INVESTIGATION OF THE POLYSACCHARIDES SYNTHESISED BY THREE GENERA OF THE PHAEOPHYCEAE.

By

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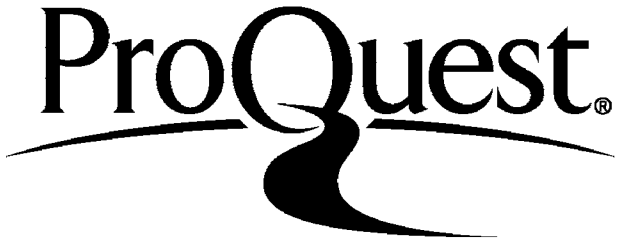

To

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ABSTRACT

1. The three brown seaweeds (*Himanthalia lorea*, *Bifurcaria bifurcata* and *Padina pavonia*) of widely different morphology are each shown to synthesise D-mannitol.

2. Aqueous or dilute acid extraction after 80% ethanol extraction led to the isolation of complex mixtures of laminaran, "fucans" and alginic acid. The separation of these polysaccharides proved impossible.

3. It was found that a 2% aqueous calcium chloride extraction before acid extraction renders the alginic acid completely insoluble and a very small proportion of the "fucans" contaminated the laminaran extracted into the aqueous solution. Subsequent extraction of the weed with dilute acid gave "fucans" virtually free from alginic acid. Further extraction of the residual material with dilute alkali gave a mixture of alginic acid and glucuronoxylofucans. Sequential extractions of the residual material with ammonium oxalate-oxalic acid and acid chlorite gave additional quantities of crude glucuronoxylofucans and the final residual algal material. The same polysaccharides in different proportions were isolated from each of the weeds.

4. Pure laminaran was obtained from each of the three weeds after fractionation of the aqueous calcium chloride extract. This was characterised as a brown algal-type glucan comprising essentially 1,3- and 1,6-linked glucose residues, but devoid of mannitol terminated chains.

5. The final residual algal material from H. lorea was characterised as a β-1,4-linked glucan admixed with protein.

6. "Fucans" extracted from all three weeds either by aqueous calcium chloride or dilute acid were separated into three distinct fractions: a uronic acid-rich and low sulphated polysaccharide a fucose-rich high sulphated fraction and a fraction with an "intermediate" composition. The respective fractions from the two extractions were similar from each of the weeds.

7. The mixture of the alginic acid and glucuronoxylofucans present in the alkali extract from all three weeds were also separated. The former was shown to contain both mannuronic acid and guluronic acid residues.

8. Attempted fractionation of the crude glucuronoxylofucans from the alkali and ammonium oxalate-oxalic acid extractions was carried out as for the other fucose-containing polysaccharides but the major fractions were found to differ from each other only in their sulphate content. These materials were shown to resemble the "intermediate" fraction of the "fucans" in their uronic acid, sulphate and carbohydrate contents as well as the mole proportions of the constituent sugars. Crude glucuronoxylofucan was also obtained by acid chlorite treatment of the residual weed.

9. Methylation studies on the various fractions of "fucans" and glucuronoxylofucans showed that free fucose, 2-O-methyl,

3-O-methyl and 3,4-di-O-methyl fucoses (in case of uronic acid-rich material 2,3,6-tri-O-methylglucose, derived from 1,4-linked glucuronic acid) were the major products from all the different fractions, although the relative proportions of these sugar derivatives varied in different extracts. In addition smaller proportions of 2,3,4-tri-O-methylglucuronic acid, xylose and fucose together with 2,3-di-O-methyl-glucuronic acid and -xylose were also detected and from the fucose-rich fraction 2,3,4,6-tetra-O-methylgalactose was identified.

10. Methylation, periodate oxidation and reduction, and Smith degradation studies on the fucose-rich fraction proved that it was a highly branched polyfucan of 1,2-linked fucose residues with appreciable proportions of 1,3- and triply linked fucose residues. As far as could be determined all the xylose and glucuronic acid residues were on the periphery of the molecule. Molecular weight analysis of the various products by Sephadex G100 confirmed that it was a highly branched molecule.

11. Methylation studies on the uronic acid-rich fraction showed that the glucuronic acid residues were largely 1,4-linked, the fucose units were linked or sulphated at C-2, and at C-3 and C-4, at C-2 and C-4 and at C-3 and at C-4, and xylose as well as glucuronic acid also occur as end groups and 1,4-linked units.

12. Repeated periodate oxidation and reduction showed that in the uronic acid-rich fraction hemiacetal formation hindered

oxidation and that in addition to end group and 1,4-linked units some other types of linkages in the glucuronic acid residues are possible. Smith degradation led to the isolation, after mild hydrolysis, of erythronic acid (major), threitol, glycerol and propane 1,2-diol derived from the oxidation of 1,4-linked glucuronic acid, galactose, xylose and 1,2-linked fucose residues respectively.

13. Molecular weight analysis on Sephadex G100 of the uronic acid-rich polysaccharide and its various alcohols and degraded polymers obtained from repeated oxidation and reduction as well as from repeated Smith degradation confirmed that it was a highly branched molecule and that degradation only occurred from the periphery of the molecule.

14. Partial hydrolysis of the uronic acid-rich fraction lead to the isolation of two aldobiouronic acids and some oligouronic acids. One of the aldobiouronic acids was identical with 3-O-β-(D-glucopyranosyluronic acid)-L-fucose isolated in a similar way from Ascophyllum nodosum. The second aldobiouronic acid comprised glucuronic acid and an "unknown" sugar. These oligouronic acids constitute important structural features of the polymers.

15. Selective degradation of the uronoxyl linkages by Hofmann reaction was attempted. This removed about 60% of the uronic acid residues from the "fucans" (0.5M KCl) with high loss of the polymer.

CONTENTS

GENERAL INTRODUCTION

SEAWEED POLYSACCHARIDES

The algae are one of the most primitive groups in the plant kingdom and even with the passage of time they have retained their primitive characters. They have a great diversity of form ranging from tiny unicellular to giant multicellular organisms. They grow in soil, fresh and salt water, but occur in greatest profusion in the latter.

Algae are essentially photosynthetic organisms and hence availability of light is an important factor for their growth. Unicellular free-floating type algae are found abundantly in the upper layer of the sea while the larger species generally grow in the continental shelf areas or where the anchorage is relatively easy. It is the latter group of algae, commonly known as the seaweeds, which have considerable industrial significance and which have been studied in more detail than the former.

The classification of algae based on, primarily, their reproductive systems and other morphological features has, however, been extended to the nature of the pigments present in the various species. This has resulted in four main classes of algae, namely brown (Phaeophyceae), red (Rhodophyceae), green (Chlorophyceae) and blue-green (Cyanophyceae) algae. Recently, as an increasing number of algal species has been studied chemically, an effort has been made to correlate the

presence or absence of a particular type of metabolite as the 'marker' for a phylogenetic classification. This appears to be consistent with respect to Phaeophyceae, Rhodophyceae and to some extent to Chlorophyceae but the number of species investigated chemically in comparison with the vast number of algal species is rather small and hence classification of algae based at least on the nature of the particular carbohydrate may be regarded as tentative.

Seaweeds offer varieties of polysaccharides which differ considerably from those obtained from land plants. This is natural as the environments are very different. Cell-walls of land plants are usually composed of cellulose, hemicelluloses and lignin materials. They provide the characteristic rigidity necessary for the land plant cells environment. In contrast, seaweeds require flexibility and ease of movement for their survival and hence have skeletal structures with a high proportion of mucilaginous materials. Cellulose when present is in small proportion, hemicelluloses and lignin as such are absent or if present are in very small quantities. Another characteristic feature of the seaweed polysaccharides is the presence of half ester sulphate groups linked to the hydroxyl groups, land plant polysaccharides are devoid of these but they are common to animal polysaccharides.

The seaweed polysaccharides can conveniently be divided into (a) food reserve (b) structural and (c) acidic polysaccharides which could be food reserve or structural. These are dealt with in detail under the appropriate class of algae.

Polysaccharides of Phaeophyceae

Since the polysaccharides of Himanthalia lorea, Bifurcaria bifurcata and Padina pavonia are the subject matter of this thesis, the polysaccharides already investigated in this group will be discussed later.

Polysaccharides of Rhodophyceae

The polysaccharides normally found in this class of algae are floridean starch, cellulose, xylans, mannans and galactans. Of these galactans constitute the major polysaccharides and these have been investigated in considerable detail.

Floridean starch. The food reserve material in the Rhodophyceae is a glucan called floridean starch.[1,2] D-Glucose is the sole monosaccharide of this type of polysaccharide and its arrangement in the macromolecule appears to be similar to that of amylopectin[1,3] of land plants. The main core consists essentially of α-1,4-linked glucose residues, in some cases occasionally interspersed by α-1,3-linkages[4a-c] and an average chain length[5,6] of 11-15. The 1,3-linkage and the somewhat shorter chain length may serve to distinguish floridean starch from amylopectin of higher plants.

Xylans. Xylans, although examined from few species of red algae are of two types. A pure water-soluble xylan from Rhodymenia palmata, thought to be food reserve, has been studied extensively [7a-c] and found to have a linear structure,[8] the chain containing β-1,4-linked and β-1,3-linked-D-xylose units. Enzymatic studies suggest that these two types of linkages are arranged in random manner in the macromolecule. Extraction with other solvents[9] led to the isolation of essentially similar xylans containing different proportions of the two types of linkages.

$$-4x(1\longrightarrow 4)x(1\longrightarrow 4)x(1\longrightarrow 3)x(1\longrightarrow 4)x(1- \ )$$

x = β-xylopyranosyl

On the other hand, xylans from Porphyra umbilicalis and Bangia fuscopurpurea are said to be built up entirely of 1,3-linked-D-xylose units.[10] This conclusion has been derived solely from X-ray diffraction studies.

Recently, xylose rich polysaccharides[9] have been investigated from R. palmata, P. umbilicalis, Laurentia pinnatifida and Rhodocorton floridulum. Polysaccharides of R. palmata extracted by different solvents are similar to the one discussed earlier. The extraction of the skeletal material with alkali gave a xylan of exclusively 1,4-linked xylose residues.

The dilute alkali extract of L. pinnatifida and the chlorite extract of P. umbilicalis are similar to one another and to that

from R. palmata. Each contains 1,3-and 1,4-links in different proportions and only a small amount of branching as judged by the yield of 2-O-methylxylose. The two fractions of xylan from R. floridulum however indicated a considerable degree of branching. Furthermore, the high yield of tri-O-methylxylose compared to 2-O-methylxylose suggest that in addition to short xylose chains from xylose there are short xylose branches, possibly on a glucan backbone.[9]

Mannans. Mannans from P. umbilicalis and B. fuscopurpurea are essentially linear polymers of β-1,4-linked D- mannose units.[10] Mannans and xylans of red algae are generally considered to serve as the structural polymers although the water-soluble xylan from R. palmata, as previously stated, may be a food reserve material.

Cellulose. Cellulose, in small proportions, has been reported from different species of red algae but suffers from the lack of any detailed structural studies.[11]

Galactans. The polysaccharides that are far more economically important than others in red algae, are the galactans. They are composed of galactose, 3,6-anhydro D- and L- galactose and 6-O-methyl galactose units and ester sulphate units. These units offer a broad spectrum of polysaccharides comprising alternate 1,3-and 1,4-linked galactose or modified galactose units. The individual polymers differ in their finer details of structure possibly due to a particular environment. The

various galactans found in this class of algae can be conveniently divided into three groups: agar, porphyran and carageenan type of polysaccharides, although there are many similarities between these groups.

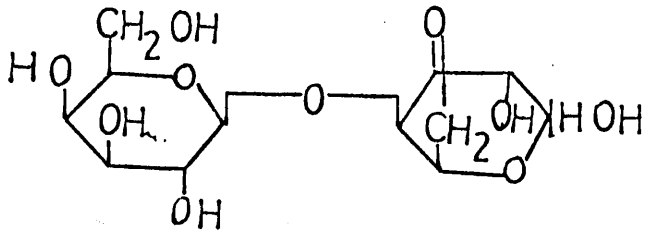
Agar. The agar-type of polysaccharide is in fact a mixture of two closely related polysaccharides: agarose and agaropectin. Agarose consists of a linear chain of alternating 1,3-linked-D-galactopyranose and 1,4-linked-3,6-anhydro-L-galactopyranose units. Evidence for this structural pattern are derived from partial hydrolysis,[12] methylation[13 a-d] and enzymatic studies.[13e] Partial hydrolysis gave a nearly theoretical yield (82%) of derivatives of the disaccharide-agarobiose, 4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose and neoagarobiose, O-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose (Fig. 1). The latter two techniques also indicated that 3,6-anhydro-L-galactose occurs at the nonreducing end, while D-galactose lies at the reducing end of the molecule. Agarose is essentially devoid of half ester sulphate group and stands unique in this respect with other galactans of the class. A general formula for agarose is shown in Fig. 2.

$$[\text{-3-D-Gal}(1\rightarrow 4)\text{-L-Agal}(1]_x\rightarrow [3)\text{-D-Gal}(1\rightarrow 4)\text{-L-Agal}(1]_y\text{—}$$

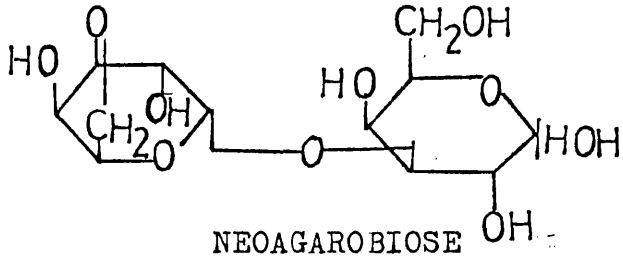
$$\text{6-OMe}$$

FIG. 2

Gal, galactopyranose; Agal, 3,6-anhydrogalactopyranose
Me, methyl.



AGAROBIOSE



NEOAGAROBIOSE

Fig. 1

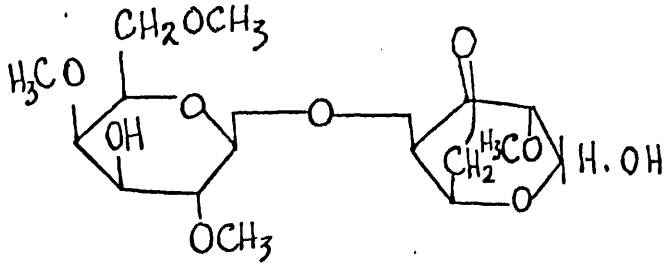
Recent studies on agarose from different species revealed the presence of variable amounts of 6-O-methyl-D-galactose[14] (and in Gelidium amansii 4-O-methyl-L-galactose[15]) but their position in the macromolecule is not yet established although it can be said with reasonable certainty that the general pattern of agarose is well established.

Agaropectin. The structure of agaropectin, on the other hand, is relatively less established than agarose. It is possibly a mixture of polysaccharides mainly built up of D-galactopyranose, 3,6-anhydroL-galactopyranose, D-glucuronic acid units and half ester sulphate groups. The basic similarity of the agaropectin structure with agarose is deduced from degradative studies.[16] These investigations have yielded similar di-and oligosaccharides to those found in agarose. In addition, pyruvic acid in acetal

linkage to D-galactose residues is found in agaropectin from G. amansii and G. subcostatum.[17] Ahnfeltia plicata also contains L-arabinose units.[18] The contribution of the glucuronic acid residues and L-arabinose units to the structure of the macromolecule is not known. The sites of all the ester sulphate groups are not established either. It seems most likely that with the advent of newer and effective fractionating techniques agaropectin will be found to be a mixture of polymers rather than a single entity.

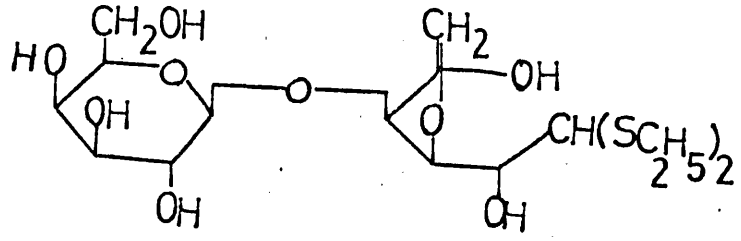
Porphyran. The porphyran type of galactan found in Porphyra and Laurentia spp. and in Bangia fuscopurpurea comprises D-galactose, L-galactose-6-sulphate, 6-O-methyl-D-galactose and 3,6-anhydro-L-galactose residues.[19,20] This is a linear polymer, like agarose, with alternating 1,3-and 1,4- glycosidic linkages. From periodate and partial hydrolysis studies[21,22] it has been established that D-galactose and its 6-O-methyl ether are linked at C-3 while L-galactose and its 3,6-anhydroderivative at C-4. The essential similarity with agarose has also been deduced from alkaline elimination[23] of the sulphate groups which results in the formation of the 3,6-anhydride, this is then followed by methylation of the alkali modified polymer. The product is identical with methylated agarose (Fig. 3). This has also revealed that the regularity of the repeating arrangements is being masked by the occasional presence of 6-O-methyl-D-galactose

Methylated agarobiose obtained in 62% yield from partial methanolysis from 'alkali-modified' porphyran.

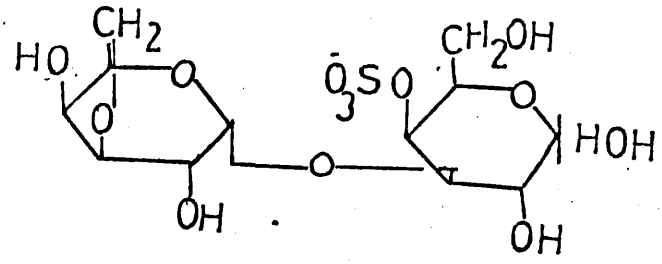


TETRAMETHYLAGAROBIOSE

[3,6-anhydro-2-O-methyl-4-O-(2,4,6-tri-O-methyl-β-D-galacto-pyranosyl)-L-galactose]



CARRABIOSE DITHIOACETAL



NEOCARRABIOSE

4 - Sulphate

Fig. 3

and L-galactose-6-sulphate. Enzymic studies[24,25] suggest that the 6-O-methyl-D-galactose is rather irregularly distributed throughout the polymer chain.

Carrageenans. The carrageenans like agar, represent a family of complex polysaccharides. The basic monomers are D-galactose and its 3,6-anhydroderivative.[26] The latter distinguishes it from the agar family where it is the L-enantiomer which is present as the 3,6-anhydride. The complexity arises from the degree and position of sulphation on the monomeric residues. Depending on the solubility in dilute potassium chloride solution[27] carrageenans may be obtained as insoluble k-carrageenan and soluble λ-carrageenan. k-Carrageenan is essentially a linear polymer having a masked repeating structure based on alternating 1,3-and 1,4- linked-D-galactose residues. This structural pattern is confirmed by the isolation of carrabiose, O-β-D-galactopyranosyl(1$\longrightarrow$4)- 3,6-anhydro-D-galactose dithioacetal[28,29] (Fig. 3) from mercaptolysis of the polymer. Enzymatic studies,[30,31] on the other hand, yield neo-carrabiose sulphate, O-α-3,6-anhydro-D-galactopyranosyl(1$\longrightarrow$3)-D-galactose-4- sulphate (Fig. 3) and an enzymic resistant fraction. The latter fraction has higher sulphate content than k-carrageenan and can undergo further enzymatic attack after alkali modification indicating the presence of D-galactose-6-sulphate (which is thereby converted into anhydride). The 1,3-linked galactose residues are often sulphated at C-4 although a portion may be

sulphated at C-6 or disulphated at C-2 and C-6. The 4-linked 6-sulphate is revealed by its convertion into the 3,6-anhydride by reduction of the reducing unit with borohydride followed by alkaline reaction,[32,33] and from spectroscopy.[34]

The potassium chloride soluble λ-carrageenans are mixtures of polymers.[35] Various fractionation experiments either before or after alkali treatment have led to the isolation of different polymers, all of which are based on an alternating sequence of 1,3-and 1,4-linked galactose units each differing from the other in the proportions of 3,6-anhydro sugar and sulphate and in the various sites of the latter.

Other sulphated galactans. Recently, sulphated galactans from Aeodes orbitosa[36,37] and Phyllymenia Cornea[38] both of which belong to the Grateloupiiaceae, have been investigated. From the former species the polysaccharide chiefly consists of D-galactose and its 2-O-methylether derivative together with small proportions of D-xylose and glycerol while the polysaccharide from the latter species mainly comprises D-galactose and its 2-O-,4-O- and 6-O-methyl derivatives. A small quantity of 3,6-anhydrogalactose and D-xylose have also been reported.[37] They are all highly sulphated polymers and the half ester sulphate groups are alkali stable. Their initial investigation[36] indicated the absence of alternating 1,3-and 1,4-linkages, an exception from the galactans so far

discussed, although equal proportions of 1,3-and 1,4-linkages had been recognised. With further understanding of the nature of the skeleton from periodate oxidation, partial hydrolysis and methylation studies,[39] however, it is clear that, at least in some regions of the molecule, alternating 1,3-and 1,4-linkages occur as with other galactans of the Rhodophyceae.

Polysaccharides of Chlorophyceae

This class of algae includes both fresh water and marine species, but most of the chemical investigations have been made on the latter. Furthermore, detailed studies on the carbohydrates are comparatively less than those on the more economically important Phaeophyceae and Rhodophyceae. The major polysaccharides that have been found so far are starch-type glucans, xylans, mannans, cellulose and water soluble complex sulphated polysaccharides.

Starch-type glucans. All the species so far investigated chemically are found to have starch-type glucans[40,41] similar to that of the land plants comprising both amylose and amylopectin. Aqueous extraction of Caulerpa filiformis, Enteromorpha compressa, Ulva lactuca and Chaetomorpha capillaris yielded these glucans together with sulphated polysaccharides. They were separated as starch-iodine complexes or as glucans

left in aqueous solution after complexing the sulphated polysaccharides with cetyltrimethylammonium hydroxide. The separated starch, apart from molecular size, had the characteristic properties of land plant starch. These starches were fractionated by thymol into amylose and amylopectin. The latter had approximately one mole of periodate uptake per anhydroglucose unit. The oxopolysaccharide recovered after periodate oxidation was devoid of any unattacked glucose units indicating 1,4-linkage and absence of 1,3-linked units. This was further confirmed from methylation studies which also proved the presence of 1,6-branch points. These amylopectins resembled potato amylopectin.

The essential similarity of green algal amylose with potato amylose has been deduced from high positive rotation, ready retrogradation in aqueous solution, β-amyloysis and periodate uptake. X-ray diffraction pattern of these starches showed a resemblance to potato-starch type materials.[42]

Xylans. The xylans constitute the main cell-wall materials of a number of green algae, although small proportions of polysaccharides comprising other sugar units may also be present.[43-48] The pure xylan, obtained by alkaline extraction of C. filiformis,[43] on methylation and hydrolysis gave largely 2,4-di-O-methyl-D-xylose indicating 1,3-linkage. Periodate oxidation studies further confirmed the results of methylation.

These results proved that the xylan of C. filiformis consists essentially of a linear chain of 1,3-linked xylose units. From the amount of periodate uptake and the quantity of formic acid released, and from the end group assay of the methylated xylan it has been suggested that it has an average chain-length of about 47 xylose residues. The negative rotation indicates that the units are β-linked. Similar results have also been obtained for Bryopsis maxima, Caulerpa brachypus, Caulerpa racemosa and Halimeda cuneata,[44,45] although the D.P. has been found to be higher than that of C. filformis. Enzymatic studies[46] on H. cuneata xylan confirmed the presence of 1,3-linkage and the absence of 1,4-linkage. All these studies merely indicate that the xylans so far investigated in green algae, are essentially linear chains of β-1,3-linked xylose units. This is, however, in contrast to Rhodymenia xylan.[8] X-ray investigations of green algae xylans suggest a helical structure.[47,48]

Mannans. The mannans comprise the main component in some Siphonaceous algae such as Acetabularia calyculus and Halicoryne wrightii.[49] A mannan from Codium fragile[50] has been studied in detail. Enzymatic degradation gave mannobiose (4-O-β-D-mannopyranosyl-D-mannose), triose, tetraose and a small quantity of oligosaccharides. Acetolysis further substantiated these results. Methylation and periodate

oxidation studies together with the negative rotation established that the C. fragile mannan, like the mannans of the Rhodophyceae and higher plants, comprises β-1,4-linked mannose units.

Cellulose. The presence of cellulose has been recognised in some species of Chlorophyceae but in most cases it has been found to be mixed with uronic acid and neutral sugar polymers. Attempted isolation of a pure cellulose[51,52] has not been achieved and so awaits its structural studies.

Sulphated polysaccharides. The characteristic mucilaginous polysaccharides, as in Rhodophyceae, constitute the major polysaccharides of this class of algae. They may be divided into two catagories depending on their sugar units. On the one hand, those from Ulva lactuca, Enteromorpha compressa and Acrosiphonia arcta are based on L-rhamnose, D-xylose and D-glucuronic acid residues. On the other hand, polysaccharides based on D-galactose, L-arabinose and D-xylose are obtained from Cladophora, Chaetomorpha, Caulerpa and Codium species.

The former, a glucurono ⊂ xylorhamnan, is found to be a heteropolymer and to contain L-rhamnose as the major sugar residue together with lesser quantities of D-xylose and traces of D-glucose. The proportion of glucuronic acid is found to be fairly constant and is about 20% while the percentage of

ester sulphate is rather variable. Partial acid hydrolysis, on all three above mentioned rhamnans, gave a relatively high proportion of an aldobiuronic acid, 4-O-β-D-glucopyranosyl-uronic acid-L-rhamnose[53a-c] (Fig. 4).

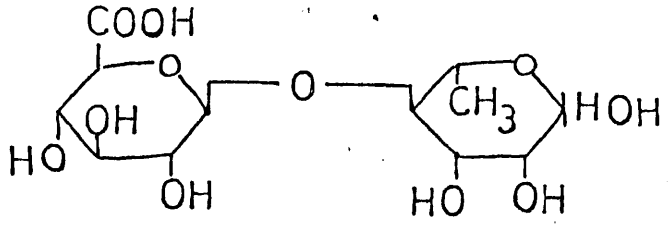


Fig 4

The polysaccharide of Ulva lactuca has been examined in greater detail. Methylation and subsequent reaction of the partially desulphated polymer has indicated that 1,4-linked and 1,3,4-linked rhamnose units, 1,4-linked xylose and 1,3-linked glucose/glucuronic acid constitute the major structural units. The possibility of 1,3,6-linked glucose, 1,4-linked glucuronic acid, 1,3-linked xylose and 1,2,3-linked rhamnose has also been recognised. Partial hydrolysis of the initial polysaccharide has given, besides the above mentioned aldobiuronic acid, smaller amounts of O-D-glucopyranosyluronic acid (1→3)D-xylopyranose, O-D-glucopyranosyluronic acid (1→4) D-xylopyranose and O-D-glucopyranosyluronic acid (1→4)L-rhamnopyranosyl (1→3)D-glucopyranosyluronic acid (1→3)D-xylopyranose. These results have confirmed that the polysaccharide is not a mixture of homopolysaccharides but is a polydisperse heteropolymer.

Periodate oxidation of the partially desulphated polymer gave a reduced rhamnose content in the derived alcohol compared with the corresponding alcohol from the fully sulphated polymer indicating that C-2/3 position in rhamnose was initially sulphated. Evidence of sulphate group at C-2 of xylose units has also been obtained from the alkaline reaction which resulted in the formation of D-arabinose. Smith degradation[55] of the initial polysaccharide has clearly revealed that a number of glucose, xylose and glucuronic acid residues throughout the macromolecule are vulnerable to periodate and that adjacent glucose and adjacent xylose units are structural features, but the overall structure of the macromolecule has not been established for this group of polymers.

The second group of polymers, so far investigated[56-62] have a high proportion of D-galactose and possibly with the exception of Caulerpa filiformis, L-arabinose. All of them contain appreciable proportions of D-xylose and very little, if any, uronic acid. Trace quantities of rhamnose have also been recognised. The half ester sulphate content is found to be fairly constant in all cases and is to some extent alkali labile. They all contain 1,3-and 1,6-linked-D-galactose units having ester sulphate at some of the C-6 of the galactose residues. Moreover, Cladophora and Chaetomorpha have sulphate groups on C-3 of arabinose units while the

evidence for C-4 sulphate both in galactose and for 1,3-linked arabinose in Codium has been established. Fractionation of these materials into simple homopolymers has not been achieved.

Recently, the polysaccharide, Cladophoran, from Cladophora rupestris has been investigated in detail.[61,62] Hydrolytic studies of the initial and partially desulphated polysaccharides have yielded a number of heterooligosaccharides containing galactose and xylose, arabinose and galactose and sulphated oligosaccharides with higher proportions of arabinose to galactose. These conclusively prove not only the heteropolymeric nature of the mucilage but also the wide diversity of the structural units present. Smith degradation studies have indicated that the polymer is highly branched and that 1,4-linked and end group xylose are present. Methylation studies have shown clearly that 1,4-linked arabinose constitutes an important structural feature of the macromolecule and that much of it is sulphated at C-3. It is most likely that no single sugar constitues the backbone of the molecule although it is apparent, from these studies, that the inner core contains a high proportion of arabinose.

I N T R O D U C T I O N

Carbohydrates of Phaeophyceae

The carbohydrates of Phaeophyceae may be divided into two broad groups: Low and high molecular weight and will be discussed under these headings.

Low molecular weight carbohydrates. Of the low molecular weight carbohydrates D-mannitol has been found to be present in all the species of brown algae so far examined. Its content in the frond of some algae may vary from a few percent of the dry weed to about 25% in late summer.[69 a-b] Besides mannitol, a seven carbon alcohol, D-volemitol,[70] 1-D-mannitol-β-D-glucopyranoside and 1,6-O-D-mannitol-di-β-D-glucopyranoside were found in Fucus vesiculosus and F. spiralis.[71,72] Trace quantities of sucrose, galactose, and mannose have also been reported.[73]

The hypothesis of a close metabolic relation between mannitol and laminaran (a high molecular weight glucan) received experimental evidence from Bidwell[74] who observed in F. vesiculosus that labelled laminaran is rapidly formed from C^{14}-labelled-D-mannitol.

High molecular weight Carbohydrates: Polysaccharides of Phaeophyceae. The brown seaweeds (Phaeophyceae) have been found to contain glucans (food reserves and structural) as well as acidic polysaccharides. The latter comprising two

distinct types of polymers. The first group is typified by alginic acid which consists solely of uronic acid residues. The acidity of the second group of polymers is due not only to the presence of uronic acid residues but also to half ester sulphate groups substituting some of the hydroxyl groups of the sugar units. It includes what is generally accepted in the literature as 'fucoidan' and the more recently coined term 'glucuronoxylofucan'.

Structural glucan: Cellulose. The presence of cellulose in brown algae was postulated by a number of workers. The insoluble residue after removal of alginic acid from the seaweeds was called 'algic cellulose' by Stanford.[63] Kylin[64] showed that it gave a colour reaction with iodine and sulphuric acid. Russell-Wells[65] showed its solubility in cuprammonium hydroxide and prepared an acetate. Dillon and O'Tuama[66] were first to prove cellulose from Laminaria digitata. They were able to prepare a viscose and chloroform soluble acetyl and methyl derivative. Structural investigation of brown algal cellulose was made by Percival and Ross.[67] They separated cellulose from L. hyperborea, L. digitata and Fucus vesiculosus. Proof of β-1,4-linkage in these celluloses was obtained from the isolation of cellobiose octa-acetate (31%). Periodate oxidation studies excluded the presence of 1,3-and 1,6-linkages. From the measurement of formic

acid released, the chain length was estimated to be about 160. X-ray diffraction studies[68] gave the same pattern as that of normal cellulose.

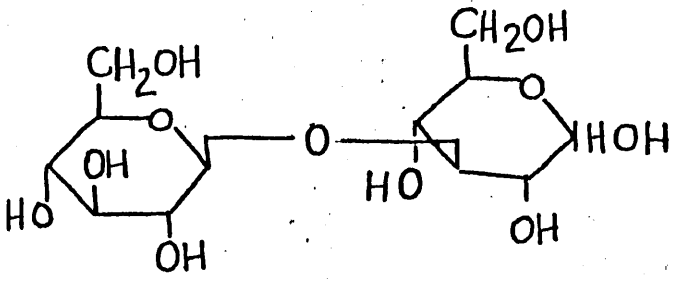
Food reserve glucan: Laminaran. Laminaran, first discovered by Schiedelberg, is present in all the brown algae so far examined. Its proportion in a species varies with season,[75-79] between autumn and winter it comprises a high proportion of dry weight of some weeds. It has been recognised as two forms:[80] soluble and insoluble, depending on their solubility in cold water. Both forms, however, dissolve in hot water and are almost indistinguishable chemically.

Laminaran is generally extracted from the weed by water, cold and hot, and by dilute acids.[81] The insoluble laminaran is spontaneously deposited from the aqueous acid extract of the weed on standing and the precipitation of the soluble form is achieved by alcohol.[82,83]

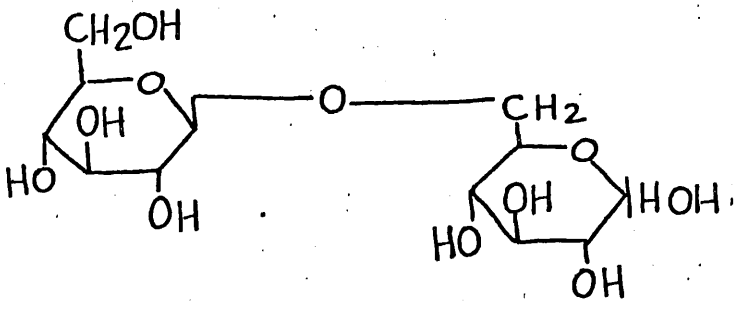
Complete hydrolysis of laminaran generally gives solely D-glucose[80,83,84] but in some cases[85,86] small proportions of mannose (and mannitol from some species, see later) have also been reported. However, examination of a range of samples showed about 0.2% mannose content and it was believed to be structurally insignificant.

