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CARBOHYDRATES OF THE BROWN SEAWEEDS *Himanthalia lorea*, *Bifurcaria bifurcata*, AND *Padina pavonia*

PART I[1]. EXTRACTION AND FRACTIONATION

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ABSTRACT

Mannitol constitutes the major carbohydrate of low molecular weight in the brown seaweeds named in the title; glucose is also present in *Himanthalia*, and glucose and *myo*-inositol were detected in *Bifurcaria*. Laminarin, alginic acid, "fucans", and cellulose were separated and characterised from each of the species investigated. The "fucans", which were present in five sequential extracts obtained with different extractants, comprised variable proportions of fucose, xylose, glucuronic acid, galactose (traces), and half-ester sulphate. Fractionation on DE-cellulose led to the isolation of highly sulphated materials having a high content of fucose, polymers having a high content of glucuronic acid and a low content of sulphate, and polysaccharides with proportions of sugars and sulphate between these two extremes. It is concluded that all three seaweeds synthesise a wide spectrum of these polysaccharides.

INTRODUCTION

Mannitol constitutes the major carbohydrate of low molecular weight and laminarin, alginic acid, fucose-containing polysaccharides, and cellulose the polysaccharides synthesised by brown seaweeds[2]. This paper describes the systematic investigation of the carbohydrates of three species having very different morphology and belonging to different families[3] of brown seaweeds. *Himanthalia lorea* (Himanthaliaceae), which is abundant near low-tide level, has a thick, leathery, perennial frond (1–2 cm broad) which may reach 2 metres in length and may be branched. The frond arises from a small, disc-like holdfast. *Bifurcaria bifurcata* (Cystoseiraceae), which is frequently found on the Atlantic shores of Europe and Africa, has a highly branched, perennial rhizome attached to the substratum by small adhesive discs. Some branches turn upward to give rise to forked, smooth shoots which grow to a height of 30–40 cm and die down in late Autumn. *Padina pavonia* (Dictyotaceae) is perennial and is found at mid-tide level on the southern shores of England and in the Mediterranean. It has numerous, stalked, paper-thin, fan-shaped fronds often loosely rolled in the shape of a cornet. The stalks arise from prostrate, perennial rhizomes which are attached to the substratum by tufts of rhizoids.

RESULTS AND DISCUSSION

Carbohydrates of low molecular weight. — The major carbohydrate of low molecular weight in all three species of weed, as in other brown seaweeds, is mannitol. It was obtained crystalline and was characterised conventionally. Compared with other species, the amounts found in *H. lorea* (0.5% of dry weight) and *P. pavonia* (very small) are low. It corresponded to ~8% of the dry weight of *B. bifurcata*. D-Glucose, found in *H. lorea* and *B. bifurcata*, was characterised by crystallisation for the latter seaweed, and confirmed with D-glucose oxidase in *H. lorea*. Chromatographic evidence for the presence of *myo*-inositol in *Bifurcaria* was also obtained.

Polysaccharides. — Preliminary extraction of *H. lorea* and *B. bifurcata* by the established methods of (*1*) dilute acid, followed by alkali, or (*2*) by water, acid, and alkali in sequence, gave rise to complex mixtures of a glucan, fucose-containing polysaccharides, and alginic acid, which defied separation into the respective polysaccharides either by fractional precipitation with ethanol, calcium salts, or cetyltrimethylammonium hydroxide, or by fractionation on resin columns.

After ethanolic extraction, the powdered seaweeds were treated therefore with formaldehyde, in order to polymerise phenolic constituents which otherwise contaminated the various extracts. Sequential extraction was then carried out as shown in

