ACTA CHEMICA SCANDINAVICA 20 (1966) 219–230

Sulphated Polysaccharides in Brown Algae

I. Isolation and Preliminary Characterisation of Three Sulphated Polysaccharides from *Ascophyllum nodosum* (L.) Le Jol.

BJØRN LARSEN, ARNE HAUG and T. J. PAINTER*

Norwegian Institute of Seaweed Research, Trondheim — N.T.H., Norway

Fractionation of extracts of *Ascophyllum nodosum* yielded three new, electrophoretically distinct polysaccharides in addition to alginic acid. They all contained, in different proportions, residues of L-fucose, D-xylose, hexuronic (mainly D-glucuronic) acids, sulphate half-ester groups, and a firmly bound polypeptide moiety.

Mild acid-hydrolysis of the major component of the group (ascophyllan) yielded a sulphate-free, acid-resistant polyuronide, a separate, non-dialysable polypeptide, and a mixture of free and sulphated mono- and oligo-saccharides based upon fucose and xylose. Alkaline treatment of ascophyllan afforded a degraded polysaccharide which, upon mild acid-hydrolysis, liberated dialysable hexuronic acid and peptide material.

This and other evidence suggests that ascophyllan contains a polyuronide backbone, to which side-chains, composed of fucose, xylose, and half-ester sulphate, are attached glycosidically. The polypeptide moiety appears to be attached, in a manner as yet undetermined, to the side-chains. The other two sulphated polysaccharides appear to have a similar type of structure.

When alginic acid is precipitated from extracts of *Ascophyllum nodosum* by acidification, considerable amounts of polysaccharidic material remain in solution.[1,2] Electrophoresis of this material has shown the presence of a new polysaccharide, named "ascophyllan", together with smaller amounts of residual alginic acid, and substances resembling fucoidan. Studies of the electrophoretic mobility of ascophyllan as a function of pH have indicated the presence of both carboxyl and sulphate half-ester groups in the molecule, and acid-hydrolysis has been found to liberate fucose, xylose, hexuronic acids, and sulphuric acid from the polysaccharide.[1,2]

The preparation of an electrophoretically pure specimen of ascophyllan is now described, together with a preliminary examination of its general structural

* Present address: University of London, Royal Free Hospital School of Medicine, London W.C.1, England.

features. Two other polysaccharides, which comprise the material referred to earlier [1,2] as "fucoidan", are also described, and are shown to be more closely related to ascophyllan than to the sulphate fucan that is commonly regarded as fucoidan.[3]

The dried alga was pre-extracted with cold, dilute hydrochloric acid to remove inorganic cations, and then aqueous sodium hydroxide, just sufficient to neutralise the acidic polysaccharides, was added to a suspension of the residue in water. The resultant viscous mixture was filtered, and the filtrate acidified to precipitate the alginic acid. When the supernatant solution was subjected to electrophoresis at pH 2, the ascending boundary pattern shown in Fig. 1 was obtained. The stationary boundary is marked with the letter δ. Peak 1 was identified as the residual alginic acid, and peak 2 is ascophyllan; peaks 3 and 4 comprise the material described earlier [1,2] as "fucoidan", and are hereinafter referred to as F_1 and F_2, respectively.

Fig. 1. Ascending pattern at pH 2 of the acid soluble fraction.

Fig. 2. Ascending pattern at pH 2 of the fraction precipitated with alcohol in the presence of 0.167 M sodium chloride.

The crude mixture of acid-soluble polysaccharides was incubated at pH 7.5 with alginase [4] to degrade the residual alginic acid, and the degradation-products were removed by dialysis. Addition of one volume of ethanol to a solution (1 % w/v) of the indiffusible residue in 0.167 M sodium chloride then yielded a precipitate which, after purification by reprecipitation, was examined by electrophoresis at pH 2 (Fig. 2). Fig. 3 shows the electrophoretic pattern given by the material that remained in solution. It was clear that an almost complete separation of ascophyllan from F_1 and F_2 had been achieved.

Fig. 3. Ascending pattern at pH 2 of the fraction soluble in alcohol in the presence of 0.167 M sodium chloride.

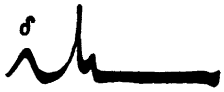

Fig. 4. Ascending pattern at pH 2 of the fraction precipitated with alcohol in the presence of 0.0067 M calcium chloride.