Laminaran from Laminaria. Laminaran particularly from the genus Laminaria[83,84,87-91] has frequently been investigated. Early methylation studies[80] on insoluble laminaran together with its negative rotation, $[\alpha]_D = -12\text{-}14^\circ$, indicated that it is essentially a linear polymer of β-1,3-linked-D-glucose units. This has been confirmed by partial hydrolysis[88] and enzymatic hydrolysis[92] which gave a homologous series of β-1,3-linked oligosaccharides from which the laminaribiose (Fig. 5) was isolated and characterised.[93] Further confirmation of 1,3-linked glucose residues has been obtained by the isolation of 40-45% D-glucometasaccharinic acid after treating the laminaran with lime water.[94](Fig 5)

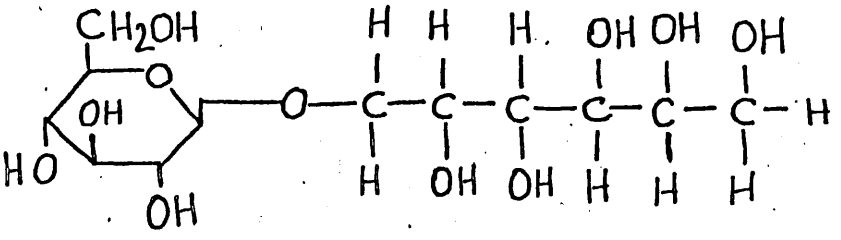
The Presence of Mannitol in Laminaran. From the results of partial hydrolysis which yielded small quantities of gentiobiose, 3-O-gentiobiosylglucose, 1-O-β-glucopyranosyl-D-mannitol (Fig. 5) and 1-O-laminaribiosyl-D-mannitol together with much higher proportions of laminaribiose (Fig. 5) and higher laminarioligosaccharides it was concluded that 1,6-links and mannitol were present in the polysaccharide. From the proportion of mannitol it was concluded that about 40% of the molecules are terminated by a mannitol residue, M-chains (Fig. 6) glycosidically linked through one of the two primary hydroxyl groups. The remaining molecules are terminated by reducing glucose residues, G-chains (Fig. 6).



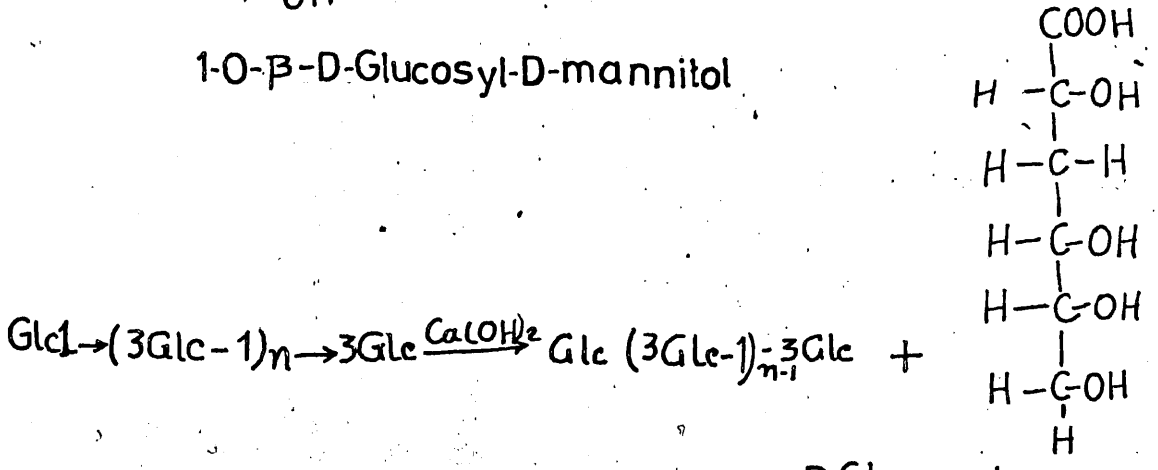
Laminaribiose



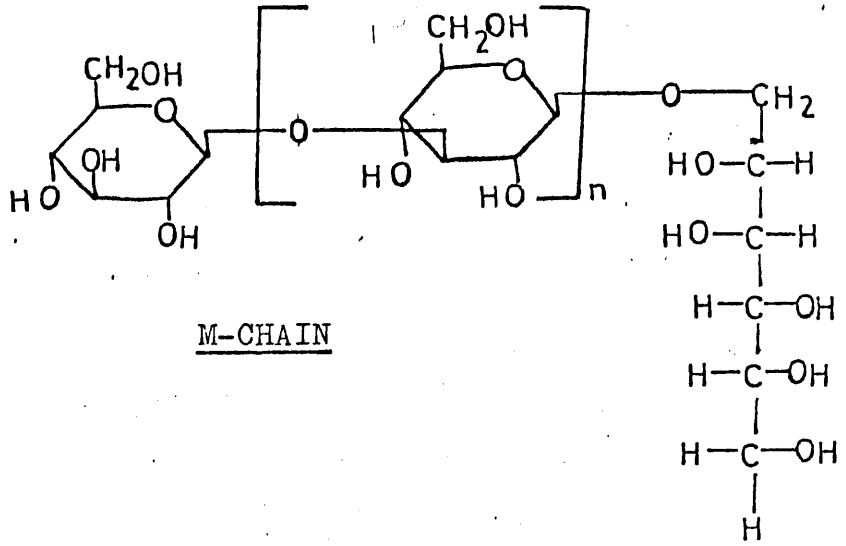
Gentiobiose



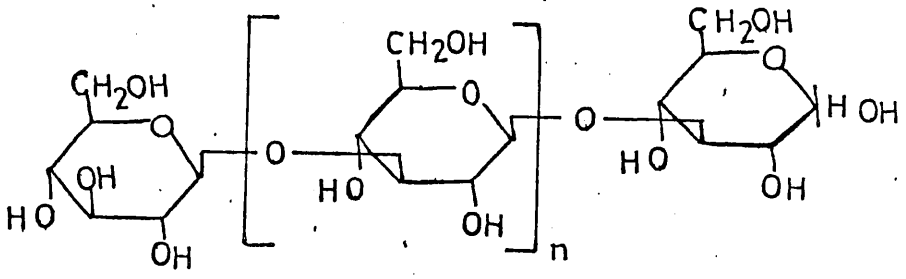
1-O-β-D-Glucosyl-D-mannitol



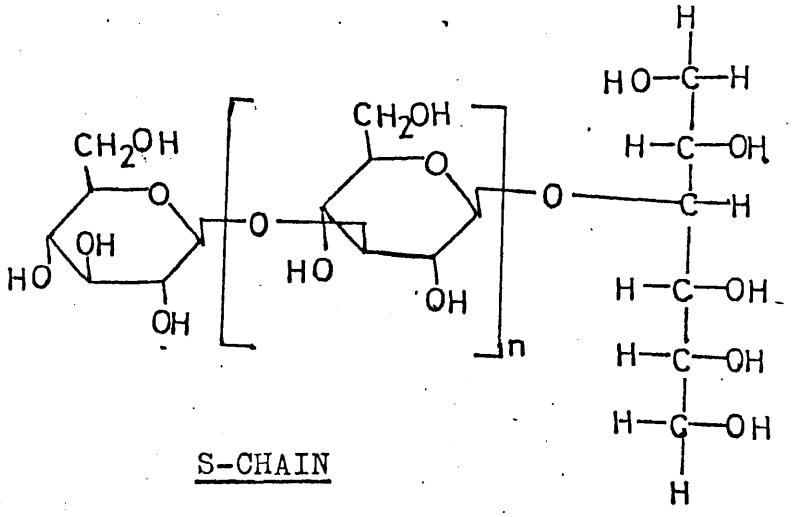
D-Glucometasaccharinic acid



M-CHAIN



G-CHAIN



S-CHAIN

Fig. 6

Further studies[95] on soluble and insoluble laminaran have led to the conclusion that in the former case about 75% of laminaran chains are terminated by a mannitol residue while in the latter case it is about 46%.

It has already been mentioned that laminaran on treatment with lime water[94] yields about 45% metasaccharinic acid. This is due to peeling action on G- chains, M-chains are alkali immune. Reduction of laminaran[96,97] on the other hand, has given S-chains from G-chains while M-chains remain unaffected. Oxidation of laminaran with bromine[98] gave rise to gluconic acid at the reducing end. M-chains are not affected under these conditions. By this reaction it has been possible to separate these two molecular species on an anion exchanger.

Periodate oxidation under mild conditions has been very useful in determining the position of mannitol in laminaran. During oxidation of about 0.4% solution of laminaran in 0.015M solution of periodate at 2°, the M-chains release one mole of formaldehyde. The G-chains, however, do not react under such conditions. Reduction and subsequent mild hydrolysis of the oxopolysaccharide led to the formation of ethylene glycol (Fig.7a). This proves that the mannitol residue is singly linked through C-1 (or C-6, due to the symmetry of the mannitol molecule).

Absence of ethylene glycol after Smith degradation studies of M-chains led to the suggestion that the mannitol residue is disubstituted[98] at C-1 and C-2. Such linkages should result in the formation of glycerol instead of ethylene glycol after Smith degradation (Fig.7b).

Re-examination[97] of an acid hydrolysate of laminaran after periodate oxidation and reduction revealed the presence of ethylene glycol. This was separated on resin and cellulose and was finally characterised as the di-p-nitrobenzoate.

Further confirmation of singly linked mannitol has been made from the estimation of formic acid[90,99] released on oxidation of laminaran under exceptionally mild conditions. M-chains singly linked at C-1 (or C-6) or doubly linked at C-1 and C-2 should release 3 and 2 moles of formic acid (Fig. 7 a & b) respectively. It was found that three different samples of laminaran[90] gave 3.0-3.1 moles of formic acid per mannitol residue.

The Presence of 1,6-linkages in Laminaran. In addition to β-1,3-linked glucose units, the presence of β-1,6-glycosidic linkages in laminaran was first suggested by Peat and his co-workers.[88] On partial hydrolysis, as already mentioned, laminaran yields not only laminaribiose and higher laminarioligosaccharides but also gentiobiose and 3-O-gentiobiosylglucose. Although they prove 1,6-glycosidic linkages in

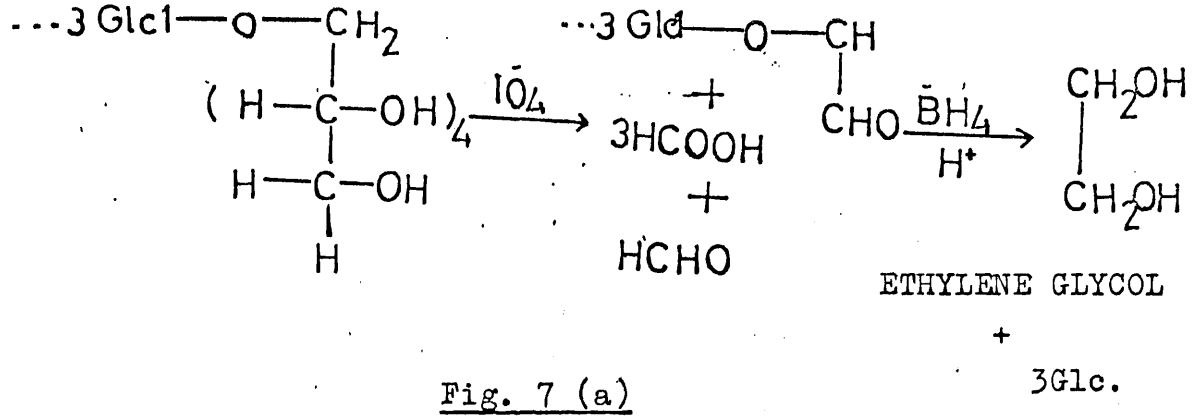

...3 Glc1—O—CH2
(H—C—OH)4
H—C—OH
H
IO4
...3 Glc—O—CH
CHO
+
3HCOOH
+
HCHO
BH4
H+
CH2OH
CH2OH
ETHYLENE GLYCOL
+
3Glc.

Fig. 7 (a)

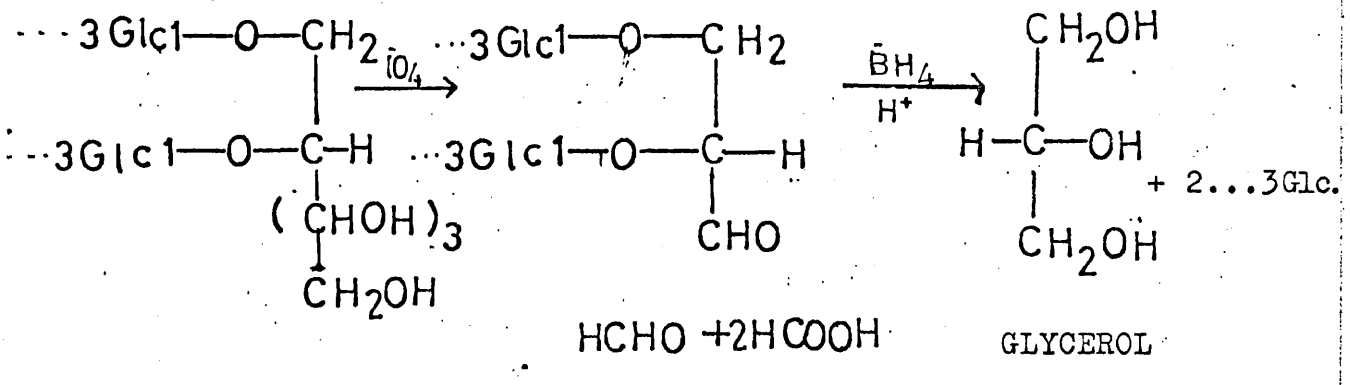

...3 Glc1—O—CH2
...3Glc1—O—C—H
(CHOH)3
CH2OH
IO4
...3 Glc1—O—CH2
...3Glc1—O—C—H
CHO
HCHO +2HCOOH
BH4
H+
CH2OH
H—C—OH
CH2OH
+ 2...3Glc.
GLYCEROL

Fig. 7(b)

they
laminaran/raise the question: are these linkages inter-residue or present as branch-points. Peat and his co-workers[87] initially assumed that 1,6-linkages were responsible for branched structure, but later on they reported[88] that laminaran is essentially a linear molecule in which the main chain of β-1,3-linked glucose residues are occasionally interrupted by β-1,6-inter-residue linkages. Absence of 3:6-Di-O-β-D-glucosyl-D-glucose in the partial hydrolysis products was the main basis for this conclusion.

Further support of 1,6-inter-residue linkages in laminaran came from methylation studies. Methylated laminaran on hydrolysis gave in addition to 2,4,6-tri-O-methyl-D-glucose, a small amount of 2,3,4-tri-O-methyl-D-glucose. Unless this resulted from the demethylation of the tetra-O-methylglucose, it could only be derived from 1,6-inter-residue linkage.

In contrast, a branched-structure for laminaran has been suggested by several workers. Anderson et al.[89] have provided evidence from methylation and degradative studies. Methylated laminaran was precipitated with light petroleum spirit (b.p. 40-60°). The precipitated material amounted to about 78% of the methylated polysaccharides. This on hydrolysis gave 2,3,4,6-tetra-O-methyl-D-glucose (4.4%), 2,4,6-tri-O-methyl-D-glucose (86.4%), unidentified di-(7.2%) and mono-O-methyl-

D-glucose (1.9%). The proportion of tetra-O-methyl to tri-O- and di-O- methyl-D-glucose corresponds to an average chain length of 23. Molecular weight determined by isothermal distillation was 12000 which corresponds to a degree of polymerisation of 58. From these two facts it may be calculated that laminaran has an average of two branch points per molecule.

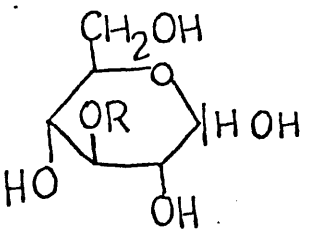
Moreover, repeated application of Barry degradation[100] on laminaran (with D.P. 58) did not give any small fragments which on dialysis could pass through a cellophane membrane. Periodate oxidation and phenylhydrazine degradation (Barry degradation) cleave only the 1,6-linkages in the laminaran molecule, as 1,3-linkages are immune to periodate attack. The presence of 1,6-inter-residue linkages in laminaran molecules having chain length on an average of 23 glucose units should lead to the fragments of dialysable size. If, however, 1,6-links occur only as inter-chain linkages then chains of approximately the same length would remain after Barry degradation. In fact after three oxidations and two degradations the residual polysaccharide retains the characteristic properties of initial insoluble laminaran. From these results the authors suggested that unless 1,6-links occur exclusively near the end of the chains, branched-structure for laminaran molecule is more likely.

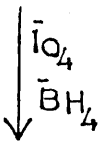
Smith degradation studies on laminaran also support the branched-structure for insoluble laminaran from L. hyperborea and for soluble laminaran from L. digitata. The sequence of oxidation, reduction and mild hydrolysis converts reducing glucose end groups to 2-O-substituted-D-arabinitol residues, at the same time 1,6-linked inter-residue glucose units are cleaved with the production of 3-O-substituted glycerol residues. A second periodate oxidation of these products should liberate one mole of formaldehyde from each original reducing glucose residue and an additional molecule of formaldehyde for every 1,6-inter-residue linkage (Fig.8). A sample of laminaran having D.P. 24 and 43% of G-chains gave 0.019 mole (required for 1,6-inter-chain linkage 0.018 moles) of formaldehyde per anhydro glucose unit. These results, therefore, provide further evidence for the absence of any significant proportion of 1,6-inter-residue linkages.

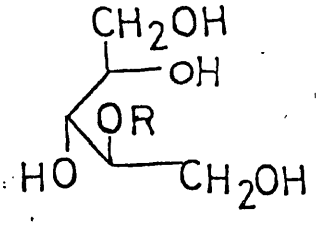
Laminaran from other genera. So far the laminaran of Laminaria have been discussed. Other genera that have been studied of the class Phaeophyceae are Fucus serratus, Eisenia bicyclis and Ishige okamurai.

Fucus Laminaran. Soluble laminaran has been investigated by Fleming and Manners[101] in an attempt to distinguish between soluble and insoluble laminarans. From the formic

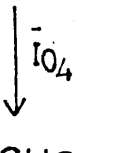
REDUCING END UNIT

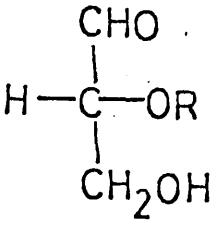






2-O-substituted-D-arabitol residue

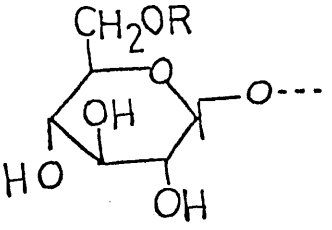


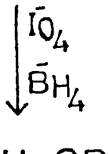


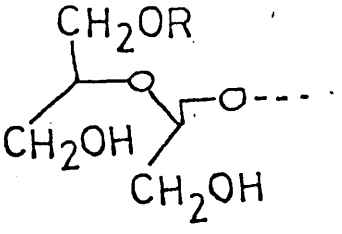
$HCHO + HCOOH$

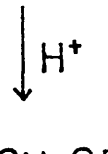
2-O-substituted glyceraldehyde

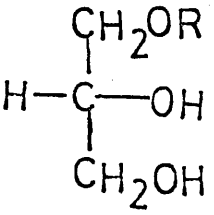
1,6-INTER-RESIDUE LINKAGE



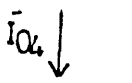








3-O-substituted glycerol



Ethylene glycol.

Fig. 8

acid released on mild periodate oxidation of the soluble laminaran with a correction for the formic acid produced from mannitol end groups, the chain length ($\overline{CL}$) was determined from various samples. This was correlated with DP determined by the measurement of formaldehyde produced on periodate oxidation of borohydride reduced laminarans, under the conditions in which both M- and S-chains yield one molecule of formaldehyde per chain. Samples of soluble laminaran from L. digitata and Fucus serratus gave $\overline{CL}$ values 7-10 and DP values 26-31 indicating an average of 2 to 3 branch points per molecule. Samples of insoluble laminaran from L. hyperborea similarity indicated an average of 1.6 branch points per molecule.

Eisenia bicyclis laminaran. Soluble laminaran, $[\alpha]_D = -45^\circ$, devoid of mannitol has been investigated by Nisizawa et al.[102,103] The absence of mannitol was confirmed by the fact that DP(18) found by measuring reducing power was approximately the same as obtained cryoscopically. It gave on partial acid hydrolysis considerable amounts of gentio-oligosaccharides in addition to laminarioligosaccharides, branched trisaccharide such as 3,6-di-O-β-glucopyranosyl-D-glucose could be detected from the partial hydrolysis products. Characterisation of gentiotetraose and laminaritetraose strongly indicate that a considerable proportion of the 1,6-linked glucose residues

occur as blocks among 1,3-linked glucose residues to form an essentially linear heteropolymer.

Alkaline degradation of Eisenia laminaran ceased after the loss of about one third of the weight. If it is assumed that peeling action is arrested at 1,6-linkage, then this indicates that 1,3 and 1,6-linkages exist at a ratio of 2:1. Later it was found from Smith degradation studies[103] to be of 3:1.

Methylated Eisenia laminaran on hydrolysis gave 2,3,4,6-tetra-O-methylglucose, 2,3,4- and 2,4,6-tri-O-methyl glucoses. No evidence of di-O-methylglucose was obtained in the hydrolysate. From these observations it has been concluded that E. bicyclis laminaran is a linear polymer containing 1,3-linked and 1,6-inter-residue linked glucose residues.

Ishige okamurai laminaran. Soluble laminaran, $[\alpha]_D = -37.5^\circ$, devoid of mannitol has been reported by Maeda et al[104,105] from I. okamurai. Hypoiodite and periodate oxidation indicated DP about 19. Partial acid hydrolysis studies revealed that laminaran contains both 1,3 and 1,6-linked glucose residues. The proportions of 1,6 to 1,3 linkages were found to be 1:6. Methylated I. okamurai laminaran yielded 2,3,4,6-tetra-O-methyl, 2,4,6-tri-O-, 2,3,4-tri-O- and 2,4-di-O-methylglucoses in the molar ratios of 1.1:6.23:1.6:0.42, respectively. Hence this laminaran has 1,3-links and 1,6-inter-residue linkages in a proportion of approximately 1:4.

Proportion of 2,4-di-O-methylglucose indicates on average no more than a single branch point but the position of branching is uncertain. Ishige laminaran, although essentially linear, is an example where 1,6-linkages occur both as inter-residue as well as inter-chain structure.

Laminaran from other algae. Chrysolaminaran obtained from mixed diatoms of Chrysophyceae has been studied by Beattie et al.[106] It is essentially a linear glucan having β-1,3- and β-1,6-linked glucose residues. It has $\overline{CL}$ 11 and DP 21. Methylated chrysolaminaran gave small quantities of 2,4-di-O-methylglucose in addition to 2,4,6-tri-O-, 2,3,4-tri-O- and 2,3,4,6-tetra-O-methylglucoses. These results indicated a single branch-point at C-6 in the molecule. From the periodate over-oxidation results it has been suggested that the branch point is situated possibly on average half way along the chain. This laminaran is devoid of mannitol terminated chains.

Studies on the laminaran[107] from Phaeodactylum tricornutum of Bacillariophyceae revealed that it closely resembles the chrysolaminaran in its structural pattern.

From the foregoing discussion it is clear that the term laminaran covers a wide range of glucans which are essentially linear molecules containing both 1,3-and 1,6-linked glucose units with minor structural differences in some species of

algae. Laminaran from some species are mixtures of two molecular forms one is terminated by mannitol residues and the other is terminated by reducing glucose residues. In contrast, some species have only reducing glucose terminated molecules. The 1,6-linkage appear to be present as inter-chain and inter-residue and/or both in some species. It is probable that variation in solubility of laminaran may be due not only to the degree of branching but also to the amount of 1,6-linkages.

Alginic Acid

A polyuronide, alginic acid, constitutes one of the principal carbohydrates of brown seaweeds. It was first discovered in about 1880 by Stanford[108] and has since then become a product of commercial interest. The hydrophillic colloidal properties and the ability, at low concentration, to form gels or viscous solutions are the main characteristics for commercial uses.

Alginic acid has been found in all the brown seaweeds so far examined but the content varies within a range of 14 to 40% from species to species and also from season to season.[109-112] It is usually low during the period of rapid growth in summer and is higher in colder months. It is insoluble in water and is usually extracted with dilute sodium carbonate solution as soluble sodium alginate with appropriate prior treatments

of the weed so as to remove salts, mannitol, laminaran, fucoidan and some colouring matter. Sodium alginate can be obtained after precipitation and reprecipitation[113,114] in alcohol and final collection after freeze-drying. It can be obtained as free acid by treating the sodium alginate solution with hydrochloric acid or as the calcium salt by treating with dilute calcium chloride solution.

Although alginic acid is known to be present in brown seaweeds and has been used for commercial purposes for some 40 years, its molecular structure has not yet been completely established. This is mainly because of its marked resistance to hydrolysis under the conditions that do not destroy the products. Initially it was considered to be a nitrogeneous organic acid[115] but later work proved it to be a polysaccharide.[64] In fact the work of Nelson and Cretcher[116] proved that mannuronic acid was a constituent of alginic acid. By decarboxylation of alginic acid with hydrochloric acid they obtained carbon dioxide (24.2%) corresponding to a uronic acid content of 100%. Further evidence was obtained by the isolation of D-mannuronic acid and its lactone from the hydrolysate of Macrocystis pyrifera.[117]

Structural investigations on alginic acid using methylation technique ~~was~~ were difficult due to the presence of carboxyl groups. However, after treatment with methanolic hydrogen

chloride (10%) a water soluble degraded polymer of considerably lower viscosity than the original polymer[118] was obtained. It was possible to methylate this with thallous hydroxide and methyliodide. Methanolysis of the methylated material gave the methylester of 2,3-di-O-methyl-D-mannuronoside which on degradative oxidation gave 2,3-di-O-methyl-erythraric acid (Fig. 9)p 40.

Methylation technique later on was extended to a less degraded alginic acid.[119] After Haworth methylation and hydrolysis with formic acid, the products were converted into the methylester methylglycosides. These were reduced with lithium aluminium hydride and the derived methylglycosides were hydrolysed. The major component of the hydrolysate was characterised as 2,3-di-O-methyl-D-mannose by converting it into 2,3-di-O-methyl-D-erythraric acid (Fig. 9). These results together with the negative rotation led to the conclusion that the main structural feature of the alginic acid molecule is a linear chain of β-1,4-linked-D-mannuronic acid residues. This conclusion was indeed in accordance with the results obtained from periodate and bromine oxidations[120] as well as x-ray diffraction studies.[121]

However, the major advance in alginic acid chemistry came from Fischer and Dörfel.[122] They, with improved chromatographic techniques, were able to show that guluronic acid in addition to mannuronic acid was present in alginic

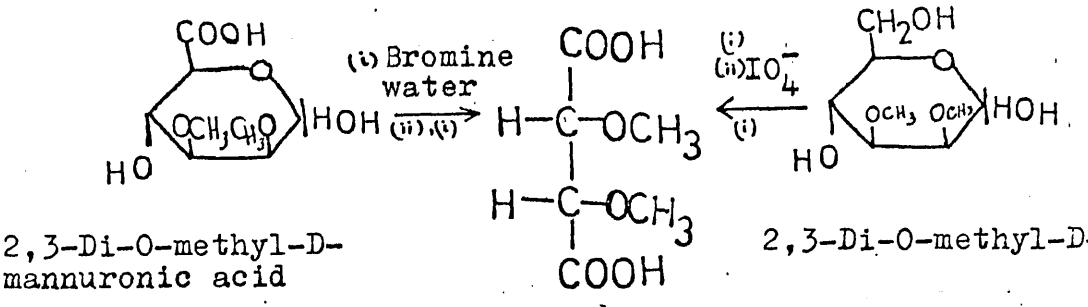
acid from various species of brown algae. They were also able to separate two crystalline lactones;[123] D-mannurono- and L-guluronolactones, from the acid hydrolysate of alginic acid and concluded from infrared measurements that both the lactones were pyranose 3,6-lactones. The ratio of the two lactones varied according to the particular weed and the conditions of hydrolysis. Prolonged heating with 0.5N-sulphuric acid, however, caused more degradation of L-guluronolactone than D-mannuronolactone.

However, further proof that guluronic acid units are indeed present in the native alginic acid and have not resulted during alkaline extraction, was obtained by the acidic hydrolysis of the weed itself where both acids were present in the hydrolysate. In order to provide additional evidence that guluronic acid was not an artefact of acid hydrolysis and also to obtain evidence of the nature of its linkage in the macromolecule, periodate oxidation followed by bromine oxidation of the derived aldehyde to carboxylic groups was carried out. 1,4-linked L-guluronic acid should under these conditions give rise to L-(+)tartaric acid while 1,4-linked D-mannuronic acid yields mesotartaric acid (Fig.10). In fact both the acids were isolated and characterised.[124] Similar results have also been obtained by the hypochlorite oxidation of alginic acid.[125]

Isolation and characterisation of 2,3-di-O-methyl-D-mannose, 1,6-anhydro-2,3-di-O-methyl-L-gulose and 2,3-di-O-methyl-L-gulose as the products of the reduced methylated polysaccharide[126] confirmed the 1,4-linkages in both D-mannuronic and L-guluronic acid residues in alginic acid. Other types of linkages were excluded by methylation studies of various samples of alginates.[127]

Despite the evidence that alginic acid is a linear molecule and is built up of 1,4-glycosidic linkages, it does not, under conditions that avoid over oxidation, consume more than 0.55 moles of periodate per anhydrohexuronic acid residue. Various explanations have been put forward to account for this discrepancy.[124,121] Recently, evidence has been advanced that the abnormal oxidation limit is due to inter-residue, inter-molecular hemiacetal formation.[128,129]

Sodium alginate on oxidation with 0.01M sodium meta-periodate consumed 0.45 moles of periodate per hexuronic acid residues. The oxopolysaccharide after reduction to poly-alcohol with sodium borohydride again became vulnerable to periodate and further oxidation proceeded smoothly up to a second oxidation limit, corresponding to a total consumption of 0.95 moles of periodate per hexuronic acid residue. A second reduction with borohydride then permitted further reduction of periodate, bringing the total consumption close to the theoretical limit of 1.0 moles per hexuronic acid

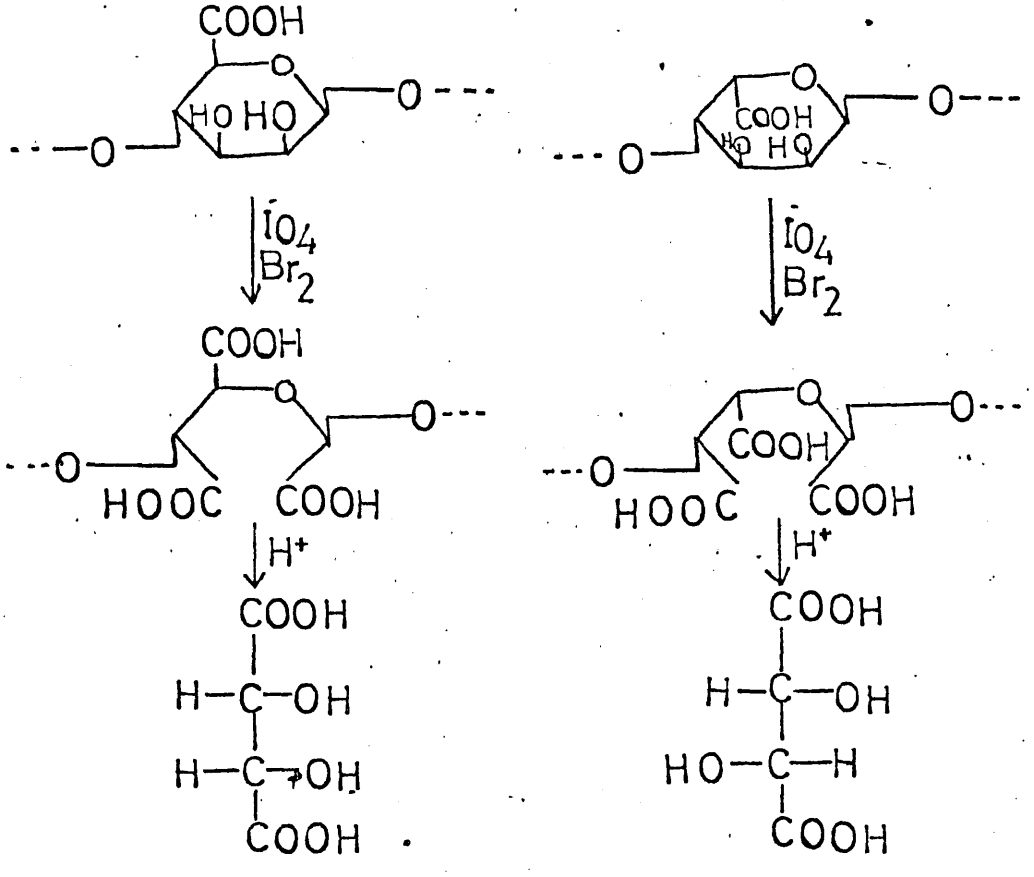


2,3-Di-O-methyl-D-mannuronic acid

2,3-Di-O-methyl-D-mannose

2,3-Di-O-erythcaric acid (see p.37)

Fig. 9



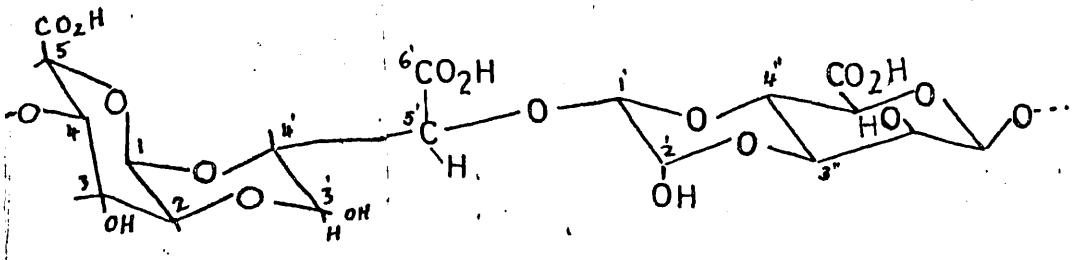
Mesotartaric acid

L-(+)Tartaric acid

Fig. 10

residue for a 1,4-linked polyuronide. Pre-treatment of the alginate with borohydride, however, did not alter the first oxidation limit of 0.45 moles per hexuronic acid residue.

Assuming (i) that oxidation initially proceeds in a random manner, (ii) that only one residue in the chain suffers oxidation at any instant of time, and (iii) that the two nearest neighbours are then instantaneously protected from the further oxidation, the oxidation limit obtained in practice was very close to the calculated value.[129] Explanations of these results were advanced on the basis of formation of an inter-residue hemiacetal ring.



An oxidised alginic acid chain showing single protection of two nearest neighbours

Although it is clear that alginic acid is built up from two types of monomers: D-mannuronic and L-guluronic acid residues, it has not been established whether it is a hetero or homoglycan. Fractionation into two homopolyuronides has not been achieved but partial fractionation into mannurone-

and gulurone-rich materials has been accomplished by precipitation of the former with potassium chloride[130] or by the addition of a mixture of manganous sulphate and potassium chloride[131] to a sodium alginate solution when the gulurone-rich fraction is precipitated.