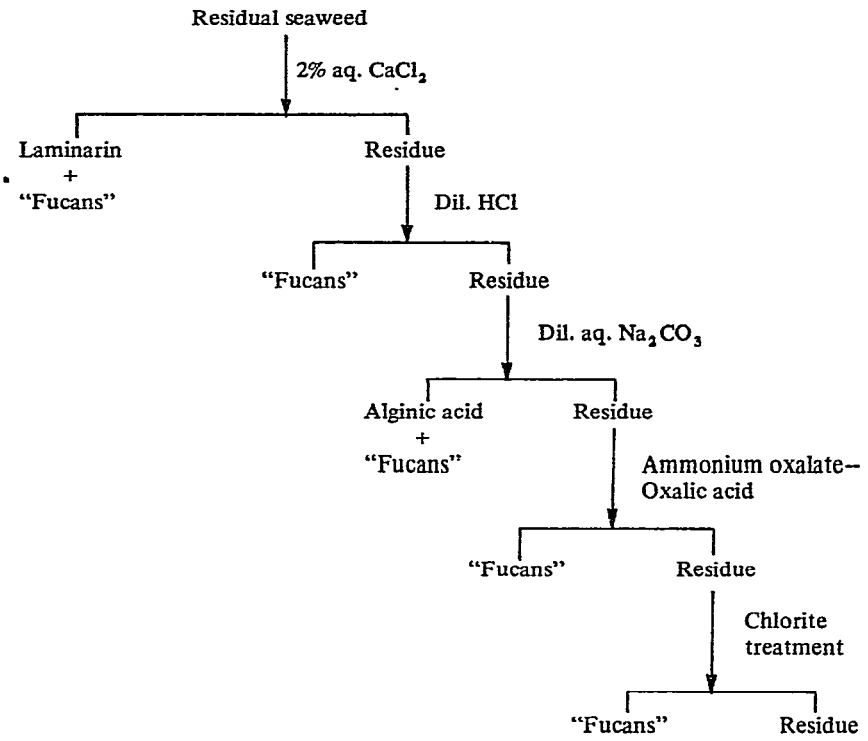

Fig. 1. Flow diagram of extraction procedure after 80% ethanol extraction and formaldehyde treatment.

Fig. 1. The presence of calcium in the initial extraction renders the alginic acid completely insoluble, whereas, in previous extractions, a small proportion of the alginic acid was extracted with water and with acid. Although a small proportion of the glucuronoxylofucans (hereinafter called "fucans" for convenience) were present in the aqueous calcium chloride extract, the major quantity of this type of polysaccharide was present in the acid extract.

TABLE I

YIELDS[a] OF SEAWEED POLYSACCHARIDES

Species	*Laminarin*	*"Fucans"*	*Alginic acid*	*"Fucans"*			*Cellulose*
				Alkali	*Ammonium oxalate*	*Chlorite*	
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

[a]Percentage yields, based on the dry weight of the different seaweeds.

Laminarin. — All the soluble glucan was found in the aqueous calcium chloride extract and this was separated from the "fucan" by elution from a DE-cellulose column with water. The percentage yields from the three algae are given in Table I. The material from each had $[\alpha]_D$ $-14°$ (*cf.* lit. values for laminarin -12 to $-14°$) and gave only glucose on hydrolysis. Parallel hydrolyses on equivalent weights of glucan from each species and of laminarin from *Laminaria hypoborea* gave a readily detectable amount of mannitol from the latter, but the three genera under investigation appeared to be devoid of mannitol. Partial hydrolyses of the glucans from *H. lorea* and *B. bifurcata* gave laminaribiose, gentiobiose, and higher laminarisaccharides. A plot of $\log [1/(R_F - 1)]$ against the degree of polymerisation for the (1→3)-linked laminarisaccharides resulted in a straight line, indicating that they belong to a homologous series. Aliquots of each glucan were methylated by using methylsulphinyl carbanion in methyl sulphoxide and methyl iodide[5], and the derived methylated glycosides gave gas–liquid chromatograms identical with those of *Laminaria hyperborea* laminarin methylated by the above technique or by the classical method[6], apart for a tiny peak for methylated mannitol in the latter. Major peaks with the same retention times (T) as those of methyl 2,4,6-tri-*O*-methylglucosides, and smaller peaks with the T values of methyl 2,3,4-tri- and 2,3,4,6-tetra-*O*-methylglucosides were obtained from all the samples; no di-*O*-methylglucosides were detected (Table II). This is the first time that methylated *L. hyperborea* laminarin has been analysed by g.l.c., and the first time that 2,3,4-tri-*O*-methylglucose has been identified. These results, together with the negative rotation and the small amount of gentiobiose, prove that the soluble glucans of *H. lorea*, *B. bifurcata*, and *P. pavonia* are β-(1→3)-linked D-glucans with a small proportion of (1→6)-linked glucose residues and are of the laminarin type[2].

