

Sulphated Polysaccharides in Brown Algae

III. The Native State of Fucoidan in *Ascophyllum nodosum* and *Fucus vesiculosus*

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Under very mild acidic conditions, a soluble, macromolecular complex (A) was isolated from *Ascophyllum nodosum*, which contained residues of fucose, xylose, galactose, traces of mannose, glucuronic, mannanuronic and guluronic acids, half-ester sulphate, and traces of firmly bound protein. The degradation of this material under mildly acidic conditions was studied by free-boundary electrophoresis, and shown to liberate a sulphated glucuronoxylifucan, a polyuronide related to alginate, and a component with a composition approaching that of a polymer of fucose monosulphate, and therefore corresponding to "fucoidan" in the classical sense.

A complex similar to (A) was also found in the extract obtained after neutralisation of the acid-extracted algal particles, and, although neither sample was compositionally homogeneous, the presence of free fucoidan in *Ascophyllum* is doubtful. On the other hand, whereas a closely similar complex was also found in *Fucus vesiculosus*, it was accompanied by substantial amounts of free fucoidan in this species.

The results suggest that, in its native state, fucoidan is mostly present as a building element of a much more complex macromolecule, and that the isolation of fucoidan entails chemical degradation as well as physical separation. This helps to explain the conflicting reports concerning the monomeric composition of fucoidans.

Since its discovery by Kylin¹ in 1913, fucoidan has been the subject of many investigations.² It appears to be a general constituent of brown marine algae, but its composition, after isolation, varies according to the species and conditions of extraction. For this reason, "fucoidan" is best regarded as a collective term for a family of polysaccharides, all approaching a polymer of fucose monosulphate in composition, but containing small amounts of residues of other sugars in varying proportions. Among these other sugars, galactose and xylose are commonly mentioned, together with uronic acids in some cases.²

Structural investigations³⁻⁵ have been carried out on the fucoidan extracted from *Fucus vesiculosus*, and have shown that it is essentially a 1,2-linked

polymer of α -L-fucose-4-sulphate, with a small proportion of linkages of other types.² This has come to be regarded as a kind of ideal structure for fucoidan generally, and there has been a tendency to attribute the presence of other sugar residues to impurities in the preparations.⁶⁻⁹ On the other hand, Quillet has held that these other sugar residues should properly be regarded as integral parts of the fucoidan molecule.¹⁰

In this laboratory, we first had reason to doubt the presence of a simple, fucan sulphate in *Ascophyllum nodosum* while examining the extract obtained by neutralising the algal particles with alkali after they had first been washed in acid. This extract, which contains most of the alginate present in the alga, and is therefore referred to here as the "alginate extract", was found also to contain three other polysaccharides, all composed of residues of fucose, xylose, hexuronic acids, half-ester sulphate and firmly bound protein.¹¹

The major component of this triad, which was named "ascophyllan", contained 26 % of xylose and 27 % of hexuronic (mainly glucuronic) acids in addition to fucose, sulphate and protein. It satisfied quite stringent tests for compositional homogeneity,¹¹ and yielded 3-O- β -D-xylopyranosyl-L-fucose upon partial hydrolysis, thus proving that the fucose-containing chains were heteropolymeric.¹² Similar findings have been reported by Percival.¹³ The other two components of the triad¹¹ (F_1 and F_2 , respectively) were intermediate in composition between ascophyllan and a pure fucan sulphate, but even the one that was richest in fucose (F_2) also yielded the above-named disaccharide on partial hydrolysis, and therefore could not be regarded as fucoidan in the accepted sense.¹¹

These findings^{11,12} prompted a systematic search for a component in *Ascophyllum nodosum* that did resemble fucoidan in composition. It was logical to begin with extraction under acidic conditions, according to the established method for preparing fucoidan,² and to use the already well-investigated species, *Fucus vesiculosus*, as a control. The results, which are now described, establish that significant amounts of fucoidan are indeed present in *Fucus vesiculosus* in the free state, whereas in *Ascophyllum nodosum*, they are not. In both species, however, most of the fucose is present in a much more complex macromolecule, from which fucoidan is liberated as a degradation-product. A preliminary report has been published elsewhere.¹⁴

Table 1. Distribution of total carbohydrate and fucose in successive extracts of 5 g of *A. nodosum*, Være 2/3.

Extraction in	Total carbohydrate ^a	Fucose mg	% of total fucose extracted
0.2 N HCl, 20°C	740	164	50.5
pH 7, 20°C {1st extract	1655	74	22.8
pH 7, 20°C {2nd extract	50	6.2	1.9
pH 7, 100°C	270	59.5	18.2
pH 13, 20°C	120	21.4	6.6

^a Phenol-sulphuric acid method, calculated as alginate.