In order to decide between homo and heteropolymeric nature of alginic acid it was subjected to partial acid hydrolysis.[132] From the hydrolysate mixtures of oligouronic and uronic acids were obtained. These results, however, gave no proof that both uronic acids occur in a single molecule. Further effort to solve this problem was made by Hirst et al.[133] Alginic acid was converted to di-O-propionylalginate which was then soluble in organic solvents and could be reduced with diborane in ether and the resulting nearly neutral polymer on partial acid hydrolysis gave some oligosaccharides. From these 4-O-β-mannosylgulose and β-1,4-mannobiose were isolated and characterised. These results indicated that at least some of the polymeric molecules contain both mannuronic and guluronic acid residues and also that mannuronic acid residues are adjacent.

Information about the sequence of uronic acid residues in the alginate molecule has been obtained by an elegant method of heterogenous hydrolysis.[134] Hydrolysis of sodium alginate from Laminaria digitata with M-oxalic acid (in which sodium alginate is insoluble hence it is considered

as a heterogenous hydrolysis) at 100° for about 10 hours dissolved about 30% of the total alginate indicating that some parts of the material were more readily hydrolysed than others. When the resistant (insoluble) material was dissolved in alkali and then the pH was adjusted to 2.85 in dilute sodium chloride solution, two fractions soluble and insoluble separated. Both the fractions were of DP about 20 units but soluble fraction was made up almost entirely of mannuronic and the insoluble fraction of guluronic acid residues.

When the resistant material was dissolved in alkali and reprecipitated as acid (but not fractionated) and hydrolysed for a further 10 hours about 19% of it dissolved, and the same happened after a second dissolution and precipitation and a third 10 hours period of hydrolysis.

The rate of formation of reducing end groups was the same during all three stages of hydrolysis, but a rapid depolymerization was observed in the first period indicating random attack on glycosidic linkages. A small reduction in the average DP of the insoluble material took place in the second and third periods suggesting the removal of hydrolysable material from the chain ends.

The hydrolysate from first ten-hour period contained both mannuronic and guluronic residues together with diuronides of two different mobilities. In contrast, the

hydrolysate of the second ten-hour or third ten-hour period contained only one diuronide and that too was of the same mobility as the minor component of the first ten-hour hydrolysis.

The major diuronide of first ten-hour period was found to be built up of mannuronic and guluronic acid residues while the major diuronide of the last two periods was composed of dimannuronic and diguluronic acid residues. These results thus indicate that alginate molecule contains blocks of mannuronic acid residues and guluronic acid residues and at the same time a portion with predominantly alternating structure.

To substantiate the results of heterogeneous hydrolysis, similar studies were extended to homogeneous hydrolysis[135] of alginate from the same species. Like the previous studies this too suggested that the alginate molecule is a block co-polymer of mannuronic and guluronic acids, 20-30 units long, separated by blocks having a high proportion of alternating mannuronic and guluronic acid residues.

_____....._____....._____

_____blocks containing essentially one type of residue, the resistant part.

.....blocks containing almost alternate residues, the hydrolysable part.

Fucoidan

Fucoidan, formerly known as fucoidin, like alginic acid, is one of the principal polysaccharides of brown algae. Its proportion in various algae depends on species and to a less extent on location and season.[136,137] Kylin[2] first isolated fucoidan from various species of Laminaria and Fucus. From the hydrolysate of fucoidan he separated fucose and characterised it as the phenyl-L-fucosazone and claimed that pentoses were also present in the hydrolysate.

Bird and Hads[138] isolated fucoidan by soaking fresh fronds of L. digitata in water and precipitating a crude fucoidan from the extract with ethanol. The material after purification gave 30.9% ash, 30.3% sulphate and 7.3% uronic acid. Since the total sulphate content was approximately double that found in the ash, fucoidan was considered to be a carbohydrate ethereal sulphate.

At the same time another water soluble polysaccharide very similar to fucoidan was isolated from the giant Macrocystis pyrifera[139] and was found to contain L-fucose, a high proportion of half ester sulphate and about 2.6% uronic acid.

Fucoidan was also obtained from the exudate from freshly collected L. digitata.[140] After purification from boiling ethanol, a product containing 26-30% ash of which the sulphate content was about half the total of sulphate in the poly-

saccharide was obtained. The main inorganic cation was sodium. Distillation with hydrochloric acid gave a value corresponding to only 33-37% of the methylpentose for a formula $(RR'-O-SO_2-OM)n$, where R, fucose; R', other sugars and M, metal.

Advancement on the structure of fucoidan was made by Percival and Ross.[141] They examined fucoidans from F. vesiculosus, F. spiralis, L. hyperborea and Himanthalia lorea by extracting with hot water. It was purified to remove alginate and protein, by lead acetate and subsequent treatment with barium hydroxide led to the separation of the fucoidan-lead complex which was decomposed with dilute sulphuric acid. The resulting solution was freed from a considerable amount of colouring matter by filtration through Filter-cel. The hydrolysate of F. vesiculosus was found to contain pentose in addition to fucose.

A crude fucoidan from H. lorea[141] was purified after dialysis and concentration, by precipitation with alcohol. Further purification was carried out by treatment with charcoal and Filter-cel. The fucoidan isolated had $[\alpha]_D = -140^\circ$, and contained approximately 44% fucose, 32.5% sulphate, 22.5% ash and 7.0% metal. In contrast to the findings of Lund et al[140] the main inorganic ion was found to be calcium. Since calcium fucan monosulphate requires fucose 66%,

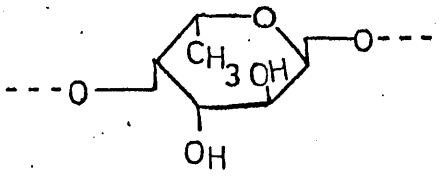
sulphate 39.0% and calcium 8.0%; 44% fucose was rather low. Prolonged search by various methods including periodate oxidation of the hydrolysis products and chromatographic analysis, failed to show the presence of more than small quantities of any other sugars. This discrepancy was accounted for when it was observed that the polysaccharides retained water (ca 10%) and ethanol (ca 6%) despite drying at 40°/ 0.1 mm for 18 hours. After making corrections for the absorbed solvents the most highly purified samples of H. lorea fucoidan gave sulphate 38.3% and metal 8.2% and from fucan hydrolysate the following percentage of sugars were obtained: fucose, 56.7%; uronic acid 3.3%; xylose 1.5% and galactose 4.1%. These values are in close agreement with calcium fucan monosulphate and these results indicate that the principal constituents of the fucoidan are fucose residues and sulphate groups. The small proportions of other sugar residues were considered to be impurities.

Similarly, fucoidan[142] was obtained from F. vesiculosus but the process of purification was relatively less intensive. It had $[\alpha]_D = -118^\circ$, fucose 38% and sulphate 32.8%. It was found by alkali treatment that approximately 10% of the ester sulphate was alkali labile indicating that only a small proportion of the sulphate groups were linked to C-2 or C-3 of 1,4-linked fucose residues. This also requires that the main linkage of L-fucopyranose units in fucoidan cannot be

1,4-linked, since this would render sulphate groups alkali labile (Fig.11a).

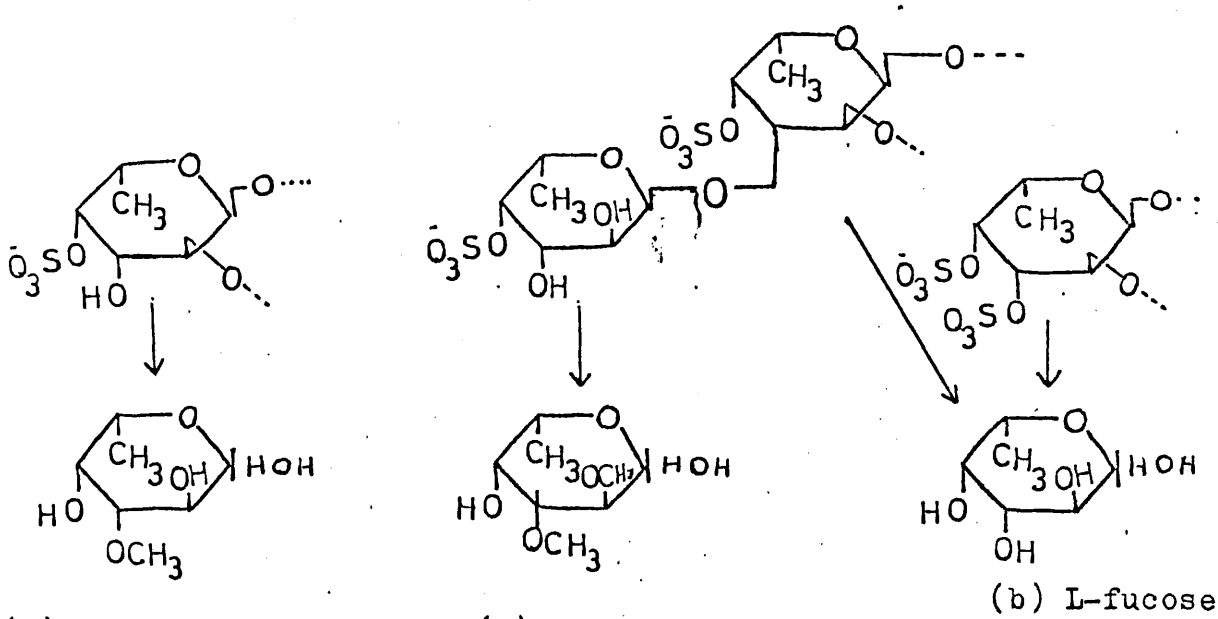
The material on methylation yielded a polysaccharide (% OCH_3, 15.8%) which on hydrolysis gave 3-O-methyl-L-fucose (57%), free L-fucose (20%) and 2,3-di-O-methyl-L-fucose (20%). Since the major proportions of sulphate groups are stable in alkali and 3-O-methylfucose is the major products of methylation, fucose residues in the native polymer must be 1,2-linked with sulphate group on C-4. The strongly negative rotations of fucoidan and its derivatives indicate that these linkages are predominantly of the α-type (Fig.11). Excluding the possibility of incomplete methylation these authors suggested that free fucose can arise from a disulphated residue (Fig.11b), from the proportion of sulphate this is necessary, at the same time it could be derived from a branch-point carrying a terminal group having free hydroxyl on C-2 and C-3. From the proportions of various fucose derivatives isolated there could be an average of one such branch for every five fucose residues (~~Fig.~~

Degradative studies by O'Neill[143] and by Côte[144] confirmed these findings. Fucoidan prepared from F. vesiculosus was subjected to mild acetolysis and the resulting product was converted to acetylated alditols. Column chromatographic separation led to the isolation of a reduced disaccharide which was deacetylated and characterised by periodate oxidation as a 2-O-α-L-fucopyranosyl-L-fucitol (Fig.13)p 51.



(a) α-1,4-linked-L-fucose Unit

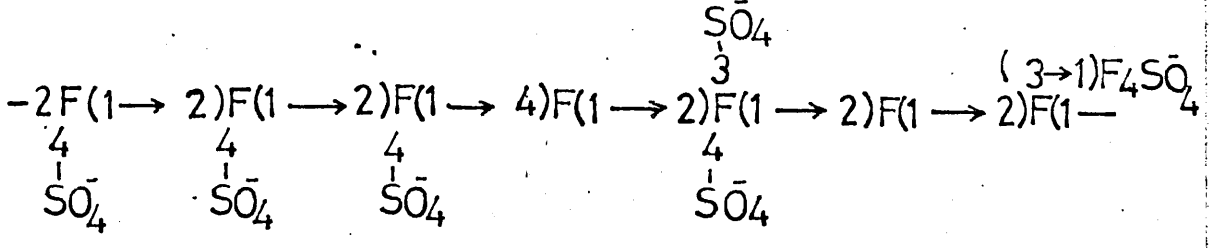
Fig. 11



(c) 3-O-methyl-L-fucose (d) 2,3-di-O-methyl-L-fucose

(b) L-fucose

Fig. 11



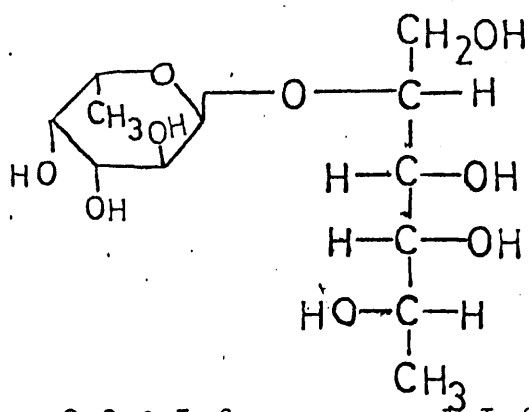
F, L-fucopyranosyl

Fig. 12

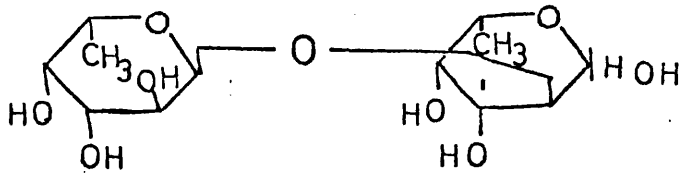
A commercial sample of fucoidan from the same species was partially hydrolysed after acetolysis. From the hydrolysate not only 1,2-linked fucobiose but also appreciable amounts of 1,4-linked fucobiose and a small proportion of 1,3-linked fucobiose were isolated by Côte. These results substantiate the structure of fucoidan as proposed by Conchie and Percival,[142] but at the same time suggest the possibility of significant proportions of 1,4-linkages in the fucoidan (Fig 13). The author, however, suggested that the two types of linkages might arise from two different polysaccharides.

From the foregoing discussion it is established that fucoidan is generally regarded to be a polymer of 1,2-linked-L-fucose residues with a small proportion of other linkages. Sulphate groups are usually at C-4 and often at C-3 and C-4. The other sugar residues are considered to be present as impurities (Fig 12. p49).

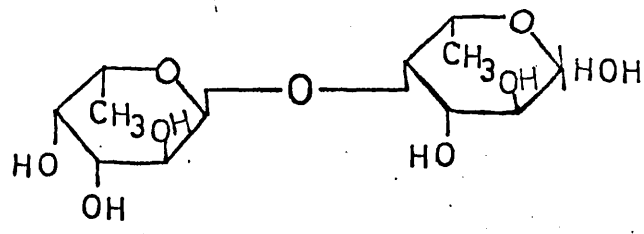
Later work on fucoidan confirmed this structure, but at the same time indicated that the other sugar residues might constitute an integral part of the fucoidan macromolecule. Dillon and his co-workers[141] isolated a sulphated polysaccharide from the seed mucilage of Ascophyllum nodosum with dilute hydrochloric acid. On hydrolysis this gave fucose and galactose in the ratio 8 to 1. Fucoidan from F. vesiculosus was fractionated in Bangor on a DEAE-cellulose column and resulted in the separation of a small quantity of pure xylan in the first instance, but in the large scale separation



2-O-α-L-fucopyranosyl-L-fucitol



1,2-linked fucobiose



1,4-linked fucobiose

Fig. 13

the xylan fraction was found to contain uronic acid residues.[146]

Fucoidan from the exudate of M pyrifera was examined by Schweiger after various purifications by precipitation in isopropylalcohol and by treatment with charcoal and Filtercel.[147] From crude and purified samples of fucoidan the proportion of fucose to galactose was almost constant at 18 to 1. The proportion of fucose to xylose, however, varied between 0.5 and 2.5%. These results indicated that the fucoidan of M. pyrifera was a heteropolymer with L-fucose and D-galactose residues. No uronic acid residue was obtained in the hydrolysate of this polymer.

About the same time Bernardi et al.[148] obtained purified fucoidan from F. vesiculosus by sequential fractional precipitation with ethanol containing 0.3% sodium acetate; ethanol and acetone, and with cetyldimethylbenzylammonium chloride. Two fractions were obtained having respectively, fucose 63 and 65%, ester sulphate 30 and 29%. Both on hydrolysis showed the presence of fucose, trace quantities of xylose and an unidentified fast moving spot with chromatographic mobility slightly different from 3-O-methylfucose. These fractions were devoid of uronic acid and galactose residues.

A highly purified fucoidan was isolated from Pelvetia wrightii[149] by dilute hydrochloric acid extraction after

methanol extraction of the weed. Purification of the fucoidan was achieved by treatment with calcium chloride solution and then by fractionation using cetylpyridinium chloride solution. The purified fucoidan had $[\alpha]_D = -141^\circ$, sulphate 36.8% and ash 26% and was comprised of fucose and galactose sugar residues in a ratio of approximately 10:1. This ratio was independent of the degree of purification. There was no evidence of uronic acid and other sugar residues in the fucoidan, and it was considered to be a galactofucan sulphate.

The heterogeneity of the fucoidan has been shown by free boundary electrophoresis on the material extracted from *F. vesiculosus*[143] and from *A. nodosum*.[150] Two bands were observed in the former case and the latter showed three components in citrate buffer at pH 2.

Recently, fucoidan has been separated from *A. nodosum* with dilute hydrochloric acid at room temperature. The isolated polysaccharide was degraded with dilute hydrochloric acid at 80° and subsequent fractionation was achieved with aqueous magnesium chloride and ethanol. The magnesium soluble fraction was the fucoidan which, the authors believe, had resulted from the degradation of a complex macromolecule in the initial acid extract. On the other hand, a similar acid extraction of *F. vesiculosus* gave free fucoidan together with the complex macromolecule. Fucoidans of both the weeds contain galactose and glucuronic acid in small proportions.[151]

Proof that C-4 of L-fucose in fucoidan is sulphated has recently been obtained by the isolation of L-fucose 4-sulphate from products of partial hydrolysis of P. wrightii fucoidan.[152]

It emerges from the above discussions that other sugar residues such as glucuronic acid, xylose and galactose in fucoidan not only vary from species to species but also from sample to sample in the same species depending on the method of extraction or fractionation. Significantly, there has been no rigorous effort to find out the arrangement nor the mode of linkages of these sugar residues in the macromolecule.

The situation has become more confused by the separation of at least three distinct fucose-containing polysaccharides from A. nodosum.[153] All these polysaccharides contain varying proportions of fucose, xylose, glucuronic acid and ester-sulphate. Since no fraction contains less than 5% uronic acid these are dealt with under glucuronoxylofucans.[153, 155]

Glucuronoxylofucans. Larsen et al[153] examined fucose containing polysaccharides of A. nodosum. Dilute sodium hydroxide treatment, after removal of the dilute acid soluble polysaccharides, of the weed gave a mixture of alginic acid and glucuronoxylofucans. The alginic acid was removed by adjusting to pH 1.3-1.5 with dilute hydrochloric acid and the glucuronoxylofucans were recovered by fractional precipitation of the supernatant. The major fraction, about 6% of the dry

weed, was termed by the authors 'ascophyllan'. It has approximately 25% fucose, 26% xylose, 19% uronic acid, 13% sulphate and 12% protein. Two other fractions F1 and F2, each about 1.5% of the weed, differ from ascophyllan only in the relative proportions of the constituents. Both ascophyllan and F2 were electrophoretically homogeneous while F1 was not pure and has a composition somewhat between ascophyllan and F2. All three fractions gave similar chromatographic patterns on autohydrolysis although variation in the intensity of various products was apparent.

Ascophyllan on hydrolysis in addition to L-fucose, D-xylose and D-glucuronic acid gave 18 common amino acids derived from the polypeptide portion. Attempts to fractionate the polypeptide from the carbohydrate were unsuccessful. This together with the electrophoretic homogeneity of ascophyllan suggest a chemical linkage between the carbohydrate and polypeptide moieties of ascophyllan. Further evidence of these linkages was derived from mild hydrolysis with 0.5M-oxalic acid. This cleaved the peptide linkages as well as the glycosidic linkages of the polysaccharide, and resulted in a mixture of dialysable mono-and oligosaccharides and non dialysable degraded polysaccharides. The latter contained almost all the uronic acid present in the ascophyllan but was almost devoid of fucose, xylose and half ester sulphate. From these results the authors deduced that ascophyllan is comprised ofaglucuronic acid backbone to which are attached

relatively long side chains of sulphated fucose and xylose residues. Later on 3-O-β-xylopyranosyl-L-fucose has been identified as one of the hydrolysis fragments indicating that the side chains are heteropolymeric.[154]

Polysaccharide material containing the same sugar residues as in ascophyllan was obtained from the same weed after extraction of the dried weed with hot water and dilute alkali. The formaldehyde treated residual weed was extracted with ammonium oxalate-oxalic acid at pH 2.8. The contaminating alginic acid (ca 10%) was removed from this crude extract as the insoluble calcium alginate. The remaining pure material, termed glucuronoxylofucan, had $[\alpha]_D = -225^\circ$ and gave fucose 49%, xylose 10%, uronic acid 12%, sulphate 21% and protein 4%.[155]

Attempted fractionation of this glucuronoxylofucan by precipitation with ethanol or copper acetate, or on columns of DEAE-cellulose, Sephadex 200G and Biogel was unsuccessful. It was noted that a considerable proportion of the polysaccharide was irreversibly bound on the columns. Since it defied fractionation it was considered to be a heteropolysaccharide.

Partial acid hydrolysis with M-oxalic acid solution led to the isolation of an aldobiouronic acid which was characterised as 3-O-(β-D-glucopyranosyluronic acid)-L-fucose(Fig14).

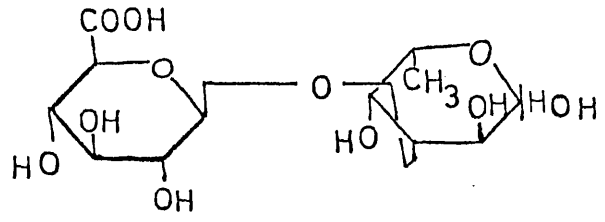


Fig. 14

Autohydrolysis of the free acid polysaccharide in a dialysis sac gave a different pattern of oligosaccharides and enabled tentative characterisation of 3-O-β-D-xylopyranosyl-L-fucose and 4-O-α-L-fucopyranosyl-D-xylose. The higher acidic oligosaccharides on further partial acid hydrolysis gave among other products the above aldobiuronic acid.

Since 0.5M-oxalic acid hydrolysis of ascophyllan cleaved most of the fucose and xylose and left a glucuronic acid backbone similar hydrolysis of glucuronoxylofucan was carried out. The degraded polymer was recovered from the hydrolysate and was compared with the materials obtained from longer hour hydrolysis as well as from autohydrolysis. Chromatographic examination of each of these degraded polymers showed the presence of fucose and glucuronic acid residues as the major constituents with lesser amounts of xylose. In addition to these degraded polymers, insoluble material was recovered after each operation, which had low carbohydrate content. On hydrolysis it gave fucose (major constituent), with appreciable proportions of glucuronic acid and xylose together with small proportions of oligouronic acids.

These results indicate that unlike ascophyllan, glucuronic acid does not appear to form the backbone of this glucuronoxylofucan and 3-O-(β-D-glucopyranosyluronic acid)-L-fucose is a major structural feature.

Glucuronoxylofucans have also been reported from L. hyperborea and from F. vesiculosus.[151 156]

These studies indicate the type and variety of algal fucose-containing polysaccharides. In the light of the newer polysaccharides - glucuronoxylofucans - it is necessary to examine not only new genera of algae but also those which have been investigated to ascertain if glucuronoxylofucans are universally present in the brown algae. This dissertation aims in this direction and includes algae of widely different morphology (as described in p.73). Apart from a study of the fucoidan in H. lorea very little is known about its carbohydrate metabolites. Part one of this thesis, therefore, deals with the overall general investigations of these algae and part two is concerned with the polysaccharides based on fucose, glucuronic acid, xylose and galactose residues.

The species investigated are: Himanthalia lorea (elongata) (family Himanthaliaceae), Bifurcaria bifurcata (family Cystoseiracea) and Padina pavonia (family Dictyotaceae).

E X P E R I M E N T A L

GENERAL PROCEDURES (G.P.)

G.P.I.

(a) Paper chromatography. Unless otherwise stated the paper chromatograms were carried out on Whatman No.1 chromatography paper in descending technique.

(b) Principal Solvents (v/v) for chromatography were:

1. Ethylacetate-acetic acid/formic acid-water (18:3:1:4)
2. Butanol-ethanol-water (40:11:19)
3. Butanol-pyridine-water (6:4:3)
4. Butanol-pyridine-water-benzene (5:3:3:1)
5. Methylethylketone-acetic acid-water-boric acid (9:1:1-sat boric)
6. Fisher and Dorfel.

   Trough: Ethylacetate-pyridine-acetic acid-water (5:5:1:3)

   Tank: Ethylacetate-pyridine-water (40:11:6)
7. Methylethylketone-water (saturated)
8. Butanol-pyridine water (10:3:3)

(c) Developing agents: The following reagents were used to develop the chromatograms either as dips or sprays:

1. Silver nitrate solution[157] (dip):

Dip (i) 1 ml saturated silver nitrate (aqueous) in acetone (200 ml). Water was added dropwise till the precipitate redissolved.

Dip (ii) Sodium hydroxide solution (5 g NaOH in 15 ml water).

Dip (iii) Aqueous sodium thiosulphate solution (10%).

2. Aniline oxalate solution (spray). Saturated solution of aniline oxalate in aqueous alcohol (50%).

Aniline oxalate solution (dip). Saturated solution of aniline oxalate (from 2) diluted with acetone (three volume).

3. Glucose oxidase.[158] (dip/spray. Worthington Biochemical Corporation)

Preparation: The content of one chromogen vial (ca 1/4 of the total) was dissolved in 0.1M phosphate buffer (20 ml, pH = 7.0). The content of one glucostat vial was dissolved in 0.1M phosphate buffer (10 ml, pH = 7.0) and was mixed with the dissolved chromogen. The final volume was made to 50 ml with the same phosphate buffer.

4. Periodate-permanganate spray.[159] (i) Sodium meta-periodate solution (2%) (ii) Potassium permanganate solution (1.0%) (i) and (ii) were mixed in a ratio of 2:1 (v/v) just before use. When the desired spots appeared (at room temperature) the chromatogram was washed with distilled water.

5. Tetrazolium spray.[160] The spray was freshly prepared 1:1 mixture of 2% triphenyltetrazolium hydrochloride and N-sodium hydroxide solution. The paper was heated at 40°

on a water saturated atmosphere for 10 minutes after it was sprayed. Intense red colour indicates the absence of 1,2-linkage.

6. Aniline-diphenylamine spray.[161] (i) Aniline (4 ml) in acetone (100 ml) (ii) Diphenylamine (9 ml) in acetone (100 ml). Solution (i) and (ii) were mixed with ortho-phosphoric acid (85%, 20 ml) and kept at 0°. The chromatogram was dipped in solution and heated at 80° for 2-3 minutes. Intense blue colour indicates 1,4-linkage and greyish-green colour indicates 1,3-linkage.

7. Deoxy sugar spray.[162] (i) 0.02M sodium metaperiodate solution in water (ii) Ethylene glycol (50 ml), acetone (50 ml) and concentrated sulphuric acid (0.3 ml) (iii) A solution of thiobarbituric acid (6%) solution (the acid is insoluble and is dissolved by adding conc. sodium hydroxide solution dropwise).

The chromatogram was sprayed with (i) and after 15 minutes with solution (ii) followed by, after 10 minutes, solution (iii). On heating at 105° for about 5 minutes intense red colour indicates 2-deoxy-sugar.

(d) Chromatographic mobilities.

$$R_G = \frac{\text{Distance travelled by sugar residue}}{\text{Distance travelled by 2,3,4,6-tetra-O-methylglucose}}$$

$$R_X = \frac{\text{Distance travelled by sugar residue}}{\text{Distance travelled by sugar X}}$$

G.P. II.

(a) Evaporations. All evaporations were carried out in vacuo on a rotatory evaporator below 40°.

(b) Hydrolysis of polysaccharides. Unless otherwise stated hydrolysis was carried out with formic acid (90%) in a sealed tube in an atmosphere of carbon dioxide for 6 h at 100°, followed by hydrolysis of the formylesters by dilution (5 vol) and further heating for 2 h at 100°.

(c) Reduction. All reductions were carried out with potassium borohydride in aqueous solution. The alkaline solution was neutralised with IR-120(H^+) and boric acid was removed by repeated evaporation with methanol.

(d) Dialysis. Dialysis of polysaccharide solutions were made in cellophane tubes in a closed system against deionised water with stirring and by changing the water from time to time. A few drops of toluene were added to prevent bacterial action whenever necessary.

(e) Melting point. Melting points were taken on a Kofler microscope melting point apparatus and were corrected.

(f) Rotation. All specific rotations are equilibrium value and unless otherwise stated are taken in aqueous solution at room temperature with sodium light.

G.P. III.

(a) Phenol-sulphuric acid method for standard graph.[163] An aqueous solution containing the respective sugar was measured

into test tubes (6 x 5/8") and diluted to 1 ml with deionised water. Phenol solution (4%, 1 ml) was added to each tube, followed by Analar conc. sulphuric acid (5 ml) from a fast delivery pipette, the stream of acid being directed on to the solution to obtain a thorough mixing resulting in a maximum rise of temperature. On cooling the optical density of the solution was measured on a Unicam spectrophotometer Sp. 500 against a blank, prepared by using water in place of sugar solution, at 487nm .

A standard graph was obtained by plotting µg sugar per ml against the optical density.

(b) The carbohydrate content. The carbohydrate content of the polysaccharide was determined by Phenol sulphuric acid method (G.P. III a). The amount of carbohydrate was determined from a standard graph of the appropriate mixture of sugars.

G.P. IV. Estimation of sulphate content.[164]

(a) Standard graph for the determinations of sulphate using potassium sulphate.

Anhydrous potassium sulphate (200 mg) was dissolved in deionised water (500 ml) and the different proportions of the solution (0.0 - 0.8 ml) were taken in test tubes and each was made to 1 ml by adding required proportions of aqueous hydrochloric acid. To each was added 1 ml of 0.19%

4-chloro-4-aminodiphenyl hydrogen chloride and a few mg of cetyltrimethyl ammonium bromide. The mixture was allowed to stand for 2 h at room temperature. Each was centrifuged for a few minutes. An aliquot (1 ml) was taken from each tube and made up to 100 ml. Optical density was measured at 254 nm (using silica cell and deuterium lamp). Plotting the difference of absorbance against the µg of sulphate per ml. of solution a straight line was obtained.

(b) Sulphate content of polysaccharides. The polysaccharides (10 mg) was digested in a sealed tube with Analar nitric acid (1 ml, plus few mg of sodium chloride) for 18 h on a boiling water bath. The solution was evaporated to dryness and treated with conc. hydrochloric acid, evaporated to dryness and left in an oven at 105° for 2 h. The residue was then dissolved in water (1 ml) and aliquots (0.1 - 0.5 ml) were taken separately and sulphate was determined as described in G.P. IV(a).

(c) Estimation of sulphate by cetylpyridinium (CPc) method.[165] Polysaccharides (0.5 - 2.0 mg) in deionised water (1-2 ml) was treated with aqueous cetylpyridinium chloride (0.1%) solution from a microburette. The solution was viewed against a dark background. As the addition was continued dropwise there was an increase of opacity and suddenly with a few drops the

coagulation settled into a precipitate. This was taken as the end point. Often the body of the solution was turbid, with further addition of a drop or two of cetylpyridinium chloride solution the supernatant was clear.

Since the polysaccharides contained both carboxylic and sulphate ester groups, two titrations were carried out in parallel (1) with cetylpyridinium chloride solution and aqueous polysaccharide solution (titration A) and (2) with polysaccharide solution in 0.025M sulphuric acid (titration B). The sulphate content was determined from titration B while uronic acid content was made by subtracting the value of B from that of A.

G.P. V. Preparation of a Cellulose column.[166]

(a) (i) Diethylaminoethyl-cellulose (DEAE-50) was suspended in 0.5N hydrochloric acid (50 g in 1L HCl solution) in a suction flask. This was then kept under vacuum for about 20 minutes to deaerate, after 20 minutes standing the supernatant was decanted, then the swelled mass filtered on a Buchner funnel and was washed with water to neutrality.
(ii) The wet material was treated in the same way as in (i) with 0.5N sodium hydroxide solution. The operations (i) and (ii) were repeated thrice. In the last alkali treatment the washings were not carried out on a Buchner funnel. The material was transferred to a column (30 x 4.5 cm) and washed with water to neutrality.

(iii) The column was equilibrated with 0.5N potassium chloride solution (2L) and washed with water till free from chloride.

(b) DEAE-52 (50 g, microgranular, preswollen, containing ca 75% moisture) was treated with 0.5N hydrochloric acid (600 ml) and deaerated for 20 minutes with occasional shaking. It was then neutralised with a saturated solution of potassium hydroxide and was transferred to a column (30 x 4.5 cm). It was equilibrated with potassium chloride solution (0.5N 1-1.5 L) and was washed with water until free from chloride.

G.P. VI. Methylation,[167] Hydrolysis and Glycosidation.

(a) Preparation of methylsulphinylcarbanion. Into a 3-necked flask fitted at one neck with a rubber serum cap and containing glass magnetic stirrer bar, was weighed sodium hydride (1.5 g, 55% coated with mineral oil). The hydride was washed with n-pentane (30 ml) and the supernatant was removed by decantation. After three washes the flask was fitted with a thermometer and a stoppered condenser. The residual n-pentane was removed by successive evacuation through an 18 gauge needle inserted into the serum cap. After each evacuation the flask was regassed with nitrogen and finally nitrogen was passed through the flask via the needle. Using a hypodermic syringe dimethylsulphoxide

(15 ml) [distilled over calcium hydride under reduced pressure and stored over dried molecular sieve (type 4A)], was transferred into the flask. The flask was heated on an oil bath at 50° with constant stirring until the solution became clear greenish coloured and evolution of hydrogen gas ceased (45-50 min.).

Concentration of the anion in dimethylsulphoxide was determined by withdrawing an aliquot (1 ml) and titrating it with 0.1N hydrochloric acid using phenol-phthalein indicator. The anion solution was preserved in a serum bottle in an atmosphere of nitrogen in the cold and was stable for 12-15 weeks.

(b) Methylation. Polysaccharide (5 mg) in 7 ml serum bottle sealed with a rubber cap, was dissolved (wherever necessary by stirring or shaking overnight, in some cases it was necessary to start with a good suspension) in dimethylsulphoxide (1.5 ml). Nitrogen was flashed through the bottle and a 2M solution of methylsulphinylcarbanion in dimethylsulphoxide (1 ml) was added dropwise with a syringe. The resulting gelatinous solution was agitated in an ultrasonic bath for 1 h and allowed to stand for six hours (or overnight) at room temperature. Methyl iodide (0.1 ml) was then added dropwise with a syringe with external cooling by tap water and the resulting turbid solution was agitated for 20 minutes in the ultrasonic

bath. A second portion of 2M methylsulphinylcarbanion (1 ml) was added and the procedure was repeated except an excess of methyl iodide (1.0 ml) was added. The solution was then poured into water (25 ml), dialysed extensively and finally evaporated to dryness.

(c) Hydrolysis.[168] The hydrolysis of the methylated polysaccharides unless otherwise stated, was according to G.P. II (b)

(d) Glycosidation.[169] This was carried out by the method of Bollenback.