TABLE II

G.L.C. ANALYSIS OF METHYLATED SUGARS[a]

Retention times (T)		*Methylated sugar*
Column 1	*Column* 2	
3.0,4.0	1.7,2.35	2,4,6-Tri-*O*-methylglucose (major peak)
2.70,3.5	1.6,2.0	2,3,4-Tri-*O*-methylglucose
1.0,1.30	1.0,1.43	2,3,4,6-Tetra-*O*-methylglucose

[a]Present in hydrolysates of the methylated, laminarin-type glucans.

Alginic acid. — The calcium-insoluble fraction of the material extracted with sodium carbonate gave only mannuronic and guluronic acids, and their respective lactones, on hydrolysis (chromatographic examination). After esterification of a portion, reduction, and hydrolysis, paper chromatography revealed only mannose and gulose. This was confirmed by ionophoresis. The yields of alginic acid from the three algae are given in Table I.

"*Fucans*". — Fig. 1 shows the distribution of these polysaccharides, and the percentage yields in each extract (aqueous and acid extracts combined) are given in Table I. Each consisted of heteropolysaccharides comprising different proportions of fucose, glucuronic acid, xylose, and half-ester sulphate, together with trace quantities of galactose. The percentage carbohydrate, sulphate, and uronic acid contents of the "fucans" separated from the aqueous calcium chloride and the acid extracts in each of the weeds are summarised in Table III. It can be seen that the fucans have similar carbohydrate contents, that both extracts from *Himanthalia* have somewhat higher sulphate and lower uronic acid contents than those from the other seaweeds, and that those from *Himanthalia* and *Padina* have similar uronic acid contents while those from *Bifurcaria* have a higher proportion of uronic acid.

TABLE III

COMPOSITION OF THE "FUCANS"[a]

	Carbohydrate (%)	*Sulphate* (%)	*Uronic acid* (%)
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
Dilute 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

[a]Extracted with aqueous calcium chloride followed by dilute acid.

TABLE IV

COMPONENTS IN THE REDUCED HYDROLYSATES OF THE "FUCANS" OF *Himanthalia lorea*

Extract	*Fucitol*	*Xylitol*	L-*Gulonic acid*	*Galactitol*
Calcium chloride	5.5	2.5	1.0	—
Dilute acid	6.0	0.6	1.0	—

The molar proportions of the components present in a reduced hydrolysate of the respective "fucans" from *Himanthalia* are given in Table IV. Whereas the proportion of fucose and glucuronic acid are similar in the two extracts, the proportion of xylose is considerably smaller in the acid extract. In both extracts, the proportion of galactose was too small to measure.

Apart from an additional "fucan" separated from the alkali extract, further quantities of this type of polysaccharide (see Fig. 1) containing fucose, glucuronic acid, and xylose, as already reported for *Ascophyllum nodosum*, were obtained from the ammonium oxalate–oxalic acid extracts and from the chlorite extracts. The latter extracts from *Padina* were very crude and resisted purification and fractionation. Apart from the determination of their constituent sugars, which were the same as those of the same extracts from the other two genera of algae, these materials were not examined further.

In an attempt to establish the homo- or hetero-geneity of the different "fucans", they were each fractionated on a DE-cellulose column by elution with increasing concentrations of aqueous potassium chloride. The aqueous calcium chloride and acid-extracted materials gave three distinct fractions at about 0.3M, 0.5M, and M concentrations of potassium chloride, which were virtually identical to each other in the two extracts from each genus of seaweed. The respective fractions from the aqueous calcium chloride and acid extracts from each seaweed were therefore combined. A summary of the specific rotations, percentage recovery, carbohydrate, sulphate, and uronic acid contents, and the molar proportions of the sugars, estimated as their alditols and L-gulonic acid, of each fraction is given in Table V.

For the extracts from *Himanthalia* and *Padina*, M potassium chloride eluted the largest amount of polysaccharide, whereas the 0.3M fraction was the major polysaccharide from *Bifurcaria*. From each seaweed, the 0.3M fraction had the highest content of uronic acid and xylose, and lowest content of fucose and sulphate (hereinafter called the uronic acid-rich fraction). In contrast, the M fractions have the highest sulphate and fucose contents. It is this fraction which closely resembles the fucoidan described in the literature[2]. The overall composition of the 0.5M fractions in all three species is intermediate in composition between the other two fractions.

