the class Bacillariophyceae. All diatoms are fundamentally unicellular, though the cells are sometimes joined into colonies. Each cell is enclosed in a rigid wall consisting of pectic material impregnated with silica. This silicified wall is known as a frustule, and there is great variation between species in its shape and construction. In the simplest cases the frustule consists of two closely fitting halves of a Petri dish. Other species have only bilateral symmetry; and the frustule is more comparable with a date-box. In both these types of diatoms the half frustule has one face which is more or less flat, known as a valve face, with an edge projecting at right angles from it. The edges of the two half frustules are called connecting bands since they form the connection between the two half-frustules of a complete diatom. The one half-frustule is slightly larger than the other, and their connecting bands overlap, forming the girdle of the cell. One therefore distinguishes between the valve view and the girdle view of a diatom, which may be as different from each other as the appearance of a Petri dish seen from above and seen from the side. Many diatoms usually come to rest with the valve face uppermost, so that this is the more familiar aspect. However, there are many species where the valve face is small in area and the girdle face much more extensive. This is so in species where the valve of each half-frustule is separated from the connecting band by one or more intercalary bands. Diatoms of this sort commonly come to rest with the girdle face uppermost. Every diatom species has a characteristic pattern of ridges, furrows, spots and other markings which are extensively used for classification.

Outside the silicified wall there is a gelatinous layer. In colonial species it is this layer which joins cells together, and some strictly unicellular forms are attached to their substratum in a similar way.

On the inside, the frustule is lined with cytoplasm containing one or more irregularly shaped Chloroplasts, a nucleus, and often droplets of oil. There is a central vacuole. Some diatoms are capable of slow, rather jerky motion. Pinnularia is a genus which shows this feature. Reproduction of diatoms is usually by cell division. This begins with nuclear division and an enlargement of the protoplast which forces the two half-frustules apart. The protoplast then divides along a plane parallel with the valve face of the frustule and each new protoplast synthesises a new half-frustule which fits inside the existing one. Thus of the two daughter cells, one is the same size as the parent but the other is slightly smaller. If this process were to continue indefinitely, the average cell size would steadily decrease. This tendency is balanced by the periodical formation of auxopores. These are sometimes the result of fusion of protoplasts from two cells, but in other cases they are produced from single cells. In either case, the protoplasts of cells which are approaching minimum size for their species discard their frustules completely; if fusion is to take place it does so at this stage. There is then enlargement up to the maximum size for the species, and the synthesis of a new frustule which is not marked in the manner characteristic of normal cells. This enlarged cell is the auxopore, which by subsequent division gives rise to new vegetative cells.

Diatoms are found in a wide range of habitats, including both fresh and salt waters. They are also common in soil and various other terrestrial habitats. Some species are extremely resistant to desiccation, and soil which has been kept dry for nearly fifty years has been shown to contain living diatoms. The silicified frustule is much more resistant to decay than the rest of the diatom cell, and bodies of water in which diatoms are plentiful accumulate a layer of empty frustules on the bottom, which may grow to a thickness of many feet under suitable conditions. In some places a layer of this sort has been raised above water level by geological changes, giving an accessible deposit of diatomaceous earth. This is quarried or mined on a considerable scale for a variety of uses such as heat insulation, the filtration of liquids, and as a mild abrasive.

Though individually small, diatoms are very numerous and they form an important part of the community of living creatures in water. Being photosynthetic, they have the important quality of adding to the organic content of water. Zooplanktons are largely dependent upon diatoms as a source of food. Both diatoms and zooplanktons are consumed by small fishes. In this food chain diatoms are of fundamental importance since only they can synthesise organic compounds.

5.1.1 Carbohydrates of the Bacillariophyceae

Diatoms are known to contain carbohydrates associated with the silica of the frustules.[162] Owing to the microscopic size and hence the difficulty of collecting appreciable quantities of diatoms, very little previous chemical investigation has been carried out. Analysis of natural crops and cells in pure culture[163,164] have been performed on organisms grown under a variety of conditions and using

different techniques for the estimation of their composition.

A comparative study of four members of Bacillariophyceae class including Coscinodiscus with seven other algae of four differnet classes namely Chlorophyceae, Chrysophyceae, Dynophyceae and Myxophyceae was carried out by Parsons et al.[165] All species were grown under similar physical and chemical conditions and cells were analysed during the exponential phase of growth. Chemical analysis consisted of a proximate analysis of each species for ash, protein, carbohydrate and lipid, and an analysis for carbon, silicon and phosphorus as well as quantitative determinations of the monosaccharides and amino acids in hydrolysates of whole cells. Based on their observations these authors reported that marine phytoplankton have very similar organic composition when grown under similar physical and chemical conditions, regardless of the size of the organisms or the class to which they belong. The different constituent sugars found by Parsons et al.[165] in members of the Bacillariophyceae are given below.

Table 5.1 Percentage of the carbohydrate constituents in the cells of the diatoms.

Species	Percentage of total carbo-hydrate dry wt. of cells	Principal monosaccharides Percentage dry wt. of cells							
		glucose	galact-ose	mannose	ribose	xylose	rhamnose	fucose	hexuronic acid
Chaetoceros sp.	9.5	3.3	1.5	0.79	0.71	0.4	2.8	+	+
Skeletonema costatum	22.1	16.4	1.8	0.87	1.2	-	1.0	0.9	+
Coscinodiscus sp.	4.11	2.1	0.4	0.41	+	-	0.7	0.5	
Phaeodactylum tricornutum	20.02	10.7	2.7	3.7	0.72	0.7	1.5	-	+

\+ detected but not examined

\- not detected.

It can be seen that while glucose, galactose, mannose and rhamnose are common constituents of all the species the proportions of these sugars vary widely. Two genera, Skeletonema and Coscinodiscus, appear to be devoid of xylose and Phaeodactylum is devoid of fucose.

The carbohydrates, free amino acids and protein composition of some marine diatoms and the limnetic diatom Melosira varians have been investigated by Kleinkauf[166] who reported the identification of six different monosaccharides in a mixture of marine diatom species (primarily Coscinodiscus concinnus, C. grani and Biddulphia sinensis), these monosaccharides included the three hexoses, namely galactose, glucose, and mannose found previously, and three pentoses, arabinose, xylose and ribose. Arabinose has not been reported previously. In contrast, in the limnetic diatom Melosira varians, only glucose and xylose were found.

5.1.2 The carbohydrates metabolised by diatoms may be classified broadly into three different types, namely:

1. Low molecular weight carbohydrates
2. Water soluble food reserve polysaccharides
3. Other polysaccharides (Alkali extract).

Besides the above three types of carbohydrates metabolised by the members of Bacillariophyceae class, many diatoms produce extracellular polysaccharides which diffuse into the surroundings. Structural investigation of some of these polysaccharides has been carried out in recent years.[167,168]

5.1.2 A Low molecular weight carbohydrates

These can be obtained from the aqueous alcoholic supernatant after precipitating the polysaccharide from an aqueous extract of the diatom with ethanol. Glucose is present as the main low molecular weight monosaccharide along with some other reducing

saccharides of slow chromatographic mobility in this extract from Phaeodactylum tricornutum.[169] Separation of these saccharides on cellulose columns and on 3MM paper resulted in six different fractions, which were identified as a disaccharide comprising xylose and glucose, sorbitol, laminitol (C-methylinositol), glucose, myoinositol and probably a mixture of O-D-glucosyl (1→3)-D-glucosyl (1→3)-D-glucose (laminaritriose) and O-D-galactosyl (1→3)-D-glucosyl (1→3)-D-glucose (galactosyl-laminaribiose).[169]

5.1.2B Water soluble food reserve polysaccharides

In 1951 Von Stosch[170] reported the presence of water soluble polysaccharides from the marine diatom Schroederella schroederi and Rhizosolenia statherfoltii which he described as Leucosin. In a parallel study with the laminaran of brown algae he also showed similarities in the physical and chemical properties of these two polysaccharides. Quillet[171] in 1955 isolated leucosin from the diatom Hydrurus foetidus and as a result of chemical analysis confirmed it as a pure glucose product. He also reported that this polysaccharide is built of 8 glucose units and that it is probably, like laminaran, (1→3)-linked; the latter being built of about 16 glucose units. A detailed structural study of crystalline leucosin supplied from a mixed culture of diatoms by Von Stosch was carried out by Beattie, Hirst and Percival.[172] They found that the polysaccharide is built of about 12 (1→3)-linked β-D-glucose units with some branching at C-6. The proportion of (1→3) to (1→6) linked glucose units was shown to be about 11:1. These authors suggested the name "Chrysolaminaran" for leucosin on the basis of the close relationship in chemical properties. The physicochemical resemblance of both the polysaccharides was also recognised by Von Stosch.

When grown under bacteria free conditions with atmospheric carbon dioxide as the sole carbon source, Phaeodactylum tricornutum[169] was found to synthesise a water soluble glucan of the laminaran type which comprised about 14% of the dry weight of the organism.

The glucan was extracted with cold water and precipitated from low molecular weight material with ethanol. Hydrolysis showed contamination with trace quantities of xylose, mannose, and rhamnose, and purification was achieved by fractionation with DEAE-Cellulose chromatography. Paper chromatography of a partial acid hydrolysate of the purified glucan revealed components with the mobilities of glucose, laminaribiose, gentiobiose, and laminaritriose, and treatment with an endo-β-1,3-glucanase gave a similar chromatographic pattern.

These findings were confirmed by methylation. The major methylated sugar, 2,4,6-tri-O-methyl glucose, was separated as a crystalline material and small quantities of 2,3,4,6-tetra-O- and 2,4 di-O-methyl glucoses were identified by paper and gas liquid chromatography and by ionophoresis.