Separation of F_1 from F_2 was accomplished by addition of one volume of ethanol to a solution (1 % w/v) of the mixture in 0.0067 M calcium chloride. The precipitated material was reconverted into its sodium salt with an ion-exchange resin, and after reprecipitation in the same way, it gave, at pH 2, the electrophoretic pattern shown in Fig. 4. The material that was not precipitated under these conditions was converted into its sodium salt, dissolved in 0.167 M potassium chloride at a concentration of 1 % (w/v), and treated

with one volume of ethanol. The electrophoretic pattern given by the precipitated material at pH 2 is shown in Fig. 5. As the figures show, the material precipitated in the presence of calcium ions was greatly enriched in F_1, whereas that precipitated in the presence of potassium chloride consisted of pure F_2.

Fig. 5. Ascending pattern at pH 2 of component precipitated with ethanol in the presence of 0.167 M potassium chloride.

Experiments were next conducted to determine whether ascophyllan, F_1 and F_2 could be detected in extracts of the alga prepared under very mild conditions, since it was desirable to ascertain whether these polysaccharides exist as such in the native state, or are merely fragments of a larger molecule, produced by degradation during drying, storage, or extraction of the alga.

A sample of freshly-harvested alga was macerated with cold (−10°) acetone, and dried under nitrogen. It was then macerated in an atmosphere of carbon dioxide, first with ice-cold water containing catalase [5] and then with ice-cold aqueous ammonium oxalate at pH 6. The highly viscous extract was centrifuged, and after dialysis against aqueous sodium acetate at 4°, three portions of the centrifugate were treated separately by the following methods for removal of alginate: (*a*) digestion with alginase, followed by dialysis; (*b*) adjustment of the pH to 2, followed by centrifugation; and (*c*) addition of excess calcium chloride, and removal of the precipitated calcium alginate by centrifugation. Electrophoresis of the residual polysaccharides at pH 6 showed in each case the presence of ascophyllan, together with a faster-moving peak, corresponding to F_1 and F_2 (which do not separate at this pH). Electrophoresis at pH 2 gave in each case a pattern similar to that shown in Fig. 1, except that no residual alginate was detected. It was concluded that ascophyllan, F_1, and F_2 either exist as such in the living alga, or form part of an extremely labile complex. For practical purposes, it was clear that the three polysaccharides should be studied separately.

Qualitatively, the composition of the three fractions described above was identical. Paper chromatography and electrophoresis [6] of acid-hydrolysates

Table 1. Composition (%) of ascophyllan and F_2.

	Fucose	Xylose	Sodium hexuronate (1) [b]	Sodium hexuronate (2) [c]	SO_3Na (1) [d]	SO_3Na (2) [e]	Protein [a]
Ascophyllan	25.3	26.0	19.2	27.1	11.9	12.9	11.8
F_2	38.6	6.3	3.3	4.5	29.6	26.9	22.6

[a] Determined with ninhydrin [9] and calculated as gelatin.
[b] Determined with carbazole [14] and calculated as glucuronic acid.
[c] Determined by titration with cetylpyridinium chloride.[15]
[d] Determined with barium chloranilate [7] after acid-hydrolysis.
[e] Determined by titration with cetylpyridinium chloride.[15]

of the materials indicated in each case the presence of fucose, xylose, and hexuronic (mainly glucuronic) acids, and sulphate was detected in the hydrolysates with barium chloranilate.[7] Tests for ester phosphate, hexosamines, and sialic acids were negative.

The presence of a non-carbohydrate moiety in the fractions was indicated by their strong absorbance at 280 mμ, and their positive reaction with the Folin-Ciocalteau reagents.[8] Moreover, the fractions had a brown colour which could not be removed by physical methods, and which could be observed to migrate with the main peaks in the electrophoresis apparatus. That this moiety was of a polypeptide nature was suggested by its positive reaction with ninhydrin.[9] The brown colour was thought to arise from the condensation of the polypeptide with adventitious phenols during extraction, since fractions prepared in the same way after treatment of the alga with formaldehyde [10,11] were colourless.

Tentative evidence for the existence of a close structural relationship between the three fractions was obtained by controlled autohydrolysis,[12] followed by paper chromatography of the products in solvents [12,13] capable of separating, and distinguishing between, free and sulphated mono- and oligosaccharides. An identical pattern of numerous spots, which comprised a well-defined "fingerprint", was obtained from each polysaccharide; differences were observed only in the relative sizes of the individual spots. Work on the separation and identification of these fragments is now in progress.

