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CARBOHYDRATES OF THE BROWN SEAWEEDS *Himanthalia lorea* AND *Bifurcaria bifurcata*

PART II. STRUCTURAL STUDIES OF THE "FUCANS"

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ABSTRACT

Methylation, periodate oxidation, and partial hydrolysis studies have revealed the essential similarity of the different fucose-containing polysaccharides separated from the title algae. The high-fucose, high sulphate-containing material more closely resembles the fucan reported in the literature and has (1→2)- and (1→3)-linked fucose residues with sulphation at C-4. The glucuronic acid and xylose residues are not sulphated and are on the periphery of highly branched molecules. In the polysaccharide having a high content of uronic acid, the fucose residues are similarly linked, and again the xylose and a large proportion of the glucuronic acid residues are present as end-group and (1→4)-linked units on the outside of the highly branched molecules. Partial hydrolysis studies proved the mutual linkage of the fucose (at C-3) and glucuronic acid, and the presence of an unidentified 6-deoxy sugar derivative which was also linked to glucuronic acid. Selective cleavage of the glucosiduronic acid linkages by Hofmann degradation of the amide derivative was unsuccessful.

INTRODUCTION

Earlier structural studies of the fucans from a variety of brown seaweeds were invariably on material that had been subjected to extensive purification[1]. Even so, the isolated polysaccharide always contained some galactose and often xylose and glucuronic acid. From methylation studies[2] of purified fucan (fucoidin) from *Fucus vesiculosus*, it was deduced that the fucose residues were mainly linked through C-1 and C-2 and were sulphated at C-4, and that there was a small degree of branching at C-3. Partial hydrolysis studies[3] confirmed the presence of (1→2)- and (1→3)-linkages, and the isolation[4] of fucose 4-sulphate from a partial hydrolysate confirmed C-4 as a site for the ester sulphate. No evidence was advanced for the part played by galactose, xylose, and uronic acid in the fucan macromolecules; in fact, they were mainly regarded as being associated with contaminating polysaccharides.

More recently, three fucose-containing polysaccharides have been separated from *Ascophyllum nodosum*[5]. The major polysaccharide, Ascophyllan, contained fucose (25%), xylose (26%), sodium uronate (19%), $NaSO_3$ (13%), and protein (12%). From partial hydrolysis studies, it was concluded that glucuronic acid formed the

backbone of the macromolecule and the isolation[6] of 3-*O*-β-D-xylopyranosylfucose provided evidence for the mutual linkage of these two sugars. In other studies[7], a "fucan" containing fucose (5 parts), xylose (1 part), and glucuronic acid (1 part), ester sulphate (20%), and protein (3.8%) was separated from *Ascophyllum nodosum*. In agreement with the earlier work on the fucan from *Fucus*, the fucose was found to be mainly (1→2)-linked with ester sulphate on C-4. In addition, partial hydrolysis provided evidence that the glucuronic acid is linked to C-3 of fucose and of the mutual linkage of xylose and fucose. No evidence for a glucuronic acid backbone in this polysaccharide could be obtained.

The preceding paper described the extraction from *Himanthalia* and *Bifurcaria* and fractionation of various sulphated, fucose-containing polysaccharides. All the fractions contained xylose, glucuronic acid, and, in some cases, traces of galactose in addition to fucose, and it was possible to separate them on DE-cellulose into high sulphate, high fucose-containing polysaccharides and high uronic acid, low sulphate-containing polymers, as well as fractions with intermediate properties.

The present paper describes structural studies of different fractions, in order to discover if they are built up on the same general plan or if they are structurally dissimilar polysaccharides, and whether they resemble the fucans from *Ascophyllum* and *Fucus*.

RESULTS AND DISCUSSION

The high uronic acid, low sulphate- (eluted from DE-cellulose with 0.3M KCl) and the high sulphate, high fucose-containing fraction (M KCl) each gave single, coincidal bands for protein and carbohydrate when subjected to gel electrophoresis, but the intermediate fraction (0.5M) gave a very diffuse pattern. From this, it may be concluded that the first two materials are reasonably homogeneous, probably with covalently linked protein. It is difficult to reach any conclusions about the 0.5M fraction.

Methylation studies. — Each of the fractions (1–16) in Tables (V–VIII) of the preceding paper (p. 133) were methylated separately. Because of the high uronic acid content of the 0.3M KCl fractions (1 and 4) and consequently the difficulty of complete methylation and of characterisation of methylated uronic acids, the uronic acid in these fractions, after partial methylation, was reduced to glucose derivatives and then remethylated. The initial, partial methylation of the fraction, before reduction, was necessary to render the polysaccharide soluble in tetrahydrofuran.

The large number of g.l.c. peaks given by the methylated glycosides derived from the methylated polysaccharides from both *Himanthalia* and *Bifurcaria* (see Table I) made complete characterisation of all the methylated sugars difficult, and any unmethylated sugars present remained undetected. To overcome these difficulties, the methylated sugars in half of the methylated hydrolysates were reduced to the alditols and converted into the corresponding acetates. Analysis by g.l.c. confirmed the tentative conclusions on the methylated glycosides and revealed the presence of

free fucose (Table I). The same methylated sugars were detected from each of the sixteen methylated materials, but the proportions of the individual sugars were widely different in the various fractions. 2,3,4,6-Tetra-*O*-methylgalactose was also detected in the products from the methylated M KCl fractions of the "fucans" (Table V, preceding paper). These results indicate that all the different extracts and fractions are built up on the same general plan, although they differ in the proportions of the different sugars, the fine details of structure, and the degree of branching. The xylose and glucuronic acid are present as end-group and (1→4)-linked units, and at least some of the galactose is end-group. The relatively high proportion of unsubstituted fucose in all the methylated hydrolysates confirms the high degree of sulphation and/or branching in the polysaccharides, although a small proportion of this could be due to under-methylation and demethylation during hydrolysis.