Parallel periodate oxidation studies of the P. tricornutum glucan and Laminaria laminaran under the same conditions revealed that the former reduces a higher proportion (0.53 mole) of periodate than the latter (0.39 mole). This could be due to the fact that 'Over oxidation' occurs in all the G-chains whereas the M-chains in Laminaria laminaran resist 'Over oxidation'. This emphasises the difference of chrysolaminaran from some of the Phaeophyceae laminarans, namely the absence of mannitol terminated chains.

The fact that glycerol was the sole cleaved product of Smith degradation is further evidence that Phaeodactylum laminaran comprises a (1→3)-linked β-D-glucan with some branching at C-6, and, as is to be expected, closely resembles chrysolaminaran; the food reserve polysaccharide of fresh water diatoms.

The water soluble polysaccharide from the marine diatom Skeletonema costatum, when examined after hydrolysis revealed glucose as the only sugar. Methylation of the polysaccharide, and analysis of the methylated alditol acetates by glc-ms revealed that this is a (1→3)-linked glucan with some branching at C-6 and C-2. Branching at the latter position has not been observed previously in glucans from other diatoms[169,171] or benthic algae.[172] By measuring the peak areas of the different methylated derivatives, the authors[173] estimated the relative amounts and calculated the average chain length of this polysaccharide as 11.

When subjected to periodate oxidation studies, the polysaccharide consumed 0.23 mole of periodate per anhydro glucose unit, corresponding to 9 residues per chain. Periodate oxidation of a non-reducing glucose end group reduces two moles of periodate and yields glycerol after reduction of the polyaldehyde followed by hydrolysis; (1→3)-linked glucose residues remain uncleaved. The ratio of glycerol to glucose was 1:10.8, giving a chain length of 11, which is consistent with the result obtained by the methylation studies.

A different food reserve polysaccharide containing only galactose units has been reported[174] from Chaetoceros decipiens.

5.1.2 C Other polysaccharides (Alkali extract)

A glucuronomannan, $[\alpha]_D$ +34° was obtained from the alkali extract of the diatom Phaeodactylum tricornutum.[175] This material was completely soluble in water and contained in addition to mannose, 27% glucuronic acid and 15% half ester sulphate. Partial acidic hydrolysis

gave mannose, glucuronic acid and three oligouronic acids. The two of thes were characterised as O-D-glucopyranosyl uronic acid-(1→3)-D-mannopyranose and O-D-glucopyranosyl uronic acid (1→3)-D-mannopyranosyl-(1→2)-D-mannopyranose respectively. The third acid contained some six units which on further hydrolysis gave mannose, glucuronic acid, glucose and the above di- and tri-oligouronic acids.

The application of Smith degradation to this polysaccharide led to the recovery of about 25% of a degraded polymer containing only mannose units and about 10% sulphate. Glycerol, glyceric acid, several slower spots which streaked badly on a paper chromatogram, and traces of glucose were found to be present in the mild acid hydrolysate from which the degraded polymer had been recovered. Further hydrolysis of this hydrolysate reduced the quantity of material of lower mobility and chromatography showed mannose in addition to the spots already given by the mild acid hydrolysis of the polyalcohol.

Methylation and periodate oxidation revealed that the degraded polymer consists of chains of about fifteen (1→3)-linked mannopyranose units with occasional residues carrying sulphate groups. This appears to constitute the backbone of the native glucuronomannan to which are attached side chains of the aldotriouronic acid, and of the third oligouronic acid, by as yet unknown linkage.

During the Smith degradation the (1→2)-linked mannose and glucuronic acid residues are oxidised by the periodate and on mild hydrolysis these are cleaved together with the unoxidised (1→3)-linked mannose and glucose units present in the side chains and are respectively responsible for the glycerol, glyceric acid, mannose and traces of glucose found in the supernatant.

Although the initial polysaccharide had a positive rotation indicating the presence of some α-linkages, no evidence for the anomeric configuration in the different fragments was advanced.

No glucose could be detected in a total acid hydrolysate of the polysaccharide. It must, therefore, be present in very small amounts, and it is unlikely that it has much significance in the overall structure of the molecule.

Any sulphate groups labile to alkali would have been lost during the extraction of this polysaccharide. However, treatment of the residual organism with sodium methoxide before extraction of the glucuronosylmannan should remove any alkali labile sulphate and result in methylation of either the carbon atom, which formerly carried the sulphate residue, or an adjacent carbon atom. Application of this procedure failed to yield a polysaccharide containing any methylated sugars and it seems unlikely, therefore, that any sulphate was lost during the extraction. In the absence of (1→3)-linked glucuronic acid, it follows that the sulphate must be linked to mannose since any linked to glucuronic acid would be alkali labile.

The alkali-soluble polysaccharides of the cell wall from five species of Chaetoceros, from Thalassiosira gravida and from Coretheon hystrix were shown[176] to contain rhamnose, fucose, galactose, mannose and xylose. The relative amounts of rhamnose and fucose varied widely from the very rhamnose-rich Chaetoceros affinis to Thalassiosira gravida; where no rhamnose was found, also the proportion of mannose to galactose varied widely from species to species.

The extracellular polysaccharide composition in all species investigated was quite different from the composition of the cellular alkali-soluble fraction.

5.1.2D Extracellular polysaccharide

It is well known that many planktonic algae excrete considerable amounts of organic material into the surrounding medium, and that this material, in many cases at least, consists partly of carbohydrates. The production of soluble, extracellular polysaccharides has been reported from several diatom species. Allan et al[177] studied eight species, and found soluble polysaccharide in the medium in all cases. The amounts were very small (less than 5 mg/l or 0.2 mg per 10^8 cells), with the exception of one species, Nitzschia frustulum, which in standard one-litre culture in enriched seawater produced 15.6 mg/l in 7 days, and in mass cultures, over two weeks, were reported to give as much as 150 mg/l, depending upon conditions like salinity and nutrient levels. Hydrolysis of the polysaccharide gave rhamnose (24%), mannose (34%), galactose (8%) and two unidentified components (14 and 20%) together with an undetermined amount of glucuronic acid. The composition of the polysaccharide however depended upon salinity.

In a study of Chaetoceros affinis Myklestad and Haug[178] reported the production of an extracellular polysaccharide in amounts of 16–40 mg/l. The production took place in the stationary growth phase, after the main production of glucan was finished. In a second paper the authors reported that they had separated a glucan and a heteropolysaccharide containing 63% of rhamnose and fucose together with less arabinose and galactose and 8.7% of half ester sulphate as $NaSO_3^-$. Analysis of the derived alditol acetates[179] showed rhamnose:fucose:galactose = 35:39:26. Methylation and periodate oxidation studies[179] on this polysaccharide indicate that most of the rhamnose is located in the outer part of the molecule whereas the fucose and galactose are present both in the inner and outer parts. Apart from being present

as end groups the rhamnose is linked through C-1 and C-2, galactose through C-1 and C-3 (major) as well as through C-1 and C-4 and some of the fucose is linked through C-1 and C-3, while the major part is present as branch points or sulphated. Fucose and galactose also occur as end groups.

Two further species of Chaetoceros have been investigated.[180] The extracellular polysaccharide from C. decipiens was shown to be very similar to that from C. affinis, while that produced by C. curvisetus is different. It is again a highly branched polysaccharide of a complex structure. Fucose is present both in furanose and pyranose forms, the former being responsible for the major part of the end groups. Fucofuranose is also present as linked through C-1 and C-2 and as branch points. Fucopyranose appears to be responsible for the main part of the branch points. Rhamnose and galactose are responsible for a small part of the end groups. Galactose linked through C-1 and C-3 is mainly present at the inner part of the molecule. Both the amounts and the composition of the extracellular polysaccharide strongly suggest that in the case of these three Chaetoceros species, the polysaccharide is excreted into the medium, and that it is not a case of leakage from dead or dying cells. The function of the polysaccharide in the life of the diatom is unknown. These extracellular polysaccharides all gave fucose, rhamnose, galactose, and sulphate on hydrolysis.

5.1.3 COSCINODISCUS NOBILIS

An unfamiliar diatom was observed in the townettings taken by research vessels of Marine Biological Laboratory, Plymouth towards the end of January 1977. It was a large member of the genus Coscinodiscus, but a species previously unknown in the English Channel. The mucilage produced by this diatom was so abundant and it was thought

sank to the bottom together with dead diatoms and caused considerable damage to nets during trawling in some areas off Plymouth.

The valve pattern of the diatom resembled that of some forms of Coscinodiscus concinnus Wm. Smith[181] with the valve having a clear centre, but the diameter was greater than that recorded for C. concinnus in the English Channel. It was unlike any other species of Coscinodiscus recorded for the Western English Channel in that 50μm deep valve mantle met the valve face at right angles. As the valve itself was almost flat the diatom had a markedly rectangular outline in girdle view. A search of the literature showed that this diatom was probably Coscinodiscus nobilis Grunow. Our material agrees essentially with the description and illustration of C. nobilis given by Simonsen[182] and agrees with the material of Grunow designated as lectotype by Simonsen, Grunow[183] described this species from the Java Sea and it has only otherwise been recorded from the Indian and Pacific Oceans,[184] although there are two doubtful records from the east coast of England[185] and one from Heligoland.[186]

The diatom was successfully cultured in the Marine Biological Laboratory, Plymouth and in our laboratory. It was observed in the Plymouth Laboratory that in culture this diatom produced much more mucilage, which collected on the bottom of the culture vessel, than other species of Coscinodiscus they have cultured in similar conditions.[187]

By the end of April the Fishery Officer at Plymouth was receiving complaints from the Plymouth fishermen that their trawls were becoming clogged or broken by a very heavy grey slime. The slime made hauling difficult and prolonged washing and air-drying did not completely remove it. The slime was most prevalent on the sea bed in areas where the unusual diatom was abundant.

Light microscopy showed that the slime was heavily loaded with clay particles and the insoluble remains of Plankton organisms, such as the skeletons of silicaflagellates.