Quantitative analytical figures for the two electrophoretically homogeneous fractions, ascophyllan and F_2, are given in Table 1. Component F_1 has not yet been obtained pure, but it appears to have a composition intermediate between that of ascophyllan and F_2.

Sulphate was determined by two different methods; in one, the total inorganic sulphate liberated by hydrolysis with hydrochloric acid was determined with barium chloranilate,[7] whereas in the other, the intact polysaccharide was titrated with cetylpyridinium chloride, in the presence of sufficient acid to suppress the ionisation of the carboxyl groups.[15] The fair agreement between the results indicates that all or most of the sulphate is present as half-ester; however, the experimental error is such that it is impossible to rule out the presence of a small proportion of sulphate diester linkages in the polysaccharides.

Hexuronic acid was determined by titration of the intact polysaccharide with cetylpyridinium chloride at pH 7, and subtraction of the titre due to sulphate half-ester groups alone.[15] In this method, a slightly higher result was obtained if the polysaccharide was pre-treated with 0.01 N sodium hydroxide at 60° for 10 min, in order to saponify any carboxylic ester or lactone linkages, and the figures in Table 1 were obtained in this way. However, the figures may be too high, due to the presence of carboxyl groups in the polypeptide moiety. Hexuronic acid was also determined by a modification [14] of the Dische carbazole method; the results, which are expressed as glucuronic acid, may be too low, since a small amount of mannuronic acid, which gives a much lower extinction with the reagents,[16] was also detected in acid-hydrolysates of the polysaccharides.

Protein was determined by application of the ninhydrin method of Moore and Stein [9] to the unhydrolysed samples, with gelatin as a standard. In the case of ascophyllan, it was also determined, with the same reagent, after hydrolysis in boiling 6 N hydrochloric acid for 24 h; by use of gelatin, hydrolysed in the same way, as a standard, a figure of 10.8 % was obtained for the polypeptide content of the polysaccharide. Hydrolysis brought about a 33-fold increase in the optical density given by ascophyllan with the reagent, and a 35-fold increase in that given by gelatin. This established the approximate validity of the choice of gelatin as a standard, and provided additional evidence that the non-carbohydrate material in the polysaccharide was of a polypeptide character.

Ascophyllan was chosen for more detailed investigation, since this was the fraction obtained in highest yield, and was most likely to yield information about the role of the hexuronic acid residues in this type of polysaccharide. L-Fucose and D-xylose were identified as components of an acid-hydrolysate of the material by separation on a carbon column and isolation of the crystalline sugars. D-Glucuronic acid was identified by paper electrophoresis,[6] ion-exchange chromatography,[17] and paper chromatography of the free acid and its lactone. The presence of a polypeptide moiety was confirmed by complete hydrolysis in 6 N hydrochloric acid, followed by two-dimensional thin-layer chromatography: the characteristic pattern of spots given by the 18 common amino-acids was obtained.

The existence of a chemical linkage between the carbohydrate and polypeptide moieties of ascophyllan was suggested by the following evidence. The polypeptide content of the material was unchanged by repeated extraction with 90 % (w/w) phenol, and no precipitate was formed on addition of trichloroacetic acid, phosphomolybdic acid, sodium tungstate, or mercuric chloride to aqueous solutions of the polysaccharide. No change in the polypeptide content was observed in samples reprecipitated with ethanol in the presence of either acid or alkali, and the material was electrophoretically homogeneous (apart from the trace of F_2 present) at all values of pH between 2 and 10.

Further evidence for the general architecture of the macromolecule was obtained by mild acid-hydrolysis. If, for example, a 1 % w/v solution of ascophyllan was treated with Dowex-50 (H^+ form) resin to remove the cations, and the resultant acidic solution (pH 2.05) was heated at 80° for 20 h, the polypeptide moiety precipitated from solution as a brown, insoluble residue. Analysis of the colourless supernatant solution now showed the absence of polypeptide, whereas its content of all the original sugars was virtually unchanged. The polypeptide material was essentially non-dialysable at all stages in the autohydrolysis prior to its precipitation; its low solubility, once liberated, was thought to be due to its association with condensed phenolic material.

When the low molecular-weight fragments that were liberated from the molecule by autohydrolysis were removed continuously from the reaction mixture by dialysis,[12] and then examined by analysis and chromatography,[12,13] it was found that they consisted almost entirely of free and sulphated mono- and oligosaccharides based upon fucose and xylose; the uronic acid-containing moiety, like the polypeptide moiety, remained essentially non-dialysable throughout the hydrolysis.