TABLE I

G.L.C. ANALYSIS OF THE METHYLATED SUGARS IN THE HYDROLYSATE OF THE METHYLATED "FUCANS" (16 SAMPLES IN ALL)

Retention times (T)[a,b]				*Identity*[c]
Methyl glycosides		*Alditol acetates*		
Column 1	*Column* 2	*Column* 4	*Column* 6	
				Fucose
—	—	1.2	2.0	Free sugar (M)
(4.0); 4.4	(1.20); (1.50)	1.1	1.5	2-*O*-Methyl (M)[d]
(2.2); 3.5; 7.3	(1.34); (1.44); (1.63)	0.88	1.83	3-*O*-Methyl (M)
(1.69); 2.69	(1.20); (1.34)	(1.0)	1.2	3,4-Di-*O*-methyl
0.73	0.61	0.72	0.69	2,3,4-Tri-*O*-methyl
				Xylose
0.43; 0.61	0.40; (0.56)	0.53	0.50	2,3,4-Tri-*O*-methyl
(1.38); (1.69); 1.82	(0.56); (0.76); (1.0)	—	1.36	2,3-Di-*O*-methyl
				Glucuronic Acid
(2.1); 3.1	(2.45); 3.9	—	—	2,3,4-Tri-*O*-methyl
—	(2.45); 3.17	—	—	2,3-Di-*O*-methyl
				Glucose[e]
3.20; (4.10)	(1.69); 2.20	1.3	2.35	2,3,6-Tri-*O*-methyl (M)
1.0; (1.37)	(1.0); (1.33)	(1.0)	1.0	2,3,4,6-Tetra-*O*-methyl

[a]Methyl glycosides relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside; alditol acetates relative to 2,3,4,6-tetra-*O*-methyl-D-glucitol 1,5-diacetate. [b]Numbers in parentheses represent incompletely resolved peaks. [c](M) = major sugar. [d]Not a major sugar in the uronic acid-rich fraction. [e]From reduced, uronic acid-rich material.

Stability of half-ester sulphate. — The sulphate groups in the high sulphate-containing polysaccharides (M KCl fractions) are essentially alkali-stable, thereby eliminating the possibility of monosubstituted half-ester sulphate groups either in (1→4)-linked or end-group xylose or glucuronic acid, and it is concluded that the majority of the sulphate is linked to C-4 of the fucose units.

Periodate oxidation and Smith-degradation studies. — It has been found that solutions of the glycuronan alginic acid are readily degraded in the presence of phenols, possible contaminants of these polysaccharides, and that 1-propanol inhibits this reaction[8]. Preliminary oxidation studies were carried out in the presence and absence of 1-propanol, and it was found that 0.6 mole of periodate was reduced in the presence of 1-propanol and 0.9 mole in its absence. All subsequent oxidations were therefore carried out in the presence of 1-propanol.

The fucose-rich "fucan" (M KCl, fraction 3) from *Himanthalia* reduced 0.36 mole of periodate for every "anhydro-hexose" unit and, after reduction, the polyalcohol was recovered in 77% yield. A repeated oxidation resulted in virtually no further reduction of periodate, indicating that, even if hemiacetal formation[9] had occurred during the first oxidation, the cleavage of the hemiacetal did not produce vicinal hydroxyl groups. Hydrolysis of polyalcohol 1 gave fucose as the only detectable, intact sugar, indicating that both the xylose and the glucuronic acid in the parent polysaccharide were vulnerable to periodate. From the carbohydrate content of the polysaccharide (120 mg) and of the polyalcohol (94 mg), it follows that 26 mg of carbohydrate were cleaved. This corresponds approximately to the proportion of xylose + glucuronic acid to fucose (3:14) (see Table V, preceding paper) in this polysaccharide and very little if any fucose can have been cleaved. The loss in weight correlates with 1.66 moles of periodate reduced for every sugar unit cleaved. This is in agreement with the methylation results which indicated end-group and (1→4)-linked xylose and glucuronic acid units in the polysaccharide. Such units would reduce two and one moles of periodate, respectively. After mild, acid hydrolysis of the polyalcohol (Smith degradation), the degraded polymer was recovered in 80% yield.

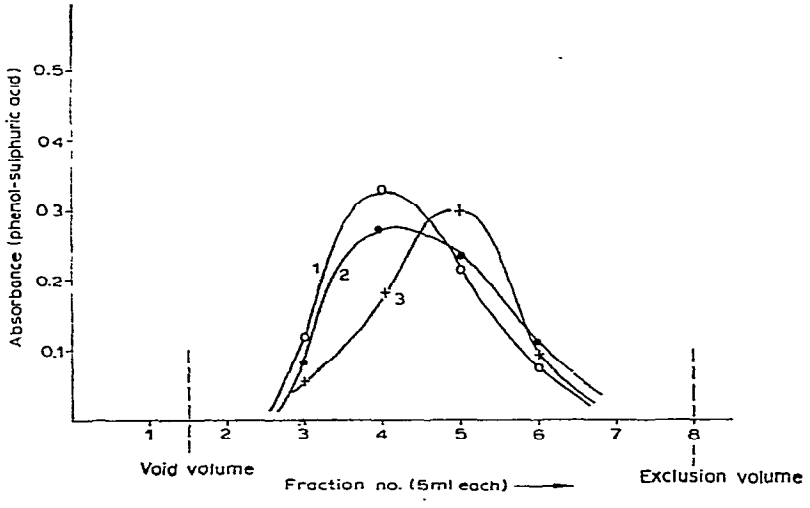

Fig. 1. Elution curves on Sephadex G100. 1, Fucose-rich polysaccharide (O—O). 2, Polyalcohol I (•—•). 3, Degraded polymer I (+—+).