The mucilage from cultured diatom used for preliminary investigation and the clay material of the affected area of the sea were kindly supplied by Dr Boalch of the Marine Biological Laboratories, Plymouth. Subsequent culture of the diatom was carried out in our Laboratory and the mucilage obtained therefrom was investigated throughout the subsequent work.

5.2.1 PRELIMINARY INVESTIGATION OF THE MUCILAGE (A AND B) AND DIATOM (D)

Experiment 1 Investigation of the mucilage elaborated by the cultured diatom (mucilage A)

The mucilage A was tested for carbohydrate [GM2.7.1]. This was treated first with Amberlite IR $120H^+$ and then with IR 45 (OH^-). The resin was filtered off and the mucilage was freeze-dried (A). 9.6 mg, carbohydrate content 20%.

Experiment 2 Isolation of the mucilage (B) from clay material

The wet clay (ca. 200 g) was extracted for 15 h at room temperature with deionised water (2 L), centrifuged off and the residual clay extracted again for six hours with deionised water. After centrifuging the clay the clear supernatants were combined, reduced to small volume and tested for carbohydrate. It was then dialysed in a closed system against deionised water (dialysate 1). The mucilageous solution was then freeze-dried (B).

Experiment 3 Composition of (A), (B) and diatom (D)

Aliquots of (A), (B) and powdered (D) were hydrolysed [GM 2.3.1] and then examined by paper chromatography [GM2.4.1 a,b and GM 2.5.1, 2.5.2, 2.5.3/4 and 2.5.7], paper ionophoresis [GM 2.4.2 a] and by glc-ms after conversion into alditol acetates.

The carbohydrate [GM 2.7.1] and protein contents of the samples were also determined.

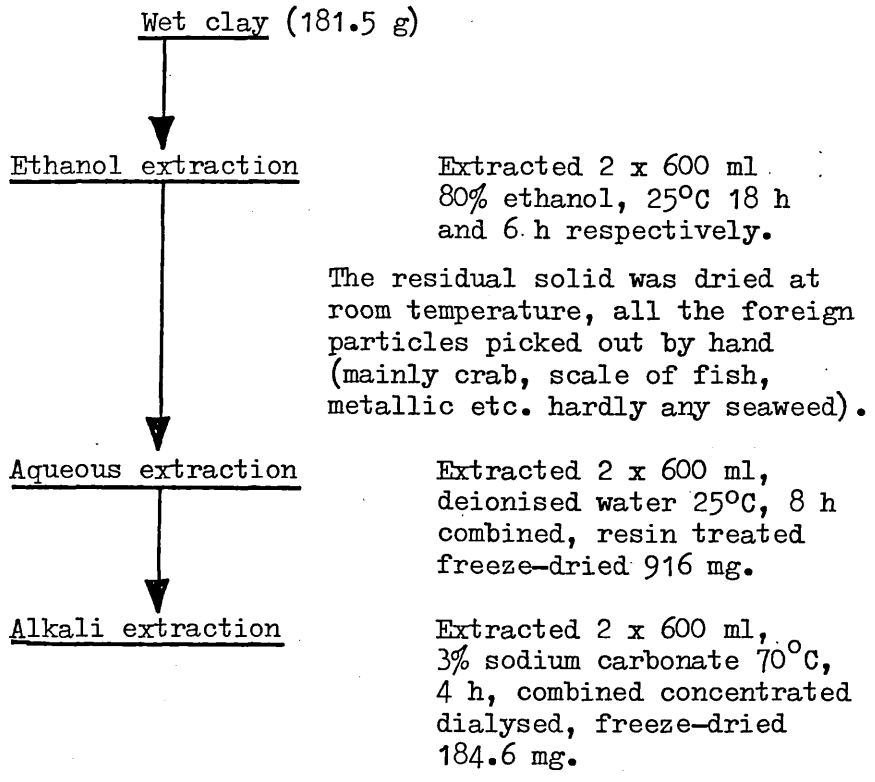
Experiment 4 Investigation of dialysate 1

The first dialysis water [dialysate 1 (3 L)] was tested for carbohydrate (+ ve). This was therefore, reduced to small volume, the solid formed during concentration was filtered off through glass fibre filter paper. The solid was tested for carbohydrate, which showed a negative test. The resulting filtrate was treated with Biodeminrolit and freeze-dried (7.2 mg).

5.2.2 SEQUENTIAL EXTRACTION OF MUCILAGEOUS CLAY

The extraction was carried out according to the flow chart IV.

Flow Chart 5.1



Experiment 5 Composition of the aqueous extract

An aliquot (28 mg) of the aqueous extract was hydrolysed [GM 2.3.1] and examined by paper chromatography [GM 2.4.1 (a), (b) and GM 2.5.1, 2.5.2, 2.5.3 and 2.5.7.

The carbohydrate content [GM 2.7.1] of the extract was also determined.

Experiment 6 Composition of the alkali extract

An aliquot (25 mg) of the alkali extract was hydrolysed and examined by paper chromatography as described in experiment 5.

5.2.3 SYSTEMATIC INVESTIGATION OF THE MUCILAGE (C) ELABORATED BY THE CULTURED DIATOM

5.2.4 Experiment 7 Preparation of the culture solution

('ERD-SCHREIBER' culture solution as prepared at the Plymouth Laboratory)

Sea water (1 L) was filtered through Whatman No.1 filter paper, glass distilled water was then added to reduce it to 95%. This water was then autoclaved at 15 lb pressure for 30 minutes to sterilise.

Soil extract was prepared from 1 kg finely sieved garden soil, adding tap water (2 L) and autoclaving for 1 h at 5 lb pressure. It was then allowed to settle and the clear liquid was used. The clear soil extract solution was again autoclaved at 15 lb pressure for 35 minutes using a separate small flask for each large flask of medium to be made up.

The salt solution ($NaNO_3$, 0.2 g/l; and $NaHPO_4 \cdot 12H_2O$, 0.02 g/l) was made up together in glass distilled water so that 1 ml of solution gives the required amount of salts for 1L culture solution. The salt solution was also autoclaved in the same way.

Required amount of salt solution was added to the cold soil extract (50 ml) and the combined solution was finally added to the cold sea water. This culture solution was allowed to reach the temperature of the cultures, which was subcultured previously.

5.2.5 Experiment 8 Culture of the diatom

Culturing was carried out in six conical flasks (250 ml) at room temperature protected from exposure to direct sunlight, violent shaking was avoided. Only a gentle swirling was applied every 24 h. Mucilage collected at the bottom of the flasks.

5.2.6 Experiment 9 Isolation and purification of the extracellular polysaccharide

The culture of the diatoms was continued for twelve weeks; aliquots of the culture solution were withdrawn at intervals of weeks and their carbohydrate content was determined [GM 2.7.1]. After the tenth week the carbohydrate content of the culture solution remained constant. The contents of the flasks were combined and centrifuged for 1 h at a centrifugal force of 740 xg . The clear supernatant was dialysed against distilled water for 3 days. The first dialysis water was examined for carbohydrate. The contents of the dialysis sac were reduced to small volume, and freeze-dried (76 mg). Carbohydrate content of the freeze-dried material was determined [GM 2.7.1].

The crude polysaccharide thus obtained was purified by ethanol precipitation. In a typical experiment the freeze-dried material (30 mg) was dissolved in water (2 ml) and ethanol (10 ml) was added slowly with continuous stirring. The resulting precipitate was centrifuged off, redissolved in water and freeze-dried (20.6 mg). The remaining portion of the polysaccharide (46 mg) was purified by ethanol precipitation in the same way. Total recovery of (C) 50 mg.

5.2.7 Experiment 10 Composition of the purified polysaccharide

The carbohydrate content [GM 2.7.1] specific rotation, the sulphate [GM 2.7.3] and the uronic acid [GM 2.7.2 (a)] were determined. After hydrolysis [GM 2.3.1], the constituent sugars were characterised by paper chromatography in solvents [GM 2.4.1 (a and b) and locating reagents [GM 2.5.1, 2.5.2, 2.5.3]. An aliquot of the hydrolysate was reduced to alditols and then acetylated, the alditol acetate was then analysed by glc and glc-ms. The relative proportions of the individual sugars present in the hydrolysate were calculated from the peak area of the different sugars and by preparative paper chromatography.

5.2.8 Experiment 11. Fractionation of the polysaccharide on DE-52 cellulose column

An aliquot of the extracellular polysaccharide (5.6 mg carbohydrate) was dissolved in water (1 ml) and layered onto a DE-52 cellulose column [2.8.9]. The column was eluted sequentially with water, 0.3M-KCl, M-KCl and finally with M-NaOH.

5.2.9 Experiment 12. Composition of the different fractions

The carbohydrate contents [GM 2.7.1] of all the fractions were determined. The aqueous fraction was tested for sulphate and its specific rotation [GM 2.1] was also determined. After hydrolysis, the sugars in the 0.3 M-KCl and M-NaOH fractions were characterised by paper chromatography in solvents [GM 2.4.1 (a-c)] and locating reagents [GM 2.5.1, 2.5.2 and 2.5.3] and by glc of the alditol acetate derivatives [GM 2.6.1/2].

5.2.10 Experiment 13. Methylation of the extracellular polysaccharide

An aliquot (5.6 mg) of the extracellular polysaccharide was methylated by Hakomori method [GM 2.8.7a and the product was divided into two portions, one portion was methylated a second time.

5.2.11 Experiment 14. Composition of the partially methylated polysaccharides

The two methylated products were hydrolysed [GM 2.3.1] and the hydrolysates were examined by paper chromatography in solvent systems [GM 2.4.1, C] and locating reagent [GM 2.5.2]. The hydrolysates were then reduced [GM 2.8.4] and converted into alditol acetates [GM 2.8.6] and analysed by glc and glc-ms [GM 2.6.1-2.6.3].