The fucans extracted by alkali, and by chlorite from *Himanthalia* and *Bifurcaria*, are very similar to each other in their sulphate and uronic acid contents (Tables VI and VIII), and they each gave two main fractions on elution from a DE-cellulose column at 0.5M and M concentrations of potassium chloride, which only differed from each other in the somewhat higher sulphate content of the molar fractions. The

TABLE V

DATA FOR THE COMBINED AQUEOUS CALCIUM CHLORIDE AND ACID FRACTIONS

Species	$[\alpha]_D$ (*degrees*)	*Recovery* (%)	*Carbohydrate* (%)	*Sulphate* (%)	*Uronic acid*[a] (%)	*Molar proportions of sugars*			
						Fuc	*Xyl*	*GlcUA*	*Gal*[e]
H. lorea									
KCl fraction:									
0.3M (1)	−87	28	50–52	2.5[b]	18.8	2.5	1.0	1.8	+
0.5M (2)	−58	16	45–47	21.0[c]	8.9	10.0[f]	1.0[f]	4.0[f]	+++
M (3)	−132	33	40–42	29.0[d]	4.0	14.0[f]	1.0[f]	2.0[f]	++
B. bifurcata									
KCl fraction:									
0.3M (4)	−84	42	48–50	4.6[b]	19.5	2.8	1.0	2.4	+
0.5M (5)	−54	8	43–45	22.7[d]	11.5	5.5	1.0	2.5	++
M (6)	−100	20	40–42	30.0[b]	2.6	13.0	1.0	2.3	+++
P. pavonia									
KCl fraction:									
0.3M	−94	31	50–52	2.5[b]	20.4	2.0	1.0	2.0	+
0.5M	−51	20	45–47	11.0[b]	9.1	3.0[f]	1.0[f]	1.2[f]	++
M	−112	38	40–42	17.0[d]	4.7	12.5[f]	1.0[f]	3.0[f]	+++

[a]Mean of modified carbazole[19] and cetylpyridinium chloride methods[20]. [b]By method *1* (ref. 17). [c]Mean of method *1* and method *2* (ref. 20). [d]By method *2* (ref. 20). [e]Quantity of galactose visually determined on paper chromatograms. [f]Determined as alditols by g.l.c., and the remainder determined as sugars and glucuronic acid and lactone after elution from a paper chromatogram and filtration through a Millipore filter.

TABLE VI

"FUCANS" EXTRACTED BY SODIUM CARBONATE

Species	$[\alpha]_D$ (*degrees*)	*Recovery* (%)	*Carbohydrate* (%)	*Sulphate* (%)	*Uronic acid* (%)	*Molar proportions of components*		
						Fucitol	*Xylitol*	L-*Gulonic acid*
H. lorea	−51.2	—	40.0	22.2	10.3	4.4	1.0	2.2
0.5M (7)	—	30	45.0	20.0	12.7			
1.0M (8)	—	50	45.0	25.0	12.6			
B. bifurcata	−48.5	—	40.0	24.0	11.3	5.0	1.0	2.0
0.5M (9)	—	30	45.0	21.6	12.2			
1.0M (10)	—	50	45.0	26.0	12.6			
P. pavonia	−58.0	—	47.0	14.5	11.0	4.7	1.0	2.4

[a]Mean value from methods *1* (ref. 19) and *2* (ref. 20).

TABLE VII

"FUCANS" EXTRACTED BY AMMONIUM OXALATE–OXALIC ACID

Species	*Recovery* (%)	*Carbohydrate* (%)	*Sulphate* (%)	*Uronic acid* (%)	*Molar proportions of components*		
					Fucitol	*Xylitol*	L-*Gulonic acid*
H. lorea	—	30.32	11.1	9.4			
0.5M (11)	40	45	9.4	21.8	4.4	1.0	3.7
1.0M	12	40	13.4	20.0			
B. bifurcata	—	35–37	9.7	11.8			
0.5M (12)	40	45	9.2	12.8	5.6	1.0	2.1
1.0M	10	37–38	14.9	9.5			

sulphate content of the "fucan" extracted from *P. pavonia* by alkali is lower than that from the other algae, but the proportions of the sugars are the same (Table VI). This material was not examined further.