Investigation of the molecular size of the polysaccharide, the derived polyalcohol, and the degraded polymer on Sagavac 6F revealed that they were all eluted near the exclusion volume, indicating a molecular size somewhat smaller than the range of this column. However, Sephadex G100, which fractionates dextrans in the molecular size-range of 1000 to 150,000, proved suitable. The polysaccharide and the polyalcohol were both very polydisperse, maximum elution occurring about midway between the void and exclusion volumes (Fig. 1). As would be expected, there was no difference in the molecular size of the two materials. The maximum elution of the degraded polymer, on the other hand, indicated a somewhat smaller molecular size, supporting the fact that no cleavage had taken place in the interior of the molecule and that the cleaved units were on the periphery of the molecule.

Free fucose, 2-*O*-, 3-*O*-, and 2,4-di-*O*-methylfucose (trace) were detected in the hydrolysate of the methylated polyalcohol 1.

The uronic acid-rich fraction (0.3M KCl fraction 1, Table V preceding paper) from *Himanthalia* reduced 0.8 mole of periodate per hexose unit, and this was followed by the reduction of 1.0 mole and 1.6 moles of periodate per "anhydrohexose" unit by polyalcohols 1 and 2, respectively. At the same time, considerable amounts of polymeric material were lost (Table II). Polyalcohol 2 was recovered in 35% yield and polyalcohol 3 in 50% yield. It is difficult, therefore, to decide how much of the reduction of periodate during the second and third oxidations was due to the release of acetals[9] which had been formed during the first oxidation and how much was due to the exposure of new units vulnerable to periodate as a result of loss of material. It was thought that the loss might be due to β-elimination of the uronic acid[10] during the alkaline conditions of reduction, but a test for 4,5-unsaturated acid in the dialysate from the reduction was negative, and the initial polysaccharide, when subjected to the conditions of reduction, was recovered in 99.5% yield.

TABLE II

PROPERTIES OF THE URONIC ACID-RICH FRACTION (1) AND THE DERIVED POLYALCOHOLS.

Material	*Weight (mg)*	*Carbohydrate content (mg)*	*Uronic acid (%)*		*Molar proportions of sugars*[b]		
			a	*b*	*Fuc*	*Xyl*	*GlcUA*
0.3M KCl fraction	1000	500	38.0	40.0	2.2	1.0	2.2
Polyalcohol 1	524	137	32.0	30.0	2.5	1.0	1.5
Polyalcohol 2	184	37	40.0	36.0	1.5	Nil	1.0
Polyalcohol 3	90	18	28.0	27.0	2.6	Nil	1.0

[a]By a modified carbazole procedure. [b]By the phenol–sulphuric acid method, after elution from paper chromatograms and filtration.

In another experiment, the polyaldehyde was recovered after periodate oxidation with a loss of ~25% by weight. When this material was reduced to polyalcohol, the loss again was ~25%, the total loss being the same as occurred in the initial

oxidation and reduction (Table II). Chromatographic analysis of the dialysates after oxidation and after reduction showed the absence of any free neutral sugars or their respective alcohols, even after hydrolysis, and the presence of erythronic acid, a trace of threitol, glycerol, and possibly propane-1,2-diol, and, in the reduction solution, gulonic acid. Gulonic acid would be derived from hydrolysed, reduced glucuronic acid, erythronic acid from (1→4)-linked glucuronic acid residues, propane-1,2-diol from 1,2-linked fucose residues, glycerol from (1→4)-linked xylose residues, and threitol from (1→4)-linked galactose residues in the polysaccharide. This is the first indication of the presence of (1→4)-linked galactose. It appears that very labile acetal-linkages are formed during the oxidation and are cleaved from the molecule, during both the oxidation and reduction stages.

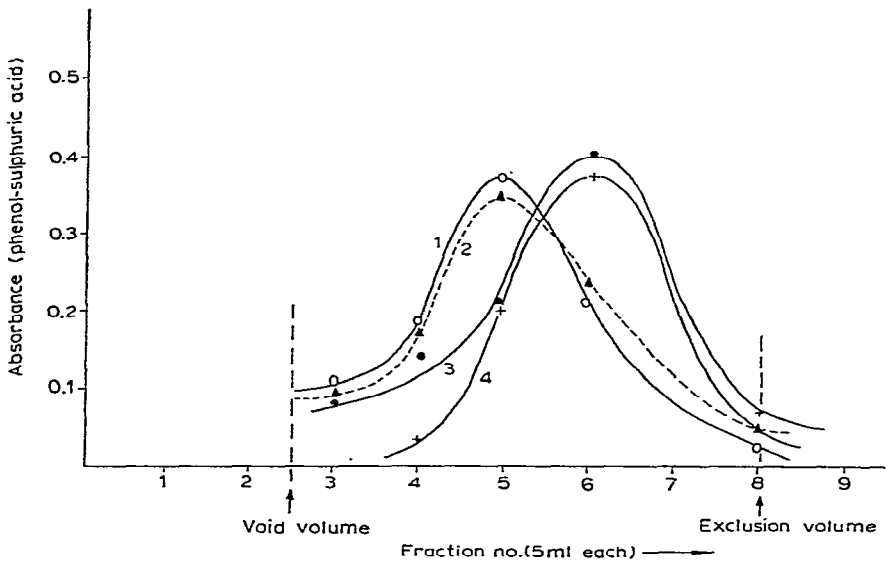

Fig. 2. Elution pattern from Sephadex G100. 1, Uronic acid-rich polysaccharide (O—O). 2, Polyalcohol I (▲—▲). 3, Degraded polymer I (●—●). 4, Degraded polymer II (+—+).