Table 2. Separate extraction of 5 g samples of *A. nodosum*, Være 2/3, at room temperature and various acidities.

Extraction	Non-dialysable		Dialysable		Non-dialysable material after isolation				
	Carbo- hydrate mg ^a	Fucose mg	Carbo- hydrate mg ^a	Fucose mg	Yield mg ^b	Fucose %	$\frac{\text{SO}_3^{2-}}{\text{COO}^-}$ ^c	Extracted amount mg	
								SO ₃ Na	Na- uronate
H ₂ O	364	54.4	196	3.4	330	16.2	1.5	49.7	64.7
pH 3	416	66	188	3.3	457	17.3	2.0	60.4	60.4
pH 2	554	143	210	4.6	514	34.0	6.0	120	38.3
pH 1	840	256	182	8.2	731	39.0	10.6	192	35.0

^a Phenol-sulphuric acid method, calculated as sulphated fucan.

^b Yield of material obtained by freeze-drying of the non-dialysable fraction.

^c Determined by titration with cetyl-pyridinium chloride (CPC).²⁸

RESULTS

Table 1 shows the distribution of fucose and total carbohydrate among fractions obtained by successive extraction of the alga with aqueous acid, water, and aqueous alkali. Total carbohydrate was determined by the phenol-sulphuric acid method, and the results are expressed arbitrarily as alginate.

It is seen that, under the conditions used, about half the total extractable fucose was removed in the acid extract. Subsequent extraction at pH 7 then yielded the alginate extract, which contained nearly 60 % of the total extractable carbohydrate and another 25 % of the fucose. Elevated temperatures and higher pH during extraction then removed further quantities of fucose.

Black *et al.*¹⁵ have previously demonstrated that the amount of fucose extracted in the acid depends strongly upon the pH and temperature. To minimize hydrolysis during extraction, our experiments were all carried out at room temperature, and the results shown in Table 2 confirm that the extraction of fucose is enhanced by higher acidity. Of the total extractable fucose, only 3 to 5 % occurred as dialysable material, and there was no tendency for this fraction to increase with decreasing pH. On the other hand, the fucose and sulphate contents of the isolated polymeric material both increased markedly with the acidity. The material extractable only under acidic conditions must therefore have contained a considerably higher proportion of fucose and sulphate than that extracted in water. In an actual experiment with successive extractions, the material extracted at pH 2 contained 44.5 % fucose, compared to only 18.4 % for the water-extractable material.

Based on these results, two different extraction procedures were used in this investigation: when it was important to assure a minimum of degradation, for instance in the case of electrophoretic studies, the extraction was carried out at pH 2 overnight; while for preparative purposes the alga was first extracted for 3 h with 0.2 N hydrochloric acid and then overnight with distilled water. The extracts were in all cases neutralised immediately after removal of the algal particles.

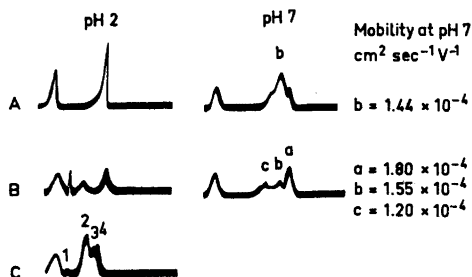


Fig. 1. Ascending boundaries obtained at pH 2 and pH 7 before and after mild acid-hydrolysis of the polysaccharidic material isolated by extraction at pH 2. A: Undegraded polysaccharide complex. B: Material A after hydrolysis in 0.02 N HCl at 80° for 60 min. C: Mixture of polysaccharides isolated from the alginate extract of *Ascophyllum*: 1. Alginic acid; 2. Ascophyllan; 3. Component F₁; 4. Component F₂.

Fig. 1A shows the typical pattern obtained by free-boundary electrophoresis of the material extracted at pH 2. A slight inhomogeneity was indicated in the run buffered at pH 7, whereas that at pH 2 only gave a skew boundary. When compared with the electrophoretic behaviour¹¹ of the fucose-containing polysaccharides of the alginate extract, reproduced in Fig. 1C, the pattern at pH 2 provides fairly strong evidence for the absence of any ascophyllan (Fig. 1C, peak 2) or alginate (Fig. 1C, peak 1) in this material. On the other hand, paper chromatography of acid-hydrolysates revealed the presence of the same monosaccharides as were found in ascophyllan,¹¹ together with mannuronic and guluronic acid, thus indicating that both ascophyllan and alginate may be present in the isolated material but not free to move in the electric field as separate entities.