G.P. VII. Determination of the uronic acid content.

(a) Modified carbazole method. Uronic acid was determined by modified carbazole method according to Bitter and Muir.[170]

Reagents. (i) 0.025M sodium tetraborate, $10.H_2O$. Analytical grade in conc. sulphuric acid (sp. grav. 1.84)

(ii) 0.125% carbazole in absolute ethanol or methanol and

(iii) Glucuronolactone solution (4-40 mg/ml) was prepared by dilution with deionised water saturated with benzoic acid.

Preparation of a standard graph of glucuronic acid. The sulphuric acid reagent [(i) 5 ml] was cooled at 4°, sample (1 ml) was carefully layered onto the acid. The tube was stoppered with a ground glass stopper and shaken first gently and then vigorously with constant cooling. The tube was then heated

for 10 minutes in a vigorously boiling water bath and cooled to room temperature. The carbazole reagent (ii, 0.2 ml) was then added, shaken well, heated on the boiling water bath for 15 minutes and cooled to room temperature. The optical density (OD) was then measured at 530 nm. The OD of the blank was less than 0.025 against conc. sulphuric acid. The quantity of the glucuronic acid against OD gave a straight line passing through the origin.

Similarly, standard graphs for two synthetic mixtures of fucose:xylose:glucuronic acid residues at ratios 3:1:1 and 5:1:1 were obtained for the estimations of uronic acid content in the respective polysaccharides.

(b) By cetylpyridinium chloride method as in G.P. IV(c).

G.P. VIII. Gas liquid chromatography (g.l.c.). This was carried out in a Pye Argon gas chromatograph using argon as the mobile gas phase. The gas flow and the temperature were varied according to the requirement for a particular case. The major columns used for analytical work were:

1. Butane-diol-succinate polyester (B.D.S.) 15% by wt.
2. Polyphenyl ether [m-bis-(m-phenoxy phenoxy)benzene] (PPE) 10%.
3. Apiezon K (Apk) 7.5%.
4. SE 30 3%
5. ECNSSM 3%
6. Ethylene glycol adipate polyester 10%.

All liquid phases were coated on Celite supports which were previously acid and alkali washed and coated with dimethyldichlorosilane.

G.P. IX (a) Preparation of Trimethylsilylether[171] (TMS). The dry material (5-10 mg) was dissolved in pyridine (1 ml) and to it was added hexamethyldisilazane (0.2 ml) and trimethylsilylchloride (0.1 ml). The solution was shaken for 2-3 minutes and then centrifuged. The supernatant was removed and evaporated to dryness. The residue was dissolved in n-hexane and analysed on g.l.c.

(b) Preparation of methyl/alditol/acetate or alditol/acetate.[172] Methylated sugars or free sugars (1 mg) were reduced in water (5 ml) with sodium borohydride (10 mg) for 2 h. After treatment with IR-120(H^+) and concentration, boric acid was removed by repeated evaporation with methanol and the product was treated with acetic anhydride-pyridine (1:1)(ml) at 100° for ten minutes. The acetylation mixture was diluted with water, concentrated to dryness and dissolved in chloroform for analysis on g.l.c.

G.P. X. Esterification, reduction and hydrolysis. The dry sample was esterified with methanolic hydrogen chloride (3-4%) by heating under reflux for 7 h. Methanolic solution was neutralised with silver carbonate, filtered and the

precipitate was thoroughly washed with methanol. The combined filtrate and washings were evaporated to dryness. Reduction was carried out according to G.P. II (c) and hydrolysis of the sugar glycosides was carried out as stated in G.P.II (b).

G.P. XI. Ionophoresis. Unless otherwise stated ionophoresis was carried out on Whatman No.3 paper using pyridine-acetic acid-(aq) buffer (pH = 6.7) under 2000 volts for time specified for appropriate analysis. The paper was developed with silver nitrate dip (G.P.I (c)(i).

G.P. XII. Thin layer chromatography. This was done by using an emulsion of Kieselgel G on a glass plate. The bed thickness was approximately 0.25 mm. Iodine was used to detect the carbohydrate.

G.P. XIII. Gel Electrophoresis. Gel electrophoresis was carried out according to the method of Steward et al.[173]

G.P. XIV. Test for unsaturated acid.[174] (a) Reagent. (i) 0.025N Sodium metaperiodate in 0.125N sulphuric acid, (ii) 2% sodium arsenate in 0.5N hydrochloric acid solution, and (iii) 0.3% 2-Thiobarbituric acid in water (pH = 2.0).

(b) Procedure. Sample solution (0.5 ml) was mixed with solution (i) 0.5 ml) and allowed to stand for 20 minutes after which solution (ii) (10 ml) was added and after shaking

was allowed to stand for 2 minutes. Then solution (iii) (4 ml) was added and the mixture heated for 10 minutes at 100°. A pink colour indicates positive test. The optical density of the resulting solution was measured at 510 to 590 nm On plotting optical density (OD) against wavelength the point of maxima was obtained at 548-554 nm for 4,5-unsaturated acid.

G.P. XV. Demethylation.[175] A portion of the material (ca. 3·0 mg) was dissolved in dichloromethane (1-2 ml) and cooled to -80°. Borontrichloride (1-2 ml), cooled to -80°, was then added. The mixture was kept at -80° for 30 minutes with calcium chloride guard tube. It was then allowed to stand at room temperature overnight under anhydrous conditions. The remaining trace of solution was removed under diminished pressure at room temperature. The residue was treated with methanol (3 x 3 ml) and was evaporated to dryness. The residue was dissolved in water for chromatographic analysis.

G.P. XVI. Determination of the Degree of Polymerisation (DP) This was done according to the method of Timell.[176]

G.P. XVII. Nitrogen analysis. Nitrogen analysis was done by Alfred Bernhardt Microanalytical Laboratory, West Germany.

Characteristics of the Species Investigated.[177]

Himanthalia lorea is abundant near low tide level on moderately exposed shores, often growing attached to cliff-faces. The thallus is sharply differentiated into sterile and fertile portions. The former is the perennial frond which arises from a small disc like hold fast, the shape of the frond being dependent upon the level of the growth. It is short and stumpy when it grows exposed at high levels, whilst it is more elongated at the lower levels when the plants are submerged for longer periods. The thallus although only 1-2 cm broad, can reach a length of 2 metres. The thallus may be considerably branched.

Bifurcaria bifurcata is frequently found on the Atlantic shores of Europe and Africa, and has a richly branched perennial rhizome attached to the substratum by small adhesive discs. Some branches bend up and give rise to forked smooth shoots which grow to a height of 30-40 cm and die down in late autumn. Despite constrictions which occur at the points of branching it is considered to be monopodial and bilateral.

Padina pavonia is a rare perennial found in mid-tide level on the southern shores of England, but it is abundant in the Mediterranean and other warm seas. The numerous stalked fan-shaped fronds, 5-12 cm high, the paper thin larger ones, often loosely rolled on their longitudinal axis like a cornet,

are distinctive of all the species of the genus. The stalk of each frond is the upward continuation of a branch of the prostrate perennial rhizome, which is richly branched and attached to the substratum by tufts of rhizoids. The stalk of the fan bears a considerable number of laterals towards the upper end.

Part I

Extraction Procedures and Overall Composition of the Carbohydrates of the different Genera

Experimental

Materials. H. lorea: The first batch was collected on the west coast of Spain in September, 1968. It was treated with dilute formaldehyde solution after collection, sun dried and was stored in the cold for about three weeks before use. Subsequently it was obtained from Millport, Scotland in September, 1969. The tips and the button or disc were removed from the healthy thallus before use.

B. bifurcata. This was also collected on the west coast of Spain in September, 1968 and was treated like H. lorea. It was stored in the cold for six months before use. A second batch was collected from Porthloo, St. Mary's Isle, Scilly in 1970 and was treated like the first batch.

P. pavonia. This was also collected in Spain at the same time and from the south of England in August. The dried material was stored at room temperature for about a year before use.

Air dried H. lorea, B. bifurcata and P. pavonia were separately ground in liquid nitrogen.

Expt. 1. Extraction of the weed with cold aqueous alcohol. The powdered weed (50 g) was extracted with aqueous alcohol (80%) with constant stirring at room temperature for 5 h.

The residue was separated by filtering through muslin, washed once with aqueous alcohol and then extracted again with aqueous alcohol for 4 h. The process was repeated four times. The filtrate and washings were combined and concentrated to a suitable volume for investigations.

Expt. 2. Extraction of the residual weed with hot aqueous alcohol. The residual weed after cold aqueous alcohol extraction was further extracted with aqueous alcohol (80%) at 70° under reflux for 4 h in the same way as in Expt. 1. The filtrates and the washings were treated similarly.

Expt. 3. Examination of the aqueous alcoholic extracts. (a) Both cold and hot aqueous alcohol extracts were separately evaporated to a suitable volume and allowed to stand at room temperature overnight. This resulted in an oily layer, a light green coloured solution and a sediment. The oily layer was separated and the sediment (fine particles of weed) was removed. The light green solution thus obtained was concentrated further and poured into excess alcohol when a precipitate (A) was instantaneously deposited, a further flocculent precipitate (B) was formed on standing. The latter precipitate was separated from the former by careful decantation. The alcoholic supernatant was concentrated and poured into alcohol and these operations were repeated until there was no further precipitation. Precipitate (A) proved

to be inorganic material. The flocculent precipitate (B) was shown to be mannitol by paper chromatography (solvent 1 and 5 with dip 1 and spray 4).

Characterisation of mannitol. The crude mannitol was dissolved in water and the light brown solution was treated with decolourising charcoal, filtered, treated with IR-120(H^+) resin and recrystallised from aqueous alcohol. It had m.p. 160-162°, mixed m.p. (with authentic mannitol) 160-162°. It had $[\alpha]_D$ = -0.25° (c, 0.5 in water).

(b) The final alcoholic supernatant after removal of crystalline mannitol was concentrated and analysed chromatographically in all solvents. Mannitol was present in all three solutions and a spot with the mobility of glucose in that from H. lorea and B. bifurcata - the latter also gave a third spot slightly slower than that from glucose.

Separations of the components from B. bifurcata. The alcoholic supernatant from B. bifurcata was separated on Whatman No.3 MM paper. The chromatogram was developed in solvent 1. The respective fractions were located with the help of guided strips. Fractions 1-3 were eluted with water.

Fraction 1. indicated to be mannitol by paper chromatography. After concentration it crystallised and had m.p. 163-64°, mixed m.p. 162-63°. This confirmed mannitol.

Fraction 2 was a neutral syrup and had the chromatographic mobility of glucose. This was confirmed by glucose oxidase. On prolonged standing crystals were deposited, m.p. 82-83°, mixed m.p. (with D-glucose) 82-83°.

Fraction 3 was a neutral syrup. Chromatographic analysis in solvent 1 gave no spots with aniline oxalate but a single substance was detected with silver nitrate having the same chromatographic mobility as myo-inositol.

Expt. 4. Treatment of the residual weed with formaldehyde solution. Each residual weed after aqueous alcoholic extraction was dipped in formaldehyde solution (40%) at room temperature and allowed to stand overnight. The excess formaldehyde solution was decanted and the residual weed was air dried.

Preliminary studies to extract Polysaccharides from the weeds

Extraction Procedure I

Expt. 5. Extraction with dilute hydrochloric acid. The residual weed after formaldehyde treatment was suspended in dil. hydrochloric acid (300 ml, pH = 2.0-2.1), and extracted for 4 h at 70° with constant stirring. The pH was maintained at about 2.0 to 2.1 by the addition of hydrochloric acid from time to time as the extraction was progressing. The mixture was centrifuged and the supernatant was collected and

neutralised with sodium hydroxide. Four extractions were made in this way. The combined supernatant (800 ml) was centrifuged and the clear solution was poured into alcohol (2.5 L). The resulting precipitate was dried in air and then dissolved in water, dialysed exhaustively, concentrated and finally recovered (as acid-soluble alcohol-insoluble materials C) by freeze-drying, yield: H. lorea, 9.2 g; B. bifurcata, 8.8 g; corresponds to 19.4 and 17.6% of the dry weed respectively. The alcoholic supernatant of each weed was preserved (acid-soluble alcohol-soluble materials).

Examination of materials C. Hydrolysis: A portion of the material (C) (100 mg) was hydrolysed (G.P. II(b)). Chromatographic analyses (solvents 1-6 using dip/spray 1-4) showed, both for H. lorea and B. bifurcata, mainly fucose, mannuronic, guluronic, glucuronic acids and small quantities of xylose, galactose and glucose. TMS derivatives (G.P. IX (d)) of the hydrolysate were analysed by g.l.c. using Apk (column 3, G.P. VIII). This confirmed the sugar residues found by paper chromatography. For other properties see Table 1(p100)

Examination of the acid-soluble alcohol-soluble materials. This was concentrated and again poured into alcohol. The precipitate (inorganic) was removed by filtration and the filtrate on concentration gave a light green coloured syrup. It was dissolved in water, treated with decolourising charcoal,

filtered and the filtrate was treated with IR-120(H^+) resin. After concentration to a small volume it was examined by paper chromatography in solvents 1-6 using dip/spray 1-4. This indicated the presence of small quantities of fucose, glucuronic acid, xylose, glucose and a trace of mannitol.

A portion of the supernatant after decolourisation was treated with biodemineralite resin and on similar examination showed the presence of all the sugar residues mentioned above except glucuronic acid. The results were similar both in H. lorea and B. bifurcata.

Expt. 6 Extraction of the residual weed with dil. sodium carbonate solution. The residual material (from 50 g weed) after dil. acid extraction was treated with 3% sodium carbonate solution (300 ml) at 50° for 4h with constant stirring. The supernatant was collected by centrifugation and the process of extraction was repeated four times. The combined supernatant (1.1L) was then poured into alcohol (4L). The precipitate thus formed was allowed to stand overnight, filtered through muslin and air dried. The filtrate (alkali-soluble alcohol-soluble material) was preserved.

The air dried precipitate was dissolved in minimum volume of water and dialysed till free from inorganic materials, concentrated and finally freeze-dried, materials (D), yield: H. lorea 8.0 g; B. bifurcata 8.2 g; corresponds to 16% of the dry weeds.

Examination of the material (D). A portion (100 mg) of each was hydrolysed and the hydrolysate was analysed in solvents 1-6 using dip/spray 1-4. Each hydrolysate showed the presence of mainly mannuronic, guluronic acids and small quantities of fucose, glucuronic acid, xylose and traces of galactose and glucose.

Examination of the alkali-soluble alcohol-soluble materials. The alcoholic supernatant was concentrated when inorganic materials were precipitated, filtered and the filtrate was dialysed. The nondialysable portion on freeze-drying yielded a light brown material (E, ca. 400 mg and 200 mg respectively for H. lorea and B. bifurcata). The dialysable part was preserved. The precipitate (E) on hydrolysis gave mainly fucose and glucuronic acid, small quantities of xylose, mannuronic acid and trace quantities of galactose, and E also contained a little 4,5-unsaturated uronic acid (G.P. XIV) in the case of B. bifurcata.

Expt. 7. Attempted separation of Laminaran from acid-soluble alcohol insoluble material C.

A portion (1.0 g) of B. bifurcata material (C) was dissolved in the minimum volume of water (10 ml). The aqueous solution was centrifuged and the clear solution was put on top of a Zeokarb 225 (H^+) column (40 x 2.5 cm). The column was washed with water (100 ml). The combined washings

(125 ml.) were passed through the column four times and finally the column was washed thoroughly with water (100 ml). The highly acidic eluant (250 ml) was poured into alcohol (800 ml). The precipitate thus obtained was centrifuged, washed with alcohol and collected after air drying (precipitate C1, 600 mg). The alcoholic supernatant was evaporated to dryness, dissolved in water and freeze-dried (precipitate C2, 300 mg).

Examination of precipitates C1 and C2. A portion (40 mg) of each precipitate was hydrolysed and analysed chromatographically in solvents 1-6 using dip/spray (1-4). The following sugar residues were detected:

Precipitate C1. Mainly fucose, mannuronic, guluronic and glucuronic acids; small quantities of xylose, galactose and glucose.

Precipitate C2. Mainly fucose and glucuronic acid; small quantities of mannuronic and guluronic acids, xylose, galactose and glucose.

Extraction Procedure II

Expt. 8. Extraction of the residual weed with water. B. bifurcata after formaldehyde treatment (from 50 g weed expt. 4) was treated with water (300 ml) at room temperature with constant stirring for 6 h. The residue was separated

and the process of extraction was repeated four times. The combined filtrates (800 ml) was centrifuged and the solution was poured into alcohol (2.5 L). The precipitate was recovered on the centrifuge, dissolved in water, dialysed thoroughly, concentrated and finally recovered by freeze-drying (5.4 g, corresponds to 10.8% of the dry weed). The alcoholic supernatant (water-soluble alcohol-soluble material) was preserved.

The residual weed after aqueous extraction was extracted with water at 70° for 4 h as described above. Yield: 2.7 g, corresponds to 5.4% of the dry weed.

Examination of the precipitates. The two precipitates from expt. 8 were separately hydrolysed and examined chromatographically in solvents 1-6 using dip/spray (1-4). Both the precipitates showed the presence of mainly fucose, mannuronic, guluronic and glucuronic acids; together with small quantities of xylose, galactose and glucose. Since both the precipitates gave the same chromatographic pattern, they were combined (material E). Percentage carbohydrate, sulphate and uronic acids are shown in Table 2, P101. Similarly, H. lorea was treated with hot water only, yield 7.4 g, other properties are shown in Table 2.

Examination of the water-soluble alcohol-soluble materials. The alcoholic supernatant of aqueous extract was treated as described in Expt. 5. It showed the presence of glucose and trace of mannitol both for H. lorea and B. bifurcata.

Expt. 9. Extraction with dil. acid. The residual material after hot water extraction was sequentially extracted with dil. hydrochloric acid at room temperature and at 70°. The procedure was as detailed in Expt. 8. Yield: H. lorea 1.8 g; 2.4 g. B. bifurcata 2.5 g; and 3.0 g., respectively at room temperature and at 70°.

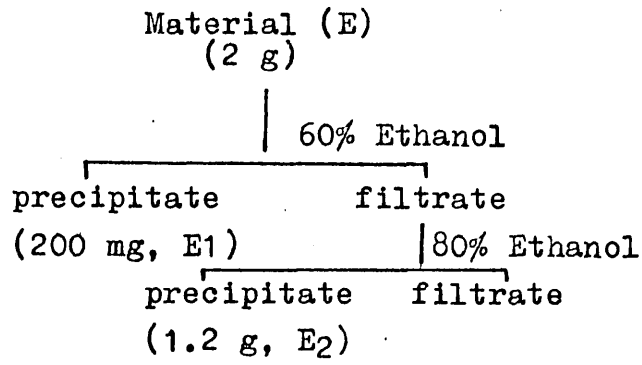
Examination of the precipitates. The precipitates both from cold and hot acid extracts were separately analysed and both showed, mainly fucose, mannuronic, guluronic and glucuronic acids, small quantities of xylose and galactose. Some properties of the extracts from H. lorea and B. bifurcata are given in Table 2.þ101.

Expt. 10. Extraction with dil. sodium carbonate. The residual material from Expt. 9, was subjected to extraction as described in Expt. 6. Yield: H. lorea 6.0 g; B. bifurcata 6.4 g; respectively corresponds to 12 and 12.8% of the dry weed. The alkali-soluble alcohol-soluble materials were similar to those found in Expt. 6.

Expt. 11. Attempted separation of a glucan from material (E).

A portion of material (E) was subjected to the following operations to separate a glucan:

(a) By selective precipitation. The material (E) was dissolved in water and treated with ethanol as in the scheme below:



Examinations of E1 and E2. Both were hydrolysed and analysed separately. Both showed the presence of mainly fucose, mannuronic, guluronic and glucuronic acids and small quantities of xylose, galactose and glucose.

Examination of the filtrate. This was evaporated to dryness, and the residue so obtained was dissolved in water and freeze-dried (560 mg, E3). On hydrolysis it gave the same chromatographic pattern as the hydrolysates from E1 and E2 except that only small quantities of mannuronic acid and no guluronic acid were detected.

(b) By anion exchange resins. (i) IR-400 (formate): IR-400(Cl^-) was converted into the formate form by passing aqueous formic acid (2N, 4L) through the resin column (24 x 2.5 cm). It was then washed with water until neutral. The precipitate E2 (0.5 g) was dissolved in water and treated with IR-120(H^+). The acidic solution (20 ml) was eluted with water from the column and the eluent was concentrated to

a suitable volume and poured into excess alcohol when precipitate E4 (ca. 400 mg) resulted. Both E4 and the alcoholic supernatant were examined.

Examination of the precipitate E4. It showed the presence of mainly fucose, mannuronic, guluronic and glucuronic acids along with small quantities of xylose, galactose and glucose as before.

Examination of the supernatant solution. The alcoholic supernatant was repeatedly evaporated with alcohol to remove formic acid, the dry residual material was dissolved in water and freeze-dried (42 mg). Hydrolysate of the product gave the same chromatographic pattern as E4.

(ii) IR-400 (Cl^-). Similarly, IR-400 (Cl^-) form was used in an attempt to separate the glucan from the other polysaccharides with the same results as with formate form column.

(c) By Zeokarb 225(H^+). The material E2 (300 mg) was treated with resin as described in Expt. 7. After passing the solution through the column several times it was finally treated with 80% ethanol and this precipitated a glucose-rich fraction (E5)(240 mg, % carbohyd. 52).

(d) By precipitation with CTA(OH) The glucose-rich fraction (E5, 100 mg) was dissolved in water (10 ml). To this cetyltrimethylammonium hydroxide (CTA-OH) was added dropwise (10 ml, 0.1N solution). The white precipitate was centrifuged off

and to the supernatant a few drops of CTA(OH) solution was added when a cloudy suspension was formed. This was centrifuged and combined with the initial precipitate. This was washed with alcohol and then with ether and dried in vacuum (49.6 mg).

The supernatant was evaporated to dryness. This and the precipitate were hydrolysed separately. The chromatographic analyses showed the presence of mainly fucose, small quantities of glucuronic acid, xylose, galactose and trace quantities of glucose in both hydrolysates.

(e) By DEAE-Cellulose column. A column (30 x 4.5 cm) was made as outlined in G.P.V. The glucose-rich material (E5, 200 mg) was dissolved in water (10 ml) and was put on top of the column. It was then eluted with water (500 ml) and the eluent was concentrated, dialysed and the polysaccharide was recovered by freeze-drying (4.0 mg). Hereafter known as the glucan. The charged material was eluted with increasing gradient of potassium chloride (see p.110a).

Extraction Procedure III

Expt. 12. Extraction of the residual weed with 2% Calcium Chloride Solution. The residual weed (from 50 g weed) after aqueous alcohol extraction and formaldehyde treatment was treated with 2% calcium chloride solution (300 ml) at room temperature with constant stirring for 4h. The residue was

removed and re-extracted in the same way twice. The combined filtrates were dialysed until free from chloride. The dialysable part was preserved. The nondialysable part was concentrated and poured into excess alcohol. The precipitate (F) thus obtained was recovered and dissolved in water and finally it was recovered by freeze-drying.

The residual material after cold aqueous calcium chloride extraction was extracted with the same solvent at 70° for 4h as described above. The precipitate (F') was analysed in parallel with precipitate (F). Both showed mainly fucose, glucuronic acid and small quantities of glucose, xylose, galactose and trace quantities of mannuronic acid. The two precipitates were combined (hereinafter called F). Yield: H. lorea 1.1g, B. bifurcata 1.3g and P. pavonia, 1.5 g. Some properties are given in Table 4, and 5. p 102.

Examination of the dialysable material. The diffusate was concentrated and again poured into alcohol when calcium chloride was precipitated. The process was repeated several times. Finally, the solution was treated with charcoal to decolourise the greenish solution, treated with IR-120(H^+), concentrated and examined by paper chromatography in solvents 1-6 using dip/spray 1-4. All three species showed the presence of mannitol. Also small quantities of glucose were detected in H. lorea and B. bifurcata.

Expt. 13. Extraction of residual material with dil. hydrochloric acid. The residual material after aqueous calcium chloride extraction was treated with dil. hydrochloric acid as described in Expt. 5. Percentage yield of the recovered polysaccharides were: H. lorea 17.0; B. bifurcata 16.0 and P. pavonia 4.0%. Some properties of acid extract are given in Table 4, p.102.

Examination of the polysaccharide. A portion (40 mg) of each polysaccharide from each weed showed the presence of mainly fucose and glucuronic acid, small quantities of xylose and galactose and trace quantities of mannuronic acid. The relative molecular proportions of different sugars were determined as the TMS derivatives of the derived alditols and L-gulonic acid (the reduction product of glucuronic acid) for H. lorea (Table 5, p.102).

Examination of the acid-soluble alcohol-soluble material. This was examined as detailed in Expt. 5, and the results were similar. In addition to fucose, xylose and glucuronic acid P. pavonia, gave a spot with mobility R_{fucose} 0.65, and 0.76. (respectively in solvent 1 and 2), which became pink (bright) on standing. This was separated on paper and had $[\alpha]_D$ = -11.5° (c, 0.25 in water). On hydrolysis gave fucose and xylose only. Determination of degree of polymerization (DP = 1.99 G.P. XVI) gave 47 µg as an equivalent

mixture of fucose and xylose, and the reduced material corresponds to 22 μg as xylose and 43 μg as fucose. It is tentatively assumed to be a xylosylfucose.

Expt. 14. Extraction of residual material with dil. sodium carbonate. The residual material from Expt. 13, was treated with 3% sodium carbonate solution as described in Expt. 6. The results for the precipitates and the alkali-soluble alcohol-soluble materials were similar to those found in Expt. 6.

Expt. 15. Fractionation of the alkali extract. It was dissolved in water to give a concentration of about 1.0% and to this 2% aqueous calcium chloride solution was added slowly with stirring until the precipitation was complete. The mixture was allowed to stand in the cold overnight and the gelatinous precipitate was removed by spinning off. It was washed with water and freeze-dried to a light brown coloured material (calcium alginate). The supernatant solution was concentrated to a suitable volume, dialysed initially for 4 days under tap water and finally against distilled water until free from chloride ion. It was then concentrated and freeze-dried to a light cream powder (hereinafter called glucuronoxylofucan) yields were: ca. 900 mg in H. lorea and B. bifurcata; 1.0 g in P. pavonia (from 50 g weed). The glucuronoxylofucan will be dealt with in Section II.

Examination of the Ca-alginate. A portion (100 mg) was hydrolysed and analysed chromatographically (solvent 1, 3 and 6) using dip or spray 1 and 2. It showed the presence of mannuronic and guluronic acids and their respective lactones. A portion of the hydrolysate was esterified, reduced and hydrolysed (G.P. X) It gave mannose and gulose (solvent 5 spray 4). Ionophoresis in borate buffers gave two spots, M_{α} 0.58 and 0.46 respectively identical with those given by mannose and gulose.

Expt. 16. Extraction with ammonium oxalate-oxalic acid solution.

The residual material obtained from all three extraction procedures was extracted with ammonium oxalate-oxalic acid solution (pH = 2.8, 250 ml) at 70° for 6 h with constant stirring. The residue was separated and the supernatant solution was dialysed till free from oxalate ion, concentrated and finally freeze-dried. This yielded a coloured powder, yield ca. 1.0% for each dry weed.

Examination of the precipitate. A portion of the precipitate on hydrolysis and chromatographic analysis revealed the presence of fucose, glucuronic acid, xylose and traces of glucose and mannuronic acid.

Expt. 17. Chlorite treatment. The residual material from Expt. 16 was suspended in water (200 ml) and heated to 70°. To it was added acetic acid (1 ml) and sodium chlorite (1.0 g)

with constant stirring. The reaction was allowed to continue for 1 h after which a second addition of acetic acid followed by sodium chlorite was made and the process was repeated four times. The white residual material was separated and washed with water until free from chloride. It was recovered after washing with alcohol, ether and petroleum ether (b.p. 40-60°) as a snow white material (ca. 4.0% for H. lorea, B. bifurcata and P. pavonia).
The supernatant was dialysed until free from chloride, concentrated and on freeze-drying gave a cream coloured powder (ca. 1.0% for H. lorea, and B. bifurcata; 2.5% for P. pavonia, hereinafter called chlorite extract.

Examination of the chlorite extract. A portion (40 mg) of the chlorite extract was hydrolysed and on chromatographic analysis showed the presence of mainly fucose, glucuronic acid and small quantities of xylose, galactose and glucose.

Examination of the residue. Like the chlorite extract, the residue was examined and found to comprise glucose with a faint trace of xylose. It had $[\alpha]_D$ about -74° (C.1 in 10% sodium hydroxide).

Expt. 18. Extraction of the residue with potassium hydroxide. The residual material was exhaustively extracted with potassium hydroxide solution (6N, 125 ml) at room temperature for 48 h in an atmosphere of nitrogen on a mechanical shaker. The

residue was removed and washed successively with dil. acetic acid, water, ethanol and ether. After drying in vacuo, a white powder was obtained (ca. 3.0%) of the dry weed. It had about 16.6% protein (for H. lorea). The combined filtrate and washings were poured into a mixture of ice cold ethanol (excess) and glacial acetic acid. The precipitate formed was collected on centrifuge and recovered after successive washing with aqueous ethanol, ethanol, ether and petroleum ether (b.p. 40-60°). After drying in vacuum a cream coloured powder (0.5 g, H. lorea) was obtained. The alcoholic supernatant was dialysed and freeze-dried (0.3 g, H. lorea). Both alcohol-insoluble and soluble materials were hydrolysed and showed the presence of mainly glucose and a very faint trace of xylose (paper chromatography only). The former had 25% protein.

Expt. 19 Final procedure to separate glucan. The material from the aqueous calcium chloride extraction was separated on a DEAE-cellulose column as described in Expt. 11(e). The glucan was obtained in the aqueous eluent and the respective yields were: 0.02, 0.20 and 0.07%. For H. lorea, B. bifurcata and P. pavonia (Table 6). p 104.

Expt. 20 Characterisation of Glucan. The glucan (10 mg) was hydrolysed with N-sulphuric acid and the hydrolysate was neutralised with barium carbonate, treated with IR-120(H^+) resin,

concentrated and analysed in solvent 1 and 2 using dip or spray (1-3). To detect mannitol in the hydrolysate methyl-ethylketone-acetic acid-water sat. boric acid (solvent 5) was used to develop the chromatogram and periodate-permanganate was used as the spray reagent. This indicated the presence of glucose only in all three weeds.

In a parallel experiment an equal quantity of laminaran from Laminaria laminaran was hydrolysed and similarly analysed. This revealed the presence of glucose and mannitol.

Partial hydrolysis. The glucans (5 mg and 10 mg, respectively from H. lorea and B. bifurcata) were separately hydrolysed with 0.1N sulphuric acid (1 ml) for 1 h at 100°. The hydrolysate was neutralised by shaking with 5% N,N-di-octylamine in chloroform solution (1 ml) three times and then once with chloroform and concentrated to a suitable volume. Chromatographic analysis gave spots with the mobility of laminaribiose ($R_{glucose}$ 0.42, solvent 1 and $R_{glucose}$ 0.59, solvent 2), and gentiobiose ($R_{glucose}$ 0.27 in solvents 1 and 2) along with other oligosaccharides.

Homologous series of 1,3-linked oligosaccharides. Chromatogram in solvent 8 for 130 h separated the oligosaccharides. Taking R_f for laminaribiose, $\log \frac{1}{R_f - 1}$, were calculated[178] for higher 1,3-linked laminarioligosaccharides. These were then plotted against the degree of polymerization of each of the oligosaccharides (including laminaribiose) when a

straight line was obtained (see Fig.15, p106a).

Methylation studies. The glucan and laminaran from Laminaria was methylated according to G.P. VI(b) and hydrolysed (G.P. VI(c) and analysed chromatographically in solvents 2 and 7. It showed the presence of

(a) 2,3,4,6-tetra-O-methylglucose (R_G 1.0, solvent 2).

(b) 2,4,6-tri-O-methylglucose (R_G 0.77, solvent 2)

Glycosidation[169] and glc. analysis. These were done according to G.P. VI (d) and G.P. VI (c); and G.P. VIII, using columns 1 and 2. The methyl glycosides identified are shown in below, with their retention time with respect to methyl-tetra-O-methyl-β-glycoside.

Methyl glycosides from the methylated glucan.

Methylated sugar glycosides	Column	H. lorea	B. bifurcata
2,3,4-tri-O-methyl glucoside (major peak)	1.	2.86, 3.5	2.60, 3.5
	2.	(1.65,) 2.0	1.50, 2.0
2,4,6-tri-O-methyl glucoside	1.	2.90, 4.0	3.0, 4.27
	2.	(1.70,) 2.3	1.64, 2.40
2,3,4,6-tetra-O-methyl glucoside	1.	1.0, 1.30	1.0, 1.37
	2.	1.0, 1.33	1.0, 1.35

Figures in parenthesis indicate T values of components which are not completely resolved.

Expt. 21 Characterisation of the final residual material.

Complete hydrolysis of an aliquot of the residual material from Expt. 18 indicated the presence of glucose with a trace of xylose.

Methylation Studies. This was methylated by the methods of Haworth[179] and Kuhn[180]. Hydrolysis and chromatographic analysis showed the presence of 2,3,6-tri-O-methylglucose (R_G 0.89 solvent 2) as the major product along with small quantities of 2,3,4,6-tetra-O-methylglucose (R_G 1.0 solvent 2). G.l.c. analysis of the methylglucosides[169] confirmed the presence of methyl 2,3,6-tri-O-methylglucosides with retention time with respect to tetra-O-methyl-β-glucoside:column (1) 3.21, 4.21 and column (2) 1.69, 2.25) as the major product together with methyl 2,3,4,6-tetra-O-methylglucosides(column 1, 1.0, 1.33 and column 2, 1.0, 1.35).

Fractionation of acid extract.

Expt. 22. By calcium chloride method. Acid extracted material (1.0 g; Expt. 5, procedure I) was dissolved in water (100 ml), centrifuged, and to the clear solution was added 2% calcium chloride solution (25 ml). The precipitate thus formed was allowed to stand in the cold overnight. It was then removed on centrifuge, washed with water, alcohol and finally with ether. On air drying it gave a brown coloured powder [530 mg, (A) calcium-insoluble material]. The supernatant was dialysed until free from chloride, filtered, concentrated and poured into excess alcohol. The precipitate was recovered after washing with alcohol and ether a light brown powder [400 mg (B) calcium-soluble material].

Examination of the Ca-insoluble and soluble materials (A and B).

Both the precipitate (A) and (B) were hydrolysed and analysed chromatographically in solvent 1, 3 and 6 using dip and spray 1 and 2. Both were found to contain mainly fucose, mannuronic and guluronic acids together with some glucuronic acid, xylose and galactose.

The acid extract (Expt. 8, Procedure II) was examined in the same way and gave similar results.