5.2.12 Experiment 15. Periodate oxidation of mucilage (C) by Avigad method.

This mucilage (2.78 mg) was dissolved in 5 x 10^{-3} M-$NaIO_4$ (5 ml); the flask was wrapped in aluminium foil to ensure complete protection from light.

The violet solution of 2,4,6-tri-2-pyridyl-s-triazine (TPTZ) was prepared as follows. TPTZ (75 mg; 0.24 x 10^{-3} M) was dissolved in acetic acid (46 ml), and M-sodium acetate (210 ml) and a freshly prepared solution of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (31.4 mg; 0.08 x 10^{-3} M in 100 ml of water) was added. The volume was then made to 1 litre with water.

Assay of the periodate. In a standard procedure 0.01 ml of oxidation solution was taken in a test tube. The violet solution (4.5 ml) was added to it and made up to 5 ml with water. A blank containing 4.5 ml violet solution and 0.5 ml water to make up to 5 ml was made and a control with 0.01 ml periodate (5 x 10^{-3} M) violet solution (4.5 ml) and water to make up to 5 ml was also prepared. The absorbances of these solutions were read at intervals of time at 593 nm to determine the amount of residual violet solution. The measurement of absorbance was continued until a constant absorbance value for the oxidation solution was obtained.

The excess periodate of the oxidation solution was then destroyed by addition of ethylene glycol (200 µl). The oxidised mucilage was then reduced with sodium borohydride (200 mg) stirring for 3 h at room temperature and then left for 18 h at 2°C. The resulting polyalcohol was dialysed till free from inorganic ions, reduced to small volume and freeze-dried.

5.2.13 Experiment 16. Composition of the oxidised and reduced mucilage (C)

The polyalcohol from the mucilage C was hydrolysed with 90% formic acid [GM 2.3.1]. The hydrolysate was then examined by paper chromatography in solvent systems [GM 2.4.1, (a), (b)] and [GM 2.5.1, 2.5.2/3].

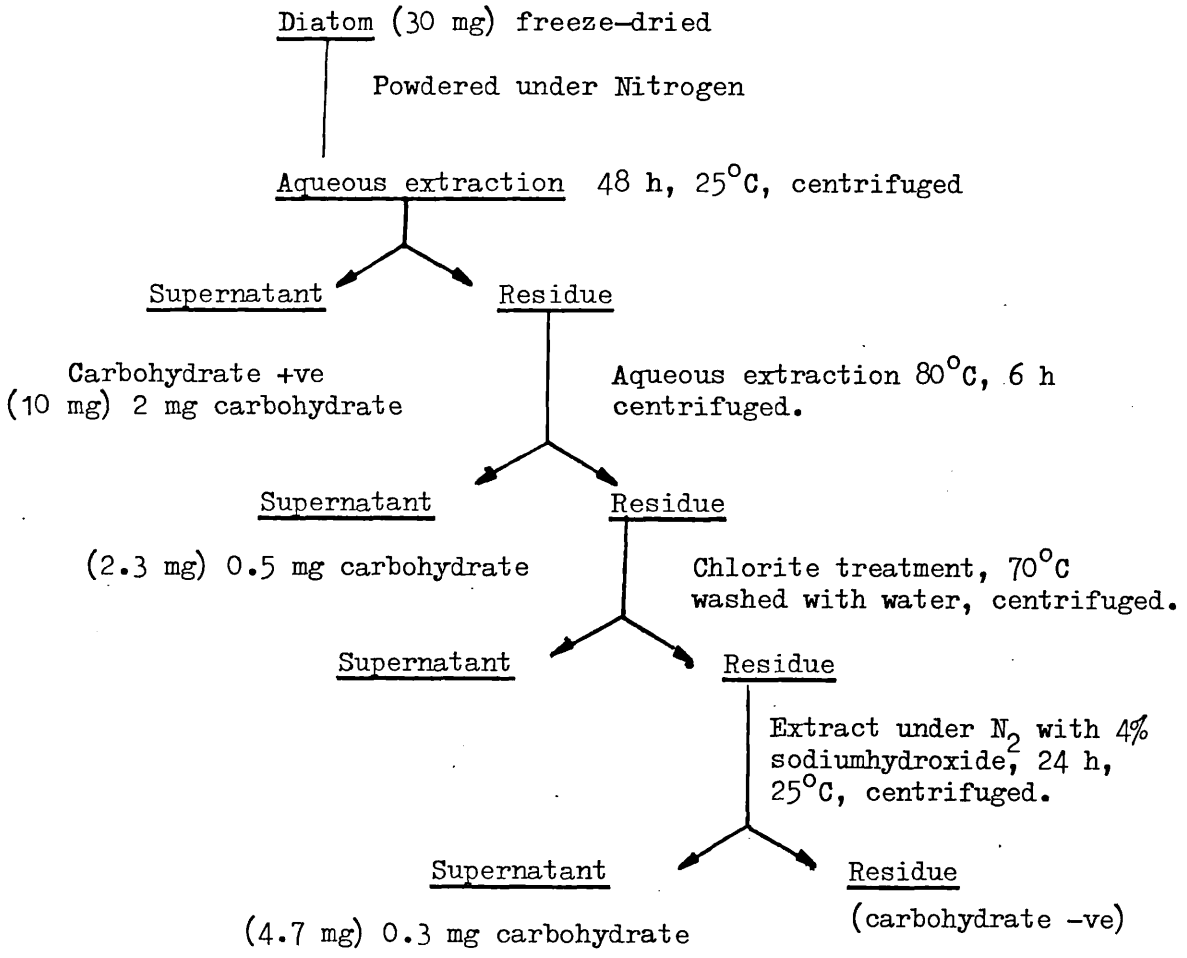
5.2.14 Experiment 17. Separation and characterisation of the slow moving components of (2 x Hakomori) methylated mucilage (C)

In order to separate the components of slower chromatographic mobility and to characterise them, the methylated material was placed on 3 MM paper and allowed to run on solvent system [GM 2.4.1(c)] for 60 h. Thereafter 4 fractions of slower mobility were cut out and eluted with water separately.

The four fractions after evaporation to dryness were dried further over conc. H_2SO_4 in vacuo. The dried samples were then esterified, reduced [GM 2.8.3, 2.8.4] and hydrolysed [GM 2.3.2]. The hydrolysates were neutralised with N-methyldioctylamine (5% in chloroform). Aliquots of the neutralised hydrolysates were examined by paper chromatography. The remaining hydrolysates were reduced and acetylated. The derived alditol acetates were examined by g.l.c. and g.l.c. - m.s.

5.2.15 SEQUENTIAL EXTRACTION OF DIATOM

Flow chart 5.2



5.2.16 Experiment 18 Extraction procedure

The washed powdered diatom (30 mg) was extracted with cold water with stirring for 48 h. After removal of water by centrifugation, the residual organism was extracted with water at 80° for 6 h. The residual material was given a chlorite treatment at 70° and then extracted with M-NaOH at room temperature under nitrogen for 24 h. The final residue was devoid of carbohydrate.

5.2.17 Experiment 19. Composition of the aqueous extracts of the diatom

Both the cold (2 mg carbohydrate) and hot (0.5 mg carbohydrate) aqueous extracts of the diatom were hydrolysed separately, and after neutralisation, the resulting hydrolysate was examined by paper chromatography, as described in experiment 12.

5.2.18 Experiment 20. Composition of the alkali extract

The alkali extract (0.3 mg carbohydrate) was hydrolysed as [GM 2.3.1] and the hydrolysate was examined by paper chromatography as in experiment 12 and by paper ionophoresis [GM 2.4.2] using buffer [GM 2.4.2 (a)].

5.3 RESULTS AND DISCUSSION

5.3.1 Preliminary investigation of the mucilage A, B and diatom D

The purpose of the preliminary investigation was to ascertain whether the mucilage (A) elaborated by the diatom cultured in the Plymouth laboratory, and the mucilage (B) obtained by extracting the clay material of the affected area of the sea, have similar composition and to find out whether the carbohydrate of the diatom (D) itself which had been freed from mucilage by repeated centrifugation has any resemblance with either of the other mucilages. The crude mucilage isolated from the clay material had a very low carbohydrate content

(see table). It was therefore, dialysed exhaustively against distilled water and after concentration to small volume the carbohydrate was precipitated by pouring on to 8 volumes of ethanol. The following table shows the yield, carbohydrate and protein content of both the crude and purified mucilage.

Table 5.2

Name of the material	Weight of the material in g.	Carbohydrate content %	Protein content %
Clay (wet)	200	-	-
Mucilage (crude)	0.906	12.4	7.7
Mucilage (purified) B	0.250	62.4	11.25

5.3.2 Constituents of the mucilages (A), (B) and diatom (D) and their Characterisation

Chromatography of the hydrolysates from (A) and (B) revealed identical components (P.C.), corresponding to fucose, rhamnose, mannose, xylose, glucose, galactose and glucuronic acid. The hydrolysate of the diatom contained fucose, xylose, glucose, galactose and glucuronic acid. The identity of the above monosaccharides as their alditol acetates was confirmed by their retention times on a gas liquid chromatograph and their e.i.-mass spectra. The presence of glucose in the hydrolysates was confirmed as the D-sugar by D-glucose oxidase. The uronic acid was confirmed as glucuronic acid by paper ionophoresis. The presence of amino acids in the hydrolysates was revealed by paper chromatography; the application of ninhydrin spray indicated the presence of similar amino acids in both the hydrolysates. On the basis of the above observations it was concluded that the extracellular mucilage of the diatom (A) and that extracted from the mud (B) have similar chemical composition.