As shown in Fig. 1B, a very limited acid-degradation of the material extracted at pH 2 had a pronounced effect upon the electrophoretic pattern. As hydrolysis proceeded, the original boundary gradually disappeared and two new boundaries were formed. No real improvement in separation was

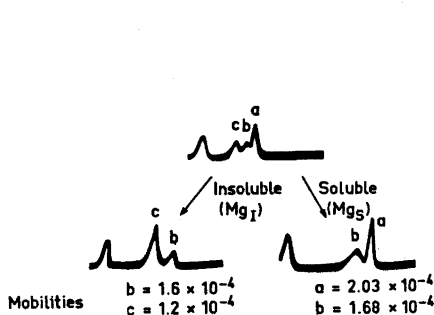


Fig. 2. Ascending boundaries at pH 7 of the degraded complex (Fig. 1B) before and after separation with ethanol-magnesium chloride into soluble (Mg_S) and insoluble (Mg_I) fractions.

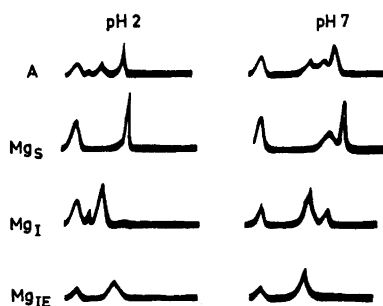


Fig. 3. Comparison of ascending boundaries at pH 2 and pH 7 of the degraded complex (Fig. 1B) and various fractions separated from it. A: Degraded complex before fractionation. Mg_S: Fraction of A soluble in ethanol-magnesium chloride. Mg_I: Fraction of A insoluble in ethanol-magnesium chloride. Mg_{IE}: Fraction Mg_I after treatment with alginase.

achieved by degradation beyond the stage shown in Fig. 1, this only serving to affect adversely the recovery of high molecular-weight material. A loss of 20–35 %, as dialysable fragments, of both total carbohydrate and fucose occurred as a consequence of the acid-hydrolysis.

A further characterisation of the components that were liberated by mild hydrolysis of the complex required a method for separation on a preparative scale. This was achieved by partial precipitation with ethanol in the presence of magnesium chloride. As shown in Fig. 2, this method effected a complete separation of the fast- and slow-moving components, but material from the intermediate peak appeared in both fractions. The distribution of total carbohydrate and fucose between the two fractions is given in Table 3. It is evident that the soluble fraction was very much enriched in fucose.

Table 3. Distribution of total carbohydrate and fucose between the fractions of the degraded complex that were soluble and insoluble, respectively, in ethanol-magnesium chloride.

	Total carbohydrate mg ^a	Fucose mg
Before fractionation	3.484	1.579
Insoluble fraction	1.314	0.184
Soluble fraction	1.957	1.267
Yield	3.271	1.451
Recovery	83.3 %	91.5 %

^a Phenol-sulphuric acid method, calculated as fucoidan.

Electrophoretic patterns for the two fractions at both pH 2 and pH 7 are shown in Fig. 3. The material from the intermediate peak contaminating the insoluble fraction had a mobility similar to alginate in both media. Its identity with alginate was demonstrated by treatment of the fraction with alginase,¹⁶ which completely removed it (Fig. 3, Mg_{IE}).

The apparent heterogeneity of the soluble fraction at pH 7 (Fig. 3, Mg_S) prompted further attempts at purification of the fast-moving component, but these were unsuccessful, and this fraction was therefore analysed as such. It should be noted that, in this case, the contaminating material was not detectable by electrophoresis at pH 2, and it was therefore not alginate.

The analytical compositions of the soluble (Mg_S) and the alginase-treated insoluble (Mg_{IE}) fractions are given in Table 4, with the corresponding figures for ascophyllan included for comparison. The analytical figures for the soluble fraction are seen to approach those of the sodium salt of a polymeric fucose monosulphate, which, if allowance is made for 7.3 % of galactose, should theoretically contain 54.1 % fucose and 38.2 % SO₃Na (fucose and galactose were the only monosaccharides detected in this fraction). The analytical

Table 4. Analytical composition (%) of the soluble (Mg_S) and the alginase-treated insoluble (Mg_{IE}) fractions with that of ascophyllan included for comparison.

Fraction	Fucose	Na-uronate ^a	SO ₃ Na ^b	Other sugars	Protein ^c
Mg_S	53.6	1	37.3	7.3 ^d	3.2
Mg_{IE}	23.2	23.5	16.3	30.1 ^e	4.1
Ascophyllan	25.3	27.1	12.9	26.0 ^f	11.8

^a Differential titration with CPC at pH 7 and pH 2 (Ref. 28).