Expt. 23 By DEAE-cellulose column. The acid extract (1.0 g, Expt. 8, Procedure II) was put on a DEAE-cellulose column. After removing the laminaran with water, the column was eluted with water with an increasing gradient of potassium chloride. Fractions were obtained at the following eluent concentrations: 0.3M KCl (300 mg), 0.5M KCl (200 mg) and 1.0M (200 mg). On examination it was revealed that 0.3M and 0.5M fractions contained alginic acid in addition to the "fucans" - and this could not be preferentially precipitated with Ca^{++}.

Part I. Results and Discussions

Low molecular weight carbohydrate. The main low molecular weight carbohydrate extracted from all three species of the weed by aqueous ethanol was mannitol. It was obtained crystalline and corresponded to about 8% of the dry weight of *B. bifurcata*, 0.5% of *H. lorea* and only a very small amount could be extracted from *P. pavonia* for comparison with other species of brown algae[111] the mannitol contents in *H. lorea* and *P. pavonia* are low. In addition to mannitol, glucose was found in *H. lorea* and *B. bifurcata*. It was separated and characterised as crystalline D-glucose in the latter while glucose oxidase confirmed its presence in *H. lorea*.

Comparison of the Extraction Procedures I - III and the different Polysaccharides.

In the first instance the established method of extraction of brown seaweeds namely sequential use of acid and alkali was carried out on H. lorea and B. bifurcata but the polysaccharide material precipitated from the acid extract from both the weeds, proved to be mixtures of a glucan, sulphated fucose-containing polysaccharides (hereinafter called 'fucans') and alginic acid (Expt. 5) which defied fractionation into homopolysaccharides. In an attempt to isolate the glucan in a single extract the weeds were extracted with water before acid and alkali but again complex mixtures were obtained in each of the extracts (see Table 2 and Expt. 9). Fractional precipitation of the aqueous extract with ethanol [Expt. 11(a)] failed to yield any separation of the different polymers. Elution from strong anion exchange resins [Expt. 11(b)] failed to give any fraction solely comprised of neutral polysaccharide. It was concluded that the proportion of charged polysaccharides was so much in excess of the glucan that it was impossible to separate the latter by these means. The addition of cetyltrimethylammonium hydroxide to a solution of the polysaccharide [Expt. 11 (d)] although it gave a precipitate, left charged material in solution and offered no significant

separation. A glucose rich fraction was, however, obtained by repeated elution from Zeokarb 225(H^+) resin [Expt. 11(c)]. Finally a small proportion of pure glucan was separated by elution with water from a column of DEAE-cellulose [Expt. 11 (e)].

It was decided that if extraction with water was done in the presence of Ca^{++} then all the alginic acid and probably some of the 'fucans' should remain insoluble. In fact this was found to be the case. The aqueous calcium chloride extract, unlike previous acid or water extracts comprised very small proportions of the dry weed. A comparison of the yields of carbohydrate by the different extraction procedures is given in Table 3.

The aqueous calcium chloride extract (Procedure III) proved to be a mixture of small proportions of the 'fucans' and the glucan. The latter was separated from the former on a DEAE-cellulose column (Expt. 19). This was the most effective procedure to obtain pure glucans from the three species of weed.

Table 1

Extraction Procedure I

Some properties of dil. acid extracted material

Species	%carbohyd. (G.P. III(b)	% SO_4'' (G.P. IV(c)	% uronic acid (G.P. VII (b)
H. lorea	40-42	21.0	18.4
B. bifurcata	40-42	24.2	18.0

Table 2

Extraction Procedure II

Some properties of aqueous and acid extracted materials

Extract	Species	% carbohydrate (G.P. III (b)	% SO_4'' (G.P. IV (c)	% uronic acid (G.P. VII (b)
water	H. lorea	40-43	16.4	23.2
	B. bifurcata	40-42	16.8	22.4
acid	H. lorea	42-43	22.1	15.2
	B. bifurcata	42-43	23.6	13.9

Table 3

Percentage yield of polysaccharides based on dry weight of the weed in the different extraction procedures.

Extraction Procedure I.

Species	dil. acid	dil. alkali	total
H. lorea	15.0	19.0	34.0
B. bifurcata	17.0	16.0	33.0

Extraction Procedure II.

Species	water			
H. lorea	16.5	4.0	10.0	30.0
B. bifurcata	15.0	5.0	12.0	32.0

Extraction Procedure III

Species	Calcium chloride			
H. lorea	1.1	17.0	18.0	36.1
B. bifurcata	1.3	16.0	18.0	35.3
P. pavonia	1.5	4.0	15.0	20.5

Table 4

Extraction Procedure III

Properties of the 'Fucans' extracted with aq. calcium chloride followed by dil. acid.

Extract	Species	%carbohydrate (G.P. III(b)	% SO_4 (G.P.IV(e)	% uronic (G.P. VII(b)
Calcium chloride	H. lorea	40-42	14.7	3.8
	B. bifurcata	40-42	10.3	7.7
	P. pavonia	42-44	7.3	3.0
Dil. acid	H. lorea	43-45	23.0	7.5
	B. bifurcata	40-42	22.2	12.3
	P. pavonia	45-47	10.0	8.2

Table 5

Mole proportions of reduced "sugars" in the 'fucans' of H. lorea.

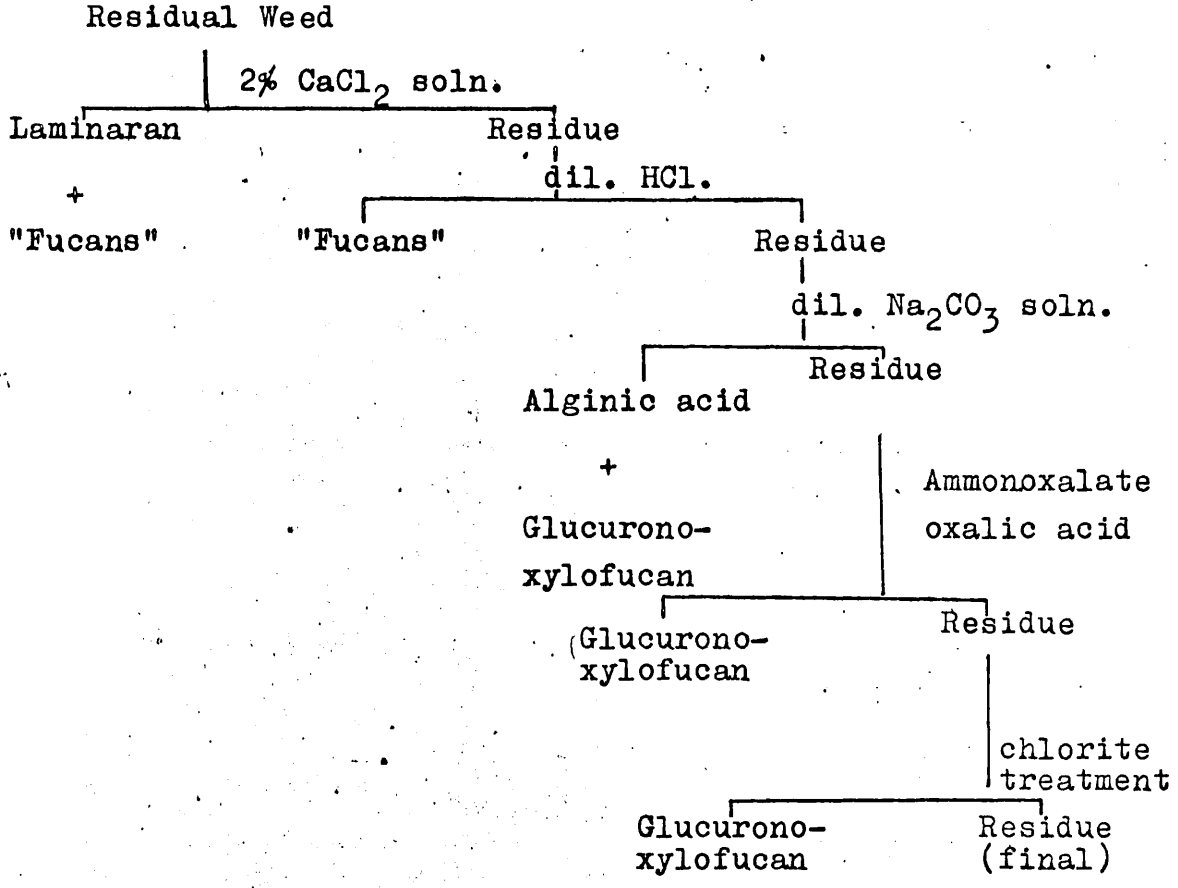
Extract	Fucitol	Xylitol	L-gulonic acid	galactitol
Aq. Ca^{++}	5.5	2.5	1.0	-
Acid	6.0	0.6	1.0	-

It can be seen from Tables 1, 2 and 4 that the general composition of extracts from the different procedures vary considerably. From all these observations it is reasonable to conclude that extraction procedure III (see flow diagram) (p.103) is better than the other two in that it gave less

complex mixtures of polysaccharides. Hence this procedure has been adopted in isolating the various polysaccharides which have been studied from H. lorea, B. bifurcata and P. pavonia. The percentage yield of the different polysaccharides are shown in Table 6.

Extraction Procedure III

Extraction of Himanthalia, Bifurcaria and Padina after 80% ethanol extraction and formaldehyde treatment



The percentage yields of the different polysaccharides are given below.

Table 6

Percentage yields of individual polysaccharide based on dry weight of different weeds.

Species	Laminaran	'Fucans'	Alginic acid	Glucuronoxylo-fucan			Cellulose
				Alk-ali	Amm-ox.	Chl-orite	
H. lorea	0.02	18.0	16.0	1.8	1.0	1.0	4.0
B. bifurcata	0.20	17.0	16.0	1.8	1.0	1.0	4.0
P. pavonia	0.07	5.0	13.0	2.0	1.0	2.5	4.0

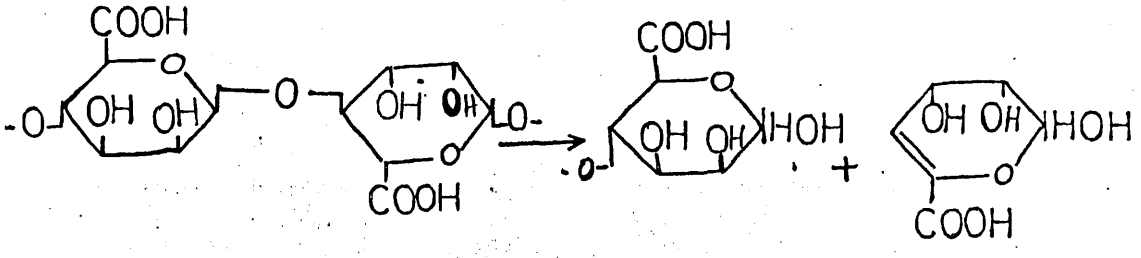
All the species have a low proportion of laminaran. The other major polysaccharides such as 'fucans' and alginic acid are comparable in H. lorea and B. bifurcata while P. pavonia has remarkably low "fucans" and alginic acid. This may be due to the striking morphological differences of P. pavonia. The distribution of the various polysaccharides which contain fucose suggests that these are present in the cell as well as in the cell-wall.

The carbohydrate composition of the alcoholic supernatants from acid and alkali extractions.

The carbohydrate composition of alcoholic supernatants of the various extracts was investigated since a knowledge of this would be useful in future biosynthetic work[156] with

C^{14}. The supernatant of all the acid extracts from all these weeds was found to contain fucose, xylose and glucuronic acid. These must have resulted from degradation of the "fucans" since if originally present in the algae they should have been extracted in the aqueous ethanol. In addition to these free sugar residues P. pavonia gave a pink spot with aniline oxalate spray having chromatographic mobilities R_{fucose}, 0.76 and $R_{arabinose}$ 0.95 (solvent 2, Expt. 13). This was separated and found to contain fucose and xylose residues. Determination of the degree of polymerization gave 47 µg as an equivalent mixture of fucose and xylose, and the reduced material corresponds to 22 µg as xylose and 43 µg as fucose (p.2) It is tentatively identified as a disaccharide, xylosyl fucose.

The supernatant of the alkali extract (Expt. 6) comprises the degraded glucuronoxylofucan[156] together with small quantities of 4,5-unsaturated acid. It is well known that alginic acid is degraded[181] to 4,5-unsaturated uronic acids by β-elimination reaction:



4,5-unsaturated uronic acid.

The Characterisation of the glucan

The neutral eluant from DEAE-cellulose column from B. bifurcata had $[\alpha]_D = -13.7^\circ$ (lit. value for laminaran -12 to -14°) and had carbohydrate content 80--82%. Since laminaran from Laminaria hyperborea contains mannitol terminated chains, mannitol was looked for in the hydrolysate of the neutral polysaccharides of all three algae. Parallel hydrolyses on equivalent weights of glucan from each species and laminaran from L. hyperborea were carried out. The presence of mannitol was readily detected on a paper chromatogram in case of L. hyperborea whereas from the three other genera of weeds no mannitol could be detected.

Analyses of the partial hydrolysates of H. lorea and B. bifurcata glucans showed the presence of laminaribiose, gentiobiose and higher laminarisaccharides. Since plotting of log $(\frac{1}{R_f - 1})$ against the degree of polymerisation for the 1,3-linked laminarisaccharides resulted in a straight line (Fig 15) ~~passing through the origin~~, the oligosaccharides belong to the homologous series. This together with negative rotation, both for H. lorea and B. bifurcata, indicates that the glucan is a laminaran and is mainly composed of β-1,3-linked glucose units. The small quantities of gentiobiose proved that 1,6-linkage is also present in the laminaran molecule.

Laminarans of H. lorea and B. bifurcata were methylated by a new technique[167] using methylsulphinyl carbanion in

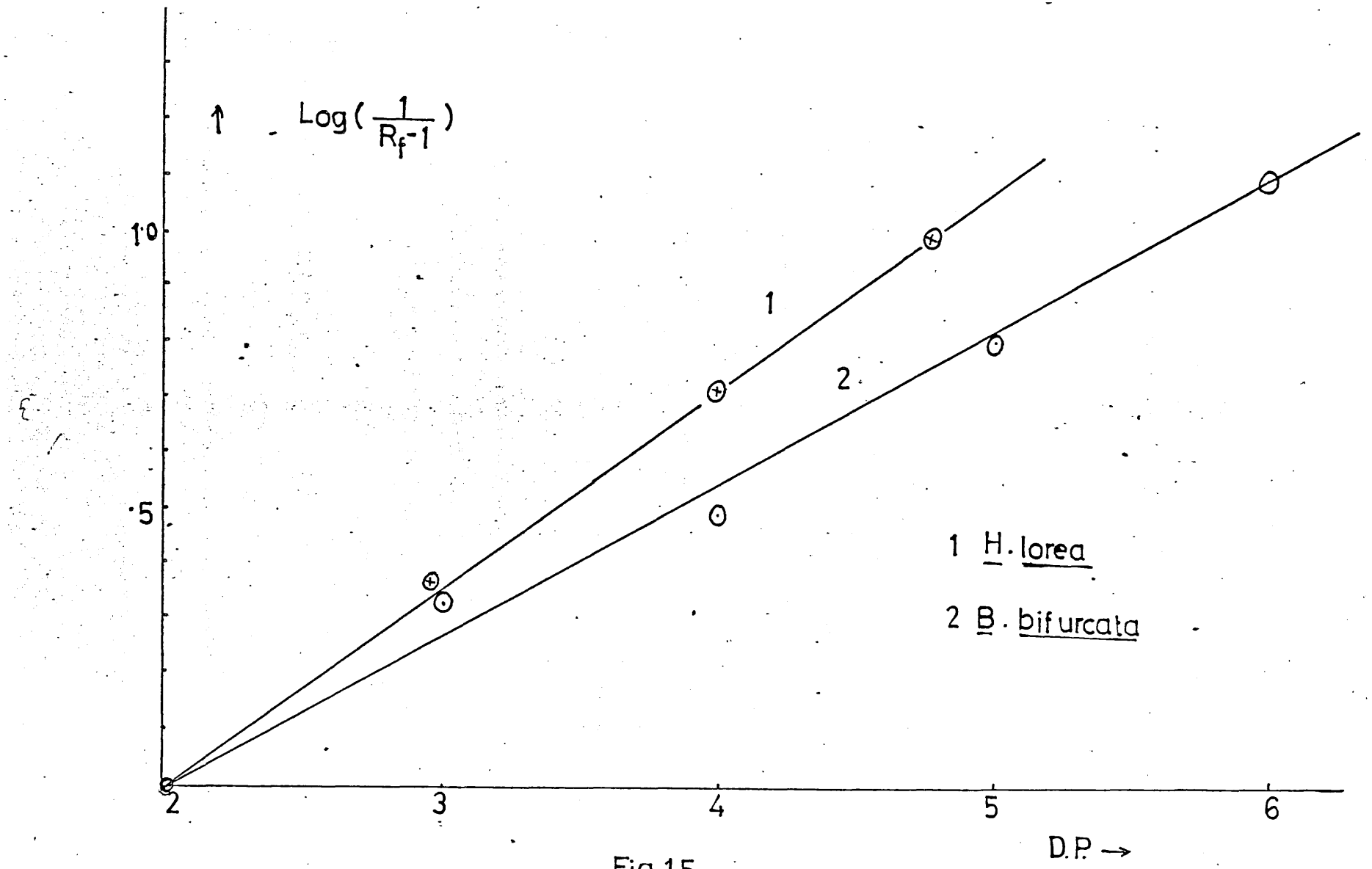


Fig 15.

1066

dimethylsulphoxide and methyliodide. Gas liquid chromatographic analyses of the methylated glycosides obtained after glycosidation of the derived methylated sugars gave identical gas chromatograms to those of Laminaria hyperborea laminaran methylated by the new technique as well as by the classical methods of methylation.[84] The major peaks had the same retention time as those of methyl-2,4,6-tri-O-methyl glucosides, smaller peaks with retention times of methyl-2,3,4-tri-O-methylglucosides and methyl 2,3,4,6-tetra-O-methyl-glucosides were obtained. This is the first time that L. hyperborea has been examined on g.l.c. and the first time 2,3,4-tri-O-methylglucose has been identified. This provides definite evidence of 1,6-linked inter-residue glucose units. No evidence of any di-O-methylglucose was obtained.

All these results clearly indicate that H. lorea and B. bifurcata laminaran are essentially linear glucans containing β-1,3-linked glucose residues with small proportions of β-1,6-linked inter-residue linkages. These laminarans appear to be devoid of mannitol terminated molecules.

The Carbohydrates of alkali extract. The polysaccharides obtained by alkali extraction (Expt. 6, 10 and 14) on hydrolysis gave mainly mannuronic and guluronic acids together with small proportions of fucose, glucuronic, xylose and galactose. It was fractionated into Ca-insoluble and Ca-soluble components.

The Ca-insoluble material on hydrolysis showed mannuronic and guluronic acids and their lactones chromatographically. A portion of the hydrolysate was esterified, reduced and hydrolysed. It gave mannose and gulose and these were confirmed by ionophoresis (Expt. 14). The Ca-soluble material was a glucuronoxylofucan.[154,156]

Cell-wall polysaccharides. An additional amount of glucuronoxylofucan was obtained from the ammonium oxalate-oxalic acid extract[155] (Table 6). Similar material was also obtained from the chlorite extract (Table 6) p 104.

The white residual material [hereinafter called (F)] obtained after chlorite treatment on hydrolysis gave predominantly glucose with a faint trace of xylose. In order to remove any hemicellulose associated with cellulosic material, the residue (F) of H. lorea was treated with potassium hydroxide solution. The alkali-soluble material had about 27% protein and on hydrolysis gave the same sugars as obtained with F. The final residue (G) after alkali extraction had approximately 16.6% protein and on hydrolysis gave the same result as (F). The residual material F may be regarded as a cellulose associated with protein.

The final residual material (G) was methylated by the method of Haworth and Kuhn. This resulted in a partially methylated material (% OCH_3 42.5) which on further methylation

by Hakomori[9] method (G.P. VI) gave a fully methylated product. This on hydrolysis, methanolysis and g.l.c. analyses showed the presence of 2,3,6-tri-O-methylglucose (major) with small quantities of 2,3,4,6-tetra-O-methylglucose. This together with the negative rotation indicate that the glucose residues are β-1,4-linked and the final material is a cellulose-type polysaccharide.

Because all the extracts isolated by procedure I and II proved to be complex mixture of laminaran, alginic acid and "fucans", various attempts were made to separate the individual polysaccharides. Preferential precipitation of alginic acid by Ca^{++} from acid and aqueous extracts (procedures I and II Expts. 22) was unsuccessful, both the Ca-soluble and the Ca-insoluble materials contained the same mixture of polysaccharides. Fractionation on DEAE-cellulose (Expt. 23) proved equally unsuccessful in separating the alginic acid and the fucans.

Part II

Structural Investigation of the Fucose-containing Polysaccharides

Expt. 24. Fractionation of the various "Fucans" isolated by Procedure III. (see Part I).

"Fucans" isolated in Expt. 12 and 13 from each of the weeds were fractionated on a DEAE-cellulose column. The elution pattern is shown in graph (Fig 16). Both "Fucans" gave three fractions at 0.3M, 0.5M and 1.0M potassium chloride concentration. The percentage composition of 0.3M, 0.5M and 1.0M fractions of aqueous calcium chloride were similar to corresponding fractions of the acid extract. The respective similar fractions were therefore combined. The rotations and percentage recoveries of the combined fractions are given in Table 7, p130. The carbohydrate, sulphate and uronic acid contents are given in Table 8, p131 and the molar proportions of the constituent sugars as the reduction products can be seen in Table 9, p134.

Expt. 25. Fractionation of the glucuronoxylofucans on DEAE-cellulose.

The glucuronoxylofucans extracted by alkali (Expt. 15), ammonium oxalate-oxalic acid (Expt. 16) and chlorite (Expt. 17) were fractionated on a DEAE-cellulose column (Expt. 23). The column was eluted in each case with 0.3M, 0.5M and 1.0M KCl. In all cases the 0.3M fraction was a very minor component. It was contaminated with alginic acid and was not examined further. Some properties of the other fractions of each extract are shown in Tables 10-14, p 135-7.

Expt. 26. Attempted removal of ester sulphate with alkali.[32] (i) The 1.0M KCl fraction of the "fucans" (carbohydrate 40%, sulphate 29.0% and (ii) the 1.0M fraction of the glucuronoxylofucans from the alkali extract (carbohydrate 45%, sulphate 26%) were treated separately. Each polysaccharide (165 mg.) in

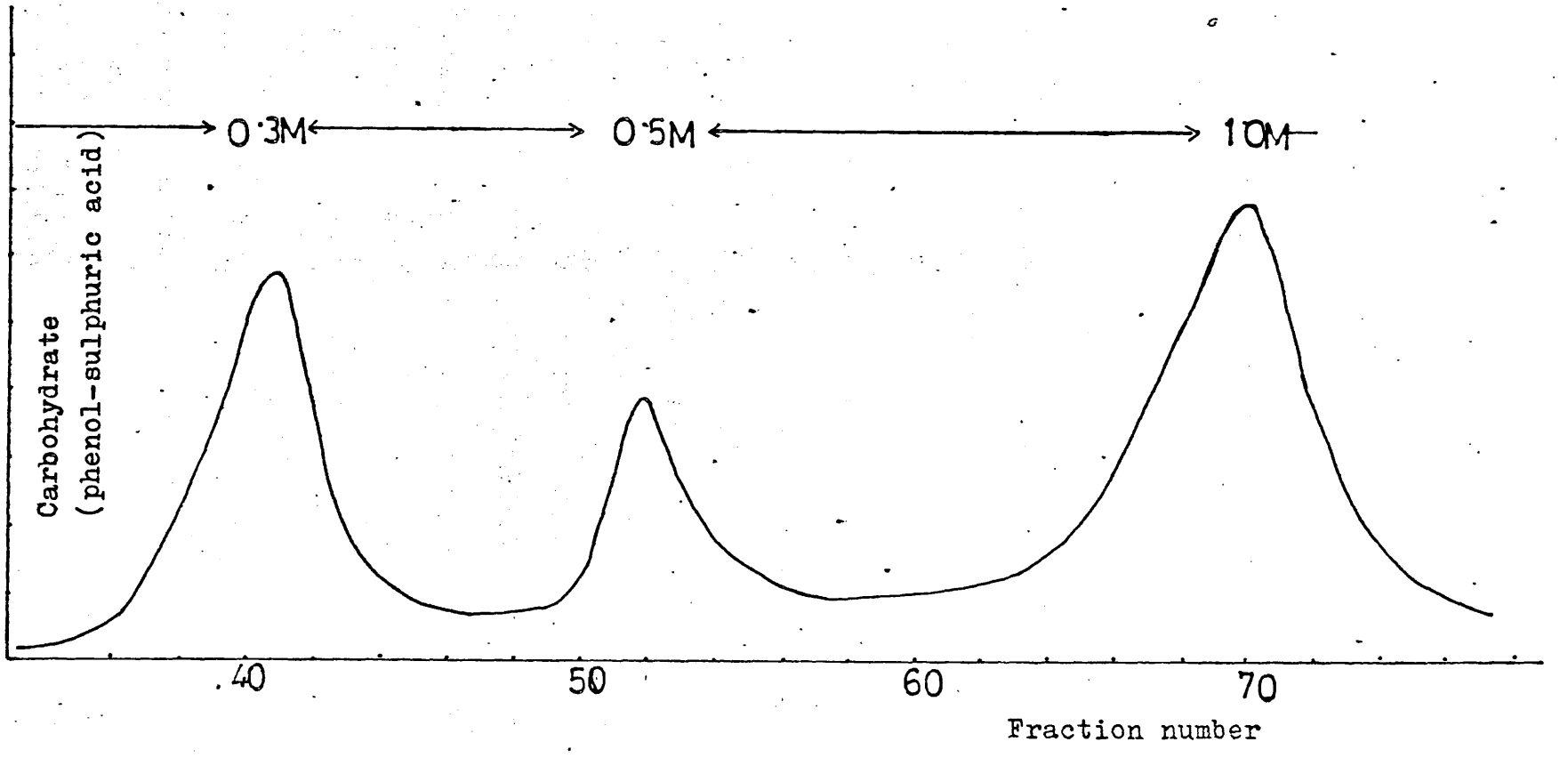


Fig.16. Elution pattern of "Fucans" from DEAE-Cellulose with a gradient concentration of potassium chloride.

water (125 ml.) was reduced at room temperature for 48 h with potassium borohydride (20 mg.). Sodium hydroxide (1.0 g.) and potassium borohydride (30 mg.) were dissolved in the solution, and the solution was heated at 80° for 2 h. The alkaline solution was neutralised with IR-120(H^+) resin. After dialysis the degraded polysaccharides ~~was~~ were recovered by freeze-drying (134 mg, found carbohydrate 40%, sulphate 27.5% for "fucans", for glucuronoxylofucan, 130 mg, carbohydrate 45% and sulphate 23.8%.

Expt. 27. Gel electrophoresis (G.P. XIII).

The polysaccharides 0.3M, 0.5M and 1.0M fractions from H. lorea (0.35 mg. each in 10% sucrose solution) was subjected to electrophoresis on a Shandon apparatus comprising two polysaccharide columns, by the method of Steward et al. for 1 h. at 250 v and 3mA. Tris-glycine, pH 8.3 was used as the reservoir buffer. One portion was stained with Naphthalene Black for protein and the other with Toluidin Blue for sulphated carbohydrate by leaving the extruded columns in the reagent for 1 h. and then washing to remove excess of stain. Single coincidal bands were obtained for protein and carbohydrate in the case of 0.3M and 1.0M fractions, but the 0.5M fraction gave a very diffuse pattern.

Expt. 28. Methylation Studies. The 0.5M and 1.0M fractions of the "fucans" and of the glucuronoxylofucans from H. lorea and B. bifurcata were methylated separately (G.P. VI(b), hydrolysed (G.P. VIc) and analysed chromatographically. Separate aliquots of the hydrolysate were converted into the methyl glycosides (G.P. VI (d) and alditolacetates (G.P.IX4) respectively and both products were analysed on g.l.c. (Table 16). The chromatographic mobilities of different methylated sugars identified from the different fractions of "fucans" and glucuronoxylofucans of H. lorea and B. bifurcata are included in Table 15, p 140137.

Expt. 29. Methylation of uronic acid-rich fraction from H. lorea.

Polysaccharide (0.3M KCl fraction, 60 mg) was methylated by G.P. VI(b). The methylated polysaccharide was then suspended in dry tetrahydrofuran (100 ml.). Lithium aluminium hydride (60 mg. in 5 ml. tetrahydrofuran) was added to the suspension gradually at room temperature with occasional shaking when a vigorous reaction took place. The mixture was allowed to stand for 0.5 h. and then heated under reflux for 2 h. Further lithium aluminium hydride (40 mg. in 5 ml. tetrahydrofuran) was added and the reaction mixture was heated under reflux for another 0.5 h. Excess lithium aluminium hydride was destroyed by addition of water to the cooled

mixture, the solution was acidified with dil. sulphuric acid to about pH 4.0, and filtered. The filtrate was concentrated to a small volume and was extracted with chloroform. The chloroform solution on evaporation yielded a yellowish syrup of the reduced methylated polysaccharide which was remethylated (Ca 20 mg). The derived glycosides and alditol acetates were analysed on g.l.c. (Table 17) p 141.

Expt. 30. Preliminary Periodate Oxidation.

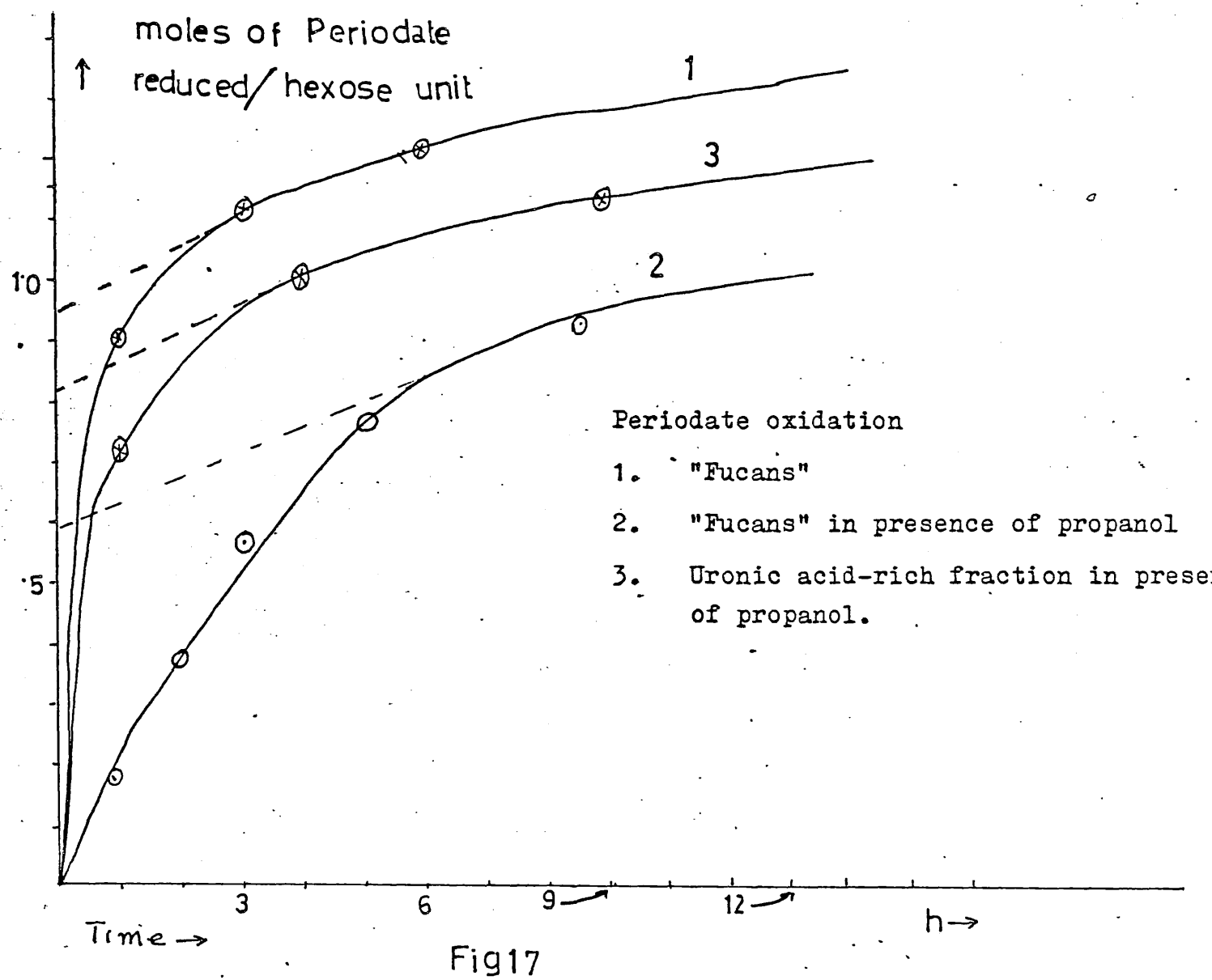
"Fucans" (acid extracted material before fractionation, Expt. 13, 600 mg) was dissolved in water (125 ml). To this sodium metaperiodate (0.03M, 125 ml) was added to bring the periodate solution to 0.015M. Aliquots (1 ml) of solution were removed at regular intervals and examined at 223 nm for reduction of periodate.[182] After 30 h the reaction was stopped by the addition of ethane diol (1 ml). Moles of periodate consumed per hexose unit were plotted against time and the primary oxidation determined by extrapolation to zero time. This was found to be 0.90 moles per hexose unit.

A similar experiment was carried out in the presence of n-propanol (1 ml per 20 ml of the solution). The primary oxidation was 0.60 moles per hexose unit (Fig. 17). Henceforth all periodate oxidations were carried out in the presence of propanol.

Expt. 31. Periodate oxidation of Fucose-rich fraction.

(a) Fucose-rich fraction (1M fraction p.134, 300 mg which corresponds to 120 mg on the carbohydrate basis) was subjected to periodate oxidation for 5 h as described in the previous experiment. The primary oxidation was found to be 0.36 moles per hexose unit (Fig. 18).

(b) The solution from Expt. 31(a) was treated with boric acid (0.05M) and 2% potassium borohydride at 0° and set aside at 0°



Periodate oxidation

1. "Fucans"
2. "Fucans" in presence of propanol
3. Uronic acid-rich fraction in presence of propanol.