In another experiment, ascophyllan was hydrolysed in 0.5 N oxalic acid at 100°, and at intervals, samples of the reaction mixture were removed, neutralised, and dialysed against water. The non-dialysable material from each sample was analysed for fucose by the cysteine method,[18] and for uronic acid by the Dische carbazole method,[14] and the results were expressed as a percentage of the original weight of ascophyllan taken (Table 2). Under these conditions, the polypeptide moiety was precipitated after 2 h of hydrolysis.

When the soluble, non-dialysable material obtained at the end of the experiment was examined by electrophoresis at pH 2, a single peak, having a mobility much lower than that of the original ascophyllan, was observed (Fig. 6). This material, which contained almost all the uronic acid present in the original ascophyllan (Table 2), was virtually free from half-ester sulphate,

Table 2. Acid-hydrolysis of ascophyllan.

	Fucose	Uronic acid [a]
Whole ascophyllan	25.0	18.0
Non-dialysable fraction after		
0.25 h hydrolysis	11.2	17.1
0.4 h „	8.2	16.5
1 h „	5.9	
2 h „	4.2	16.7

[a] Determined with carbazole [14] and calculated as glucuronic acid.

and was shown by potentiometric titration to have a hexuronic acid content of 74 %. In Fig. 7, its electrophoretic mobility at different values of pH is compared with that of the original ascophyllan, and is shown to be lower throughout; this clearly indicates its lower charge density, following removal of the sulphate half-ester groups, and the much sharper rise in its mobility, as the pH increases from 2 to 6, is a behaviour to be expected from a simple polyuronide.

Fig. 6. Ascending pattern at pH 2 of the acid-resistant fraction of ascophyllan.

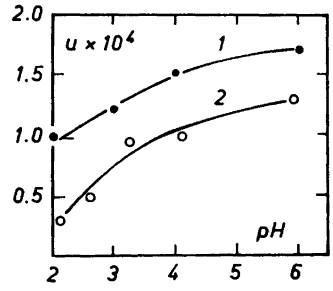

Fig. 7. Mobility of ascophyllan (1) and non-dialysable fraction (2) as a function of pH.

The non-dialysable material present in the reaction mixture at intermediate stages in the hydrolysis of ascophyllan was also examined electrophoretically at pH 2. As hydrolysis progressed, the single peak given by the starting material (Fig. 2) decreased in mobility until, just before precipitation of the polypeptide, it divided into two peaks, both of which migrated very slowly towards the anode. However, after precipitation of the polypeptide, which occurred very suddenly, the material remaining in solution gave only one peak, as shown in Fig. 6.

The behaviour of the polysaccharide upon degradation with alkali was also studied. In one experiment, ascophyllan (100 mg) was dissolved in N-sodium hydroxide (4 ml), and potassium borohydride (100 mg) was added to minimise degradation by the alkaline peeling reaction.[19] The solution was divided into two equal parts, which were heated at 80° for 15 and 40 h, respectively. Each portion was then neutralised and analysed, both before and after exhaustive dialysis against water. The results are shown in Table 3; the figures are percentages, based upon the weight of the original ascophyllan.

Table 3. Alkaline degradation of ascophyllan.

	Fucose	Uronic acid	SO_3Na [a]	Protein [b]
Whole ascophyllan	25.4	18.2	12.9	13.8
Alkali-treatment, 15 h				
Recovered	26.7	12.1	12.0	8.0
Non-dialysable	23.9	9.0	12.0	2.4
Alkali-treatment, 40 h				
Recovered	18.4	11.9		6.0
Non-dialysable	16.0	9.2		2.2

[a] Determined with cetylpyridinium chloride.[15]
[b] Determined by the Folin-Ciocalteau method,[8] and calculated as ovalbumin.

Additional analyses were carried out to ascertain the chemical nature of the changes brought about by the alkaline treatments. The polypeptide moiety was expected to suffer alkaline hydrolysis, and accordingly, the 15-h and 40-h samples showed, respectively, a 4.45-fold and a 3.49-fold increase in the optical density given with the ninhydrin reagent.[9] However, the true extent of alkaline hydrolysis was probably much greater than these figures indicate, due to the concomitant destruction of the liberated amino-acids. Of the ninhydrin-positive material present in the two samples, 85.7 % and 87.5 %, respectively, was dialysable.