^b Titration with CPC at pH 2 (Ref. 28).

^c Folin-Ciocalteu method.^{29,30}

^d Galactose, determined by difference.

^e Mainly xylose, with some mannose, determined by difference.

^f Xylose, determined directly.

composition of the alginase-treated insoluble fraction (Mg_{IE}) is very close to that of ascophyllan, and partial acid-hydrolysis, followed by paper chromatography, revealed the characteristic pattern of mono- and oligo-saccharides noted earlier for ascophyllan.¹¹ In this connection, it is particularly significant that the disaccharide previously isolated from ascophyllan¹² was also a major component in partial acid-hydrolysates of this insoluble fraction. Additional evidence for the identity of the alginate-free, insoluble fraction (Mg_{IE}) with ascophyllan is furnished by the identical electrophoretic mobilities obtained both at pH 2 and pH 7 (Fig. 3).

Of the fucose-containing polysaccharides isolated from the alginate extract of *Ascophyllum*, component F_2 showed, upon free-boundary electrophoresis, a mobility very similar to that of the material isolated from the acid extract. A preparation of component F_2 , similar to the one described earlier,¹¹ was therefore subjected to mild acid-hydrolysis followed by free-boundary electrophoresis at pH 7. The resulting patterns are given in Fig. 4 and clearly show the appearance of the same type of electrophoretic heterogeneity after mild hydrolysis as was found for the acid-extractable material (Fig. 1). Fractionation of the degraded material with ethanol-magnesium chloride again provided

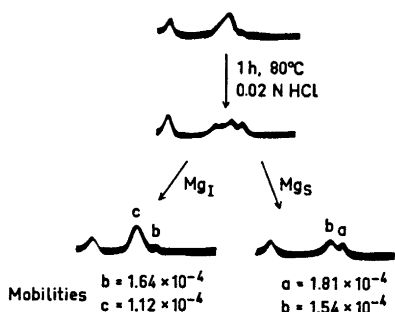


Fig. 4. Hydrolysis and fractionation of component F_2 . Electrophoretic patterns at pH 7 before and after acid-hydrolysis and after separation of the degraded material, with ethanol-magnesium chloride, into soluble (Mg_S) and insoluble (Mg_I) fractions.

two fractions, with a clear-cut separation of the fastest and slowest moving components. The analytical compositions of the two fractions are given in Table 5. The fractionation is obviously of the same type as that obtained for the acid-soluble material, although there is a marked difference in content and composition of sugars other than fucose.

Table 5. Analytical composition (%) of the soluble (Mg_S) and insoluble (Mg_I) fractions obtained by fractionation of the acid-degraded component (F₂) with ethanol-magnesium chloride.

Fraction	Fucose	Na-uronate ^a	SO ₃ Na ^b	Other sugars	Protein ^c
F ₂ Mg _S	40.4	1	31.5	21.7 ^d	1.5
F ₂ Mg _I	23.4	18.2	15.8	27.0 ^e	2.6

^a, ^b, ^c, and ^d Same significance as in Table 4.

^e Xylose and galactose, determined by difference.

Experiments similar to those reported for *A. nodosum* were also carried out with *F. vesiculosus*, and the distribution of material obtained by successive extraction with distilled water, and 0.2 N hydrochloric acid for 3 h, followed by extraction with water overnight, is shown in Table 6. The data for the aqueous extract are directly comparable with those for *A. nodosum* in Table 2. To compare the yields obtained by acid extraction, the data in the appropriate columns of Table 6 must be summed and compared with the results shown for extraction at pH 1 in Table 2. Except for the higher amount of fucose extracted from *F. vesiculosus*, the data are quite similar for the two algae.

For reasons to be discussed later, the two extracts were in this case separated into soluble and insoluble fractions with ethanol-magnesium chloride prior to any degradation with acid, and the analytical figures for the fractions are given in Table 7. The soluble and insoluble fractions obtained in this way from the acid extract were subjected to mild acid-hydrolysis (*cf.* Fig. 1), followed by

Table 6. Successive extractions of 5 g of *F. vesiculosus*, Røberg 5/5, with distilled water and 0.2 N HCl. The superscripts, *a*, *b*, and *c*, refer to the analytical methods named in Table 2.

Extraction	Non-dialysable		Dialysable		Non-dialysable material after isolation				
	Carbo- hydrate mg ^a	Fucose mg	Carbo- hydrate mg ^a	Fucose mg	Yield ^b mg	Fucose %	SO ₃ ^{a-} COO ⁻ ^c	Extracted amount mg	
								SO ₃ Na	Na- uronate
H ₂ O	530	157	342	3	338	33.5	1.66	67.0	78.2
0.2 N HCl	369	211	70	0	380	50.2	21.5	108.1	9.7

Table 7. Analytical composition (%) of fractions obtained from water- and acid-extracts of *F. vesiculosus* by fractionation with ethanol-magnesium chloride.