Fig17

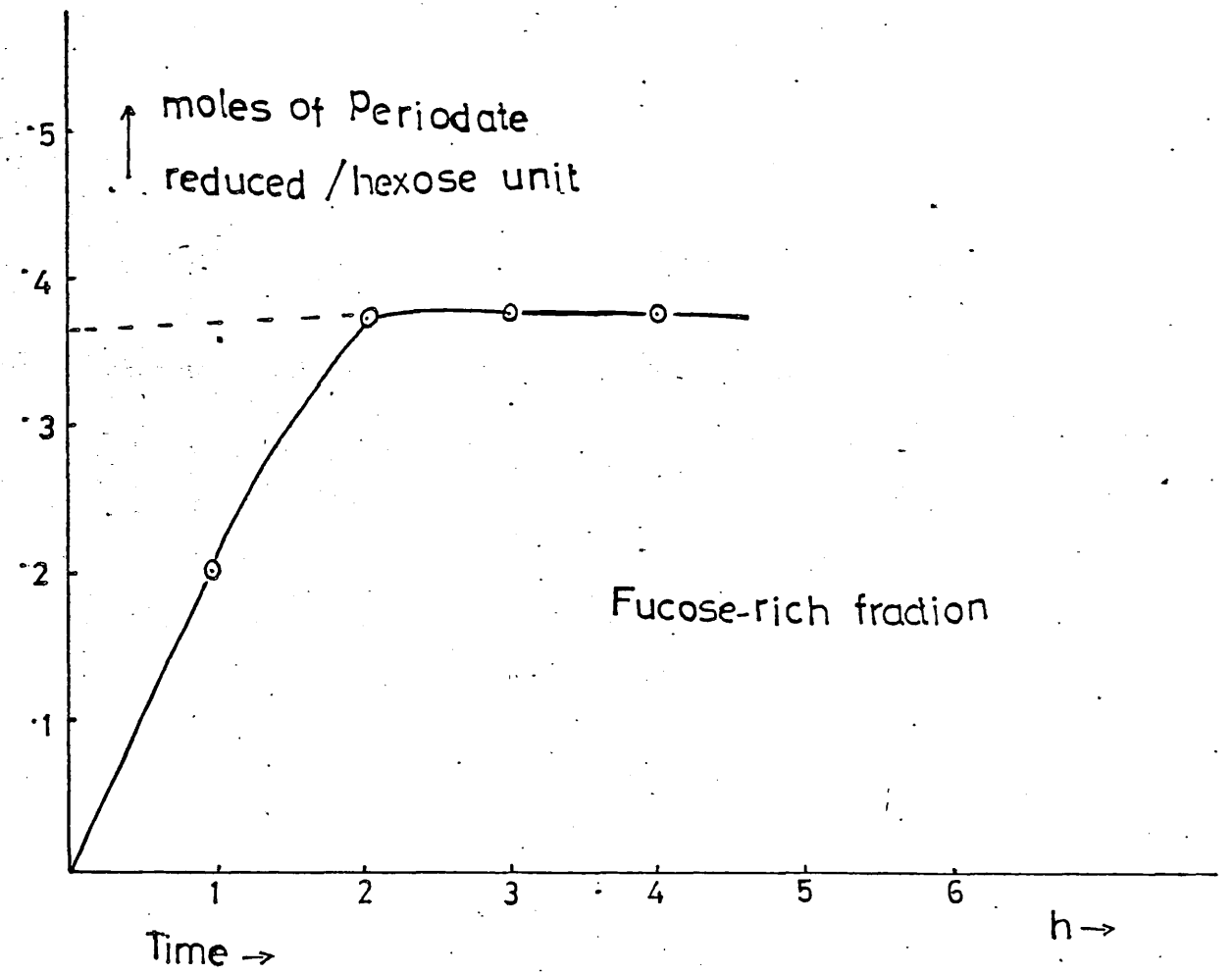

moles of Periodate
reduced /hexose unit
·5
·4
·3
·2
·1
Fucose-rich fraction
1
2
3
4
5
6
h →
Time →

Fig 18.

overnight. Total reduction was determined by a negative Fehling test. The solution was neutralised with glacial acetic acid and exhaustively dialysed against distilled water. The polyalcohol solution was concentrated to a small volume and freeze-dried (230 mg corresponds to 94.0 mg on the carbohydrate basis).

Examination of the polyalcohol (hereinafter called polyalcohol I). The polyalcohol I was hydrolysed and analysed for the surviving sugar residues. Only fucose was found to be present and this was confirmed by g.l.c. analysis as the sugar and as fucitol.

Reoxidation of the polyalcohol I. The polyalcohol I (62.0 mg) was treated as in Expt. 31(a) and the primary oxidation was found to be 0.06 moles per hexose unit. Polyalcohol 2 (60.0 mg) was recovered as in Expt. 31(b).

Methylation of polyalcohol I. The polyalcohol I was methylated as in Expt. 27 and methanolysed and g.l.c. analysis of the derived methyl glycosides and methyl alditol acetates gave peaks corresponding to free L-fucose, 2-O- and 3-O-methyl fucose, trace of 2,4-di-O-methyl fucose and some smaller unidentified fragments of the oxidation.

Mild hydrolysis of polyalcohol I. The polyalcohol was treated with 1N sulphuric acid at room temperature for 11.5 h (see details in Expt. 34(c)]. The degraded polymer was recovered in about 80% yield.

Expt. 32. Periodate oxidation of uronic acid-rich fraction.

The uronic acid-rich fraction (1.0 g corresponding to 500 mg carbohydrate) was subjected to repeated oxidation and reduction as in Expt. 31(a) and (b) and the consumption of periodate for each oxidation is shown in Fig. 19 and Table 19, p157. The respective weight and carbohydrate content were for polyalcohol I 524 mg and 137 mg, for polyalcohol 2 184 mg and 37 mg, and for polyalcohol 3 90 mg and 18 mg. Some properties of polyalcohol I, 2 and 3 are given in Tables 19 and 20, p157.

Expt. 33. (a) Reductions of polysaccharide under the conditions of Expt. 31(b). The uronic acid-rich fraction (40 mg) was treated as in Expt. 31(b). The reduced polysaccharide was recovered, after neutralization and dialysis, by freeze-drying (38.2 mg).

(b) Oxidation of the polysaccharide and isolation of the polyaldehyde. The uronic acid-rich material (41.2 mg) was oxidised as in Expt. 31(a). The reaction was stopped after 5 h by the addition of ethane diol, dialysed extensively and the polyaldehyde was recovered by freeze-drying (31.0 mg). The dialysable material (C') was examined by paper chromatography and shown to contain erythronic aldehyde, threose and glyceraldehyde. This was reduced with potassium borohydride and was thereafter preserved.

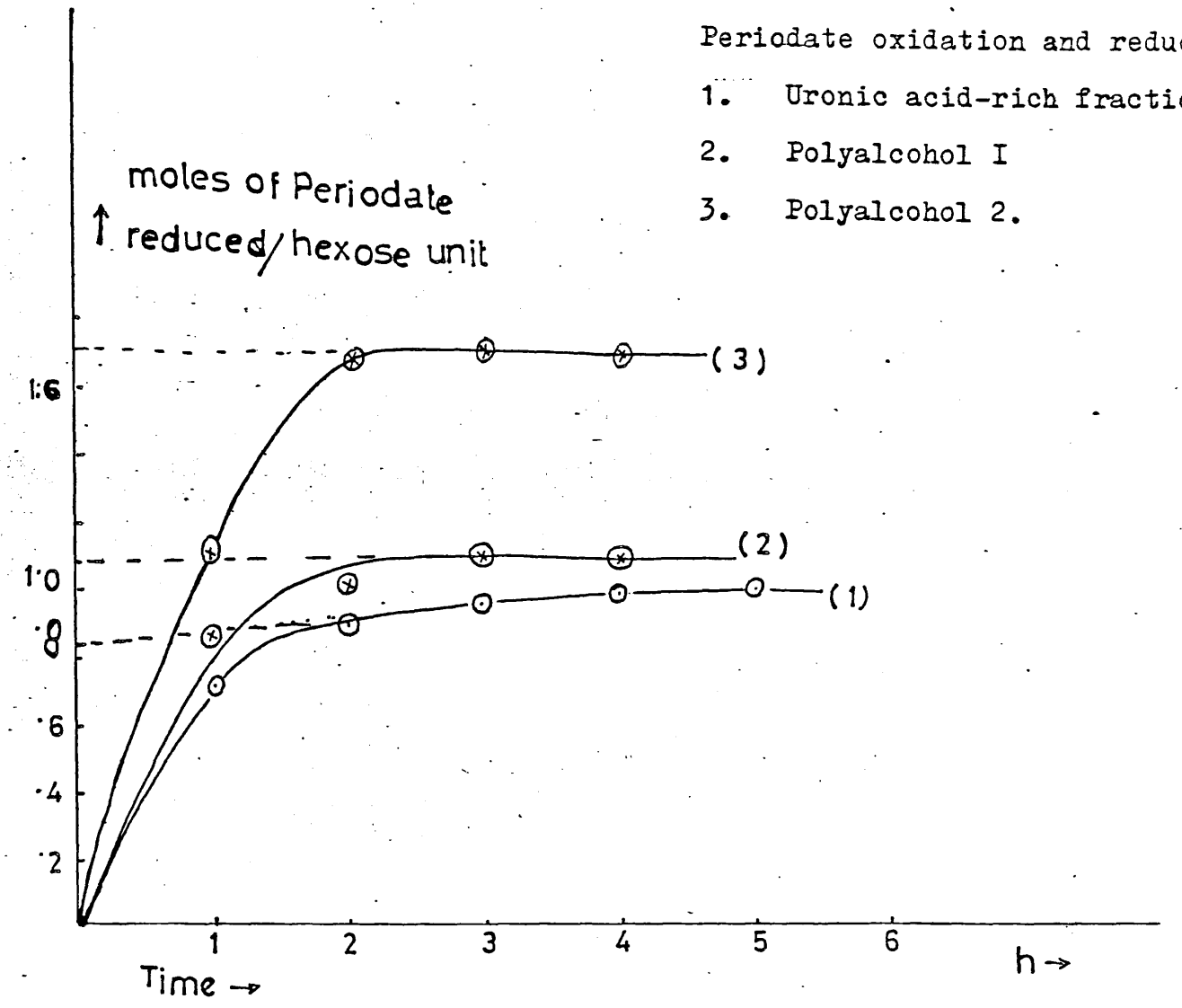

Periodate oxidation and reduction
1. Uronic acid-rich fraction
2. Polyalcohol I
3. Polyalcohol 2.
moles of Periodate reduced/hexose unit
1·6
1·0
·8
·6
·4
·2
(3)
(2)
(1)
1
2
3
4
5
6
Time →
h →

Fig19.

(c) Reduction of the polyaldehyde to polyalcohol. The polyaldehyde (30.2 mg) was treated as in Expt. 31(b) and the polyalcohol I (22.2 mg) was recovered in the same way as in Expt. 31(b). The dialysable material (C") was preserved.

Examination of the dialysable material, (C").

This was treated with biodeminrolit resin and examined chromatographically in solvents 1 and 3 using dip and spray 2. The following components having R erythronic acid in solvent 1 were identified: gulonic acid (0.42) derived from glucuronic acid, erythronic acid (1.0, major), threitol (2.3), glycerol (3.3) detected from an aliquot before dialysis, and possibly propane 1,2-diol (4.0). Ionophoresis in acetic acid-pyridine (pH 6.7) showed gulonic and erythronic acids. Test for 4,5-unsaturated acid was negative.[174]

In a separate experiment the uronic acid-rich fraction was treated as in Expt. 33(a-c) but the excess periodate was destroyed by sulphur dioxide and the dialysable materials were examined at each stage and found to be similar to C' and C".

Expt. 34. Smith degradation studies on Uronic acid-rich fraction

First Smith Degradation.

(a) Periodate oxidation as in Expt. 31(a).

(b) Reduction of the polyaldehyde to polyalcohol as in Expt. 31(b).

(c) Mild hydrolysis: Preliminary hydrolysis: Polyalcohol I (25 mg) was treated with N-sulphuric acid (6 ml) at room temperature. An aliquot (2 ml) was withdrawn after 2h, 4h and 6h hydrolysis. Each aliquot was neutralised with barium carbonate, filtered and the filtrate was thoroughly dialysed. The dialysate was treated with IR-120(H^+) resin, concentrated and analysed for free sugars (aniline oxalate, dip. 2) and for fragments (silver nitrate, dip. 1). Free sugars were detected after 2h hydrolysis.

A similar experiment with 0.1N sulphuric acid was carried out except that aliquots were withdrawn hourly. This showed that the hydrolysis can proceed up to 5h and thereafter free sugars could be detected. Henceforth mild hydrolysis was carried out for 5h.

Second Smith Degradation. The degraded polymer I was subjected to a second Smith degradation as under Expt.34(a), (b) and (c).

Third Smith Degradation. Degraded polymer II was subjected to a third degradation. The Smith degradations are shown in Fig.24, p151 and some of the properties of the different products of Smith degradations are given in Tables 21 and 22, p158. The periodate uptake at different stages can be seen in Fig.20.

Smith Degradations.

1. Uronic acid-rich fraction.
2. Degraded polymer I
3. Degraded polymer II.

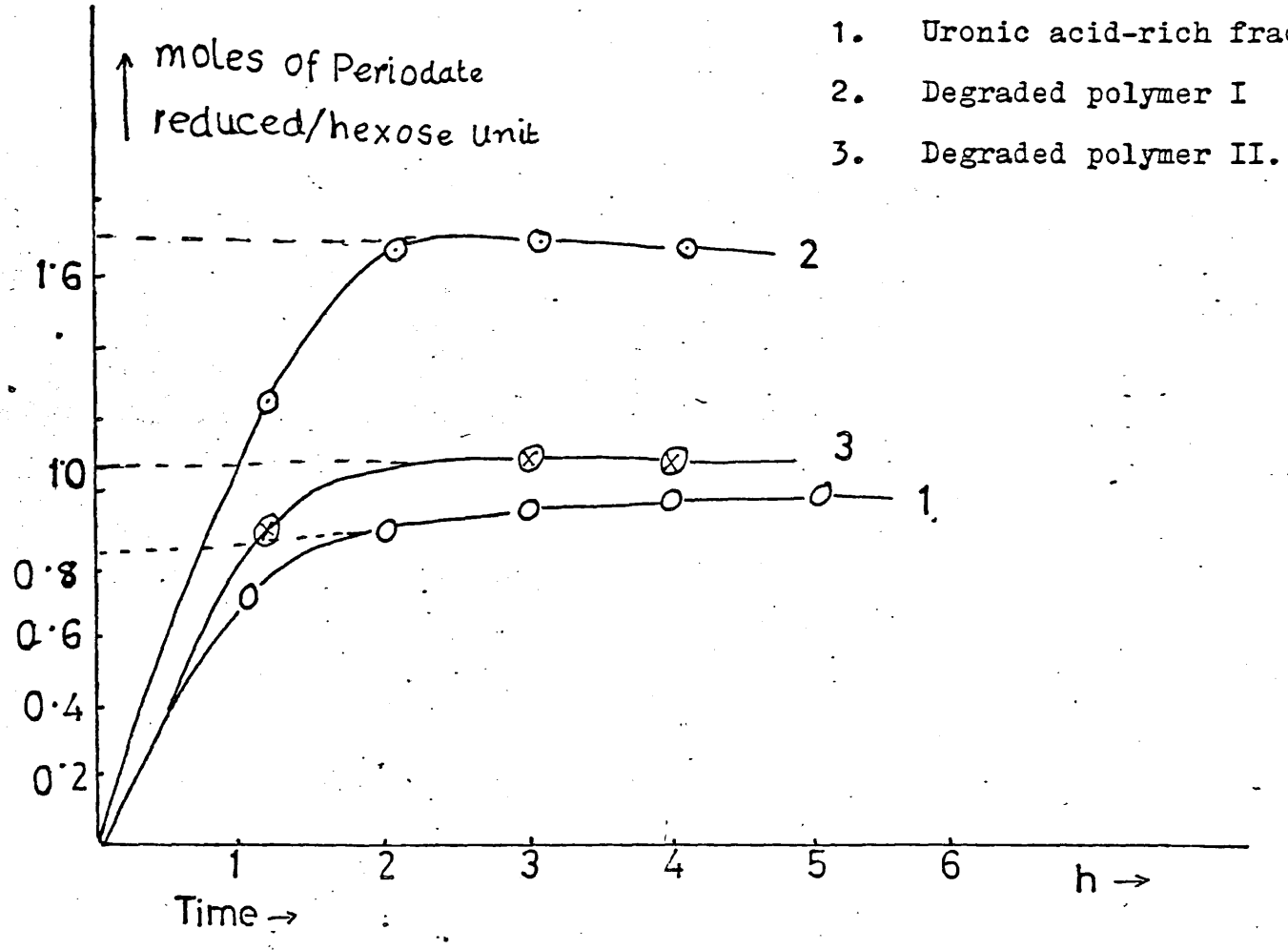


Fig 20.

Expt. 35. Examination of the fragments I. Chromatographic analysis of the fragments I (Fig. 24) [p151] in solvent 1 and 3 using dip. 1 and 2 showed the presence of erythronic acid (major), propane 1,2-diol, glycerol (detected from an aliquot before dialysis), threitol and glucuronic acid. The erythronic acid was confirmed by g.l.c. analysis and comparison with an authentic sample. Propane 1,2-diol and glycerol were confirmed by comparing the products on g.l.c. obtained by periodate oxidation of a standard methylglycoside of 2-O-methyl L-fucose followed by borohydride reduction and hydrolysis.

The chromatographic pattern of the fragments II was similar to that of fragments I. Hydrolysis of the fragments I and II did not alter the initial chromatographic patterns.

Expt. 36. Molecular Weight Analysis[183] of Polymeric materials from Smith Degradation by Sagavac Column chromatography.

(i) A column (90 x 1.5 cm made by Wrights) of Sagavac 6F (by Sarvac Ltd.) was prepared. Preswollen Sagavac 6F was placed in 1M sodium chloride solution containing sodium azide (0.02%) and was de-aerated for 30 minutes after which it was packed in the column by gravity. The completed column was eluted with 1M sodium chloride until the level of the Sagavac at the top of the column remained stationary (ca. 4 days). Both ends of the column were fitted with stainless steel mesh

(80 mμ pore diameter) to prevent blockage by the fine beads.

The column was calibrated to determine the void volume and exclusion volume by passing down a suspension of lyophilised E. coli cells (2 mg) in 1.5M sodium chloride (1 ml). This mixture contained the cellular fraction which was eluted at the void volume (90 ml) and the low molecular weight proteins, which were eluted at the exclusion volume (138 ml). Aliquots (2 ml) of the eluate were collected over 12 h and each of these fractions was analysed at 280 nm on an S.P.500. A graph was drawn of intensity measurement against aliquot number.

Samples of fucose-rich fraction (i.e. polysaccharide, derived polyalcohol I and degraded polymer I) and uronic acid-rich fraction (i.e. polysaccharide, derived alcohols I, II, III, 2 and 3, and degraded polymers I and II), about 2 mg each, were dissolved in 1.5M sodium chloride solution and applied on top of the column. The column was eluted with 1M sodium chloride solution and fractions (2 ml) were collected. Every fifth tube was examined for carbohydrate (G.P. IIIb) and once detected the aliquots to either side were examined. The intensity of the colouration produced was measured at 487 nm using S.P. 500 spectrophotometer and these values were plotted against the relevant fraction number (Figs 21,22) (p 149 a, b).

(ii) Sephadex G.100 column (12 x 0.25 cm) was calibrated with blue dextran 2000 (M. wt. 200,000 lot No. 1328 pharmacia) to

find the void volume (40 ml) and with sucrose to get the exclusion volume (15 ml). Samples of fucose-rich (i.e. polysaccharide, derived polyalcohol I and degraded polymer I) and uronic acid-rich fractions (i.e. polysaccharide, derived polyalcohols I, II, III, 2, 3, degraded polymers I and II), each (0.5 mg) was dissolved in 1M sodium chloride solution and were applied on the column. Fractions (2 ml) were collected and the carbohydrate was detected by phenol-sulphuric acid. Elution patterns are shown in Fig$ 23,27 (p 150a , 154a).

Expt. 37. Selective cleavage of the glycuronosidic linkages. (a) Preparation of the polysaccharide amide.[188] The "fucan" [0.5M, 500 mg carbohydrate content 45%, uronic acid ca. 10%]. The infrared spectrum showed bands at 1600-1500 cm^{-1} and 1400 cm^{-1} (carboxylic)] was placed in a stainless steel bomb. It was cooled to -40° for 2h after which ammonia gas was passed into the bomb for 1h at -40°. This was then allowed to stand for 0.5h at that temperature and again ammonia was passed into the bomb for another 1h. The flow of ammonia was stopped and the mixture allowed to stand at room temperature for 24h. The excess ammonia was slowly released from the bomb and the polysaccharide was transferred to a Soxhlet apparatus and extracted with methanol to remove adhering ammonia. The material (554 mg) after drying under vacuum for 6h contained 5.43% nitrogen. [The I.R. spectrum gave bands 3450, 1700 and 1650 cm^{-1} (amide) and weak bands at 1600-1500 cm^{-1}]

(b) Hofmann degradation of the polysaccharide amide.
The amide (240 mg) was suspended in water (10 ml) and to it was added sodium hypochlorite solution [(10 ml. 14%, sp. gr. 1.26), the pH of the mixture was about 13] at 0°. This was allowed to stand at 0° for 1 h, then heated for 5 minutes at 100°. The cooled solution was acidified with acetic acid to pH 5 and allowed to stand overnight at room temperature. It was dialysed exhaustively, the solution in the dialysis sac was concentrated and freeze-dried (42.0 mg). The dialysable material gave a negative test for carbohydrate. The material (42.0 mg) was dissolved in water and reduced with borohydride as in Expt. 31(b). The degraded polymer (40.8 mg) was recovered after neutralisation, dialysis and freeze-drying.

(c) Mild hydrolysis of the degraded polymer was carried out with 0.1N sulphuric acid at room temperature for 5 h. It was then neutralised with barium carbonate, dialysed, concentrated and finally freeze-dried [(38.1 mg), % carbohydrate 32, spectrum showed weak bands at 1600 cm^{-1} (carboxyl) % carboxyl 4.2]. On hydrolysis this gave mainly fucose, together with small proportions of xylose and glucuronic acid.

The dialysable part was treated with IR-120(H^+) resin and the recovered material was acetylated and analysed on g.l.c. (column 5). A number of peaks were obtained, one of which had the same retention time as an authentic pentitol acetate.

Similar treatment of the methylated polysaccharide amide (50 mg) gave a degraded polymer (ca. 10% yield). This gave only methylated fucoses on hydrolysis.

Expt. 38. Partial hydrolysis. The uronic acid-rich fraction (423 mg) was treated with formic acid (90%) in an atmosphere of carbon dioxide for 2 h in a sealed tube at 100° after which the insoluble material was removed on centrifuge, washed with formic acid (90%) twice. The supernatant and the washings were combined, diluted (5 vol) with water, hydrolysed for 2 h at 100° and then evaporated to dryness. The hydrolysate (hereinafter called 2 h hydrolysate) was preserved.

The insoluble material left after 2 h hydrolysis was retreated with formic acid (90%) and hydrolysed as before for a further 2 h at 100°. The hydrolysate (hereinafter called 4 h hydrolysate) was obtained as described earlier. The insoluble material (K) left after 4 h hydrolysis was successively washed with formic acid (90%, 3 times), alcohol (twice) and ether (twice). The material on air drying gave a sticky mass which was dissolved in water, dialysed thoroughly and finally recovered by freeze-drying as a snow white powder (87.0 mg).

Separation of the 2 h hydrolysate. The separation was carried out on Whatman No.3 MM chromatographic paper in solvent 1. The following 5 fractions were obtained.

Fraction 1. A neutral syrup $(\alpha)_D = -50^\circ$ [(c 0.20 in water), concentration determined by phenol-sulphuric acid read off a standard graph of fucose] gave a yellow coloured spot on a paper chromatogram with aniline oxalate spray. It had R_G 0.64 (solvent 2, compared with 2,6 dideoxylyxose R_G 0.74 in the same solvent) and had R_{fucose} 1.50 and 1.46 (solvents 1 and 2). Only on one chromatogram did this material give a pink colour with deoxy spray although 2,6-dideoxylyxose run as control always gave the pink colour. On demethylation it showed no change in its chromatographic mobility in different solvents indicating that no methyl group is initially present in the sugar residue. Periodate oxidation was carried out on the free sugar and the release of formaldehyde was estimated using acetylacetone reagent.[190] This showed that there was no formaldehyde release on oxidation. The free sugar was converted into methyl glycoside which on periodate oxidation reduced 1.6 moles of periodate per anhydro unit based on weight from phenol-sulphuric and fucose graph (methyl fucoside reduced 2.3 moles per anhydro unit in a parallel experiment) indicating that C-3 of the sugar unit must have a free hydroxyl group. The retention time (T) of the T.M.S. derivative of the free sugar was 1.45, 1.6 (shoulder) (relative to the corresponding xylitol derivative, column 3 at 160°, cf. 2,6-dideoxylyxose T 2.5, 3.3 and 4.7). (T) of the derived alditol acetate was 1.70 (relative to that of xylitol acetate cf. 2,6-dideoxylyxitol acetate 1.06 on column 5). Hereinafter this material is tentatively assumed to be a "dideoxy sugar".

Fraction 2, was a neutral syrup and was found to be fucose by paper chromatography and by g.l.c. analysis of the TMS derivative.

Fraction 3 was also a neutral syrup and by chromatographic and g.l.c. analysis proved to be xylose.

Fraction 4 was an acidic syrup and gave a broad yellowish orange coloured spot on a paper chromatogram with aniline oxalate spray. It had R_{fucose} 0.65 (solvent 1). This was subjected to ionophoresis for 6 h at 2000 volts at pH 6.7 (pyridine-acetic acid buffer) and gave four spots. These were eluted separately with water from a number of similar ionophoretograms and examined as described below:

(a) was an acid syrup and gave no change in chromatographic pattern on hydrolysis. The chromatographic and ionophoretic mobilities were identical with those of glucuronic acid.

(b) was an acidic syrup, $[\alpha]_D = -20.7^\circ$ (c 0.15 in water) had R_{fucose} 0.61 and 0.65 (solvents 1 and 2). It gave a red colour with tetrazolium hydroxide indicating the absence of 2-O-substituent. Aniline diphenylamine-phosphoric acid failed to detect it. Hydrolysis of an aliquot and paper chromatographic analysis of the hydrolysate showed the presence of glucuronic acid and the "dideoxy" sugar. A second aliquot had a D.P. 1.5 (since it gave 220 μg as an equivalent mixture of glucuronic acid and fucose, and the reduced material corresponds to 72 μg as glucuronic acid). A third portion on reduction and

hydrolysis gave only glucuronic acid (paper chromatography with aniline oxalate).

(c) was an acidic syrup, $[\alpha]_D = -18.5^\circ$ (c 0.2 in water) and had R_{fucose} 0.58 and 0.61 (solvent 1 and 2). (Cf. Aldobiouronic acid from Ascophyllum nodosum[155] R_{fucose} 0.56 and 0.63 in solvent 1 and 2). It gave an intense red colour with tetrazolium hydroxide indicating the absence of 2-O-substituent. A greyish-green colour was given with aniline diphenylamine-phosphoric acid from which it can be deduced that 1,3-linkage[161] is probable. Hydrolysis of an aliquot and chromatographic analysis showed glucuronic acid and fucose as the sole products. Determination of D.P. gave 80 μg as equivalent mixture of glucuronic acid and fucose, and the reduced material corresponded to 40 μg as glucuronic acid. Another portion on reduction and hydrolysis gave only glucuronic acid (paper chromatography using aniline oxalate spray).
(d) was a neutral syrup and on hydrolysis it showed no change in chromatographic mobility in different solvents. Chromatographically it had the mobilities of galactose in a number of solvents.

Fraction 5 was an acidic syrup, $[\alpha]_D = -24^\circ$, which gave a yellowish-orange coloured spot on a paper chromatogram with aniline oxalate spray and had R_{fucose} 0.15 (solvent 1). It had a D.P. 4.3, and on hydrolysis gave glucuronic acid, fucose and "dideoxy" sugar.

(a) Partial hydrolysis. An aliquot of fraction 5 was hydrolysed for 1 h in a sealed tube at 100° with 90% formic as before and the hydrolysate was analysed as described previously. This showed on paper chromatography in solvent 2 the presence of "dideoxy" sugar, fucose, and some fractions of slower mobility with R_{fucose} 0.65 (major) 0.25 and 0.15 (unchanged materials, solvent 1). These fractions were separated on 3MM chromatographic paper and each fraction was examined. The major fraction (R_G 0.65) was subjected to ionophoresis and gave a similar pattern to that of fraction 4.[125] Each fraction was eluted from the ionophoretogram and examined as described in 4(a-c). They proved to be identical with the fractions 4(a-c). The fraction with R_G 0.25 was hydrolysed and analysed chromatographically and was found to contain glucuronic acid, fucose and "dideoxy" sugar residues.

(b) Methylation of an aliquot was carried out as in Expt. 27. G.l.c. analysis, after hydrolysis and glycosidation, gave peaks corresponding to methyl 2,3,4,6-tetra-O-methylglucoside (1.0, 1.43 column 1 and 1.0, 1.32 column 2), methyl 2,4-di-O-methyl-fucoside (1.62, 1.70 column 1 and 0.89, 1.0 in column 2) and unidentified peaks of T value 2.54, 2.82 [(major) column 1)] and 1.54 (major) and 0.70 (column 2).

Examination of 4 h hydrolysate. This was analysed as described in 2 h hydrolysate. It showed identical fractions to those of the 2 h hydrolysate except for the presence of a fraction with R_{fucose} 0.25 (solvent 1). This fraction ~~(R_G 0.25)~~ on hydrolysis and chromatographic analysis showed the presence of glucuronic acid, fucose and "dideoxy" sugar.

Examination of the insoluble material (K).

The insoluble material was hydrolysed and on chromatographic analysis revealed the presence of glucuronic acid, fucose (major) and "dideoxy" sugar.

PART II. RESULTS AND DISCUSSIONS

This part of the thesis deals with the constitution of the fucose-containing polysaccharides isolated from the three genera of algae. The ammonium oxalate-oxalic acid extract from *P. pavonia* proved to be a crude intractable material which defied purification and fractionation. Apart from an examination of its constituent sugars which were the same as those of the same extract from the other two genera, this material was not examined further. The chlorite extract from *P. pavonia* also had the same constituent sugars as found in the same extract of the other two genera and was not investigated further.

The flow diagram (p.103) shows the distribution of these polysaccharides in the three brown seaweeds. The "fucans" extracted in the aqueous calcium chloride solution and in the acid solutions proved to be very similar, both showed negligible contamination with alginic acid. In order to establish the homo- or hetero-geneity of these extracts they were fractionated separately on a DEAE-cellulose column. Both gave three fractions at about 0.3M, 0.5M and 1.0M aqueous potassium chloride concentrations which were virtually identical to each other from each of the genera of the weeds. The respective aqueous calcium chloride and acid extracts from each weed were therefore combined and their properties are given in Tables 7 and 8. Of these the molar fractions comprise the

Table 7

Some Constants of the combined aqueous Calcium chloride and Acid Extracts (Procedure III)

Species	Fraction	$[a]_D$	C in water	% Recovery
H. lorea	0.3M	-87^o	0.25	28
	0.5M	-58^o	0.22	16
	1.0M	-131.7^o	0.50	33
B. bifurcata	.3M	-84^o	0.50	42
	0.5M	-54^o	0.25	8
	1.0M	-100^o	0.5	20
P. pavonia	.3M	-94^o	0.20	31
	0.5M	-51^o	0.10	20
	1.0M	-112^o	0.16	38

Table 8

Percentage composition of the combined aqueous Calcium chloride and Acid Extracts

Species	Fraction KCl	% Carbohyd.	% CPC	SO_4 Col.	% CPC*	Uronic Carbazole	Mean
H. lorea	0.3M	50-52	-	2.5	17.6	20.0	18.8
	0.5M	45-47	20.0	21.7	7.5	10.3	8.9
	1.0M	40-42	29.0	-	3.6	4.3	4.0
B. bifurcata	0.3M	48-50	-	4.6	19.1	20.0	19.5
	0.5M	43-45	22.7	-	12.0	11.0	11.5
	1.0M	40-42	30	-	1.5	2.8	2.6
P. pavonia	0.3M	50-52	-	2.5	19.2	21.7	20.4
	0.5M	45-47	-	11.0	8.2	10.1	9.1
	1.0M	40-42	17.0	-	4.5	5.3	4.7

* CPC = Cetylpyridinium chloride

major component in H. lorea and P. pavonia while 0.3M fraction is the major fraction from B. bifurcata. The overall composition (Table 8) shows that 0.3M fraction in all these algae has the highest uronic acid and lowest sulphate content (hereinafter called uronic acid-rich fractions). On the other hand, 1.0M fraction in all cases has the highest fucose (Table 9, p134) and sulphate contents (Table 8) (hereinafter called fucose-rich fraction). The overall composition of the 0.5M fraction in all three species has a somewhat intermediate composition between the 0.3M and 1.0M fractions although the proportion of fucose in this fraction from P. pavonia is rather low. The percentage SO_4^{2-} and fucose composition of the fucose-rich fraction in all three algae closely resembles fucoidan as described in the literature. [141-144]

The 0.3M fraction was analysed for nitrogen and found to contain 0.25% nitrogen which corresponds to 1.6% protein. In order to obtain information on the homogeneity of the polysaccharide fractions of the "fucans" and to determine if protein was covalently linked, these polysaccharides were subjected to gel electrophoresis (Expt. 27). The 0.3M and 1.0M fractions appeared to be homogeneous and protein which moved at the same speed as the polysaccharide may therefore be covalently linked. The 0.5M fraction gave a diffuse pattern.

The glucuronoxylofucans extracted by alkali (Expt. 15) and by chlorite (Expt. 17) from H. lorea and B. bifurcata

were found to be similar to one another (Tables 10 and 14)(p135, 137) in their uronic acid and sulphate contents. They also had similar sulphate and uronic acid contents to the respective 0.5M KCl fractions described under the "fucans" (Table 8)(p131), but the proportion of sugars in the "fucans" and the alkali extracts were somewhat different (Tables 9 and 11)(p 134, 135).

Fractionation of these materials on a DEAE-cellulose column gave only two main fractions at about 0.5M and 1.0M concentrations of KCl solution (Tables 10 and 14)(p135, 137) and these only differed from one another in the somewhat higher sulphate content of the molar fractions. The sulphate content of P. pavonia glucuronoxylofucan from the alkali extract was lower than that of the other two algae (Table 10) but the proportions of sugars were similar. No attempt was made to fractionate this.

The glucuronoxylofucan extracted by ammonium oxalate-oxalic acid from H. lorea (Table 12)(p136) has a lower sulphate and higher uronic acid than the alkali extracted glucuronoxylofucans and in this respect resembles the 0.3M fraction of the "fucans". On the other hand from B. bifurcata had a low sulphate but the uronic acid content was similar to that of the alkali extracted glucuronoxylofucans from B. bifurcata and H. lorea. The proportions of the constituents are in agreement with these findings.