As the constituent monosaccharides of the mucilage (B) showed many similarities with those found in the polysaccharide of the brown seaweeds, it was suspected that the mucilage obtained from the clay material might be contaminated with seaweeds. Keeping this fact in mind an attempt was made to remove all the visible foreign particles present in the clay. It was first extracted with 80% aqueous ethanol as outlined in flow chart 5.1

The residual solid was dried at room temperature and the foreign particles were removed by hand, these were mainly crab, scales of fish, metallic wires, etc. and included hardly any seaweeds. Subsequent sequential extraction of the dry material with water and dilute alkali and examination of these extracts after hydrolysis for constituent monosaccharides revealed the same chromatographic patterns to those observed previously. This observation confirmed the conclusion that the mucilage (B) was elaborated by the diatoms.

The dialysis water from the crude mucilage when tested for carbohydrate gave a positive response, it was therefore, reduced to small volume and after removal of inorganic ions was freeze-dried (7.2 mg). An aliquot of this solid was examined by paper chromatography which revealed the presence of glucose, along with a fast moving component. The fast moving component was not detected on the paper sprayed with aniline oxalate which indicates that this component is not due to a reducing sugar. The presence of D-glucose was confirmed by its identical mobility with authentic glucose and by glucose oxidase.

The residual clay material after aqueous extraction was extracted with 3% sodium carbonate as outlined in flow chart 5.1. After dialysis and freeze-drying an amorphous white solid (184.6 mg) was obtained from this extract. An aliquot of the extract after hydrolysis was investigated by paper chromatography which revealed the presence of four different monosaccharides, the mobility of which corresponded with galactose, mannose, xylose and fucose.

These interesting preliminary results on the mucilage (A) prompted a systematic investigation to study more completely the extracellular polysaccharides elaborated by the diatom. For this purpose the diatom was cultured in our laboratory. By monitoring the carbohydrate content of the culture solution, at intervals of weeks, it was observed that the production of mucilage continued for ten weeks, after which the carbohydrate content of the mucilage remained constant. The mucilage was then separated from the diatoms and freeze-dried. The freeze-dried material was then purified by ethanol precipitation as described in experiment 9. Yield of polysaccharide (C) 50 mg.

5.3.3 Composition of the purified extracellular polysaccharide (C)

The percentage contents of carbohydrate, uronic acid and sulphate in the polysaccharide are tabulated below.

Table 5.3

Specific rotation $[\alpha]_D^{25}$	-4.6° (C = 0.54)
Carbohydrate content	63%
Uronic acid content	9.3%
Sulphate content	16.7%

The negative rotation of the polysaccharide indicates a fairly high proportion of β-linkages.

Acid hydrolysis of the mucilage and analysis of the hydrolysate by paper chromatography and by gas liquid chromatography of the derived alditol acetates showed six different monosaccharides. Their relative molar proportionsas calculated from the peak areas of the glc are given in the table.

Table 5.4

Sugar	Approximate molar proportion
Fucose/rhamnose	3
Mannose	1.15
Glucose	1.0
Xylose	0.3
Galactose	trace

The relative proportion of the glucuronic acid in the polysaccharide could not be determined by the above method. As the solvent system pyridine : acetic acid : water in the proportion of 6:4:3 offers better separation by paper chromatography of the acidic sugars from the neutral ones, than that previously used an aliquot (5 mg) of the hydrolysate was separated on Whatman No.1 paper in the above solvent system, the uronic acid fraction and the combined neutral fractions were eluted separately. The amount of glucuronic acid was determined by comparison with a standard glucuronic acid graph and that of the combined neutral fraction was determined by comparing a standard graph made of an artificial mixture of fucose, mannose, xylose and glucose in the proportion of 3:1.15:0.3:1. The relative proportion of the uronic acid with respect to combined neutral fraction was 1:10 which is in reasonable agreement with the 9.3% determined by the carbazole method.

As the retention time of the alditol acetates of fucose and rhamnose were the same, relative proportions of these sugars could not be obtained by measuring the peak area of glc chromatogram. In order to determine the relative proportion of the fucose and rhamnose in the polysaccharide, an aliquot (5 mg) of the hydrolysate was separated on

Whatman No. 1 paper in the solvent system ethyl acetate : acetic acid : formic acid : water in the proportion of (18:3:1:4) as this solvent system separates these two deoxy-sugars. The proportion of fucose : rhamnose was found to be 2.3 : 1.0. The relative proportion of the individual sugars as obtained by the different methods is given in the table.

Table 5.5

Sugar	Approximate molar proportion
Fucose	2.3
Mannose	1.15
Rhamnose	1.0
Glucose	1.0
Glucuronic acid	0.7
Xylose	0.3
Galactose	trace

Although the relative proportions are different, a similar apart from xylose, mixture of monosaccharides was reported for a hydrolysate of whole cells of *Coscinodiscus sp* by Parson et al.[165] Whereas *Phaeodactylum tricornutum*[169] was devoid of fucose, it contained the other sugars present in the *Coscinodiscus nobilis* extracellular polysaccharide.

Differences are the absence of fucose from the mucilage from *Nitzschia frustulum*[170] and the absence of mannose from the *Chaetoceras sp.*[171,172,173]

5.3.4 Fractionation of (C) by DE-52 Cellulose column

The recoveries after fractionating an aliquot (5.6 mg) of (C) on the cellulose column (experiment 8) is shown in the following table.

Table 5.6

Fraction	Recovery of carbohydrate	
	weight (mg)	%
Water	2	35.7
0.3 M-KCl	2.5	44.6
M-KCl	Nil	Nil
M-NaOH	0.8	14.3

The overall recovery of carbohydrates (5.3 mg) from the column was about 94%. The different fractions were then hydrolysed and examined for constituent saccharides (P.C.). The aqueous fraction was found to contain mainly glucose, and the 0.3 M-Potassium chlroide fraction indicated the presence of all the constituent sugars of the parent polysaccharide except glucose (confirmed by negative response to glucose oxidase). The M-sodium hydroxide fraction revealed the presence of two sugars, corresponding to the paper chromatographic mobility of mannose and fucose. Due to shortage of material further structural investigations of these fractions were not carried out.

5.3.5 Characterisation of the glucose (aqueous fraction)

The aqueous eluant from the column had $[\alpha]_D$ -7.5° (C = 0.2) was devoid of sulphate and gave mainly glucose on hydrolysis. Its

structure as a (1→3)-linked glucan (Chrysolaminaran) was shown by the characterisation of 2,4,6 tri-O-methyl-glucose as a major constituent of the hydrolysis products from methylation of the whole mucilage C. (Tables 5.8 and 5.9A) small degree of branching at C-6 was indicated by the presence of a trace of 2,4-di-O-methyl glucose in the hydrolysate of the methylated polysaccharide (Tables 5.8 and 5.9). The presence of a fairly high proportion of tetra-O-methyl glucose in this hydrolysate indicates a relatively small molecule in keeping with the Chrysolaminaran found in other diatoms. The structure of the glucan was confirmed by periodate oxidation studies on the mucilage (Table 5.7). The major constituent of the hydrolysate of the polyalcohol after periodate oxidation and reduction of the mucilage was D-glucose (+ve glucose oxidase), confirming the (1→3)-linkage of this sugar. The low negative specific rotation is evidence of β-linkage.

It follows that Coscinodiscus nobilis metabolises a chrysolaminaran similar to that characterised from other diatoms.

Table 5.7

Constituent saccharides identified in the hydrolysates of mucilage (C) before and after periodate oxidation.

Constituent saccharides	Hydrolysates of mucilage (C)	Hydrolysates of mucilage (C) after periodate oxidation and reduction.
Fucose	+++++	++
Mannose	+++	++
Rhamnose	+++	++
Glucose	+++	+++++
Oligouronic acid	++	+
Xylose	++	-
Galactose	+	-

+++++ major component

++ minor component.

It should however be noted that this was not extracted from the diatom as in other species but separated from the extracellular mucilage. It could possibly have been derived from dead diatoms present in the culture. In fact the presence of a glucan in the aqueous extracts of the diatom itself was also observed (p.221) although detailed structural studies were not carried out due to small amounts of material isolated.

5.3.6 Methylation studies of the mucilage (C)

Due to insufficient material it was not possible to methylate the separate fractions from the column. Instead a portion of the whole mucilage was methylated by the Hakomori method. The methylated product was divided and one portion subjected to a second Hakomori methylation.

5.3.7 Investigation of the sulphated uronic acid containing polysaccharide(s) mucilage (C).

These contain mannose, rhamnose, fucose, xylose and galactose in the molar ratios of approximately 1.2:1.3:2.5:0.5:trace [although a proportion of the fucose and mannose appears to be in a separate polysaccharide (alkali eluant from the column)] together with about 10% glucuronic acid and approximately 20% of half ester sulphate ($O\text{-}SO_3.H$). A hydrolysate, after a single Hakomori methylation contained a large proportion of unmethylated saccharides although fully methylated saccharides were also present. A second Hakomori methylation resulted, after hydrolysis, in the methylated sugars detailed in Tables 5.8 and 5.9 and glc chromatogram, fig. 5.1.

Table 5.8

Methylated sugars tentatively identified by paper chromatography[168] in the hydrolysate after 2 Hakomori methylation of mucilage(C).

RTMG[a]	Possible Sugars
0 - 0.04	Oligouronic acid.
0.11	Aldotriouronic acid.
0.24	Aldobiouronic acid.
0.41	4-O-methyl fucose.
0.53	2,4-di-O-methyl fucose
0.58	3,4-di-O-methyl mannose.
0.61	2-O-methyl rhamnose/fucose
0.67	2,4-di-O-methyl glucose.
0.71	2,3-di-O-methyl xylose.
0.76	2,3,6-tri-O-methyl mannose.
0.78	2,4,6-tri-O-methyl glucose.
0.88	3,4-di-O-methyl rhamnose
0.93	2,3,4-tri-O-methyl xylose.
1.0	2,3,4,6-tetra-O-methyl glucose.