Fraction	Water extract					Acid extract				
	Yield mg	Fucose	SO ₃ Na	Na-uronate	Protein ^a	Yield mg	Fucose	SO ₃ Na	Na-uronate	Protein ^a
Soluble	162	48.0	25.2	2.9	1.5	208	59.2	28.4	0	0.5
Insoluble	176	20.5	14.9	42.0	1.5	172	39.4	28.7	5.7	1.1

^a Determined by Micro-Kjeldahl.

free-boundary electrophoresis at pH 7, and the patterns given by the degradation products are shown in Fig. 5. The fragments of the insoluble fraction gave a pattern closely resembling that obtained after degradation of the total acid-extractable material from *Ascophyllum*. Partial precipitation of the degraded, insoluble material also gave two distinctly different fractions (Fig. 5), and the conspicuous similarity revealed by comparison with Fig. 2 strongly suggests that *F. vesiculosus* contains polymers of essentially the same composition as those of *Ascophyllum*. Acid-hydrolysis of the two fractions, followed by paper chromatography, essentially confirmed this suggestion: fucose, together with small amounts of galactose, were the only detectable monosaccharide components of the magnesium-soluble fraction (IMg_s of Fig. 5), while the insoluble fraction (IMg_i) gave a pattern of components characteristic of ascophyllan.

The data in Table 7 illustrate the effect of fractionating the extracted polysaccharide complex with ethanol-magnesium chloride prior to acid-degradation. In both the aqueous and acidic extracts, there was a marked tendency for accumulation of material rich in fucose and sulphate in the magnesium-soluble fractions, while uronic-acid containing material was accumulated in the insoluble fractions. The insoluble fractions also contained most, and in the case of the acid extract, all of the xylose present. It is note-

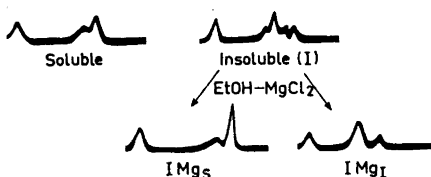


Fig. 5. Fractionation of the material extracted with acid (Table 7) from *F. vesiculosus* without previous acid hydrolysis. Both fractions were subjected to mild hydrolysis before electrophoresis at pH 7, and the insoluble fraction was subsequently fractionated again in the same system.

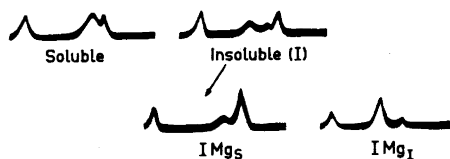


Fig. 6. Same as Fig. 5, but with polysaccharide from *A. nodosum*.

worthy that the soluble fraction from the acid extract seemed to approach the composition of a sulphated fucan. The electrophoretic pattern obtained with this fraction (Fig. 5), after acid-treatment, clearly points to the same conclusion. The insoluble fraction, on the other hand, was of very complex nature, but was, after partial hydrolysis and further fractional precipitation, resolved into components closely resembling those obtained from *Ascophyllum*.

Similar experiments were carried out with the acid extract of *Ascophyllum*, and the electrophoretic boundaries shown in Fig. 6 leave no doubt about the similarity of fractions, obtained both before and after acid-treatment, with those from *F. vesiculosus*. The distribution of material was, however, very different for the two algae. Of the total acid-extractable material, only 8.7 % remained soluble by fractional precipitation of the *Ascophyllum*-material compared to 55 % for *F. vesiculosus* (Table 8). Except for a slightly higher amount in the soluble fraction in both algae, the distribution of fucose was very similar to that of total carbohydrate.

Table 8. Distribution of material (%) by fractionation of acid extracts of *A. nodosum* and *F. vesiculosus* with ethanol-magnesium chloride before acid degradation.

Fraction	<i>A. nodosum</i>		<i>F. vesiculosus</i>	
	Total carbohydrate.	Fucose	Total carbohydrate.	Fucose
Mg _s	8.7	10.9	55	60
Mg _i	91.3	89.1	45	40

DISCUSSION

The data in Tables 1 and 2, and the earlier results of Black *et al.*,¹⁵ are not readily reconciled with the idea that the extraction of fucoidan is a simple, physical process. Once isolated, the fucose-containing polysaccharides are freely soluble in water at any pH, and in the presence of any of the inorganic cations that are present in the alga, and yet only a small fraction of them can be extracted from the finely milled alga with distilled water alone.