Table 9

Mole proportions of fucose-containing polysaccharides from acid extract

Species	Fraction	Fucitol	Xylitol	L-gulonic	Galactitol
H. lorea	.3M	2.5	1.0	1.8	+
	.5M	*10.	1	4.0	+++
	1.0M	*14.0	1	2.0	++
B. bifurcata	.3M	2.8	1.0	2.4	+
	.5M	5.5	1.0	2.5	++
	1.0M	13.0	1.0	2.3	+++
P. pavonia	.3M	2.0	1.0	2.0	+
	.5M	*3.0	1.0	1.2	++
	1.0M	*12.5	1.0	3.0	+++

* Determined as alditol by g.l.c., and the remainder were determined by phenol-sulphuric after elution from paper chromatograms.

+ indicates quantity of galactose visually ascertained in different fractions.

Table 10

Some properties of the glucuronoxylofucans from alkali extract

Species	Fraction	$[\alpha]_D$	% Carbohyd.	% SO_4		% Uronic			% Recovery
				CPC	Col.	CPC	Carbazole	Mean	
H. lorea	Unfractionated	-51.2°	40.0	22.2	-	10.3	-	-	-
	0.5M	-	45.0	20.0	21.0	10.0	15.4	12.7	30
	1.0M	-	45.0	24.6	25.0	10.3	15.0	12.6	50
B. bifurcata	Unfractionated	-48.5°	40.0	24.0	-	11.3	-	-	-
	0.5	-	45.0	21.6	22.0	9.5	15.0	12.2	30
	1.0M	-	45	26.0	24.0	10.0	15.2	12.6	50
P. pavonia	Unfractionated	-58.0°	47	14.5	-	11.0	-	-	-

Table 11

Mole proportions of unfractionated glucuronoxylofucans from alkali extracts

Species	Fucitol	Xylitol	L-gulonic
H. lorea	4.4	1.0	2.2
B. bifurcata	5.0	1.0	2.0
P. pavonia	4.7	1.0	2.4

Table 12

% Composition of the different fractions of Ammonium oxalate-oxalic acid extract

Species	Fractions	% carbohyd.	% SO_4	% uronic	% Recovery
H. lorea	0.5M	45	9.4	21.8	40
	1.0M	40	13.4	20.0	12
	Unfractionated	30-32	11.1	9.4	-
B. bifurcata	0.5M	45	9.2	12.8	40
	1.0M	37-38	14.9	9.5	10
	Unfractionated	35-37	9.7	11.8	-

Table 13

Mole proportions of Ammonium oxalate-oxalic acid extract (0.5M fraction)

Species	Fucitol	Xylitol	Gulonic
H. lorea	4.4	1.0	3.7
B. bifurcata	5.6	1.0	2.1

Table 14

Some properties of glucuronoxylofucans from chlorite extract

Species	Fraction	% carbohyd.	% SO_4	% uronic	% Recovery
H. lorea	0.5M	45	17.7	10.6	50
	1.0M	40-42	24.2	11.3	30
	Unfractionated	40-42	19.2	..	-
B. bifurcata	0.5M	45	18.7	10.6	45
	1.0M	43	24.0	11.3	30
	Unfractionated	40-42	21.2	..	-

Table 15

Chromatographic mobilities of different methylated sugars obtained from different fractions of "fucans" and glucuronoxylofucans of H. lorea and B. bifurcata (R_G are respectively in solvent 2 and 4).

2-O-methyl-L-fucose R_G 0.56 and 0.60
3-O-methyl-L-fucose R_G 0.41 to 0.5 and 0.56
3,4-di-O-methyl-L-fucose R_G (0.69) and 0.78
2,3-di-O-methyl-D-xylose R_G (0.70)
2,3-di-O-methyl-D-glucuronic acid R_G 0.17 and 0.17.
2,3,4-tri-O-methyl-L-fucose R_G (0.92)
2,3,4-tri-O-methyl-D-xylose R_G (0.92)
Free-L-fucose R_G 0.41 and 0.48.

Figures in the parenthesis indicate R_G values of components which are not completely resolved.

These studies show that the three different genera of brown seaweeds synthesise the same fucose-containing polysaccharides as one another, and that these comprise a wide variety of polymers based on fucose, xylose, glucuronic acid and galactose. However, the proportions of each type of polymer varies in the different genera. It was decided to apply methylation, periodate oxidation, Smith degradation and partial hydrolysis to each of the extracts and fractions in order to see if they are each built up on the same general plan or if there are major structural differences between them.

Methylation Studies. The 0.5M and 1.0M KCl fractions of the "fucans" and of the glucuronoxylofucans from H. lorea and B. bifurcata were each methylated separately (Expt. 28). The methylated products after hydrolysis and chromatographic analysis (Table 15 p137) were divided into two. One half was converted into the methylated glycosides and analysed by g.l.c. (Table 16 p140). The different methylated sugars were characterised by comparison with authentic materials. However, the large number of peaks obtained from the methylated glycosides made complete characterisation of all the methylated sugars difficult and any free sugars would not be detected. To overcome these difficulties the other half of the methylated hydrolysates were reduced to the methylated sugar alcohols (alditols) and acetylated. The derived methylated acetylated (in case of free sugars only acetylated) alditols were analysed on two,

different columns (Table 16, p140) and their identity confirmed by comparison with authentic samples.

Because of the high uronic acid content of the 0.3M KCl fraction of the "fucans" and the difficulty of characterising methylated uronic acids, the uronic acid in these materials, after partial methylation, was reduced to glucose derivatives and remethylated. The product was hydrolysed and analysed by paper and g.l.c. chromatography as before.

Free fucose, 2-O-methyl, 3-O-methyl and 3,4-di-O-methyl-fucose were major products from all the different fractions, although the relative proportions of these derivatives varied in different fractions. In addition smaller proportions of 2,3,4-tri-O-methylfucose, glucuronic acid and xylose were detected in all the fractions, and trace quantities of 2,3-di-O-methylxylose and 2,3-di-O-methyl glucuronic acid could be detected in some fractions such as the glucuronoxylofucans isolated from alkali and ammonium oxalate-oxalic acid extracts. 2,3,4,6-Tetra-O-methyl galactose was also detected in the products from the methylation of the 1.0M KCl fraction of the "fucans". The reduced methylated uronic acid-rich fraction (Expt. 29) gave 2,3,6-tri-O-methylglucose as the major product (Table 17, p141). The usual methylated fucose derivatives were also present, but some of the peaks of the methylated glycosides were partly masked by those of the tetra-O- and tri-O-methyl glucose peaks. Conversion to the alditol acetate, however, confirmed their presence.

These results indicate that all the different extracts

Table 16

Methylation results of fucans and glucuronoxylofucans (1.0M) of H. lorea

Retention times (T) are relative to 2,3,4,6-tetra-O-methyl-β-D-glucoside and 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-O-glucitol respectively.

	As methyl glycosides		As alditol acetate	
Major products	Column 1	2	ECNSSM	ApK
Free-L-fucose			2.0	1.20
2-O-methyl-L-fucose	(0.73)	(1.0)	1.50	(1.0)
	4.0	(1.20)		
	4.4	(1.50)		
3-O-methyl-L-fucose	(2.2)	(1.34)	1.83	(0.55)
	3.5	(1.44)		0.88
	5.3	1.64		
Other products				
2,3,4-Tri-O-methyl-L-fucose	(0.73)	0.61	0.69	0.72
2,3,4-Tri-O-methyl-D-xylose	0.43	0.40	0.50	(0.53)
	0.61	(0.56)		
2,3,4-Tri-O-methyl-D-glucuronic acid	(2.1)		-	-
		(2.45)		
	3.1	3.9,		
2,3-Di-O-methyl-D-glucuronic acid	-	(2.45)		
		3.17		
2,3-Di-O-methyl-D-xylose	1.38,	(0.56,)	1.36	-
	(1.69)	0.76		
	1.82	(1.0)		
3,4-Di-O-methyl-L-fucose	(1.69)	0.94,	1.20	(1.0)
		(1.0)		
	2.69	(1.20,)		
		(1.34)		

Figures in parenthesis indicate T values of components which are not completely resolved.

Table 17

Methylation results of Uronic acid-rich fraction

T are relative to 2,3,4,6-Tetra-O-methyl-β-D-glucoside and 2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl glucitol respectively.

	As methyl glycosides		As alditol acetate	
Major products	Column 1	2	ECNSSM	ApK
2,3,6-tri-O-methyl-D-glucose	3.20	(1.69)		
	(4.10)	2.20	2.35	1.3
Free-L-fucose	-	-	2.0	1.2
2-O-methyl-L-fucose	(4.1)	(1.0)		
	4.4	1.18	1.52	1.1
Other products				
2,3,4,6-tetra-O-methyl-D-glucose	1.0	(1.0)	1.0	1.0
	1.37	(1.33)		
3-O-methyl-L-fucose	2.0	(1.33)	1.81	0.55
	3.5	(1.69)		0.89

Figures in parenthesis indicate T values of components which are not completely resolved.

and fractions are built up on the same general plan. They are also in agreement with the earlier methylation studies on "fucoidan". They confirm the presence of fucose linked or sulphated at C-2, C-3 and C-4, and at C-2 and C-4, and at C-2, C-3 and C-4. The xylose and glucuronic acid are present as end groups and 1,4-linked units, and at least some of the galactose as end group.

In an attempt to give some idea of the different proportions of the methylated derivatives in the different fractions and extracts from H. lorea a rough estimate of the average peak areas on g.l.c. of the derived methylglycosides and methylated alditol acetates was made and the approximate quantity in decreasing order of magnitude of each of the methylated sugars identified in each of the extracts is given in Table 18, p 143. These are not to be regarded as absolute amounts but since the g.l.c. of each fraction was done under the same conditions it serves to confirm the quantitative differences between the different fractions.

It is interesting to see that in all the fractions, except in the uronic acid-rich material, 2-O-methylfucose is the major sugar indicating a reasonably high proportion of linkage or sulphation at C-3 and C-4 of the fucose units. This does not invalidate the 1,2-linkage of fucose as the major structural feature of fucoidan since all the other methylated fucose derivatives could be derived from 2-linked fucose.

Table 18

Proportions of Methylated sugars in H. lorea fractions

key; L-Fucose: 2-O-methyl = A, 3-O-methyl = B

Free fucose = C, 3,4-di-O-methyl = D

2,3,4-tri-O-methyl = E

D-xylose: 2,3-di-O-methyl = F, 2,3,4-tri-O-methyl = G

D-Glucuronic acid: 2,3,4-tri-O-methyl = H,

2,3-di-O-methyl = I, 2,3,6-tri-O-methylglucose = J,

2,3,4,6-tetra-O-methylglucose = K.

"Fucans"

0.3M KCl fraction (after reduction) J>A>C>B>K

0.5M KCl fraction A>B>C>D>I>E>G>F>H

1.0M KCl fraction A>C>B>D>E>G>H

Glucuronoxylofucans		
alkali extract	0.5M KCl fraction	A>C>F>I>D>H>G>E
	1.0M " "	A>C>D>F>B>G>E>H
Ammoniumoxalate-oxalic acid extract	0.5M KCl fraction	A>C>F>I>D>B>E>G>H
	1.0M " "	A>C>H>D>I>E>G
Chlorite extract	0.5M " "	A>C>D>F>E>G>H
	1.0M " "	A>C>D>I>F>B>E>H>G

The relatively high proportion of free fucose in all the fractions confirms the high degree of sulphation and/or branching in these polysaccharides. It is somewhat surprising that this is the second largest fraction in the uronic acid-rich material in view of the low sulphate content of this fraction but it should be emphasised that the peaks given by free fucose are relatively small compared with that given by the 2,3,6-~~tetra~~tri-O-methyl glucose (derived from 2,3-di-O-methyl glucuronic acid).

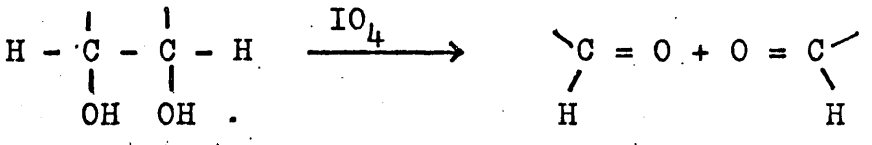
A further point that emerges from this comparison, apart from the major fractions, is the difference in order of the methylated sugars from the different polysaccharides. This again illustrates that in spite of the fact that they are all built up on the same general plan, there are very significant differences in the proportion of the different linkages and/or site of ester sulphate.

The negligible loss of sulphate on alkaline treatment (Expt. 26) of the fucose-rich fraction and 1.0M glucuronoxylofucan from alkali extraction indicated that the sulphate groups in both the cases are essentially alkali stable. This eliminates the possibility of monosubstituted half ester sulphate groups either in 1,4-linked xylose or glucuronic acid units and it is concluded that the majority of sulphate groups are linked to the fucose residues.

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. Periodate oxidation and reduction as well as Smith degradation on the two extreme structures namely the uronic acid-rich and fucose-rich polymers were, therefore, carried out in the hope that further insight into the macromolecular structure would be obtained. Partial hydrolysis was carried out on the uronic acid-rich material for further structural information of this polymer.

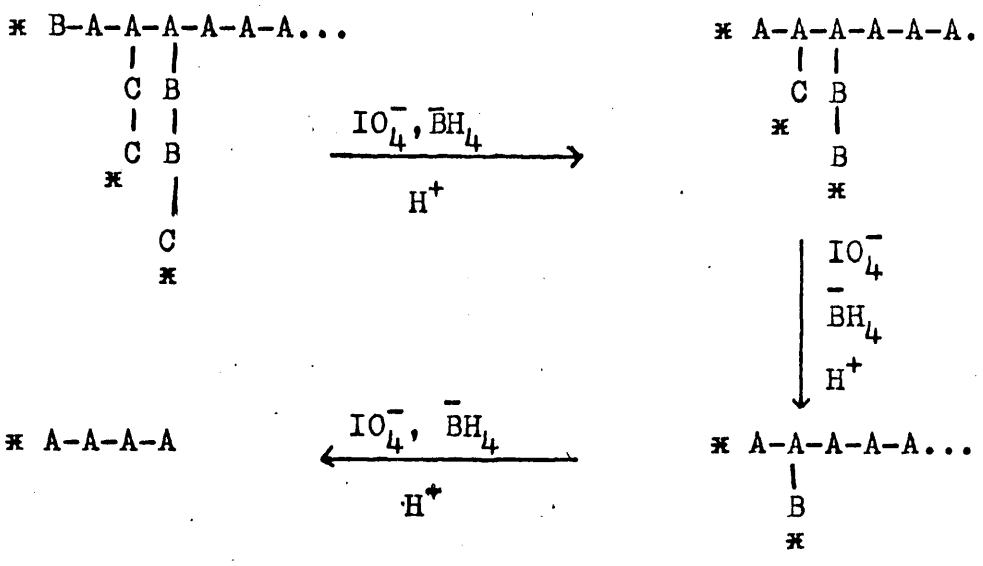
Periodate oxidation and Reduction, Smith degradation and Molecular weight analysis.

Periodate oxidation studies have been used for some considerable time, as a powerful tool in obtaining structural information on polysaccharides. Wherever 1,2-diol systems occur in constituent sugar residues oxidation with sodium metaperiodate causes cleavage of the carbon-carbon bond with resultant conversion to the dialdehyde.



Hydrolysis of the polyaldehyde gives cleavage products diagnostic of the type of units and linkages present in the polymer molecule. This system, however, has some drawbacks.

Firstly, some of the products are unstable in the acidic conditions used during hydrolysis and secondly, in some cases, intra-and intermolecular acetal formation between free aldehydic and hydroxyl groups may occur whichhinders complete oxidation. To overcome these problems the polyaldehyde is converted to the polyalcohol by reduction.[184] Further structural information of the initial polysaccharide can be obtained (1) by subjecting the polyalcohol to mild hydrolysis which will cause hydrolysis of the acetal linkages leaving the glycosidic linkages intact.[185] Thus the sequential periodate oxidation, reduction and mild hydrolysis (Smith degradation) of a polysaccharide removes the residues in the molecule which contain α-glycol groups and new vicinal hydroxyl groups are exposed in the residual polymer. Repeated Smith degradations of this material will gradually erode the molecule down to a core:



* Unless sulphated at C-3 these residues are vulnerable to periodate.

The system above shows that it is possible, under the conditions which avoid over oxidation, for all the A core to be revealed as long as 'in chain' A residues are not attacked by periodate.

(2) By subjecting the polyalcohol to further periodate oxidation which will attack the molecules containing α-glycol groups previously blocked by hemiacetal formation in the polyaldehyde stage, further information on the structure of the molecule is obtained. Repeated oxidation and reduction of

a macromolecule where the primary oxidation is hindered by hemiacetal formation will give at the end a core that is immune to periodate or a molecule where no surviving sugar unit is present. Both the approaches in ascertaining the structure of a polymer are in use. Because periodate reacts very readily with phenols (possible contaminants of these polysaccharides) and that certain aliphatic alcohols inhibit this reaction[187] and it was found that considerably less periodate (0.6 instead of 0.9 moles per anhydro unit in the presence of n-propanol (Fig.17, p114a) was reduced in the case of "fucans"} all periodate oxidations were, therefore, carried out in the presence of n-propanol.

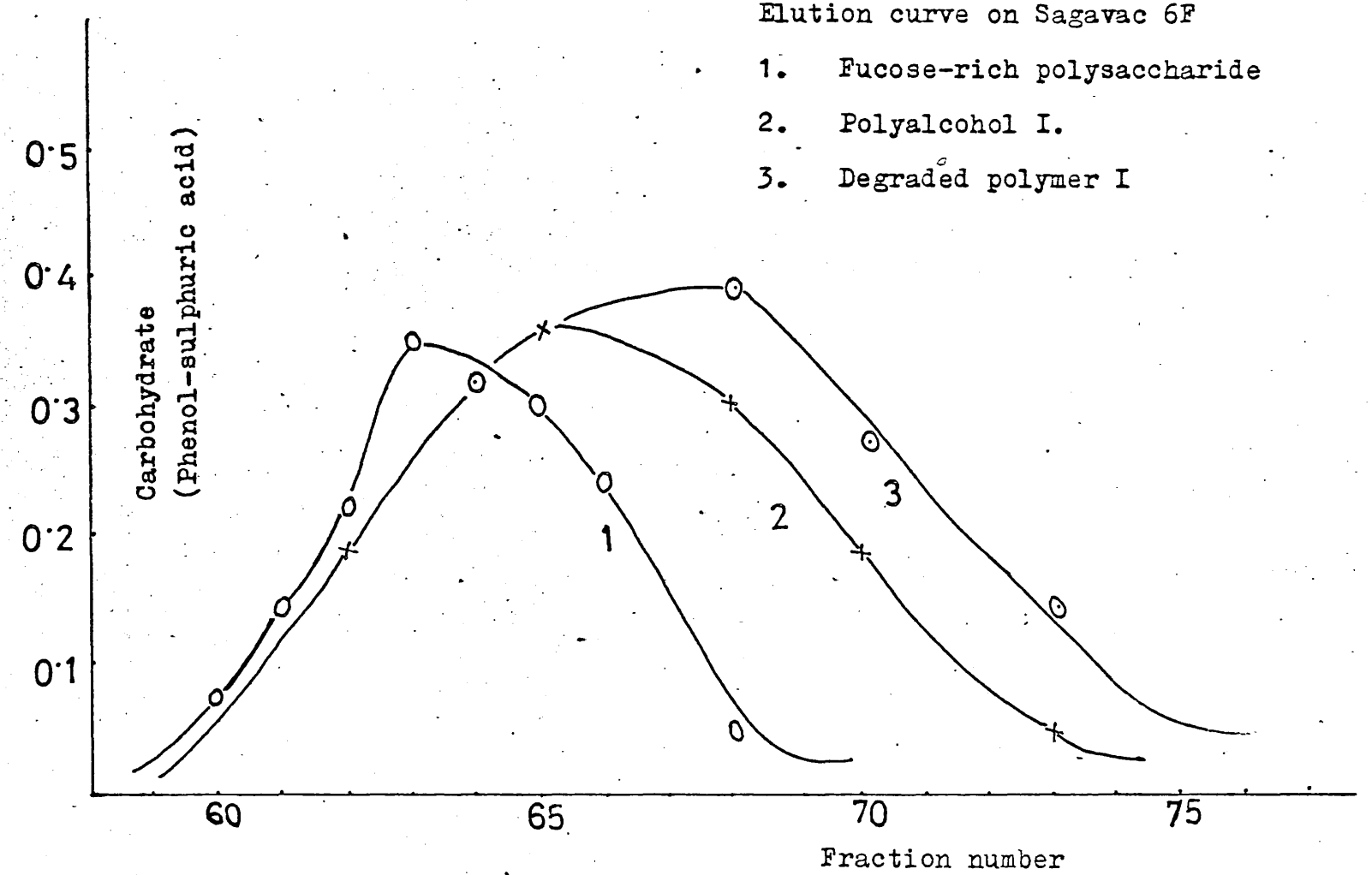
The fucose-rich fraction (1M "fucan") was subjected to periodate oxidation and found to reduce 0.36 moles periodate per anhydro unit (Fig.18, p114b). The derived polyalcohol (recovered in 80% yield) on hydrolysis gave fucose residues as the only surviving sugar units which could be detected. The polyalcohol I on further oxidation reduced virtually no periodate indicating that even if hemiacetal formation had occurred during the oxidation of this polysaccharide then the cleavage of the hemiacetal did not produce vicinal hydroxyl groups. The high sulphate content of this polysaccharide could explain this.

From the molar proportions of the sugar residues and the

carbohydrate content of the polysaccharide and the polyalcohol, it is possible to express the proportions of the constituent sugars in terms of weight. The reduction of periodate per hexose unit can then be correlated with the loss of carbohydrate. This amounts in the present experiment to 0.34 moles per hexose unit cleaved only if each of these units reduces 2 moles periodate. This is in reasonable agreement with the observed value (0.36 moles per hexose unit), and indicates that the greater part of the oxidation occurs at non-reducing end groups and that the macromolecule must be highly branched. The difference of 0.02 moles, if it is at all significant, shows a small amount of oxidation occurs in the core of the molecule.

Preliminary experiments on a column of Sagavac 6F to determine the molecular size of these materials and to substantiate these findings, showed that they were all eluted round the exclusion volume (Figs, 21, 22, p. 149 a,b), indicating that the molecules were somewhat smaller than the range of this column and that the Sagavac 6F was unsuitable for the detection of any change in molecular size of these materials during oxidation and reduction.

A column of Sephadex G100 which will fractionate dextrans with molecular size of 1000 to 150,000 was next investigated and it was found that although the polysaccharide and polyalcohol were both very polydisperse maximum elution occurred.



Void vol 90 ml (Fraction no 45)
exclusion vol 138 ml (Fraction no 69). Fig 29.

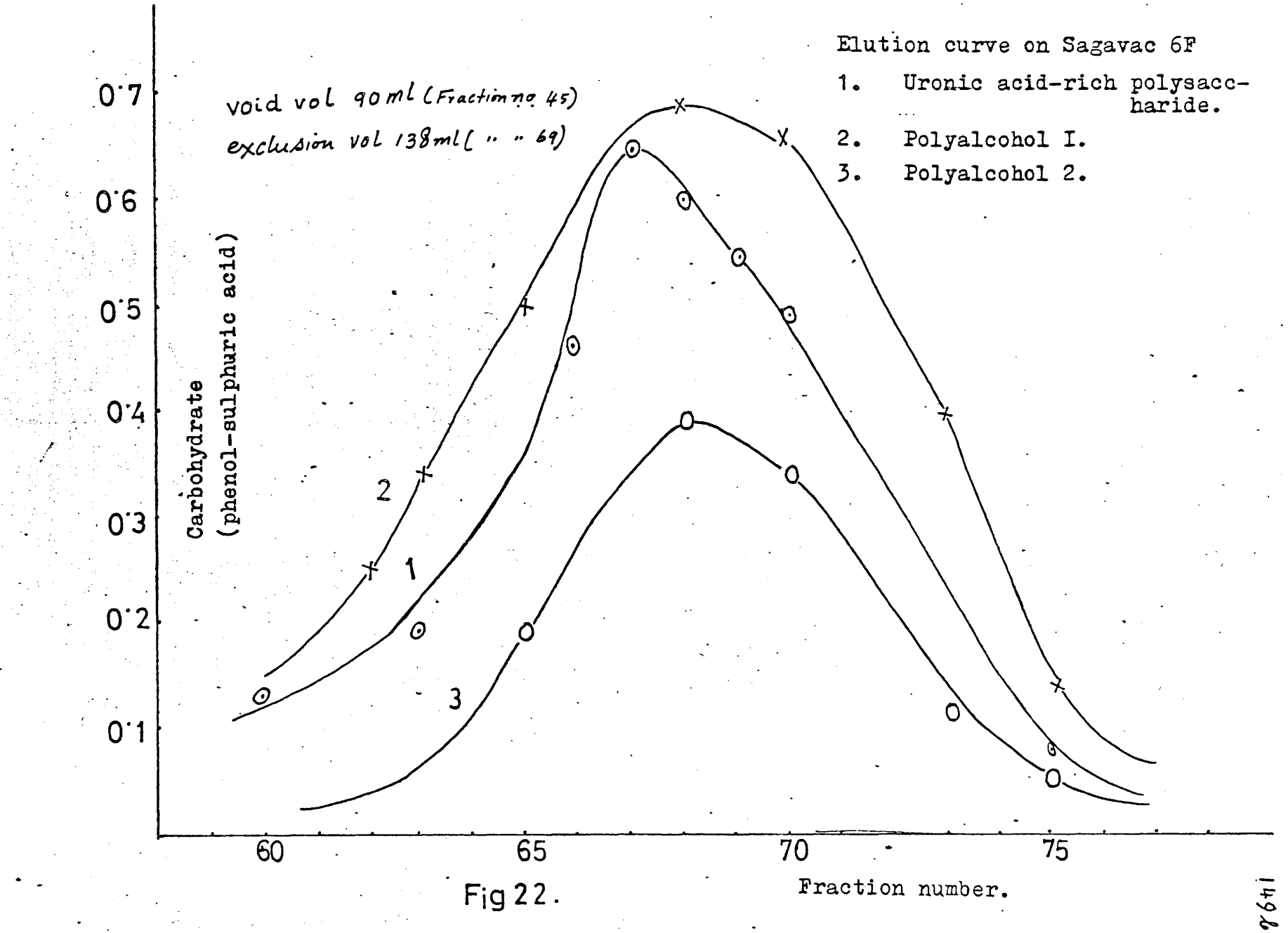

Elution curve on Sagavac 6F
1. Uronic acid-rich polysaccharide.
2. Polyalcohol I.
3. Polyalcohol 2.
void vol 90 ml (Fraction no 45)
exclusion vol 138 ml (" " 69)
Carbohydrate (phenol-sulphuric acid)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
60
65
70
75
Fraction number.
1
2
3

Fig 22.

about midway between the void and exclusion volumes on this column (Fig.23, p150a). The maximum of the degraded polymer on the other hand indicated a somewhat smaller molecular size, although it too was very polydisperse (see Fig.23.). This is in excellent agreement with the periodate oxidation figures and confirms that a small amount of internal cleavage takes place during Smith degradation. Similar studies were carried out with the uronic acid-rich fraction. The scheme of Smith degradations can be found in Fig. 24, p151. It is to be noted that there is a very considerable loss of the carbohydrate during oxidation and reduction. To ascertain at what stage the loss occurs three experiments were set up [Expt. 33(a-c)]. Firstly, the initial polysaccharide was subjected to the alkaline conditions of reduction with borohydride and it was found that virtually no loss of polysaccharide occurred. In a second experiment the polyaldehyde recovered after periodate oxidation showed a loss of about 25%. In the third experiment the polyaldehyde was reduced to polyalcohol and the loss again was about 25%, the total loss during the process of oxidation and reduction being about 50% of the initial material. Chromatographic analysis of the dialysable material (after reduction of the aldehyde) at each stage showed mainly erythronic acid, gulonic acid, trace of threitol, glycerol and possibly propane 1,2-diol and complete absence of free sugar or their respective alcohols even after hydrolysis of this

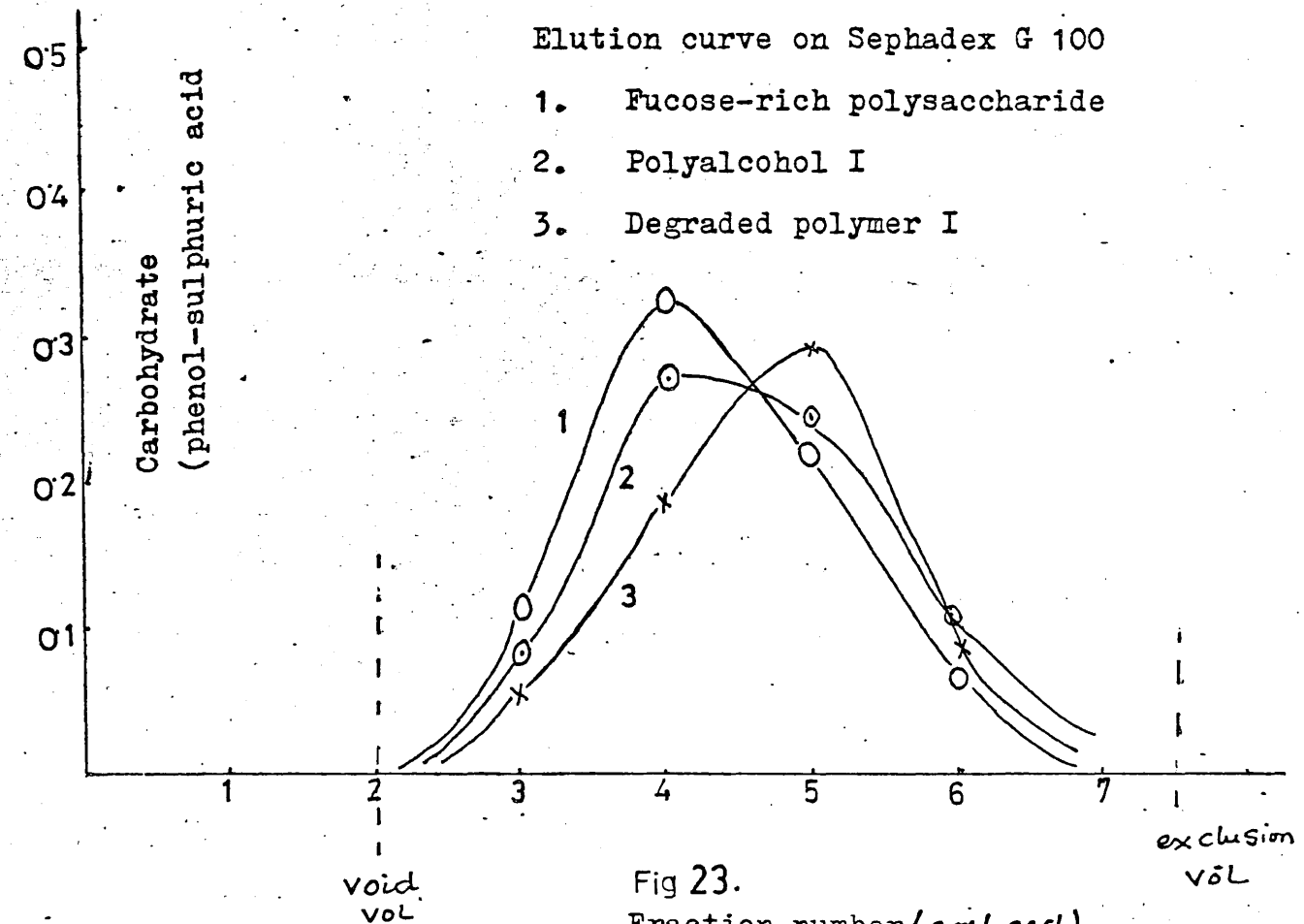

Elution curve on Sephadex G 100
1. Fucose-rich polysaccharide
2. Polyalcohol I
3. Degraded polymer I
Carbohydrate (phenol-sulphuric acid
0·5
0·4
0·3
0·2
0·1
1
2
3
4
5
6
7
1
2
3
void vol
exclusion vol
Fraction number (2 ml each)

Fig 23.

Smith Degradations

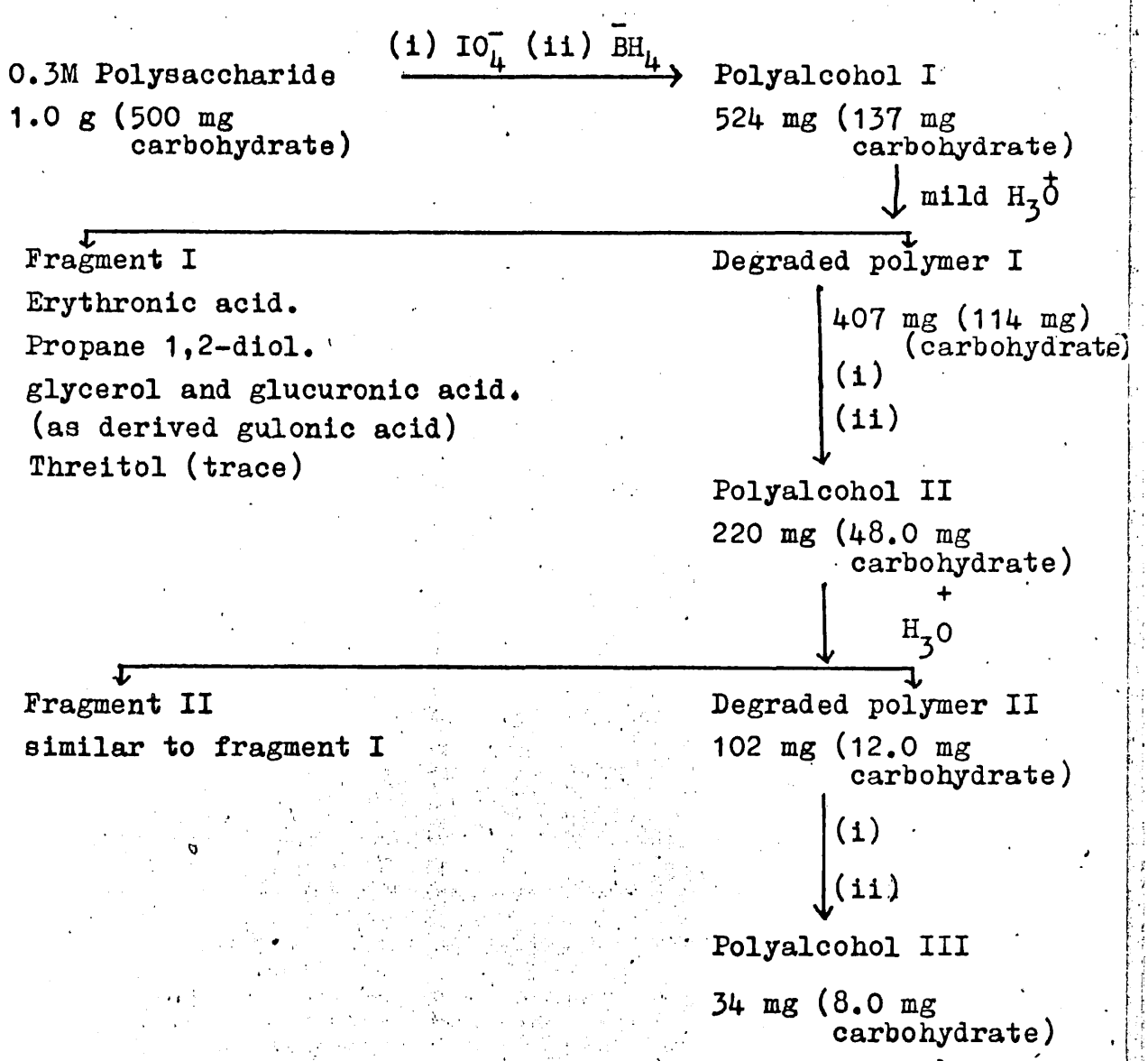


Fig. 24

dialysate. The gulonic acid is derived from unoxidised glucuronic acid, the erythronic acid from 1,4-linked glucuronic acid residues, the propane diol from 1,2-linked fucose, the glycerol from 1,4-linked xylose and the threitol from 1,4-linked galactose in the polysaccharide. These results confirm other structural findings and indicate the presence of 1,4-linked galactose (Figs 25,26).