[a] RTMG is the mobility of sugars relative to that of 2,3,4,6-tetra-O-methyl glucose.

Table 5.9

Methylated sugars identified in the hydrolysate after 2 Hakomori methylations of mucilage (C).

Retention time[a]	Peak No.	Possible Sugars	Confirmed by ms
0.47	1	2,3,4-tri-O-methyl rhamnose (m)	
0.55	2	2,3,4-tri-O-methyl xylose (m)	
0.61	3	2,3,4-tri-O-methyl fucose (vl)	
0.88	4	3,4-di-O-methyl rhamnose (m)	
1.02	5	2,4-di-O-methyl fucose (vl)	
1.16	6	2,3,4,6-tetra-O-methyl glucose (l) 2,3,4,6-tetra-O-methyl mannose 2,3-di-O-methyl xylose	
1.27	7	2-O-methyl rhamnose (m)	
1.41	8	2-O-methyl fucose (m)	
1.59	9	4-O-methyl rhamnose (s)	
1.71	10	3-O-methyl fucose/rhamnose (l)	
1.81	11	2,4,6-tri-O-methyl glucose (l)	
1.98	12	2,4,6-tri-O-methyl mannose (s)	
2.16	13	2,3,6-tri-O-methyl mannose (s)	
2.25	14	2,3,4-tri-O-methyl mannose (l)	
2.55	15	2,3,6-tri-O-methyl glucose (s)	
3.88	16	3,6-di-O-methyl glucose/mannose (s)	
4.3	17	3,4-di-O-methyl mannose 2,4-di-O-methyl glucose } (s)	
4.79	18	2,4-di-O-methyl mannose (s)	

Size of peaks - vl = very large; l = large; m = medium; s = small.

[a] Retention time relative to that of 1,5-di-O-acetyl 2,3,4,6-tetra-O-methyl glucitol.

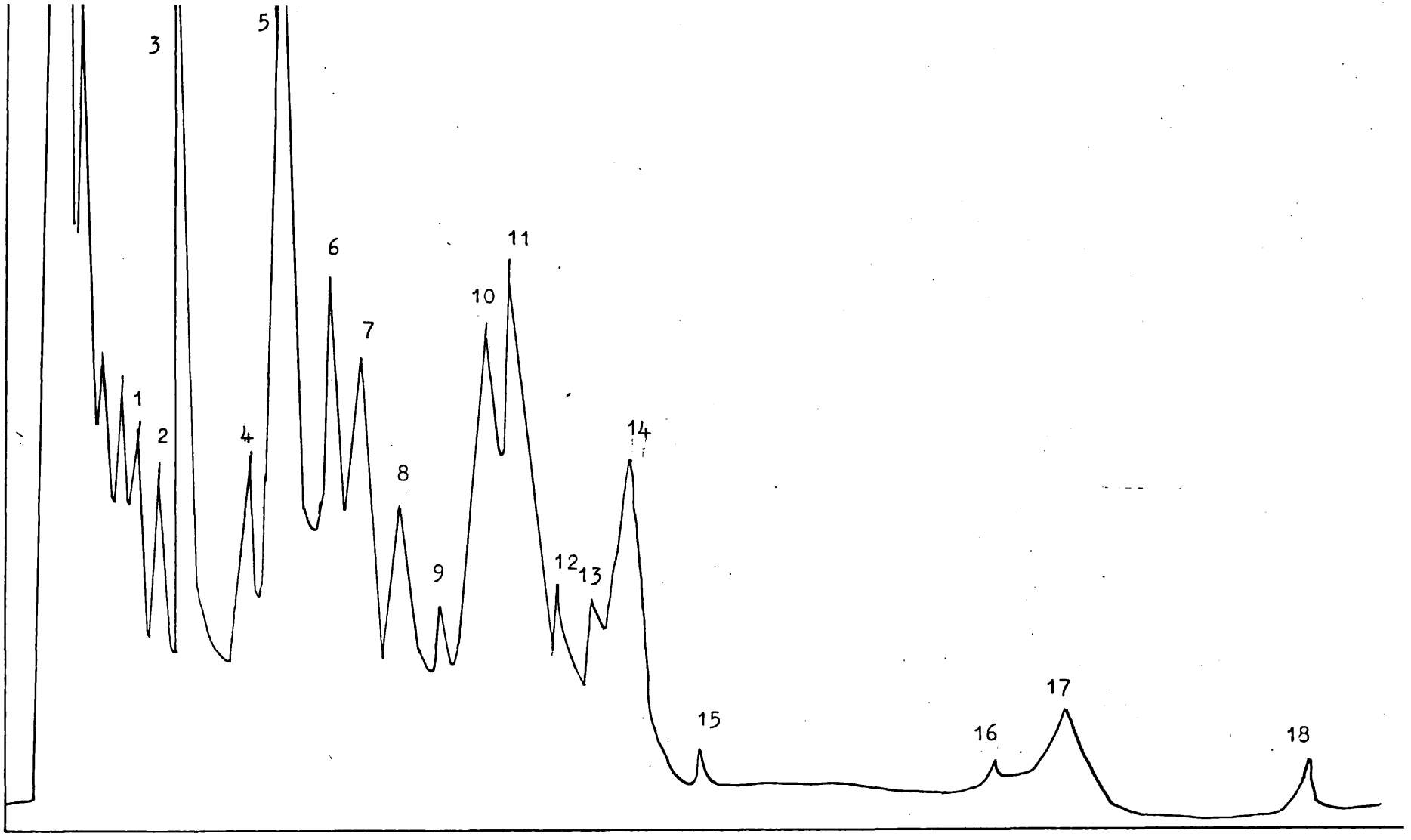


Figure 5.1 Gas liquid chromatogram of the hydrolysate of twice methylated mucilage (C) after conversion into alditol acelate derivatives. Retention time relative to that of 1,5 di-O-acetyl 2,3,4,6-tetra-O-methyl glucitol.

The methylated glucose derivatives have already been discussed under the glucan.

While the high proportion of fully methylated sugars probably result to a certain extent from degradation during the second Hakomori methylation - their presence after a single methylation indicates a high degree of branching.

The presence of (1→3)-linked fucose is indicated from these results. Both fucose and rhamnose are also present as branch points or carrying half ester sulphate ($O-SO_3H$). Evidence for mannose linked through C-1 and C-6 as well as through C-1 and C-4 and C-1 and C-3 as well as a little doubly linked or sulphated mannose was also obtained. The major methylated xylose derivative was the 2,3,4-trimethylether or end group xylose although some (1→4)-linked xylose was also indicated. In addition slow moving spots (Table 5.8) were detected indicating uronic acid containing oligosaccharides.

5.3.8 Characterisation of oligouronic acid

It appears that any uronic acid which had not been degraded during the methylation is incompletely hydrolysed and present as oligouronic acid (Table 5.8). These were separated into 4 fractions from a paper chromatogram. Each fraction was separately esterified, reduced and hydrolysed. Paper chromatography revealed the similarity of the four fractions; each contained free glucose (derived from glucuronic acid) xylose, fucose, rhamnose and traces of methylated monosaccharides. Each fraction was analysed by g.l.c. and g.l.c.-m.s. after conversion into alditol acetates. (Table 5.10). The characterisation of free fucose/rhamnose, xylose and glucose indicates that these oligouronic acids were either highly branched or that incomplete methylation had occurred.

It can, however, be concluded from these results that these monosaccharide units are mutually linked and present in a single polysaccharide. The complete absence of mannose in these oligouronic acids suggests that it is present in a separate polysaccharide as indicated by the fractionation studies (p213), at the same time its presence on the periphery of the complex heteropolysaccharide cannot be ruled out.

Table 5.10

The monosaccharide components identified from each fraction from their relative retention time with 2,3,4,6-tetra-O-methyl glucitol 1,5 diacetate (R_{TMG}) by g.l.c. and g.l.c.-m.s.

R_{TMG}	Monosaccharides identified	Fractions			
		1(0.0)	2(0.5)	3(0.11)	4(0.24)
2.21	Fucose/rhamnose	✓	✓	✓	✓
2.83	Xylose	✓	✓	✓	✓
3.3	2,3 di-O-methyl fucose	–	–	✓	–
4.8	2,3 di-O-methyl glucose	✓	–	–	–
6.33	2-O-methyl glucose	✓	✓	✓	–
7.08	Glucose	✓	✓	✓	✓

Figures in the parenthesis indicates the paper chromatographic mobility w.r.t. 2,3,4,6-tetra-O-methyl glucose.

5.3.9 Periodate oxidation studies

Because of the very small amount of material available it was decided to periodate oxidise the polysaccharide by the micro method devised by Avigad (expt. 15). After 48 h the oxidation was complete and the polysaccharide was reduced to the alcohol. This was hydrolysed and examined by p.c. (Table 5.7). Glucose was the major monosaccharide component but smaller proportion of mannose, fucose and rhamnose were also detected in the hydrolysate. The proportion of these monosaccharides relative to that of glucose was considerably smaller than in the original

mucilage, thereby confirming the methylation results that some of these residues are vulnerable to periodate. Some oligouronic acids were also detected in the hydrolysate of the polyalcohol.

Had sufficient material been available removal of the glucan from the sulphated material would have been carried out. After a single methylation the glucuronic esters would then have been reduced and after a second methylation the derived glucose derivatives characterised.

Unfortunately this work had to be terminated since the "bloom" on the sea vanished and neither Dr Boalch nor ourselves were able to reculture the diatom.