It is unlikely that they are rendered insoluble in the native state by a purely ionic association between the sulphate half-ester groups and insoluble protein, because such a complex should be stabilised, rather than dissociated, in dilute acid. Similarly, it is difficult to accept that they are insoluble because of physical inaccessibility to the water used for extraction. Any cells that had not been ruptured by milling should certainly have been destroyed by removal of the alginates, and yet the essentially isotropic paste of cellular debris that remains still contains 25 % of the total extractable fucose.

On the other hand, it is well known that fucosidic linkages are very sensitive to acid-hydrolysis, and certain that the conditions required for extraction of fucoidan in appreciable yield must bring about acid-degradation. It was also known from the earlier work^{11,13,16} that some of the fucose-containing

polysaccharides, after isolation, contain firmly bound protein and polyuronide material. It was therefore suspected that degradation of the polymeric matrix of the alga was an essential part of the extractive process.

On this hypothesis, the data in Table 1 are more readily explained. In the initial extraction with acid, the bulk of the alginate is retained in the algal particles as insoluble alginic acid, while fucose-containing fragments that are split out from the polymer matrix by acid-hydrolysis are extracted, together with the small amount of similar material that is present in the free state.

Upon neutralisation of the residual algal particles to pH 7, the alginate is extracted as its soluble sodium salt, together with any fucose-containing chains that are still linked to sufficient polyuronide to have been insoluble in acid, at least in the presence of adventitious alginate. Under the conditions used (see Experimental, and also Ref. 11), this extraction at pH 7 is known to be accompanied by free-radical depolymerisation, brought about by autoxidation of the phenols that are present in the alga.^{17,18} The fucose-containing materials, ascophyllan, F₁ and F₂ described earlier¹¹ were isolated by acidification of such an extract of *Ascophyllum nodosum*, which precipitated the alginate, leaving them in solution.

The principles underlying the remainder of the extractions shown in Table 1 are less easily envisaged, but it can be supposed that they also include degradation. An increase in temperature at pH 7 would accelerate free-radical depolymerisation, and, at 100°, β -alkoxycarbonyl elimination in any polyuronide chains containing esterified carboxyl functions would occur at a significant rate.^{19,20} An increase in pH to 13 would then lead to β -alkoxycarbonyl elimination in unesterified polyuronide chains,^{11,20} cause significant cleavage of peptide bonds, and increase the solubility of polypeptide fragments that are linked to the polysaccharides.

Confirmation of these ideas required the isolation of some soluble intermediate in the degradative process, whose further degradation could be conveniently studied by physical methods. Such an intermediate (A) was readily found in very mildly acidic extracts of *Ascophyllum*, and a further examination of the component F₂ described earlier¹¹ showed that it was closely similar to A. Both materials exhibited slight heterogeneity upon electrophoresis at pH 7 (Figs. 1A and 4), but gave single peaks with sharpened boundaries at pH 2 (Fig. 1A and Ref. 11).

Although both A and F₂ contained the monomeric building units of ascophyllan and alginate (together with galactose and additional amounts of fucose and sulphate), no trace of these polymers was found in either by electrophoresis at pH 2 (Fig. 1 and Ref. 11). In the case of F₂, the total absence of free ascophyllan or alginate was further assured by the fact that it had been clearly separated from ascophyllan by fractional precipitation, and freed from alginate by digestion with alginase.¹¹

In studying the effect of further acid-degradation on A and F₂, it was convenient to hasten the process by raising the temperature. However, on the basis of the temperature-coefficient of reaction that is usually assumed for hydrolysis of polysaccharides (10 % per degree), the conditions chosen (0.02 N acid at 80° for periods of up to 60 min) were expected to show the changes occurring during routine preparations of fucoidan.^{2,15}

Upon degradation of A under these conditions, the electrophoretic pattern rapidly broadened, and became distinctly trimodal, at both pH 2 and pH 7 (Fig. 1B). The mobilities of two of the liberated components corresponded exactly to those of ascophyllan and alginate, respectively, while the mobility of the third component was slightly higher than that of the starting material (Fig. 1). In this connection, it should be mentioned that alginate is not necessarily insoluble at pH 2, and that the pH at which it becomes insoluble depends upon the arrangement of mannuronic- and guluronic-acid residues in the chains.^{21,22} A fraction of the alginate in *Ascophyllum* is known to be freely soluble at pH 2 (Refs. 16, 22, 23).