TABLE VIII

"FUCANS" EXTRACTED BY CHLORITE TREATMENT

Species	*Recovery* (%)	*Carbohydrate* (%)	*Sulphate* (%)	*Uronic acid* (%)
H. lorea	—	40–42	19.2	—
0.5M (13)	50	45	17.7	10.6
1.0M (14)	30	40–42	24.2	11.3
B. bifurcata		40–42	21.2	—
0.5M (15)	45	45	18.7	10.6
1.0M (16)	30	43	24.0	11.3

The 0.5M and M fractions of the "fucans" extracted by ammonium oxalate–oxalic acid from *Himanthalia* (Table VII) have a lower sulphate and higher uronic acid content than the alkali-soluble material and, in this respect, resemble the 0.3M fraction of the acid-soluble "fucan". In contrast, that from *Bifurcaria*, although it has a low sulphate content, has a uronic acid content similar to the alkali-soluble "fucans". The proportions of sugars to acid in the 0.5M fractions (Table VII) are in agreement with these findings.

These investigations illustrate the extreme complexity of the fucose-containing polysaccharides synthesised by the Phaeophyceae. It is not considered that the different fractions of "fucans" represent distinct polysaccharides, but that an arbitrary fractionation of a whole spectrum of polysaccharides based on fucose has bene achieved; the high uronic acid, low sulphate-containing polymers represent one end of the spectrum and the high fucose, high sulphate-containing fractions the other end.

Cellulose. — The material remaining after the chlorite treatment gave mainly glucose with traces of xylose on hydrolysis. In order to remove any hemicellulose associated with the cellulose, it was treated with potassium hydroxide. The alkali-soluble material from *Himanthalia* contained 27% of protein and gave glucose and traces of xylose on hydrolysis. It was separated into ethanol-soluble and -insoluble materials, and hydrolysis revealed that this was merely a separation by molecular weight of otherwise identical materials consisting mainly of glucose with traces of xylose. They were not examined further.

The final residue was also contaminated with 16.6% of protein, and on hydrolysis gave glucose as the only sugar. Permethylation and hydrolysis gave 2,3,6-tri-*O*-methylglucose with small quantities of 2,3,4,6-tetra-*O*-methylglucose, indicating that the glucose residues are (1→4)-linked and that the final residue is probably a cellulose-type polysaccharide. All three species appear to synthesise the same proportion of cellulose (Table I).

These investigations show that these three genera of brown seaweeds synthesise the same polysaccharides as other members of the Phaeophyceae. Since no other species have been subjected to this systematic extraction, it is not possible to compare the distribution of the "fucans" with other members of this Class. It must be remembered that the percentage yields of the individual polysaccharides, based on the dry weight of seaweed, vary with the season of harvesting. In the present work, all the species examined were harvested in late summer.

Padina, apart from cellulose, appears to synthesise a lower proportion of polysaccharides, particularly of the "fucans" soluble in acid. It is considered that the polysaccharides which require more-drastic conditions of extraction are laid down as permanent structural features, and these comprise a much higher proportion of the "fucans" synthesised by *Padina*. It is probable that this finding is related to the different morphology of this species.

The composition of the dialysates and the alcoholic supernatants of the various extracts was investigated since a knowledge of this would be useful in future biosynthetic studies[8] with ^{14}C. The dialysates of the aqueous and the acid extracts from all three seaweeds were 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 would have been extracted by aqueous ethanol. In addition to these free sugars, the acid dialysate from *Padina* also contained a disaccharide comprising fucose and xylose residues, indicating the mutual linkage of these sugars in the polysaccharides.

The supernatant of the sodium carbonate extract contained the same free sugars as the acid and aqueous dialysates, together with small quantities of a 4,5-unsaturated acid. It has been reported[9] that alginic acid is degraded to a 4,5-unsaturated uronic acid by a β-elimination reaction under alkaline conditions.

EXPERIMENTAL

All solutions were evaporated under diminished pressure below 50°. Specific rotations, unless otherwise stated, were measured in water at 20° with a Perkin–Elmer 141 polarimeter. Paper chromatography was carried out on Whatman No. 1 and 3MM paper with the following solvent systems v/v: (*1*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*2*) 1-butanol–ethanol–water (40:11:19); (*3*) 1-butanol–pyridine–water (6:4:3); (*4*) 1-butanol–pyridine–water–benzene (5:3:3:1); (*5*) butanone–acetic acid–water (9:1:1) saturated with boric acid; (*6*) ethyl acetate–acetic acid–pyridine–water (5:1:5:3, upper phase), with ethyl acetate–pyridine–water (40:11:6) in the bottom of the tank; (*7*) butanone saturated with water; (*8*) 1-butanol–pyridine–water (10:3:3). Papers were sprayed with (*A*) saturated 50% ethanolic aniline oxalate; (*B*) 2 parts of 2% sodium metaperiodate + 1 part of 1% potassium permanganate[10]; (*C*) tetrazolium hydroxide[11]; (*D*) aniline–diphenylamine[12]; or dipped in (*E*) silver nitrate[13]; (*F*) D-glucose oxidase[14]. T.l.c. was carried out on plates coated with Kieselgel G. Iodine vapour was used to detect the carbohydrate. Ionophoresis was