Owing to the presence of borohydride, the polysaccharide part of the molecule was not expected to undergo appreciable degradation by the alkaline peeling reaction,[19] and it is noteworthy that the portion containing fucose and sulphate was still virtually intact and non-dialysable after 15 h. The loss of fucose observed after 40 h may be due to the elimination of sulphate, with formation of anhydro-derivatives of fucose. On the other hand, the relatively rapid degradation shown by the polyuronide moiety, with the liberation of

dialysable uronic acid, suggested that cleavage of internal glycosidic linkages in the chains had occurred. This can be expected to take place, by the β-alkoxycarbonyl elimination mechanism, if the hexuronic acid residues are linked glycosidically through positions 1 and 4.[19,21] The products would then contain 4,5-unsaturated derivatives of hexuronic acids, which can be detected, after periodate oxidation, with thiobarbituric acid.[20,21] Analysis of the 15-h sample in this way confirmed that this type of degradation had occurred, and showed that 84.6 % of the total unsaturated uronic acid formed was dialysable.

Electrophoresis of the non-dialysable material obtained from the 15-h sample showed, at pH 2, a single, very broad peak, with an average mobility intermediate between that of intact ascophyllan and that of the polyuronide obtained by acid-hydrolysis. At pH 6, a single peak was also observed, but it was more compact, showing that at this pH the material was less highly polydisperse with respect to charge density. Upon hydrolysis in 0.02 N hydrochloric acid at 80°, this material liberated substantial amounts of dialysable uronic acid, as shown in Table 4.

Table 4. Liberation of dialysable uronic acid from alkali-treated ascophyllan by hydrolysis with acid (0.02 N HCl, 80°).

Time (h)	0	2	5	16
Dialysable (%)	0	24.4	38.4	38.6

A solution (*ca.* 1 % w/v) of the non-dialysable material from the 40-h sample was converted into its acidic form with Dowex-50 resin, and heated at 80° for 20 h, as described earlier for intact ascophyllan. The solution was then dialysed against water, and the dialysable and non-dialysable materials were analysed by the ninhydrin method. The results indicated that all the residual peptide material in the alkali-treated ascophyllan was now freely dialysable.

Finally, the degradation of ascophyllan under very severe alkaline conditions was studied. A solution of ascophyllan (17 mg) and potassium borohydride (40 mg) in 5 N sodium hydroxide (1 ml) was allowed to stand overnight at 20°, and then heated at 80° for 48 h. After neutralisation, the products were analysed, both before and after dialysis against water. The results showed that 75 % of the original uronic acid was destroyed, and that the remainder was completely dialysable. On the other hand, 57 % of the original fucose was destroyed, and, of the remainder, 29 % was still non-dialysable.

DISCUSSION

The foregoing results establish the presence in *Ascophyllum nodosum* of three hitherto unrecognised polysaccharides, in addition to alginic acid. They appear to be separate constituents of the living alga, rather than artefacts formed during extraction. All three are very similarly constituted, but there are marked differences in their quantitative composition. The differences in their relative contents of hexuronic acid and sulphate half-ester groups allowed them to be readily differentiated by electrophoresis at pH 2. The three poly-

saccharides all contain a firmly bound polypeptide moiety, and to our knowledge, this is the first occasion upon which the existence of such polysaccharide-protein complexes in a marine alga has been clearly recognised.

The major component of the group, ascophyllan, contains three well-defined moieties, namely, a high-molecular weight polyuronide, a high-molecular weight polypeptide, and a moiety composed of fucose, xylose, and half-ester sulphate. It is likely that the other two polysaccharides, F_1 and F_2, are also built in this way, but this is not yet fully established.

The electrophoretic homogeneity of ascophyllan at all values of pH, together with a variety of other circumstantial evidence, strongly suggests that its three parts are covalently linked together, in a single, complex macromolecule. Perhaps the most conclusive evidence for this is provided by the experiment in which alkali-treated ascophyllan is shown to liberate dialysable hexuronic acid and peptides when hydrolysed with acid under conditions that do not, with the intact polysaccharide, bring about appreciable cleavage of hexuronosidic or peptide linkages. It is established that the alkaline treatment cleaves internal linkages in the polyuronide and polypeptide chains, and therefore the alkali-degraded polysaccharide should contain short segments of these chains, which are non-dialysable as a consequence of some chemical linkage with the relatively intact fucose-containing moiety. Subsequent breakdown of the fucose-containing moiety by mild acid-hydrolysis can then be expected to liberate these segments as dialysable material.