Separation of these components was achieved by fractional precipitation with ethanol in the presence of magnesium chloride (Fig. 2 and Table 3). By digestion with alginase,¹⁶ the slowest-moving component at pH 2 was identified as alginate (Fig. 3). The component of intermediate mobility was identified as ascophyllan by its analytical composition (Table 4), and the characteristic pattern of mono- and oligo-saccharides liberated from it by partial acid-hydrolysis.¹¹ The fastest-moving component was electrophoretically homogeneous at pH 2, but separated out as two components at pH 7 (Fig. 3). A separation of the two components on a preparative scale was not achieved, but the material contained fucose, galactose, and sulphate as the only detectable constituents, and its composition (Table 4) allowed it to be classified as fucoidan. Galactose residues have previously been reported as constituents of materials regarded as typical fucoidans.²

It was therefore concluded that A consisted of ascophyllan, a polyuronide related to alginate, and fucoidan, chemically linked together. The ascophyllan also contains firmly bound protein;¹¹ and traces of mannose residues not previously reported¹¹ were also found to be associated with the samples of ascophyllan isolated in the present work.

The component F₂, isolated from the alginate extract, had an electrophoretic mobility and an analytical composition similar to A. It should be mentioned, however, that preparations of F₂ show some variation in composition, and the one isolated in the present work had a higher carboxyl content than that described previously.¹¹ The electrophoretic boundaries obtained after mild acid-hydrolysis and subsequent fractionation with ethanol-magnesium chloride (Fig. 4), together with the analytical data for the fractions (Table 5), leave no doubt as to the close similarity of this material with A. The principal difference between the two materials lies in the galactose content of the fractions that were soluble in the magnesium chloride-ethanol mixture.

As pointed out earlier, F₂ is freely soluble in acid after isolation, and the reason for its retention by the alga during the extraction that removed A, and its subsequent appearance in the alginate extract, is most probably that further degradation of the polymer matrix of the alga was needed to liberate it. It seems a reasonable extrapolation to suppose that the fucose residues that are retained in the insoluble residue, after the extraction of alginate and the accompanying fucose-containing polysaccharides, are present in structural elements similar to those that comprise A and F₂, namely, ascophyllan and fucoidan.

Although it was quite clear that neither A nor F₂ contained significant amounts of free ascophyllan or alginate prior to their degradation by acid, neither fraction was compositionally homogeneous. An attempt was therefore made to determine whether A contained any free fucoidan, by fractionating it, before hydrolysis, with ethanol-magnesium chloride under conditions that were known to separate fucoidan in the degraded material. It was found (Table 8) that 8.7 % of the total carbohydrate did indeed appear in the appropriate fraction, and its essential identity with the fucoidan liberated from the remainder of the material in A by acid-hydrolysis was demonstrated by the experiment illustrated in Fig. 6.

Since the amount of material in A that could be regarded as fucoidan was so small, and the preparation of A itself contained less than half of the total fucose in the alga, the presence of free fucoidan in *Ascophyllum* is doubtful. The small amount of fucoidan in A could clearly have been liberated during its isolation.

Since it was not known to what extent *Ascophyllum nodosum* can be regarded as typical of the *Fucaceae* generally, similar experiments (Fig. 5) were carried out on *Fucus vesiculosus*, which is accepted to be an excellent source of fucoidan. With this species, a similar acid-extract contained about 50 % of material that could be described as fucoidan by the same criterion (Tables 7 and 8). Although it is not known to what extent degradation could have occurred during drying and storage of the alga, it must be accepted that substantial differences between species very probably do exist, and that some probably do contain free fucoidan, where this term is understood to refer to a fucan sulphate that contains variable amounts of galactose residues.

The remaining 50 % of the acid-extract of *Fucus vesiculosus* contains its fucose in the form of a complex similar to A and F₂ (Fig. 5), and it is reasonable to assume that the fucoidan not extracted by acid was also present in a combined state. Even in this species, therefore, most of the fucoidan is very probably linked to the polymer matrix as in *Ascophyllum*. Previous reports by Black,⁸ Percival and Ross,⁷ Quillet,¹⁰ and Schweiger²⁴ suggest that residues of xylose are widely distributed in the brown algae. It is therefore tempting to suggest that similar complexes are of fairly general occurrence, at least in the *Fucaceae*.

Some comment is finally necessary about the possible nature of the linkages between the separate building units in complexes such as A and F₂. At first sight, it might be supposed that these linkages are very unstable to acid, even in comparison with fucosidic linkages. This, however, is unlikely. The conditions that are required to break down A into its separate building units are those that are required to cleave fucosidic linkages. This is clearly shown by the liberation of free fucose and dialysable oligosaccharides during that process. If the linkages that bind ascophyllan, alginate, and fucoidan together in the native state were very much weaker than fucosidic linkages, it would have been impossible to isolate A in the way described.