carried out on Whatman No. 4 paper in pyridine–acetic acid buffer (pH 6.7), and in borate buffer (pH 10). G.l.c.[15] was performed on columns of acid-washed Celite coated with 15% by weight of poly(butane-1,4-diol succinate) (column *1*), 10% of polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] (column *2*), and 10% of poly(ethylene glycol adipate) (column *3*) at an operating temperature of 175° for methylated sugars. Retention times (*T*) are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-*β*-D-glucopyranoside. For the trimethylsilylated sugars and alditols, the liquid phase was 7.5% of Apiezon K (column *4*) at 155° and 175°, and 3% of S.E.30 (column *5*) at 175°, and *T* values are expressed relative to that of the Me_3Si derivative of xylitol. The methylated alditol acetates were run on Chromosorb W coated with 3% ECNSS-M (column *6*), and *T* values are expressed relative to that of 2,3,4,6-tetra-*O*-methylglucitol 1,5-diacetate.

Reductions were carried out with potassium borohydride in aqueous solution. Excess borohydride was destroyed and the cations were removed with Amberlite IR-120(H^+) resin. Boric acid was removed from residues by methanol distillation. The carbohydrate content of polysaccharides was determined by the phenol–sulphuric acid method[16]. Unless otherwise stated, hydrolyses were effected with 90% formic acid in sealed tubes in an atmosphere of carbon dioxide for 6 h at 100°, followed by hydrolysis of the formic esters by dilution with water (5 vol.) and further heating for 2 h at 100°. The molar proportions of the sugars in the different polysaccharides were determined on hydrolysates by (**1**) elution of the appropriate areas from a paper chromatogram, filtration through a Millipore filter, and application of the phenol–sulphuric acid method, or (**2**) reduction to the alditols and aldonic acid and calculation of the peak areas of the Me_3Si derivatives on g.l.c. (columns *4* and *5*).

Sulphate (expressed as percentage of carbohydrate) was determined by the method of Jones and Letham[17] after digestion of the poly- or oligo-saccharide[18], and with cetylpyridinium chloride[20]. Uronic acid was determined by a modified carbazole method[19] and with cetylpyridinium chloride[20]. Methylation was by a modification of the Hakomori method[5]. Conversion of methylated sugars into the methylated glycosides was effected with anhydrous methanol and Amberlite IR-120(H^+) resin[21]. D.p. values were determined by the method of Timell[22]. Demethylation was accomplished by the method of Bonner and co-workers[23].

Extraction of the seaweeds. — In a typical experiment, the powdered seaweed (50 g) was extracted exhaustively with cold and hot (70°) 80% aqueous ethanol with constant stirring. The residual seaweed, recovered by filtration, was immersed overnight in 40% formaldehyde. The formaldehyde was decanted off and the air-dried weed was then sequentially extracted, with constant stirring, with the following reagents. *1.* 2% Aqueous calcium chloride (300 ml) for 4 h (twice at room temperature and once at 70°). *2.* Dilute hydrochloric acid (300 ml, pH 2.0) for 4 h at 70°, during which time the pH was maintained at 2.0–2.1 by the addition of hydrochloric acid; this extraction was repeated four times. *3.* 3% Aqueous sodium carbonate (300 ml) for 4 h at 50° (five times). *4.* Ammonium oxalate–oxalic acid (0.25% with respect to each, 250 ml, pH 2.8) for 6 h at 70°. *5.* A mixture of water (200 ml), acetic acid (1 ml),

and sodium chlorite (1 g) at 70°; in all, four additions of acetic acid and sodium chlorite were made at hourly intervals. *6.* Water, until free from chlorite, and then exhaustively with 6M potassium hydroxide (125 ml) for 48 h at room temperature in an atmosphere of nitrogen. *7.* The residual solid was washed with dilute acetic acid, water, ethanol, and ether, and was recovered as a white solid.