Since the polyuronide obtained by mild acid-hydrolysis still contains some 17 % of the fucose present in the original ascophyllan (Table 2), but is essentially free from polypeptide and sulphate, it is very probable that the linkage between the fucose-containing moiety and the polyuronide is glycosidic. This would also account for the stability of the linkage to alkali. It would follow that all or part of the fucose residues in ascophyllan are present in side-chains, attached to a polyuronide backbone. Probably there are several side-chains, because it is unlikely that a single one could render such a large part of the alkali-fragmented polyuronide non-dialysable (Table 4). However, at least some of the side-chains must be quite long, since after very severe alkaline treatment, a small part of the fucose in ascophyllan is obtained as non-dialysable material free from uronic acids. In another study (to be published), it is shown that a disaccharide containing one fucose residue and one xylose residue is obtained in quite high yield by mild acid-hydrolysis of ascophyllan; it follows, therefore, that the xylose residues are glycosidically linked to fucose residues in the side-chains, and are not present in separate side-chains.

The nature of the carbohydrate-peptide linkage is not yet known. It appears that there is more than one type of linkage, since some polypeptide can be removed from the molecule by very mild alkaline treatment, whereas the remainder is much more firmly bound. The fact that the polypeptide liberated by mild acid-hydrolysis of ascophyllan migrates, just before it becomes insoluble, towards the anode upon electrophoresis at pH 2, suggests that at this stage it is still linked to part of the sulphated fucose-containing moiety, but not to the polyuronide, which travels as a separate peak. The polypeptide is therefore probably linked to the rest of the molecule through the fucose-containing moiety. Further support for this idea is provided by the analytical

figures for F_2 (Table 1); this polysaccharide contains twice as much polypeptide material as ascophyllan, but very little uronic acid.

In the light of the present results, some comment is required upon the meaning of the term "fucoidan". It has been known for 50 years that fucose-containing polysaccharides are present in many brown algae,[22] and on the basis of detailed structural work,[3] carried out on a sulphated fucan obtained from a single species *(Fucus vesiculosus)*, it has become widely accepted that the fucose residues in other species are present as homopolymers of the same type. Although residues of xylose and uronic acids have previously been detected in numerous preparations of "fucoidan", they have hitherto been attributed to the presence of impurities.[23] More recently, however, Quillet [24] has challenged this view, and on the basis of an examination of a wide range of brown algae, has claimed that xylose and a number of other sugars besides fucose must be regarded as normal structural units of "fucoidans". In the present work, no evidence for the presence in *Ascophyllum nodosum* of any fucan other than the three heteropolymers just described was obtained. It is therefore possible that the other algal species studied by Quillet contain their fucose in the form of heteropolymers more closely related to ascophyllan, F_1, and F_2 than to the fucan of *Fucus vesiculosus*. A semantic problem thus arises; the term "fucoidan" should either be reserved for the sulphated fucan that is present in *Fucus vesiculosus*, and quite probably also in other species,[23] or it should be used as a general term for all fucose-containing polysaccharides from brown algae, or it should be discarded completely.

EXPERIMENTAL

Paper chromatography was carried out by the ascending method, on Schleicher and Schüll No. 2043 b paper. The following solvent systems were used: (*a*) acetic acid-ethyl acetate-water (1:2:3 v/v) for separation of fucose and xylose; (*b*) pyridine-ethyl acetate-water (2:5:7 v/v) for uronic acid lactones; (*c*) pyridine-ethyl acetate-acetic acid-water (5:5:1:3 v/v) for uronic acids; (*d*) butan-1-ol-acetic acid-water (50:12:25 v/v) for sulphated sugars; and (*e*) butan-1-ol-ethanol-water (3:1:1 v/v), containing cetylpyridinium chloride (3 % w/v),[13] for sulphated sugars. An aniline trichloro-acetate dipping reagent (2.5 % solution in glacial acetic acid) was used to locate the sugars.

Two-dimensional thin-layer chromatography was carried out on plates (20 × 20 cm) coated with silica gel (250 μ); they were irrigated first in phenol-water (3:1 w/w), and then in the other dimension with butan-1-ol-acetic acid-water (4:1:1 v/v). The amino-acids were located with ninhydrin (0.5 % w/v) in butan-1-ol.

Free boundary electrophoresis was carried out with a Perkin-Elmer Model 238 instrument, equipped with a standard analytical cell; the experimental conditions and buffer systems are described elsewhere.[3] All patterns and mobilities refer to the ascending boundary. In all experiments, the current (10 mA) was passed for 30 min.