Investigation of the changes in molecular size by elution of the initial polysaccharide and the derived polyalcohol from Sephadex G100 showed them to have the same molecular size (Fig. 2). It follows that either some molecules had been completely degraded or that all the cleavage had taken place from the periphery of the molecule. It must be remembered that changes in the shape of the molecule, which play an important role in the elution pattern of polymers, may have occurred during conversion into the polyalcohol and this may have masked a change in size. Furthermore, accurate calculation of the loss in carbohydrate (apparently 363 mg from 500 mg) is difficult and may not be as high as it appears.

Mild hydrolysis of polyalcohol 1 (Smith degradation) gave a degraded polymer (I), and the hydrolysate contained the same fragments (Fig. 3) as the dialysates from polyalcohol 1. Complete hydrolysis of the degraded polymer revealed (Table III)

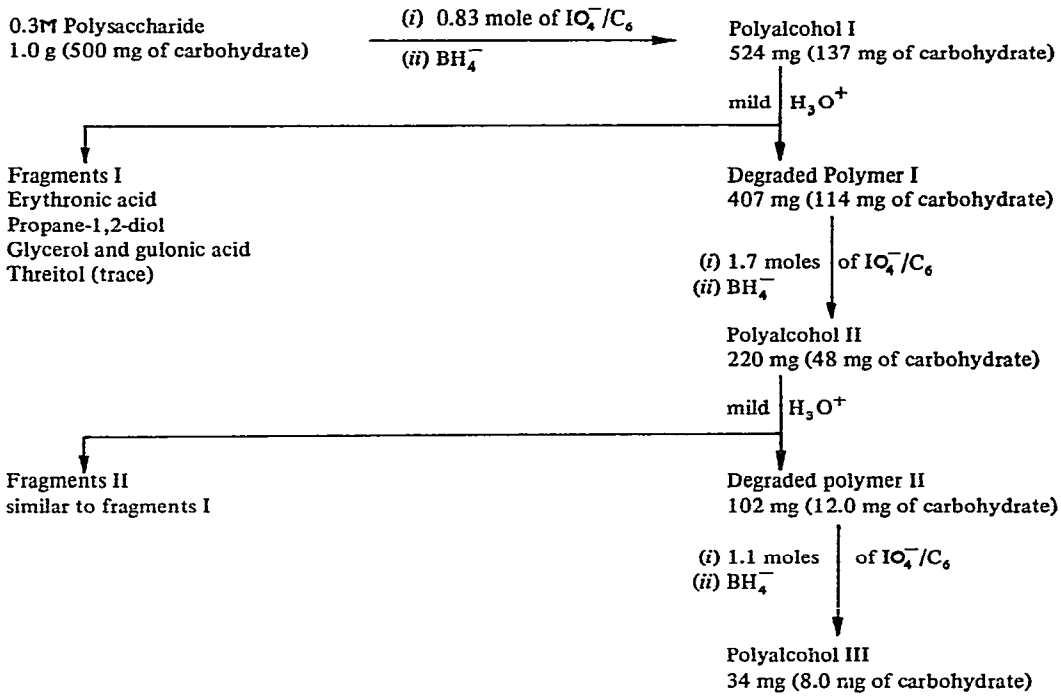

Fig. 3. Smith degradations.

that proportionally more xylose had been oxidised than fucose or glucuronic acid although, from the nature of the fragments present in the dialysate, both these sugars must also have been oxidised. The degraded polymer was eluted from Sephadex G100 somewhat later than the initial polysaccharide (Fig. 2), indicating a reduction in molecular size, but little further change occurred during a second Smith-degradation (Fig. 2).

TABLE III

PRODUCTS OF SMITH DEGRADATIONS OF URONIC ACID-RICH FRACTION

Material	*Carbohydrate* (%)	*Uronic acid* (%)		*Molar proportions of sugars*[b]		
		[a]	[b]	*Fuc*	*Xyl*	*GlcUA*
0.3M KCl Fraction	50.0	38.0	40.0	2.2	1.0	2.2
Polyalcohol (I)	26.0	31.0	30.0	2.5	1.0	1.5
Degraded polymer (I)	28.0	45.0	43.0	4.0	1.0	3.8
Polyalcohol (II)	20.0	40.0	36.0	1.8	Nil	1.0
Degraded polymer (II)	10.0[c]	20.0	22.0	3.4	Nil	1.0
Polyalcohol (III)	20.0	32.0	32.0	2.2	Nil	1.0

[a]By a modified carbazole procedure. [b]By the phenol–sulphuric acid method, after elution from paper chromatograms and filtration through a Millipore filter. [c]The low carbohydrate content is unaccounted for.