5.3.10 Carbohydrate constituents of the diatom

Sequential extraction of the powdered diatom (30 mg) with cold and hot water and with alkali resulted in the isolation of three polysaccharide materials. The total recovery of carbohydrate from the diatom was approximately 2.8 mg (ca. 9%) cf. Parsons[165] who found that Coscinodiscus species synthesised about 4% carbohydrate. The composition of the cold and hot aqueous extracts appeared to be similar by paper chromatographic examination of the hydrolysate. Both aqueous extracts consist of three sugars which had the mobility of glucose, mannose and fucose. The presence of glucose in the aqueous extracts indicates the presence of chrysolaminaran the common glucan of diatoms. Mannose and fucose were also reported[165] to be constituents of the polysaccharide of Coscinodiscus species.

The alkaline extract of the diatom was found to contain mannose and uronic acid, the sulphate content was not determined due to insufficient material. The presence of a sulphated glucuronosylmannan in a similar extract from Phaeodactylum tricornutum[175] (p.193) shows a possible similarity between these two genera of diatoms. Lack of material prevented further study of this diatom.

5.4 Conclusion

It can be concluded from these experiments that three separate polysaccharides were present in the extracellular mucilage isolated from *C. nobilis*. The major polysaccharide comprised a highly branched acidic heteropolymer containing fucose, rhamnose, mannose, xylose glucuronic acid and half ester sulphate. A small amount of chrysolaminaran, a β-1,3-linked glucan, which is the common food reserve material of diatoms, was also present. It is thought that this might have arisen from dead cells in the culture. Evidence was also obtained that at least a proportion of the mannose occurred in a separate polysaccharide although this did not appear to be related to the glucuronosylmannan present in *Phaeodactylum tricornutum*.

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CARBOHYDRATES OF THE SEAWEEDS, *DESMARESTIA LIGULATA* AND *D. FIRMA**

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Key Word Index—*Desmarestia ligulata*; *D. firma*; Desmarestiaceae; seaweeds; carbohydrates; low molecular weight carbohydrates.

Abstract—Crystalline mannitol and some oligosaccharides were separated from ethanolic extracts of *Desmarestia ligulata* and *D. firma*. Laminaran, 'fucans' and alginic acid were also isolated from both species. The laminaran from *D. ligulata* comprised both M- and G-chains but no M-chains were found in the laminaran from *D. firma*. In both species the amount of 'fucan' was small, particularly in *D. firma*. Both 'fucans' contained glucuronic acid, galactose, xylose and fucose and that from *D. ligulata* also contained mannose. After sequential extraction of *D. ligulata* with water, acid and alkali evidence was obtained for the presence of cellulose, a uronan, and protein in the residual material.

INTRODUCTION

Both *Desmarestia ligulata* and *D. firma*, members of the Desmarestiaceae, grow below low water tide mark and can only be collected by divers. *D. ligulata* has a branched thallus whereas in *D. firma* the thallus is largely unbranched. The two species may also be distinguished by the delicate papery texture of *D. firma* at the end of the growing season, in contrast with the much coarser cartilaginous texture of *D. ligulata* [2]. Both species contain free sulphuric acid, thought to be present in the vacuolar sap [3], which causes the weeds to break down rapidly on exposure to air. Neither species has been previously investigated chemically, partly because of the difficulty of collection, and also because of the need to neutralise or destroy the sulphuric acid immediately on removal of the weed from seawater. It seemed desirable therefore to repeat experiments on *Desmarestia ligulata* and *D. firma* which have been carried out on *D. aculeata* [1] a species which is devoid of sulphuric acid. Comparison of the results should demonstrate any differences in the carbohydrates among the different species of *Desmarestia* and reveal any effect that free sulphuric acid might have on these metabolites.

RESULTS AND DISCUSSION

Even in the presence of solid barium carbonate the ethanolic extracts of *Desmarestia ligulata* (A) were very acid. It was found subsequently that this difficulty could best be overcome by using 80% ethanol containing 1% triethanolamine which had a pH 10, and which remained at this pH after the addition of fresh weed of A. Removal of the weed into water gave a neutral solution indicating that the amine had removed all the free sulphuric acid. This method was also used for the extraction of freeze-dried *Desmarestia firma* (B) weed. Gravimetric determination of the sulphate in the ethanol gave 5.8% of free sulphuric acid in the dry weight of this weed.

Crystalline mannitol, 3.4% of the dry wt of A and 5.4% of B, was separated from the ethanolic extracts of both species. The residual syrups (*ca* 5 mg carbohydrate from A) was shown by PC and GLC to contain xylose, mannose and galactose together with some oligosaccharides. The last were combined and after hydrolysis gave galactose (major) xylose, mannose and myoinositol. The residual syrup from B. contained D-glucose, monouronic acid and its lactone (with the same PC and ionophoretic [4] mobilities as mannuronic acid and mannuronolactone) and oligosaccharides consisting of mannitol, xylose (major), glucose and galactose (trace). Although both 1-*O*-D-mannitol-β-D-glucopyranoside and 1,6-*O*-D-mannitol di-(β-D-glucopyranoside) have been found in a number of brown seaweeds [5] the above oligosaccharides appear to be unique to the present algae. They are possibly either precursors of 'fucans' or fragments hydrolysed by sulphuric acid from these saccharides during extraction. Sprays specific for glucose, ketoses and heptuloses gave negative results (except for glucose in B) indicating the absence of these carbohydrates in the ethanolic extracts. This is in contrast to the extract from *D. aculeata* [1] where sucrose, fructose and sedoheptulose were all present.

The aqueous extract from A (2.7% of the dry wt) had a carbohydrate content of 76% (glucose graph) and contained glucose, galactose, mannose, fucose and xylose. Glucose and galactose were confirmed as the D-sugars by the appropriate oxidase spray. L-Fucose, D-xylose and D-galactose are normally found in 'fucans' from brown seaweeds, but as mannose is rare it was separated from a hydrolysate and characterised as D-mannose by its mobility on ionophoresis in borate buffer and as the crystalline phenylhydrazone with mp and mmp with authentic material of 188°. The presence of glucuronic and mannuronic acids in the hydrolysate of

* Part IV in a series 'Carbohydrates of the Brown Seaweeds'. For Part III see Ref. [1].

Table 1 Fractionation of the aqueous extract from *Desmarestia ligulata*

Fraction	Recovery %	Carbohydrate %	Uronic acid content %	Sulphate content %
Aqueous	2.5	85*		
0.1 M KCl	12.5	83†	14.8	3
0.2 M KCl	41	70†	22.5	3
0.3 M KCl	18	87†	17.0	7.5
0.5 M KCl	11	71‡	3.6	20
	85	90		

* Compared to a glucose graph.

† Compared to a graph based on the carbohydrate composition of the 0.2 M KCl fraction.

‡ Compared to a graph based on the carbohydrate composition of the 0.5 M KCl fraction.

this extract was confirmed by PC and ionophoretic mobility [4]. After fractionation of the aqueous extract on DE- cellulose five fractions were separated (Table 1). The aqueous fraction, after hydrolysis, contained glucose with a trace of a non-reducing substance with the mobility of mannitol indicating the presence of laminaran with M- and G-chains (i.e. chains terminating in mannitol and glucose respectively). Treatment of a hydrolysate with glucose oxidase converted the glucose into gluconic acid allowing the detection of mannitol by PC to be more definitive. Similar treatment of a synthetic mixture of glucose and mannitol in the ratio of 25:1 gave an identical chromatogram to that from the aqueous fraction, confirming the presence of M-chains in the laminaran from A. The total yield of laminaran was 0.08% of the dry wt of the weed. Similarly the aqueous fraction from the separated aqueous extract of B (Table 2) contained mainly glucose with traces of mannuronic acid and appeared to be devoid of mannitol. To confirm the absence of mannitol a sample was analysed by elution from a DEAE-cellulose-molybdate column [6]; no fractionation occurred. The presence of M-chains is not invariable although the laminaran obtained from *D. aculeata* also contained M-chains while that from *Bifurcaria bifurcata, Himanthalia lorea* and *Padina pavonia* [7] did not. All four KCl fractions from the aqueous extract of A contained fucose, galactose, mannose, xylose and glucuronic acid. The ratios of these sugars in the 0.2 M KCl fraction was 15:1.5:0.4:1:11,

Table 2. Fractionation of the aqueous extract from *Desmarestia firma*

Fraction	Recovery (mg)	Carbohydrate content (%)*	Uronic acid content (%)	Sulphate content (%)
Aqueous	11	83	—	—
0.3 M KCl	466	53	17†	1
0.5 M KCl	22	78	7‡	8
1.0 M KCl	—			
	499	90		

* Glucose graph; † Mannuronolactone graph; ‡ Glucuronic acid graph.

and in the 0.5 M KCl fraction 9.7:2.7:1.4:1:trace respectively. These proportions are in agreement with the uronic acid and sulphate contents (Table 1) since in all previous studies of 'fucans' the higher proportions of sulphate are always accompanied by a higher proportion of fucose and a lower proportion of glucuronic acid.

The aqueous extract from B isolated as a white solid in 3.2% yield of the dry wt of the weed contained glucose (major), galactose, fucose, xylose, glucuronic and mannuronic acids but was devoid of mannose. The results of fractionation on DE-cellulose are given in Table 2. The 0.3 M KCl fraction contained glucose (major), fucose, galactose, xylose, glucuronic and mannuronic acids. Quantitative GLC analysis of this hydrolysate revealed that at least half of it was glucose. This makes the total laminaran content of the aqueous extract about 1.8% of the dry wt. The 0.5 M KCl fraction contained fucose (major), galactose, xylose and glucuronic acid.

The acid extract from A isolated in 2.7% yield of the dry wt, had a carbohydrate content of 72% (read off a graph made from a soln with the composition of the 0.2 M KCl fraction of the aqueous extract). A hydrolysate contained fucose, galactose, xylose, mannose and glucuronic acid together with a small amount of mannuronic acid. Fractionation of an aliquot of the hydrolysate on a cellulose column resulted in the same elution pattern as obtained from the aqueous extract, except that the water eluate contained no carbohydrate.