Most of the analyses were carried out by the published procedures referred to in the main text. In addition, xylose was determined by the orcinol method, with a heating time of 40 min;[25] sialic acid by the resorcinol [26] and direct Ehrlich [27] methods; and hexosamine by application of the Elson-Morgan method [28] to samples hydrolysed in 0.5 N hydrochloric acid at 100° for 18 h. Analyses for phosphate and sulphate were carried out by modifications of the Fiske-Subbarow [29] and barium chloranilate [7] methods, respectively, after hydrolysis of the sample (5 mg) in N hydrochloric acid (1 ml) at 85° for 24 h, neutralisation (to pH 4) with sodium hydroxide, and dilution to 4 ml. In the method for protein analysis entailing acid-hydrolysis, ascophyllan (*ca.* 10 mg) was boiled under reflux for 24 h in 6 N hydrochloric acid (5 ml). The mixture was then cooled and neutral-

ised with N sodium hydroxide (*ca.* 30 ml), and the pH was adjusted to 5 by addition of sodium bicarbonate or citric acid. The volume of the solution was then made up to 50 ml. As a standard, gelatin (2 mg) was treated in an identical manner. For calibration of the ninhydrin reagent, dilutions of the standard were prepared with 0.6 M sodium chloride, which was also used in the reagent blank.

Preparation of ascophyllan, F_1 and F_2. A dried and milled sample of *Ascophyllum nodosum* (20 g; 85 % dry matter) was shaken overnight with 0.2 N hydrochloric acid (500 ml), collected by filtration, and extracted again in the same way for 4 h. The residue was then stirred with water (1 l), and N-sodium hydroxide was added very slowly until the solution was just alkaline (*ca.* 25 ml was required). The resultant viscous mixture was stirred at 20° for 20 h, then filtered. The residue was again extracted by stirring with water (1 l) at 100° for 2 h. The two extracts were combined and brought to pH 1.3—1.5 by addition of 0.2 N hydrochloric acid. The precipitated alginic acid was removed by filtration and the filtrate was neutralised, concentrated by evaporation under diminished pressure, and dialysed against water. The resultant solution was finally concentrated to a volume of 100 ml, and a portion (5 ml) of the concentrate was dialysed against the appropriate buffer [2] prior to examination by electrophoresis.

The remainder of the concentrate was mixed with 0.13 M phosphate buffer (pH 7.5; 50 ml), sodium chloride was added to give a concentration of 3 % w/v, and a suitable amount of alginase was added to the solution. After incubation at 30° for 24 h, the solution was dialysed exhaustively against water. The concentration of the solution of non-dialysable material obtained was adjusted to 0.7—1.0 % w/v, and to this solution (5 vols.) was added 1.0 M sodium chloride (1 vol.) and ethanol (6 vols). The precipitate was collected by centrifugation, redissolved in water to a concentration of 0.7—1.0 % w/v, and reprecipitated, after addition of 0.8 M sodium chloride (1 vol.), with ethanol (6 vols.); it was finally dissolved in water, dialysed, and freeze-dried, to yield 1.2 g of ascophyllan as a light brown solid. The mobility of this material upon electrophoresis at pH 2 was 1.13×10^{-4} cm²/sec volt.

The supernatant solution remaining after precipitation of ascophyllan was concentrated, dialysed against water, and concentrated again to 0.7—1.0 % w/v of total carbohydrate. To this solution (5 vols.) was added 0.04 M calcium chloride (1 vol.) and ethanol (6 vols.). The precipitate was dissolved in water (25 ml), and the solution was passed through a column (1.4 × 20 cm) of Dowex-50 (H^+ form) cation-exchange resin. The acidic effluent, with washings, was just neutralised with sodium hydroxide, concentrated to 0.7—1.0 % w/v of total carbohydrate, and treated with calcium chloride and ethanol as before. The precipitated material was reconverted into its sodium salt, dialysed, and freeze-dried. The yield of brown solid was 0.3 g. This fraction was enriched in F_1.

The supernatant solution remaining after the first precipitation in the presence of calcium chloride was concentrated, dialysed against water, passed through a column of Dowex-50 (H^+ form) resin, neutralised with sodium hydroxide, and concentrated to give 0.7—1.0 % w/v of total carbohydrate, as before. To this solution (5 vols.) was added M-potassium chloride (1 vol.) and ethanol (6 vols.). The precipitate was dissolved in water, dialysed, and freeze-dried, to give 0.3 g of light brown solid (F_2).