Examination of the various extracts. — 80% Aqueous ethanol. Both cold and hot ethanolic extracts were evaporated separately to ~250 ml and allowed to stand at room temperature overnight. Fine particles of seaweed which had passed through the filter settled out, and an oily layer separated on top of the light green solution. These were both removed. The solution was concentrated further and poured into excess of ethanol. Inorganic salts were immediately deposited, and a further, flocculant precipitate formed on standing. The latter precipitate gave a light-brown solution in water, which was decolourised with charcoal and then treated with Amberlite IR-120(H^+) resin, and the precipitate was recovered by addition of ethanol. Recrystallisation from aqueous ethanol gave D-mannitol, m.p. and mixed m.p. 160–162°, $[\alpha]_D$ −0.25°. The mother liquors were concentrated to a syrup, and analysed by paper chromatography in solvents *1*, *2*, and *5*, with development by sprays *A*, *B*, *E*, and *F*. Mannitol was present in the syrups from all three seaweeds. Glucose was also present in the syrups from *Himanthalia* and *Bifurcaria*, and that from the latter showed a third spot with reagent *E*. This syrup was fractionated on 3MM paper to give mannitol, m.p. and mixed m.p. 162–163°; a neutral syrup with the chromatographic mobility of glucose (confirmed with D-glucose oxidase), which yielded D-glucose, m.p. and mixed m.p. 82–83°; and a non-reducing, neutral syrup with the chromatographic mobility of *myo*-inositol.

Calcium chloride extract (1). The two extracts collected at room temperature were combined and dialysed against distilled water in a closed system until free from chloride ions. The concentrated dialysate was then poured into excess of ethanol, and a solution of the resulting precipitate in water was freeze-dried. The extract collected at 70° was similarly treated. Aliquots of the two freeze-dried products were analysed and found to be similar. They were therefore combined (Material *F*). The two dialysates were concentrated, the bulk of the calcium chloride was precipitated with ethanol, the last traces were removed by treatment with resins, and the resulting solution was concentrated and examined by paper chromatography.

An aliquot (200 mg) of *F* dissolved in water (10 ml) was applied to the top of a cellulose column (DE-52, micro-granular; 30 × 4.5 cm) which had been equilibrated with 0.5M potassium chloride. The column was eluted with water (500 ml) and then with an increasing gradient of 0.1M potassium chloride. Fractions (10 ml) were collected and monitored for carbohydrate[16].

Acidic extract (2). The combined extracts were treated in the same way as extract *1*, and the non-dialysable product was fractionated on a column of DE-52 microgranular cellulose.

The polysaccharides in extracts *1* and *2* gave the same elution pattern with potassium chloride, fractions of polysaccharide being eluted at 0.3M, 0.5M, and

M potassium chloride concentrations. The bulk of these two extracts was therefore separated into three main fractions by graded elution with 0.3M, 0.5M, and M potassium chloride from a column of DE-cellulose. Estimation of carbohydrate, sulphate, and uronic acid contents, and paper chromatography of hydrolysates of each fraction showed the essential similarity of the same fractions from the two extractions, and they were therefore combined.

Sodium carbonate extract (3). The combined extracts were poured into ethanol (4 vol.) and, after standing overnight, the derived precipitate was filtered through muslin and air-dried. The filtrate was concentrated, inorganic salts were removed by filtration, and the filtrate was dialysed. On concentration and free-drying, the non-dialysable portion gave a solid (*G*) (*ca.* 400 mg from *H. lorea*, and 200 mg from *B. bifurcata*), hydrolysis of which gave mainly fucose and glucuronic acid, small quantities of mannuronic acid, and traces of galactose. The dialysable material was preserved. A solution of the ethanol precipitate in the minimal volume of water was dialysed until free from inorganic salts, concentrated, and freeze-dried. An aliquot was hydrolysed and examined by paper chromatography. It appeared to be a mixture of alginic acid and a glucuronoxylofucan. In order to separate these two polysaccharides, the rest of the freeze-dried material was dissolved in water to give a concentration of alginic acid of $\sim 1\%$, and to this 2% aqueous calcium chloride was added slowly with stirring until precipitation was complete. The mixture was set aside in the cold overnight, and the gelatinous precipitate of calcium alginate was then removed by centrifugation, washed with water, and freeze-dried. The supernatant solution containing the "fucan" was dialysed until free from chloride ion, and then concentrated, freeze-dried, and analysed (Table VI).