A summary of the loss of material and amount of periodate reduced during a second oxidation and reduction of the degraded polymer (I) is given in Fig. 3. Smith degradation then gave rise to the same fragments as obtained in the first degradation. A third oxidation and reduction is summarised in Fig. 3. and considerable loss of material again occurred, which makes interpretation difficult. Methylation studies indicated that the xylose and glucuronic acid residues are end-group or (1→4)-linked, and therefore should be vulnerable to periodate. However, a proportion of both xylose and glucuronic acid survived the first oxidation and ~4.0% of the glucuronic acid residues survived three oxidations (Table III). While this result could be explained as partly due to hemiacetal formation, it is possible that the glucuronic acid is attached to units other than through C-4, particularly since some unoxidised glucuronic acid residues were present in the dialysates and in the mild, acid hydrolysates (Fig. 3).

Comparison of the polyalcohols (II) and (III), derived after Smith degradation with 2 and 3 obtained from repeated oxidation and reduction (Tables II and III), shows considerable similarity and can be considered as providing further evidence of the extreme lability of the acetal links formed during the oxidations.

The periodate-oxidation and Smith-degradation studies of the uronic acid-rich "fucan" illustrate the dangers inherent in the interpretation of the results from such experiments, particularly when the yields of the different products are ignored.

Partial hydrolysis. — Partial, acid hydrolysis of the uronic acid-rich polysaccharide gave a neutral, reducing syrup which had a chromatographic mobility faster than fucose; this corresponded to the sugar reported previously in the "fucan" from *Fucus vesiculosus*[11] with a mobility slightly different from that of 3-*O*-methylfucose. However, attempted demethylation[12] gave only unchanged material. No formaldehyde was produced when this sugar was oxidised with sodium metaperiodate, indicating that it is a 6-deoxy sugar. It had a chromatographic mobility and retention time as the Me_3Si derivative on g.l.c. different from those of 2,6-dideoxy-D-*lyxo*-hexose and gave a different mass spectrum. Although it was also isolated linked to glucuronic acid in an aldobio- and a poly-uronic acid fraction, insufficient pure material was obtained to allow characterisation.

Glucosyluronic acid-(1→3)-fucose, previously separated[7] from the glucuronosylxylofucan from *Ascophyllum*, was separated and characterised. An oligouronic acid, which on partial hydrolysis gave spots with the chromatographic mobilities of the above two aldobiouronic acids, was also separated. Methylation, reduction, and remethylation of this saccharide, with hydrolysis of the product, gave methylated sugars which confirmed the presence of end-group glucuronic acid and (1→3)-linked fucose. No tri-*O*-methylglucose could be detected, indicating that the glucuronic acid is not within the chain of the oligouronic acid. This may indicate that the oligouronic acid is branched.

Selective cleavage of the glycuronosidic linkage in the polysaccharide, leaving the glycosidic linkages intact, was attempted using the Hofmann reaction. In reported work[13] on birch xylan (11.7% of uronic acid), ~80–85% of the uronic acid units were cleaved in this way without effective hydrolysis of the glycosidic linkages. The

uronic acid residues in the xylan were first converted into 5-aminopentopyranose residues by the action of sodium hypochlorite on the amides. Mild, acid treatment converted the amino derivative into a dialdehyde and cleaved it from the polysaccharide, and it was subsequently stabilised by reduction to the pentitol.

Application of these reactions to the "fucan" (0.5M KCl fraction, 10% of uronic acid) gave an amide containing 5.4% of nitrogen, even after extensive extraction with methanol. This result indicates that either a considerable amount of ammonia was absorbed by the polysaccharide or that it had reacted with the half-ester sulphate groups. The residual polysaccharide was recovered after the Hofmann degradation and mild hydrolysis in only 16% yield from the amide, and had a uronic acid content of ~4%, indicating that ~60% of the uronic acid residues had been removed from the "fucan" by this method. The residual polysaccharide also contained fucose and a little xylan. A peak with the retention time of xylitol penta-acetate was obtained on g.l.c. analysis of the hydrolysed fragments, confirming that the reaction was to some extent successful. The incomplete cleavage of uronic acid could be explained by incomplete amide formation due to the inaccessibility of some of the uronic acid units in this highly branched polysaccharide and is in agreement with the Smith-degradation studies. The degraded polymer was eluted from Sephadex G100 somewhat later (see Fig. 4) than the initial polysaccharide, indicating that the cleaved units had been removed from the periphery of the molecules and that little cleavage in the interior had taken place. At the same time, the high loss of polysaccharide must indicate the complete degradation of the majority of the molecules during these reactions.

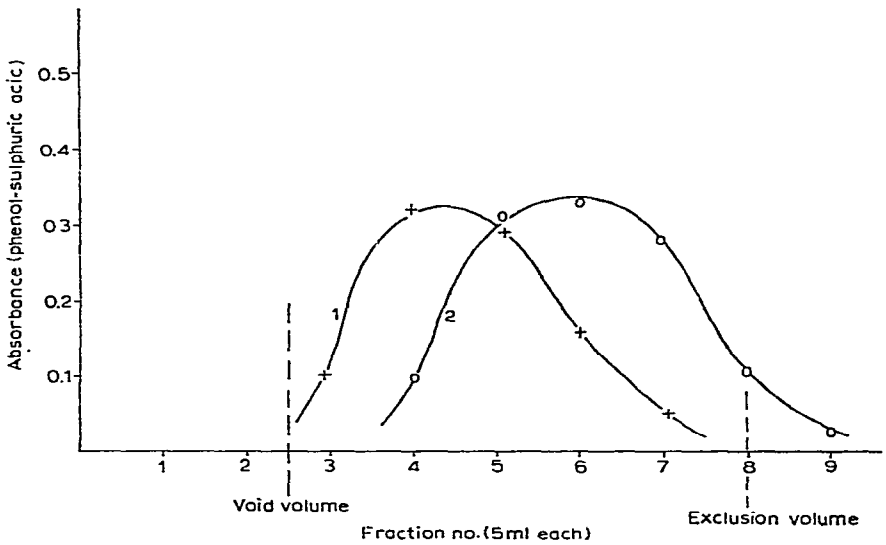

Fig. 4. Elution of "Fucan" 0.5M KCl fraction from Sephadex G100. Before (+—+) and after Hofmann (O—O) degradation.