It was thought at first that the high losses incurred with this polysaccharide might be due to β-elimination of glucuronic acid residues but the standard test for 4,5-unsaturated acid, the product of β-elimination, proved negative. Analysis of the supernatant solutions also revealed the absence of free sugars both before and after hydrolysis. In fact erythronic acid, threitol, glycerol and possibly propane 1,2-diol appeared to be the sole detectable materials. These would be derived by cleavage of the acetal linkages of oxidised glucuronic acid, galactose, xylose and fucose residues respectively as previously shown. It can only be assumed that very labile acetal linkages are formed and these are cleaved as the oxidation and reduction proceed.

These high losses (50% by weight) make the interpretation of the oxidation and reduction results difficult. Investigation of the changes in molecular size by elution of the initial and derived polyalcohol from a Sephadex G100 column gave similar elution patterns for the two materials showing that either some molecules had been completely degraded or

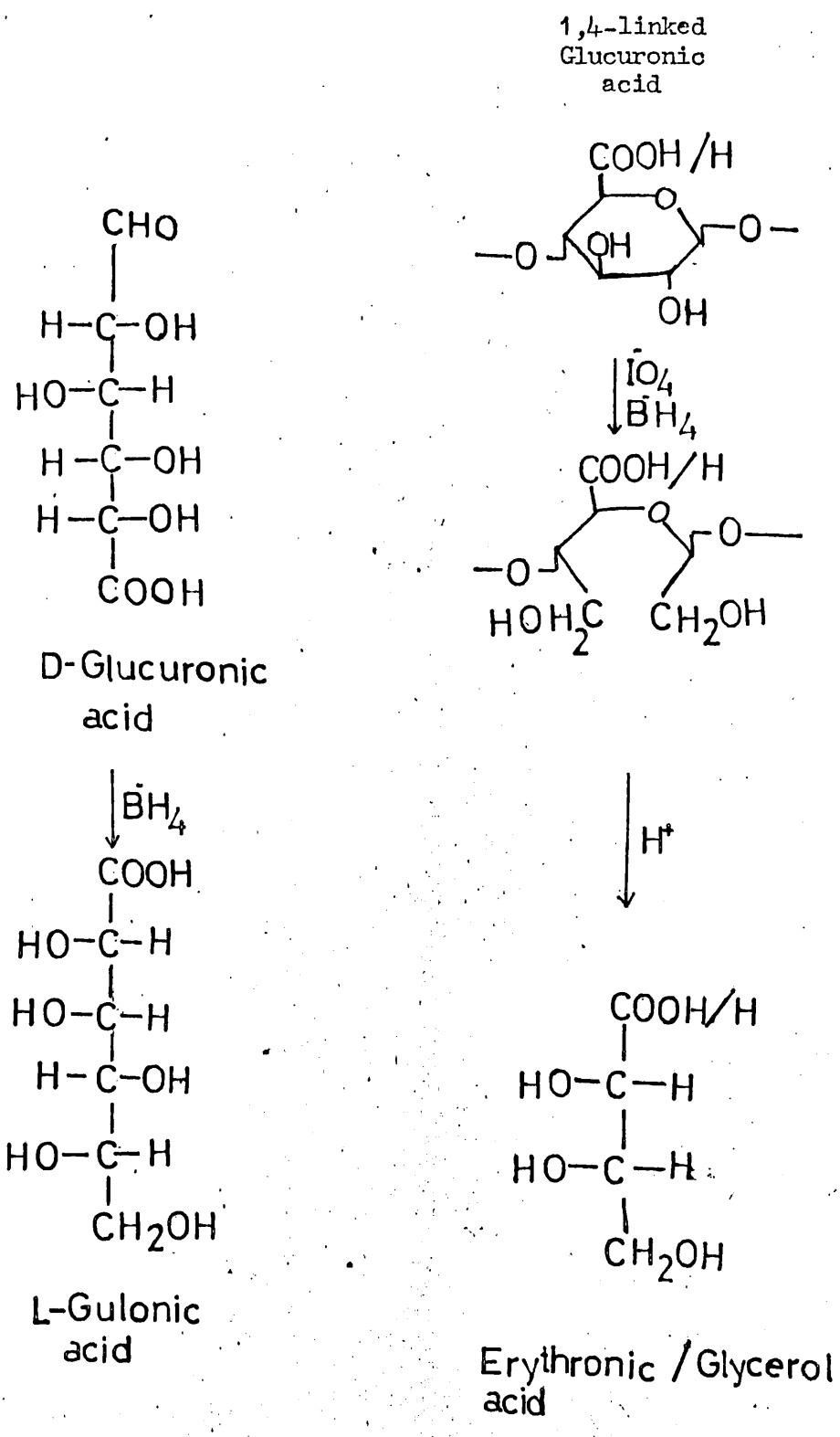

1,4-linked
Glucuronic
acid
COOH/H
O
OH
O
O
OH
IO_4^-
BH_4^-
COOH/H
O
O
O
HOH_2C
CH_2OH
D-Glucuronic
acid
BH_4^-
COOH
HO—C—H
HO—C—H
H—C—OH
HO—C—H
CH_2OH
L-Gulonic
acid
H^+
COOH/H
HO—C—H
HO—C—H
CH_2OH
Erythronic / Glycerol
acid

Fig 25.

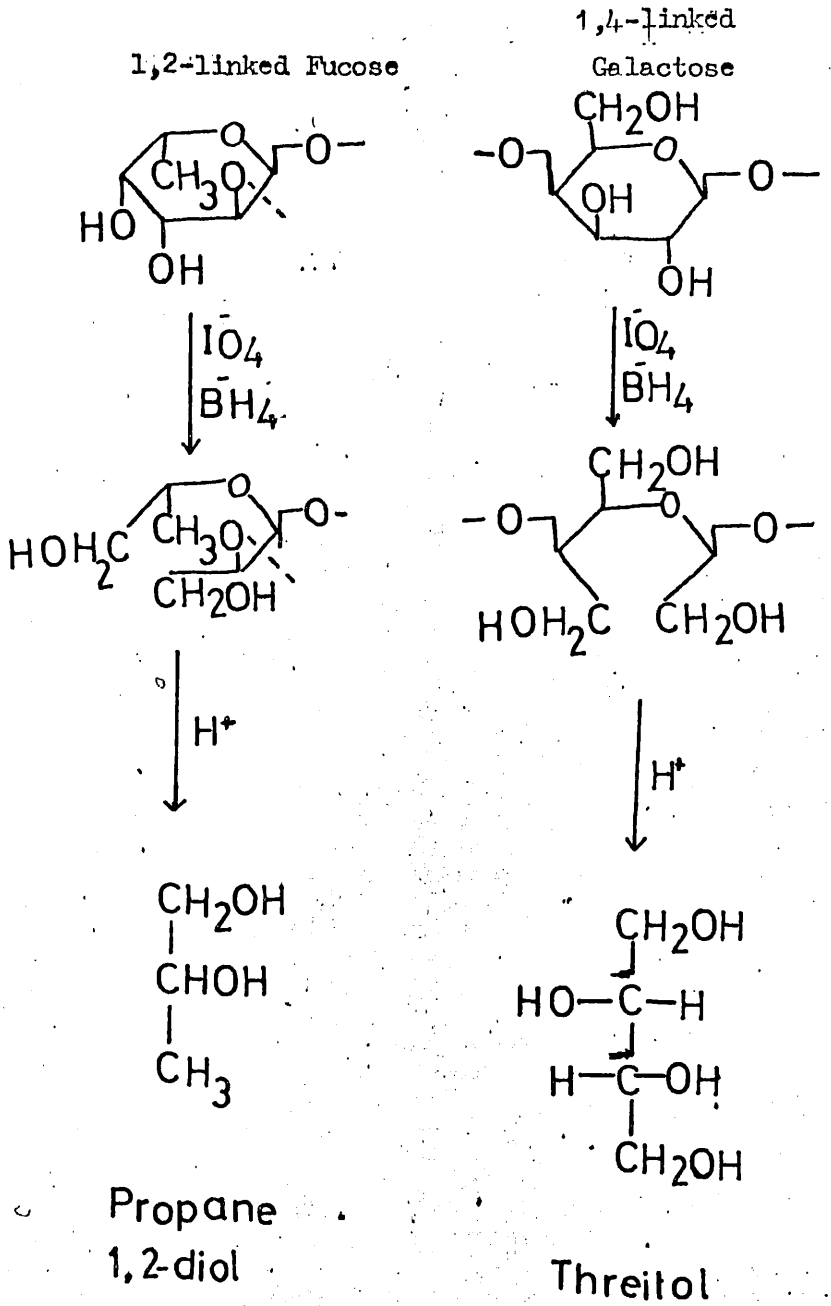

1,2-linked Fucose
1,4-linked
Galactose
IO4
BH4
H+
Propane
1,2-diol
Threitol

Fig 26.

Elution curves for Sephadex G 100

1. Uronic acid-rich polysaccharide

2. Degraded polymer I

3. Degraded polymer II

4. Polyalcohol I

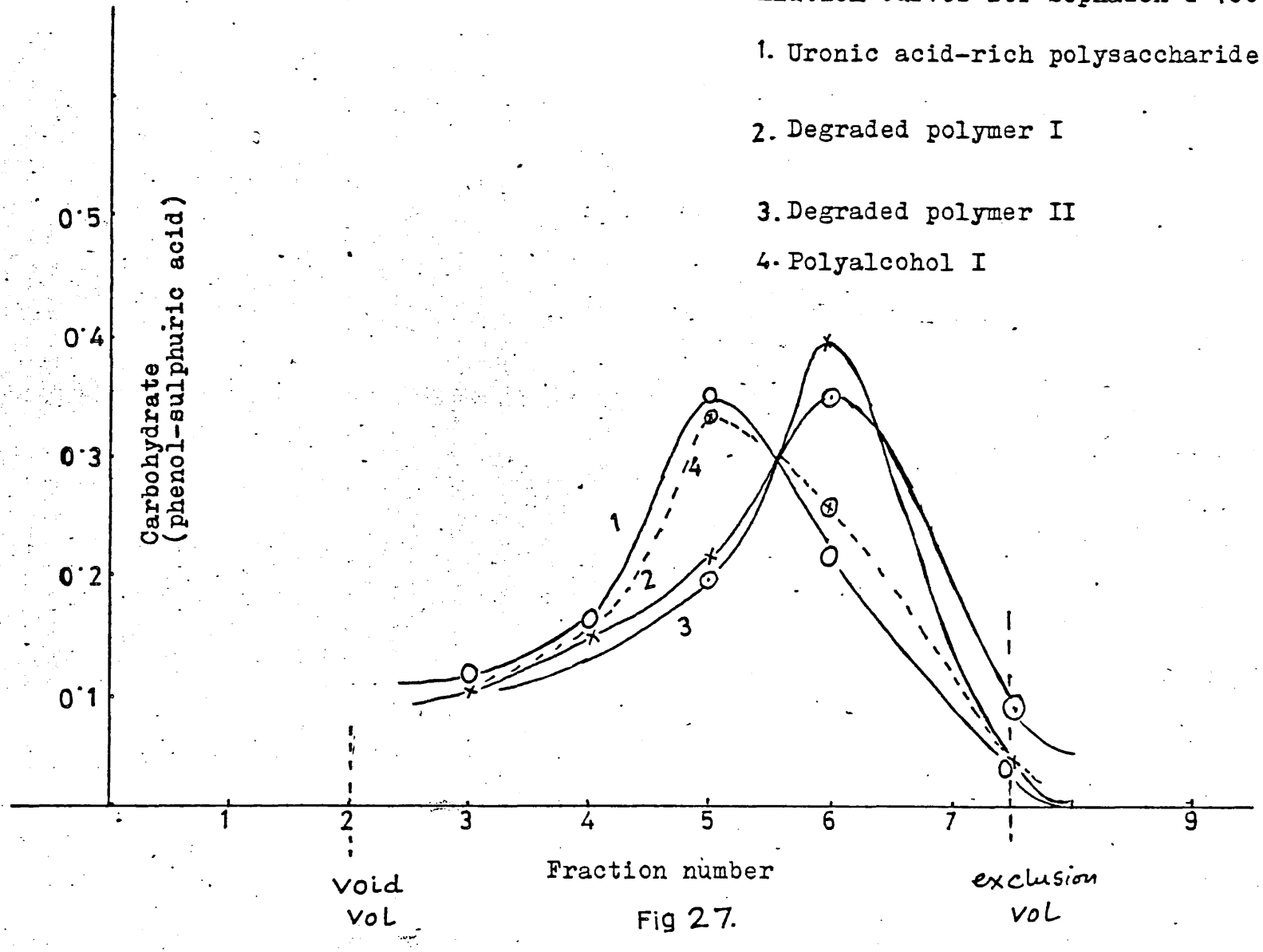


Fig 27.

that cleavage had taken place from the periphery of the molecule (Fig 27 p 154a.). On the other hand accurate calculation of the loss in carbohydrate is difficult and this may not be as high as appears from the weight. It is possible that changes in the shape of the molecule have occurred during conversion to the polyalcohol which might mask any changes of size in the elution pattern. It must be remembered that shape as well as size play an important role in the elution of polymers from these columns.[183]

Application of Smith degradation to this polyalcohol cleaved similar fragments to those cleaved during oxidation and reduction and the degraded polymer was eluted from Sephadex G100 column somewhat later than the polyalcohol indicating some reduction in molecular size (Fig 27, p 154a).

Reoxidation of the polyalcohol I (without previous mild hydrolysis) reduced 1.1 mole of periodate per anhydro unit and gave polyalcohol 2 in 20% yield. A third oxidation reduced a further quantity of periodate (1.7 moles per anhydro unit) and the polyalcohol was recovered in a 50% yield. Again these high losses make interpretation difficult but at least it is clear that with this polysaccharide hemiacetal formation hinders complete periodate oxidation.

Earlier methylation results showed that most of the xylose and glucuronic acid residues are vulnerable to periodate since

these are present as end groups and 1,4-linked units. The xylose residues, however, survived oxidation up to the first oxidation limit. By the second oxidation all the xylose is apparently cleaved and in each of the oxidations uronic acid is more susceptible to oxidation than the fucose residues. However, the polysaccharide even after three oxidations was found to contain about 30% of the glucuronic acid residues and it seemed very unlikely that this proportion would have survived three oxidations through hemiacetal formation. So the presence of some other types of linkages for the glucuronic acid cannot be ruled out although from the methylation studies it was only possible to confirm end groups and 1,4-linkage.

It is of interest to compare the polyalcohols derived after Smith degradation with the corresponding ones obtained from repeated oxidation and reduction (i.e. polyalcohols 2 and 3). The polyalcohols 2 and II (Smith degradation) are virtually identical in their carbohydrate and uronic acid contents and in the relative mole proportions of their constituent sugar residues. So also were polyalcohols 3 and III. This clearly indicates that both straight forward oxidation and reduction as well as Smith degradation lead to the similar products in this polysaccharide and it can be concluded that the acetal linkages are so labile that they are cleaved during subsequent oxidation. This is in keeping with the losses during the first oxidation and reduction.

(Tables 19-22), p 157 & 158.

Table 19

Uronic acid-rich fraction: Some properties of oxidation-Reduction products

Material	% carbohyd. (G.P. IIIb)	% uronic (By carbazole based on carbohyd.)	% uronic (from mole proportions)	Periodate consumption/ hexose unit.
0.3M Poly-saccharide	50.0	38.0	40.0	0.83
Polyalcohol I	26.0	32.0	30.0	
Polyalcohol 2	20.0	40.0	36.0	1.1
Polyalcohol 3	20.0	28.0	27.0	1.7

Table 20

Mole proportions of constituent sugars* in the various polyalcohols

Material	Fucose	Xylose	Glucuronic acid
0.3M Poly-saccharide	2.2	1.0	2.2
Polyalcohol I	2.5	1.0	1.5
Polyalcohol 2	1.5	-	1.0
Polyalcohol 3	2.6	-	1.0

* Determined by phenol-sulphuric after elution from paper chromatography.

Table 21

Some properties of the different products of Smith degradations

Fraction	% carbohyd. (G.P. IIIb)	% uronic (by carbazole based on carbohydrate)	% uronic (from mole proportions)	Periodate consumption/ hexose unit.
0.3M Polysaccharide	50.0	38.0	40.0	0.83
Polyalcohol I	26.0	31.0	30.0	
Degraded polymer I.	28.0	45.0	43.0	1.7
Polyalcohol II.	20.0	40.0	36.0	
Degraded polymer II.	10.0 *	20.0	22.0	1.1
Polyalcohol III	25.0	32.0	32.0	

* Low carbohydrate cannot be accounted for.

Table 22

Mole proportions* of constituent sugars in the various products of Smith degradations

Fraction	Fucose	xylose	Glucuronic acid
0.3M Polysaccharide	2.2	1.0	2.2
Polyalcohol I	2.5	1.0	1.5
Degraded polymer I	4.0	1.0	3.8
Polyalcohol II	1.8	-	1.0
Degraded polymer II	3.4	-	1.0
Polyalcohol III	2.2	-	1.0

* Determined by the phenol-sulphuric after elution from paper chromatogram.

The overall conclusions from these series of experiments are that in the fucose-rich fraction little, if any, hemiacetal formation occurred and that all the xylose and glucuronic acid residues are cleaved during the first oxidation confirming that these groups are present as end groups and 1,4-linked units. Molecular size determined on Sephadex G100 shows that very little change in the molecular size had occurred during oxidation and reduction of the polysaccharide to polyalcohol I. From this it may be concluded that these residues are on the periphery of the molecule.

In the case of the uronic acid-rich fraction where considerable hemiacetal formation occurs, the results are not so clear, particularly with regard to the glucuronic acid residues. It is possible that some of these units are in the centre of the molecule and are not accessible to periodate or even it may be that some other types of linkages make it immune to periodate.

It is, however, quite clear in both the fucose-rich and uronic acid-rich polymers that a high degree of branching occurs since extensive reduction of periodate causes little change in molecular size even after repeated degradations.

Selective cleavage of the glycuronosidic linkages.

Recently, the Russian workers[189] after preliminary experiments on model monosaccharides, attempted selective cleavage by the Hofmann reaction of the glycuronosidic linkages in polysaccharides, leaving the glycosidic linkages intact. This involves the conversion of the uronic acid residues in the polysaccharide into their amides followed by their transformation into 5-aminopentopyranose residues by sodium hypochlorite. These moieties are cleaved easily by acids under mild conditions with concomitant release of the aglycone and the formation of a dialdehyde from the uronic acid residue. If this is followed by reduction the dialdehyde is converted into a pentitol (Fig.28).

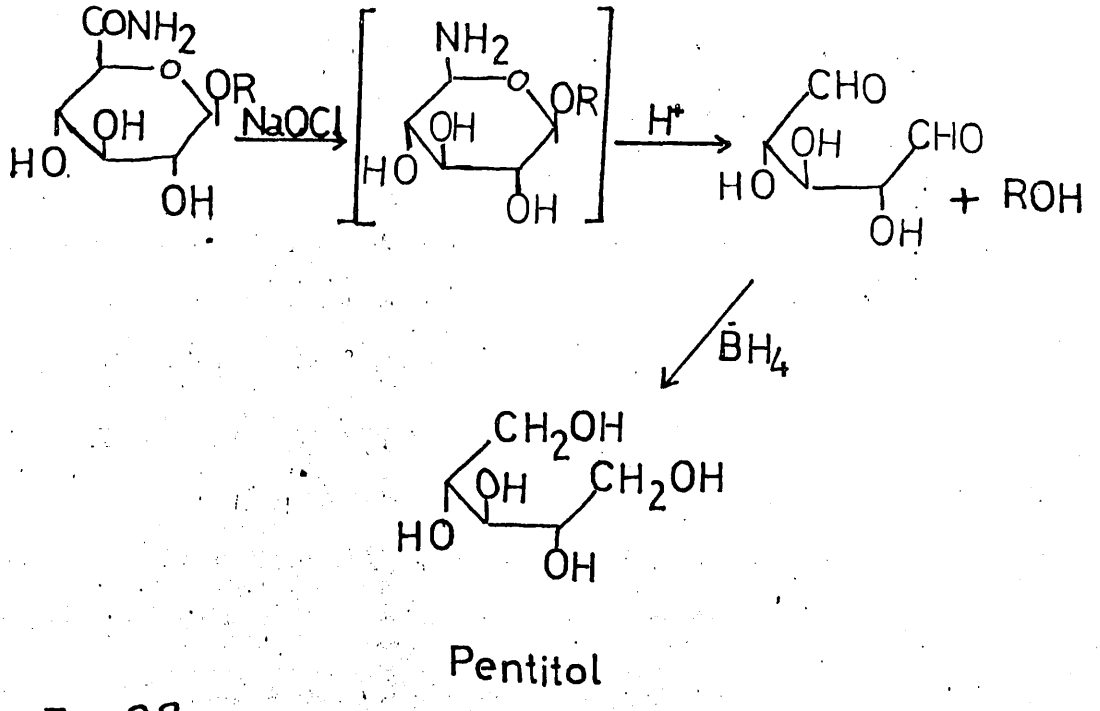


Fig 28.

Applying this technique to birch xylan (11.7% uronic acid) the Russian workers were able to cleave about 80-85% of the uronic acid residues without effective hydrolysis of the other glycosidic linkages.

This sequence of reactions was applied to 'fucan' (0.5M containing ca. 10% uronic acid). A 10% uronic acid content requires about 0.8% nitrogen for a complete conversion to the acid amide. Even after extensive extraction with methanol the derived amide had a nitrogen content of 5.4%. This shows that either a considerable amount of ammonia is still absorbed in the polysaccharide or that some reaction between the half ester sulphate group on the fucose units and the ammonia has occurred. As far as the author is aware this reaction has never been investigated. The amide after Hofmann degradation and reduction gave on mild hydrolysis and acetylation of the dialysable fragments evidence for the presence of a pentitol acetate. This is the expected product from the cleavage of uronic acid residues (Fig.28). The residual polymeric material (16% of the initial) from the mild hydrolysis had a uronic acid content of about 4.0%. This indicates that only about 60% of the uronic acid residues are removed from the "fucan" by this method. This can only be explained as due to non amide formation due possibly to the fact that these residues are present in the centre of this highly branched macromolecule and are inaccessible.

The initial and degraded polysaccharides were passed separately through the Sephadex G100 column to obtain an idea of the extent of degradation which had occurred. The elution pattern (Fig. 29, p 162a), indicates clearly that both the materials are very polydisperse and that some cleavage had taken place during the Hofmann reaction. It is possible that some of the molecules had been completely degraded since a considerable loss had occurred, at the same time only loss from the periphery of the recovered polysaccharide molecule can have taken place. If all the uronic acid was present on the periphery of the molecule then this series of reactions should result in the recovery of at least 70% of the original weight. It follows that a considerable proportion of the fucose and xylose units must also have been lost as fragments during these reactions. If this is the case it is difficult to account for the absence (negative phenol-sulphuric acid test) of carbohydrate in these fragments. Until some experiments with model sugar sulphates and the degradation procedure have been carried out further interpretation of these results is not possible, although it might be deduced that a high proportion of the molecules contain chains of mutually linked glucuronic acid, xylose and fucose and that the latter two sugars are completely degraded during the reaction.

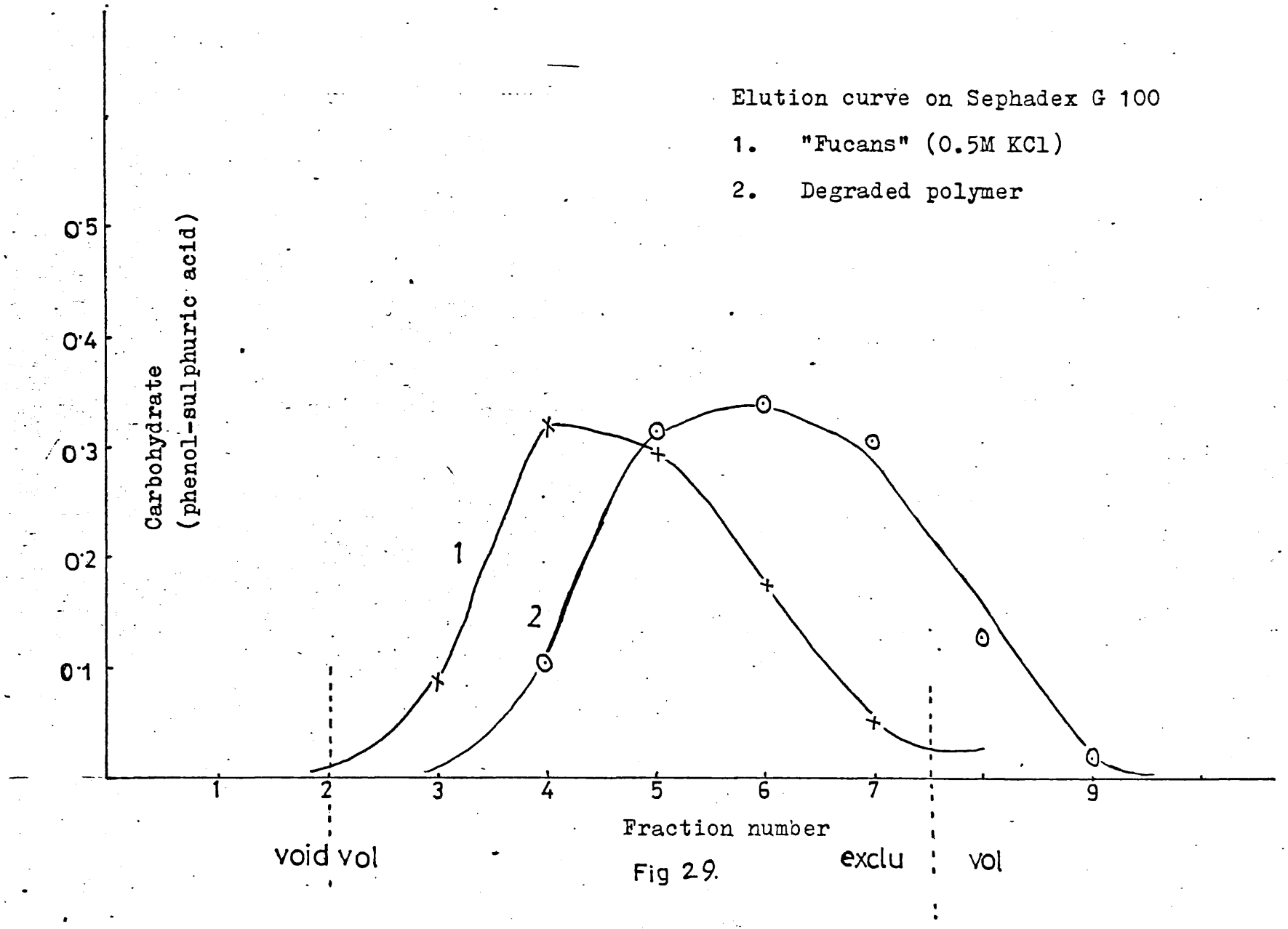

Elution curve on Sephadex G 100
1. "Fucans" (0.5M KCl)
2. Degraded polymer
Carbohydrate
(phenol-sulphuric acid)
0·5
0·4
0·3
0·2
0·1
1
2
1
2
3
4
5
6
7
9
Fraction number
void vol
exclu
vol

Fig 2.9.

Partial hydrolysis

Because uronic acid and neutral sugars are present in the same macromolecule, partial hydrolysis should result in the production of aldobiouronic acid, oligouronic acids together with monosaccharides. The uronic acid-rich fraction was hydrolysed in a sealed tube for 2 h and 4 h periods. Except that apparently more monosaccharides were present in the 4 h hydrolysate, the chromatographic patterns of the two hydrolysates were very similar.

Monosaccharides

The monosaccharides characterised from the hydrolysate are glucuronic acid, xylose and fucose. In addition to these there is a small quantity of another fast moving sugar residue present in the hydrolysate. Its chromatographic mobility (R_G 0.64 cf. 2,6-dideoxylyxose 0.74) in solvent 2, is in the range of 2,3-di-O-methylrhamnose (0.62) and 2,3,4-tri-O-methylgalactose or 2,3-di-O-methylarabinose (0.64). Bernardi and Springer [148] obtained from a purified fucoidan a fast moving spot with chromatographic mobility slightly different from 3-O-methylfucose. However attempted demethylation of the present material showed that there is no methoxyl group present in the molecule. It has a negative rotation calculated as $[\alpha]_D = -50^\circ$, but it should be pointed out that the small quantity of syrup obtained was difficult to weigh accurately and the weight was calculated from a fucose graph

after phenol-sulphuric acid.

With aniline oxalate it gives a colour on paper chromatogram similar to that given by hexoses, but at the same time no formaldehyde was produced when it is oxidised with sodium metaperiodate indicating that it is a 6-deoxy sugar. From the amount of periodate reduction of its methylglycoside it is possible that it is either a 2,6 or 4,6-"dideoxyhexose".

Aldobiouronic acids.

The partial hydrolysis led to the isolation of two aldobiouronic acids and an oligouronic acid. One of the aldobiouronic acids consists of glucuronic acid and fucose and the other is of glucuronic acid and "dideoxy" sugar. The former (D.P.2) has the same specific rotation, chromatographic mobilities and colour reactions to spray as found for the aldobiouronic acid from A. nodosum[155] and hence is considered to be 3-O-β-(D-glucopyranosyluronic acid) L-fucose.

The second aldobiouronic acid (D.P. 1.5) had chromatographic mobilities (0.61 and 0.65 in solvents 1 and 2) typical ~~mobilities~~ for an aldobiouronic acid. The determination of an accurate D.P. was difficult since as already mentioned no standard graph of "dideoxy" sugar and glucuronic acid was possible. Hence the standard graph for an equivalent mixture of glucuronic acid and fucose was used in determining the D.P. Because on hydrolysis it gave solely glucuronic acid and "dideoxy" sugar apparently in equal proportions this

was considered to be an aldobiouronic acid. It gave a red colour with tetrazolium hydroxide indicating that there is no O-2 substituent. The periodate oxidation of the glycoside of the "dideoxy" sugar revealed that O-3 cannot be the site of the second deoxy group in the "dideoxy" sugar residue, this suggests that this aldobiouronic acid is also 1,3-linked.

Oligouronic acid.

The oligouronic acid R_{fucose} 0.15 consists of glucuronic acid, fucose and "dideoxy" sugar. It had an apparent DP 4.3 but again exact determination was impossible. The partial hydrolysis of this oligouronic acid yielded, besides the constituent monosaccharides, two aldobiouronic acids similar to those obtained by the partial hydrolysis of the polysaccharide and an oligouronic acid (R_{fucose} 0.25, solvent 1). The latter was separated and on hydrolysis gave the same constituent sugars as found with the initial oligouronic acid.

The oligouronic acid (D.P. 4.3) was methylated, reduced and remethylated. The product, after hydrolysis, glycosidation and g.l.c. analysis gave methyl 2,3,4,6-tetra-O-methylglucoside and methyl 2,4-di-O-methylfucoside and two unidentified peaks having (T) 2.54, 2.82 [(major) column 1] and 1.54 (major) and 0.70 (column 2). The tetra-O-methylglucose must have resulted from the end groups of glucuronic acid, and 2,4-di-O-methyl fucose is obtained from 1,3-linked fucose. No methyl tri-O-methylglucosides peaks were detected on g.l.c. indicating that

glucuronic acid is not within the chain of the oligouronic acid. This could only be explained if this is a branched oligouronic acid.

Degraded (insoluble material, K) polymer (p. 128).

In view of the fact that hydrolysis of ascophyllan with 0.5N oxalic acid for 1 h at 100° cleaved most of the fucose and xylose and left a glucuronic acid backbone,[153] the degraded polymer of the present uronic acid-rich fraction (ca. 20% of the initial material) left after 4 h 90% formic acid hydrolysis in a sealed tube was examined. The material was recovered after dialysis in a cellophane tube indicating that the degraded polymer did not consist of very small fragments. On hydrolysis and chromatographic analysis this showed the presence of glucuronic acid, fucose (major) and "dideoxy" sugar residues. In view of these constituents in this degraded polymer it is very unlikely that a glucuronic acid backbone is a structural feature in the present polymer.

Overall Conclusions

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: laminaran, fucose-containing polymers, alginic acid and cellulose and the only discernible difference was in the proportion of these materials in the different species. The fucose-containing polysaccharides from each of the species examined comprise a wide spectrum of polymeric carbohydrate, all based on a similar structural pattern, but with different proportions of ester sulphate, uronic acid, xylose, galactose and possibly "dideoxy" sugar residues. At one extreme lies, what is generally accepted in the literature as, "fucoidan", a highly sulphated polysaccharide comprising mainly fucose and not more than 5% glucuronic acid and small but variable proportions of xylose and galactose, both of which appear to be present as end group residues. At the other extreme ~~is~~ are the "fucans" with about 20% glucuronic acid, low ester sulphate, fucose and small proportions of xylose, galactose and "dideoxy" sugar residues. The main structural feature of this latter polysaccharide is the mutual linkage of glucuronic acid and fucose as well as glucuronic acid and the "unknown" sugar. Most of the xylose and galactose again appear to be on the periphery of the molecules. Bridging these two extremes are

a mixture of polysaccharides of intermediate structure containing the same sugar residues.

It is interesting that the three different species of brown seaweed from widely different families synthesise this same spectrum of fucose-containing polysaccharides. The only detectable difference was in the proportion of the fucoidan to the other polysaccharides. This is also true for laminaran and alginic acid. The difference is most noticeable in Padina pavonia which has a very different morphological form.

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