The dialysates from the calcium chloride and acid extractions and the alcoholic supernatant of the sodium carbonate extractions were separately concentrated and examined by paper chromatography. The acid extract from *Padina* gave an unusual pink spot with R_{Fuc} 0.76 and R_{Ara} 0.95 (solvent *2*, spray *A*). This was separated, the d.p. determined, and an aliquot hydrolysed. The product from the sodium carbonate supernatant gave a positive test for 4,5-unsaturated acid with thiobarbituric acid, after treatment with an acidic solution of sodium periodate[9].

The ammonium oxalate–oxalic acid extract (4). The solution was dialysed until free from oxalate ion, concentrated, and freeze-dried. A portion was hydrolysed, and the hydrolysate was examined by paper chromatography and g.l.c. The "fucans" extracted by alkali and by ammonium oxalate from *Himanthalia* and *Bifurcaria* were fractionated as for the acid extracts (*2*) on a DE-cellulose column, major fractions being eluted with 0.5M and M concentrations of potassium chloride. These fractions were analysed for carbohydrate, sulphate, and uronic acid contents, and the molar proportions of the constituent sugars of the larger fraction were determined (Tables VI and VII).

The chlorite extract (5). The solution was dialysed until free from chloride ions, and the non-dialysable material was concentrated and freeze-dried. A portion of the residue was hydrolysed, and the hydrolysate was examined by paper chromatography

and g.l.c. The remainder of this extract from *Himanthalia* and *Bifurcaria* was fractionated as for extract *4*, and the products were analysed for carbohydrate, sulphate, and uronic acid contents (Table VIII).

The potassium hydroxide extract (6). The combined extracts and washings were poured into a mixture of ice-cold ethanol (excess) and glacial acetic acid. The precipitate was collected by centrifugation, washed successively with aqueous ethanol, ethanol, and ether and then dried *in vacuo*. The alcoholic supernatant, after removal of the ethanol, was dialysed and freeze-dried. Aliquots of the alcohol-soluble and insoluble materials were hydrolysed and examined by paper chromatography in solvents *1*, *2*, and *5*, with detection by reagents *A*, *B*, *E*, and *F*. The hydrolysates were also analysed by g.l.c.

Characterisation of the glucans. — (*a*) The material separated from the aqueous calcium chloride extract (*1*) of each weed by elution of the DE-cellulose column with water was examined as follows. An aliquot was hydrolysed with 0.5M sulphuric acid for 1 h at 100°, and the hydrolysate was neutralised ($BaCO_3$), freed from barium ions with Amberlite IR-120(H^+) resin, concentrated, and analysed by paper chromatography in solvents *1*, *2*, and *5*, with development by sprays *A*, *B*, and *E*. A second aliquot and *Laminaria* laminarin (10 mg of each) were hydrolysed separately with 50mM sulphuric acid (1 ml) for 1 h at 100°. The hydrolysate was neutralised by shaking with 5% dioctylamine in chloroform, and analysed by paper chromatography (130 h in solvent *8*, developed with reagent *E*). A third aliquot and *Laminaria hyperborea* laminarin were methylated separately. The two methylated materials were examined by t.l.c. They, together with a sample of the *Laminaria* laminarin (methylated by the method of Haworth[6]), were hydrolysed and analysed by paper chromatography in solvents *2* and *7* and then converted into the methyl glycosides and analysed by g.l.c.

(*b*) An aliquot of the white solid, remaining after extraction *7*, was hydrolysed with 72% sulphuric acid. A second aliquot was methylated by the method of Haworth[24], followed by that of Kuhn[25]. The product was hydrolysed and examined by paper chromatography and by g.l.c. of the methyl glycosides of the derived methylated sugars.

Characterisation of alginic acid. — The calcium-insoluble material extracted with sodium carbonate (extract *3*) was hydrolysed. Paper chromatography of the hydrolysate (solvents *1*, *3*, and *6*), with development by reagents *A* and *E*, revealed the presence of mannuronic and guluronic acid and their respective lactones. A portion of the hydrolysate was esterified, reduced, and hydrolysed, and the resulting syrup was analysed by paper chromatography in solvent *5* with spray *B*, and by ionophoresis in borate buffer.

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