It can be concluded, therefore, that the different fucose-containing materials extracted and fractionated from these two brown seaweeds all belong to the same family of highly branched polysaccharides and, as indicated in Part I, the fractionations are somewhat arbitrary. All the materials are built up on the same general plan, but at one end of the spectrum is the highly sulphated polysaccharide comprising mainly fucose and $>5\%$ of glucuronic acid. At the other extreme, the polysaccharides have a high content of glucuronic acid, low contents of ester sulphate and fucose, and small proportions of xylose and possibly galactose. A very small proportion of an unknown sugar derivative appears to be present in all the fractions. The xylose and galactose and the majority of the glucuronic acid occur in the outer branches of the polysaccharides. Glucuronic acid is linked to C-3 of fucose and is also linked to the unknown sugar. The fucose units are linked through C-2 and C-3 and sulphated at C-4, as already found in the fucans from *Fucus* and *Ascophyllum*.

EXPERIMENTAL

Details of analytical methods are given in the preceding paper.

Gel electrophoresis. — The polysaccharides isolated from the 0.3M, 0.5M, and M KCl fractions (1, 2, and 3, respectively) of the combined aqueous and acid extracts from *H. lorea* detailed in the previous paper (0.35 mg of each in 10% sucrose solution) were subjected to electrophoresis (Shandon analytical polyacrylamide apparatus, Model SAE 2734) using Tris–glycine buffer (pH 8.3). Three tubes of acrylamide gel were run for each polysaccharide. One gel was stained for sulphate[14], one for protein with amido black, and a third for any charged material with 1-ethyl-2,3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenylnaphtho[1,2-*d*]thiazolium bromide (Serva, Feinbiochemica, Heidelberg). Bromophenol Blue was used as a marker in each tube. The extruded columns were left in the respective reagent for 1 h and then washed to remove excess stain.

Attempted removal of sulphate with alkali. — The M KCl fractions of the "fucans" from (*a*) the combined acid and aqueous extracts (3, Table V, preceding paper; carbohydrate, 40; sulphate, 29%) and (*b*) the alkali extract (8, Table VI, preceding paper; carbohydrate, 45; sulphate, 25%) from *Himanthalia* (165 mg of each) in water (125 ml) were reduced with potassium borohydride for 48 h at room temperature. Sodium hydroxide (1.0 g) and potassium borohydride (30 mg) were then added and the mixture was heated for 2 h at 80°. The solution was neutralised with Amberlite IR-120(H^+) resin and, after dialysis, the degraded polysaccharides were recovered by freeze-drying [134 mg from (*a*) (Found: carbohydrate, 40; sulphate, 27.5%) and 130 mg from (*b*) (Found: carbohydrate, 45; sulphate, 23.8%)].

Methylation studies. — (*a*) The 0.5M and M fractions (2,3,5–16; 5 mg of each, 14 samples in all) of the "fucans" from *Himanthalia* and *Bifurcaria* detailed in Tables V–VIII of the preceding paper were methylated separately. The solutions were then poured into water (25 ml), dialysed extensively, and finally concentrated to dryness. The products were then examined by t.l.c. If more than a single spot was

detected, the methylation procedure was repeated. The methylated polysaccharides were hydrolysed with 90% formic acid[15] and analysed by paper chromatography. Separate aliquots of the hydrolysates were converted into the methyl glycosides and into the alditol acetates and examined by g.l.c.

(*b*) The 0.3M KCl fractions (1 and 4, 60 mg of each) of the "fucans" from *Himanthalia* and *Bifurcaria* (Table V, preceding paper) were methylated as above. After a single methylation, the product was suspended in dry tetrahydrofuran (100 ml), and lithium aluminium hydride (60 mg in 5 ml of tetrahydrofuran) was added gradually with shaking. A vigorous reaction took place and, after standing for 0.5 h, the mixture was heated under reflux for 2 h. Further lithium aluminium hydride (40 mg in 5 ml of tetrahydrofuran) was added and the mixture heated under reflux for a further 0.5 h. Excess of hydride was destroyed by the addition of water to the cooled mixture, and the solution was brought to pH 4 with sulphuric acid, filtered, concentrated to small volume, and extracted with chloroform. Removal of the chloroform left a yellowish syrup (*ca.* 20 mg) of the reduced, methylated polysaccharide which was remethylated. The derived glycosides and alditol acetates were analysed by g.l.c.

Periodate-oxidation studies. — (*a*) *Preliminary studies.* Two samples (600 mg each) of the "fucan" from *Himanthalia* extracted with acid were oxidised separately with 15mM sodium metaperiodate at room temperature in the dark, one in the presence, and the other in the absence, of 2.5% of 1-propanol. Aliquots (0.5 ml) were withdrawn at intervals and the reduction of periodate was measured[16]. The primary oxidation was complete after 7 h.