If this is accepted, it may be questioned why the complex breaks down in such an apparently specific way. For example, it has been shown¹¹ that ascophyllan consists of a polyuronide backbone, containing mainly glucuronic-acid residues, to which heteropolymeric side-chains, composed of sulphated

fuco- and xylose residues, appear to be glycosidically attached. Despite this, neither a polyglucuronic acid, nor a sulphated xylofucan, appeared as a separate entity in the reaction mixture by the time that the complex had been degraded sufficiently to liberate alginate and fucoidan.

We suggest that the answer must be sought, not in specific cleavage, but in the way in which the building elements are arranged in the macromolecule. In ascophyllan, there is reason to believe that the fucoxylian side-chains are fairly short, and that a number of separate side-chains is attached to every glucuronan backbone.¹¹ This would mean that more fucosidic (and xylosidic) linkages would have to be cleaved in this moiety, than for example in a singly branched molecule, in order to broaden the composition-distribution sufficiently for the separate parts to be detected as discrete entities by a given physical method. For the more simple, but closely analogous case of linear, block copolymers, this kind of phenomenon has been investigated in some detail, both theoretically^{25,26} and experimentally.²⁷ Briefly stated, we propose that the complex cleaves as it does, not because the bonds between the different elements are weaker than fucosidic linkages, but because they are few in number.

EXPERIMENTAL

All experiments were carried out with dried and milled samples of *Ascophyllum nodosum*, harvested at Være, March 2, 1959, and *Fucus vesiculosus*, harvested at Røberg, May 5, 1958. Methods of analysis, paper chromatography and free-boundary electrophoresis have been published previously.¹¹ Electrophoresis at pH 7 was carried out in a M/15 phosphate buffer.

Distribution of fuco-. The seaweed sample (5 g) was extracted overnight in a mechanical shaker with 0.2 N HCl (250 ml). The extract was filtered off, neutralised and analysed. The residue was then stirred with water (250 ml) and N sodium hydroxide was added very slowly to keep the solution at a pH between 6 and 7. The extraction was allowed to proceed overnight in the shaker. After filtration the residue was extracted once more for 24 h with distilled water (250 ml), and the two extracts were analysed separately. The residue was again extracted by stirring with water (250 ml) at 100° for 2 h, and finally with 0.1 N sodium hydroxide (250 ml) overnight at room temperature. The latter extract was neutralised with 1 N hydrochloric acid prior to analysis.

Extraction at different pH-values. Prior to extraction, the seaweed samples (5 g) were treated overnight with aqueous formaldehyde solution (2 %, 25 ml). They were then suspended in distilled water (250 ml), adjusted with hydrochloric acid (0.1 N) to the desired pH, and extracted overnight in a mechanical shaker. The extract was filtered off and neutralised immediately, and the residue extracted for another 5 h with distilled water. The extracts were pooled, dialysed thoroughly against distilled water, and lyophilised.

Isolation of polysaccharide. The seaweed sample (40 g) was stirred to a paste with aqueous formaldehyde (2 %, 200 ml) and left overnight in a tightly closed 5 l flask. Dilute hydrochloric acid (0.2 N, 2 l) was then added and the mixture stirred for 3 h at room temperature. The extract was immediately neutralised with 2 N sodium hydroxide and the residue extracted overnight with distilled water (2 l). The two extracts were pooled, dialysed thoroughly against distilled water, and lyophilised. In some experiments the formaldehyde-treated sample was extracted with water prior to the acid extraction described above. The two extracts were then freeze-dried separately.

The detailed procedure for extraction and purification of component F₂ has been described previously.¹¹

Degradation and fractionation. The isolated polysaccharide was dissolved in distilled water to give approximately a 1 % solution. To nine parts of this solution was added one part 0.2 N hydrochloric acid and the solution treated in a water bath at 80°C for

60 min. After cooling in tap water, the solution was neutralised with 1 N sodium hydroxide and dialysed against distilled water. After analysis, the solution was evaporated under diminished pressure to give a final concentration of 1 % with respect to carbohydrate. Fractional precipitation was carried out by mixing equal volumes of the concentrated solution and aqueous magnesium chloride (0.1 M) and adding ethanol to 50 % concentration. The precipitate (Mg_I) was removed by centrifugation and redissolved in distilled water. Both fractions were dialysed against distilled water and lyophilised. The soluble fraction (Mg_S) obtained from *Ascophyllum* by this procedure (Table 4) had $[\alpha]_D^{24} = -144^\circ$ ($c=1.0$, water). Omitting the acid-treatment, the same procedure was used for direct fractionation of the extracted material (Tables 7 and 8).

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