The acid extract from B (1.4% of the dry wt) consisted of mainly mannuronic acid together with traces of glucose, fucose, galactose and xylose.

The alkaline extracts of the residual material from A contained 13.9% and from B 16% of alginic acid. Ionophoresis [4] of hydrolysates confirmed the presence of guluronic acid and mannuronic acid. The viscosities of 1% aqueous solns of the derived sodium alginates at 25° from A and B were 1.7 and 1.5 cps respectively. These very low values indicate that the alginic acid has been much degraded. An estimation of the mannuronic to guluronic acid in the sodium alginate of A gave an M/G ratio of 0.06. The high guluronic acid content supports this degradation since mannuronic acid residues are more readily hydrolysed by acid. Degradation is also confirmed by earlier findings in similar extracts. The so-called 'fucan' separated from the alkaline extract of B was mainly degraded alginic acid.

In an attempt to avoid degradation the alginic acid was extracted from the algae directly after neutralisation of the free sulphuric acid. Two samples of alginate were separated from each weed; those from A had viscosities of 300 (0·1% NaOH extract) and 150 cps (Na_2CO_3 extract) and from B 56 and 68 cps respectively. The M/G ratios [13] of the former extracts were 0.47 and 0.40 indicating a guluronic acid content of 67 and 71%. While it is recognised that the method overestimates the guluronic acid content it is still considered that a certain amount of degradation of the alginic acid has occurred. Nevertheless these results indicate a guluronic acid content of more than 50% for the alginates from both weeds. The loss of mannuronic acid is supported by the presence of this acid and low molecular weight alginic acid in the 'fucans' separated from the direct alkaline extracts.

The residue remaining after the sequential extraction of A contained 27% protein (based on a N_2 content of 4.4%) and gave a red/brown colour with Herzberg's

Table 3. Approximate percentages of the dry weight of the polysaccharides isolated from three species of *Desmarestia*

Species	Laminaran	'Fucan'	Alginic acid
D. aculeata			
August col.	5.6	14	12
March col.	1.8	9	16
D. ligulata	0.08	6	19 (16)
D. firma	1.8	1.9*	23 (17)

Figures in parentheses are the yields from the sequential extractions.

* Derived from aqueous and acid extracts only.

stain indicating the presence of cellulose, confirmed by the presence of D-glucose in a hydrolysate. Uronic acid was also detected in the hydrolysate. Quantitative determination of the 25° hydrolysate gave 11% and of the 100° hydrolysate 1.3% uronic acid.

The approximate percentage yields of the different carbohydrates based on the dry wt of three species of *Desmarestia* are given in Table 3. The most striking feature of these results is the low yield of 'fucans' particularly from B. This low yield cannot be explained by the hydrolytic effect of the sulphuric acid after death of the alga since if this were so fragments of these 'fucans' would have been found in the ethanolic extracts. It can only be concluded that A, B and *D. aculeata* synthesise less 'fucan' than most other brown seaweeds [7]. However, *Durvillia* species [9] also appear to be virtually devoid of 'fucans'.

The presence of mannose in the 'fucan' from A is in striking contrast to the 'fucan' of other species of *Desmarestia*, although it has been reported in the 'fucans' of *Sargassum linefolum* [10] *S. pallidum* [11] and *Pelvetia wrightii* [12].

It must be stressed that the alginate content in the growing weed of the species under investigation is probably considerably higher than the estimated value (Table 3), since the results clearly show that some degradation does occur during storage and extraction.

Finally it can be concluded that the presence of sulphuric acid in the two species of *Desmarestia* has no effect on their carbohydrate metabolism.

EXPERIMENTAL

Algal material. Desmarestia ligulata (A) was collected on 17 July 1973 from deep water at Port Erin, Isle of Man, and immediately plunged into boiling EtOH containing solid $BaCO_3$.

Desmarestia firma (B) was collected from False Bay, Cape Town, South Africa from 10 m depth on 27th March 1975, and was freeze-dried immediately after removal from seawater.

General methods. Details of analytical methods are given in Parts 1 [7] and III [1].

Isolation of carbohydrates. Samples of both weeds were sequentially extracted as detailed in Part I [7] except that the $C_2O_2(NH_4)_2$ extraction and chlorite treatments were omitted and alginic acid was extracted directly from the dried weed with alkali.

Ethanol extract. All the EtOH extracts of A were combined. The residual weed was air-dried and weighed (dry wt 55.7 g). During the EtOH and H_2O extractions of A, solid $Ba(CO_3)_2$ was added in an attempt to keep the pH between 5 and 7. Freeze-dried B (50 g) was extracted with 80% EtOH (500 ml) containing 4% triethylamine at 25% pH 7.0, acidified with M HNO_3 and the sulphate ppted with $BaCl_2$.

The combined EtOH extracts from each weed were treated as in a previous publication [1]. Crystalline mannitol (1.9 g from A and 2.7 g from B) was separated and the residual syrups analysed by PC solvents (1) and (3), and (A) (E) (F) [7] and galactose oxidase, heptose and ketose sprays [1], and by GLC of the sugar and alditol TMSi derivatives on column 4. Spots with $R_{mannitol}$ less than 1.0 were eluted from PC, combined and the mixture hydrolysed. The hydrolysates were analysed by PC and GLC as above.

Aqueous extract. The combined extracts, after dialysis, were freeze-dried to a white powder (1.62 g from A, and 1.60 g from B). An aliquot of each was hydrolysed and the hydrolysate examined by PC and GLC as for ethanolic extracts and by ionophoresis at pH 6.7 and 10.

An aliquot of the extract from A (100 mg) was hydrolysed and the sugars separated on 3 MM chromatography paper in solvent (3). The 'mannose' fraction was eluted and mixed with phenylhydrazine. The derived phenylhydrazone was recrystallised from EtOH.

Fractionation of the aqueous extracts. Aliquots of the white powders (430 mg from A and 480 mg from B) were fractionated on DE-cellulose columns [7]. The carbohydrate, sulphate and uronic acid contents were determined for each fraction, and after hydrolysis each fraction was analysed by PC and GLC as before. Hydrolysates of the 0.2 M KCl and 0.5 M KCl fractions from A were separated on 3 MM paper in solvents (3) and (1), eluted and the relative proportions of the sugars measured.

The acidic extracts. After appropriate treatment [7] the combined extracts from each weed were freeze-dried to white powders (1.5 g from A and 0.7 g from B), analysed and the A extract fractionated as for aqueous extracts above.

Alkaline extracts. (1) The residual solids remaining after the acid extractions were exhaustively extracted with 3% Na_2CO_3 soln. The derived Na alginates were treated with a 2% soln of $CaCl_2$ [7]. From this Ca alginate (9.3 g from 55.7 g A and 8.6 g from 50 g B and 'fucans' 345 mg A and 960 mg B) were separated. (2) The dry powdered alga (5.0 g A and 5.4 B) was suspended in a saturated soln of $CaCO_3$ (150 ml) with stirring and pH maintained at 5–7 by the addition of solid $CaCO_3$. After dialysis (3 days) the supernatant was freeze-dried to a white solid (20 mg from A and 22 mg from B). A soln of 1.8% formalin (2 ml) was added to each residual alga, the mixture stirred and stood for 30 min. The supernatants were decanted off solid Na_2CO_3 (1.5 g) and 0.1% NaOH soln (1.5 ml) added to the residual weeds, and the mixtures kept at 60° for 2 hr with stirring. After dilution the residues were filtered and the solns neutralised (pH 6.5) with dilute HCl and diluted to 800 ml. A 2% $CaCl_2$ soln (100 ml) was added to each mixture with stirring and the ppt gelatinous Ca alginates were removed by centrifugation, washed with dilute $CaCl_2$ and suspended in H_2O and freeze-dried to a brownish powder of Ca alginate (692 mg from A and 1.22 g from B). The supernatants from the centrifugations were dialysed until the dialysate was free from Ca^{++}, and the solns freeze-dried to white solids ('fucans', 173 mg from A and 30 mg from B). The residues were extracted with 3% Na_2CO_3 solns (150 ml each) at 70° for 3 successive 3 hr periods. The combined solns were treated as before and gave Ca alginates (223 mg from A and 90 mg from B) and 'fucans' (45 mg from A and negligible amount from B). (3) A second portion of B (10.9 g) was exhaustively extracted with 80% EtOH containing triethylamine to maintain pH 7–8, and treated *ca* 18 hr with formaldehyde. The recovered material was then subjected to alkaline extraction as above. From the 0.1% NaOH extraction 2.15 g of Ca alginate and 119 mg of 'fucan' were separated. From the subsequent Na_2CO_3 extract 823 mg of Ca alginate and 302 mg of 'fucan' were separated.

Conversion of calcium alginate to sodium alginate. The respective Ca alginates were converted to Na alginates by

leaching with acid. The derived alginic acids were suspended in H_2O with vigorous stirring and titrated with 0.1 M NaOH to pH = 7 so that all the alginic acid dissolved. The solns were dialysed for 2 days and freeze-dried.

Estimation of the guluronic to mannuronic acid ratio. The Na alginates were subjected to sequential partial hydrolysis [13]. The guluronic acid contents of the supernatants and the residues (the latter converted into soluble Na alginate) were determined by the carbazole method measuring the chromophore produced in H_2SO_4 with and without added borate at 55° [14]. Standard carbazole graphs were prepared for polyguluronic acid and mannuronolactone.

Examination of the residual solid. The solid remaining after the sequential extraction of the A [7] was tested for cellulose by Herzberg's stain. Aliquots of the residue (2 × 50 mg) were hydrolysed with 72% H_2SO_4 (5 ml each) at 25° and at 100° for 1 hr. The uronic acid contents of the hydrolysates were determined and the hydrolysates were investigated by PC and ionophoresis.

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