(*b*) The "fucan" [M fraction (3) from *Himanthalia* (300 mg, corresponding to 120 mg of carbohydrate); Table V, preceding paper] was oxidised as above with 15mM periodate containing 2.5% of 1-propanol; the primary oxidation was complete after 7 h. The reaction was stopped by destruction of the excess of periodate with ethylene glycol. After standing for 2 h, the cooled (0°) solution was mixed with 50mM boric acid (25 ml) and potassium borohydride (500 mg), and the mixture was set aside for 18 h at 0°. Total reduction was indicated by a negative Fehlings' test. The solution was neutralised with glacial acetic acid and exhaustively dialysed against distilled water. The polyalcohol 1 (230 mg, 94 mg of carbohydrate) was recovered after concentration and freeze-drying. Aliquots were examined as follows. The polyalcohol (62 mg) was re-oxidised with 15mM periodate; after 7 h, the reduction of periodate was 0.06 mole per hexose residue. Polyalcohol 2 (60 mg) was recovered as for polyalcohol 1. Polyalcohol 1 (10 mg) was hydrolysed and analysed by paper chromatography (eluants 1–4, reagents *A*, *B*, and *E*). Polyalcohol 1 (10 mg) was methylated as above, and the product was analysed by g.l.c. as the derived methyl glycosides and alditol acetates. Polyalcohol 1 (25 mg) was treated with 0.5M sulphuric acid (6 ml) at room temperature. Aliquots were withdrawn at hourly intervals, neutralised with barium carbonate, filtered, and dialysed. The dialysate was treated with Amberlite IR-120(H^+) resin, concentrated, and analysed by paper chromatography for free sugars (spray *A*) and for fragments (dip *E*). After 11 h, trace quantities of free sugars were detected. The hydrolysis was then repeated on the remainder of

polyalcohol 1 for 10 h, and the degraded polymer was recovered by precipitation with ethanol.

(*c*) The uronic acid-rich polysaccharide from *Himanthalia* (1 g corresponding to 500 mg of carbohydrate) [0.3M fraction (1), Table V; preceding paper] was subjected to repeated oxidation and reduction as in (*b*). Some properties, the weights, and the molar proportions of the uncleaved sugars in the respective polyalcohols are given in Table II. In one experiment, the oxidation mixture, after the addition of ethane-1,2-diol, was dialysed in a closed system against distilled water, and the polyaldehyde (31 mg from 41.2 mg of polysaccharide) was recovered from the dialysis sac and freeze-dried. It was then reduced to the polyalcohol (22.5 mg). The dialysable materials from both the polyaldehyde solution and the polyalcohol solution in this experiment, after treatment with Biodeminrolit resin (carbonate form) to remove inorganic ions, were examined separately by paper chromatography (solvents 1 and 3, spray *A* and dip *E*) and by ionophoresis at pH 6.7. They were also tested for unsaturated acid[17]. In a separate experiment, the excess of periodate was destroyed by sulphur dioxide, and the dialysable materials were separated and examined as before.

The initial polysaccharide (40 mg) was subjected to the reduction conditions used on the oxidised polysaccharide. The product (38.2 mg) was recovered, after neutralisation and dialysis, by freeze-drying.

(*d*) *Smith degradation.* The uronic acid-rich fraction (1) (1 g, 500 mg of carbohydrate) from *Himanthalia* was oxidised with periodate and the derived polyaldehyde reduced to polyalcohol under the conditions used above. Preliminary hydrolyses on separate aliquots of the polyalcohol (25 mg each) with 0.5M and 50mM sulphuric acid at room temperature, with chromatographic analysis of aliquots withdrawn at hourly intervals and treated as under (*b*), revealed that free sugars were released after hydrolysis for 2 h with 0.5M sulphuric and 6 h with 50mM sulphuric acid. The remainder of the polyalcohol was hydrolysed with 50mM sulphuric for 5 h at room temperature. The degraded polymer was recovered, after dialysis of the neutralised ($BaCO_3$) solution, by freeze-drying. The fragments in the dialysate were analysed as in the preliminary hydrolyses, and also hydrolysed and analysed by paper chromatography. Smith degradation of the degraded polymer was repeated and the degraded polymer II was subjected to a third oxidation and reduction. The amount of periodate reduced, the yields of the respective polyalcohols and of the degraded polymers, and the identity of the fragments are given in Fig. 3; a summary of some of their properties and the molar proportions of the uncleaved sugars are given in Table III.

Gel-column chromatography. — A column containing Sephadex G100 (12 × 0.25 cm) was calibrated with blue dextran 2000 (Mol. wt. 200,000; lot No. 1328 Pharmacia) to find the void volume (15 ml), and with sucrose to find the exclusion volume (40 ml). Samples of the M fraction of the "fucan" [oxidised under (*b*)], the derived polyalcohol, and degraded polymer, and the uronic acid-rich material oxidised under (*c*), the derived polyalcohols (I), and degraded polymers (I) and (II) (0.5 mg of each) were dissolved in M sodium chloride and applied to the column.

Fractions (5 ml) were collected and monitored for carbohydrate. Elution patterns are illustrated in Figs. 1 and 2.

Partial hydrolysis. — The uronic acid-rich fraction (1, 423 mg) from *Himanthalia* was hydrolysed with 90% formic acid in an atmosphere of carbon dioxide for 2 h at 100° in a sealed tube. Insoluble material was removed by centrifugation, washed twice with 90% formic acid, and then re-hydrolysed for a further 2 h and re-centrifuged. The combined supernatants and washings, diluted to five times the original volume, were hydrolysed for 2 h at 100° and evaporated to dryness (residue *A*). The insoluble material remaining after the second hydrolysis was dissolved in water, and the dialysed solution was freeze-dried to a white powder (87 mg). An aliquot (10 mg) of this material was hydrolysed for 6 h with 90% formic acid and analysed by paper chromatography.

Examination of the hydrolysate. The hydrolysate (*A*) was analysed by paper chromatography and separated on 3MM paper into three neutral and two acidic fractions.

Fraction 1. A neutral, reducing syrup, $[\alpha]_D$ −50° (*c* 0.2; concentration determined as fucose by the phenol–sulphuric acid method), which had R_{FUC} 1.50 and 1.46 (solvents *1* and *2*) and gave a yellow spot (spray *A*). After attempted demethylation, paper chromatography showed a single spot with the same mobility as the original material. An aliquot was oxidised with periodate and analysed for the release of formaldehyde with pentane-2,4-dione[18]. A second aliquot was converted into the methyl glycosides which reduced 1.6 mol. of periodate based on an original weight determined as fucose by the phenol–sulphuric acid method. Methyl fucoside reduced 2.3 mol. of periodate in a parallel experiment. G.l.c. analysis of the Me_3Si derivative of the free sugar gave peaks with *T* 1.45 and 1.6 (shoulder) (column 4) at 160° (*cf.* 2,6-dideoxy-D-*lyxo*-hexose, *T* 2.5, 3.3, and 4.7). The derived alditol acetate on column (6) gave a peak with *T* 1.70 (relative to that of xylitol acetate); 2,6-dideoxy-D-*lyxo*-hexitol acetate gave a peak at T 1.06.

Fraction 2. A neutral syrup, characterised as fucose.

Fraction 3. A neutral syrup, characterised as xylose.

Fraction 4. An acidic syrup with R_{FUC} 0.65 (solvent *1*). It was separated into 4 fractions by electrophoresis at pH 6.7 for 6 h at 2000 volts. Fraction 4_1 was chromatographically and ionophoretically identical with glucuronic acid and its lactone. Fraction 4_2 was an acidic syrup, $[\alpha]_D$ −20.7° (*c* 0.15), which had R_{FUC} 0.61 and 0.65 (solvents *1* and *2*) and gave a red spot with spray *C*; hydrolysis of an aliquot and paper chromatography of the hydrolysate revealed the presence of glucuronic acid and the unknown neutral fraction 1; after an aliquot had been reduced and hydrolysed, paper chromatography of the hydrolysate (spray *A*) gave a single spot with the mobility of glucuronic acid. Fraction 4_3 was an acidic syrup, $[\alpha]_D$ −18.5° (*c* 0.2), R_{FUC} 0.58 and 0.61 (solvents *1* and *2*) [*cf.* glucosyluronic acid-(1→3)-fucose, from *Ascophyllum nodosum*[7] glucuronoxylofucan, $[\alpha]_D$ −18°, R_{FUC} 0.56 and 0.63 in these solvents]. With spray *C*, it gave a red spot, and a greyish green spot with spray *D*; paper chromatography of a hydrolysate revealed glucuronic acid and fucose; deter-

mination of the d.p. gave 80 μg as an equivalent mixture of glucuronic acid and fucose, and the reduced material corresponded to 40 μg as glucuronic acid; an aliquot was reduced and hydrolysed, and paper chromatography of the hydrolysate (spray *1*) gave spots for glucuronic acid and its lactone. Fraction 4_4 was a reducing, neutral syrup with the chromatographic mobility of galactose (solvents *1–3*).

Fraction 5. An acidic, reducing syrup with $[\alpha]_D$ −24° and R_{FUC} 0.15 (solvent *1*). After hydrolysis, paper chromatography gave spots having the mobilities of the unknown sugar (fraction 1), fucose, and glucuronic acid. Partial hydrolysis of an aliquot for 1 h in a sealed tube at 100° with 90% formic acid, followed by paper chromatography and ionophoresis of the hydrolysate, revealed spots with the mobilities of the unknown fraction 1, fucose, glucuronic acid and fractions 4_1–4_3. An aliquot of fraction 5 was methylated, reduced, and remethylated as for the 0.3M KCl fraction (1), except that the methylated product was separated from the methylating reagents by extraction into chloroform. It was hydrolysed and the methylated sugars, after conversion into the glycosides, were analysed by g.l.c. (columns *1* and *2*). Peaks corresponding to methyl 2,3,4,6-tetra-*O*-methylglucosides (Table I), methyl 2,4-di-*O*-methylfucoside [*T* 1.68, 1.47 (column *1*); 0.87, 1.0 (column *2*)], and unidentified peaks having *T* 2.5, 2.82 (major) (column *1*), and 1.54 (major) and 0.70 (column *2*) were obtained.

Cleavage of glucuronosidic linkages. — The fucan (0.5M fraction 2; 500 mg; carbohydrate 45%, uronic acid 9%) from *Himanthalia* was converted into the polysaccharide amide[19]. The recovered material was extracted with methanol in a Soxhlet apparatus and then dried *in vacuo* for 6 h. The product (554 mg, N, 5.43%) showed i.r. bands at 3450, 1700, and 1650 cm^{-1} (amide), and weak bands at 1600–1500 cm^{-1}. It was subjected to a Hofmann degradation and reduction with borohydride[13]. After dialysis, the modified polysaccharide was recovered by freeze-drying (42.0 mg). The dialysable material was tested for carbohydrate. An aliquot was hydrolysed and then examined by paper chromatography. The dialysate was treated with Amberlite IR-120(H^+) resin and then acetylated and examined by g.l.c. (column *6*).

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