Isolation of monosaccharides from ascophyllan. Ascophyllan (1.01 g) was heated at 100° in 0.5 N oxalic acid (40 ml) for 1 h. The hydrolysate was cooled and dialysed against water (4 × 300 ml). The dialysates were combined and neutralised with calcium carbonate, centrifuged, and concentrated to 10 ml. This solution was washed on to a column (25 × 2 cm) of carbon (Norit FQP): Celite (1:1 w/w), which was then eluted with a linear gradient (0—20 % v/v) of ethanol in water, the total volume of eluant being 1 l. The elution pattern was followed by paper chromatography, and fractions containing pure fucose and pure xylose, when pooled separately, afforded 170 mg and 100 mg, respectively, of the two sugars.

A sample (743 mg) of non-dialysable material, obtained in a hydrolysis experiment similar to that just described, was taken up in ice-cold sulphuric acid (80 % w/w; 5 ml) and kept at 20° overnight. The mixture was then diluted to give an acid-concentration of 2 N, heated in a sealed ampoule at 100° for 5 h, neutralised with calcium carbonate, and filtered. The filtrate, with washings, was concentrated to 5 ml, adjusted to pH 8 with N-sodium hydroxide, and kept at room temperature for 30 min. It was then applied to a column of Dowex-1 (× 8) anion-exchange resin, and eluted with a linear gradient of acetic acid in water as described earlier [17,30]. The elution pattern obtained indicated that

the main component of the hydrolysate was glucuronic acid, but small amounts of mannuronic acid,[17] were also detected. The fractions containing glucuronic acid were pooled and freeze-dried, to yield 40 mg of syrup.

The isolated sample of fucose had $[\alpha]_D = -74°$ (*c*, 0.3 in water). It crystallised from absolute ethanol, and had m.p. 143°, unchanged on admixture with authentic α-L-fucose.

The isolated sample of xylose had $[\alpha]_D = +15° \pm 2°$ (*c* 0.5 in water). A solution in absolute methanol deposited crystals, having m.p. 148°, undepressed on admixture with authentic α-D-xylose. The derived phenylosazone had m.p. and mixed m.p. 166°, and $[\alpha]_D = -28° \rightarrow -65°$ (24 h) (*c*, 0.4 in pyridine).

The identity of the sample of glucuronic acid was confirmed by paper electrophoresis,[6] and paper chromatography of an aqueous solution indicated the presence of both glucuronic acid and glucurone. After drying over phosphoric oxide, the syrup had $[\alpha]_D = +12$ (*c*, 2.3 in water).

One of us (T.J.P.) is much indebted to the *Royal Norwegian Council for Scientific and Industrial Research*, for a grant which made his contribution to the work possible.

REFERENCES

1. Larsen, B. and Haug, A. *Proc. 4th Intern. Seaweed Symp.*, Pergamon Press 1963, p. 338.
2. Larsen, B. and Haug, A. *Acta Chem. Scand.* **17** (1963) 1646, 1653.
3. Conchie, J. and Percival, E. G. V. *J. Chem. Soc.* **1950** 827; O'Neill, A. N. *J. Am. Chem. Soc.* **76** (1954) 5074; Cŏté, R. H. *J. Chem. Soc.* **1959** 2248.
4. Eimhjellen, K. E., Rosness, P. A. and Hegge, E. *Acta Chem. Scand.* **17** (1963) 901.
5. Smidsrød, O., Haug, A. and Larsen, B. *Acta Chem. Scand.* **17** (1963) 2628.
6. Haug, A. and Larsen, B. *Acta Chem. Scand.* **15** (1961) 1395.
7. Lloyd, A. G. *Biochem. J.* **72** (1959) 133.
8. Folin, O. and Ciocalteau, V. *J. Biol. Chem.* **73** (1927) 627.
9. Moore, S. and Stein, W. *J. Biol. Chem.* **211** (1954) 907.
10. Haug, A. and Larsen, B. *Report No. 22*, Norwegian Institute of Seaweed Research, Trondheim 1958.
11. Haug, A. *Report No. 30*, Norwegian Institute of Seaweed Research, Trondheim 1964.
12. Painter, T. J. *Chem. Ind. (London)* **1959** 1488.
13. Rees, D. A. *Nature* **185** (1960) 309.
14. Bitter, T. and Ewins, R. *Anal. Biochem.* **4** (1962) 330.
15. Scott, J. E. *Methods Biochem. Anal.* **8** (1960) 163.
16. Dische, Z. *Methods Carbohydrate Chem.* **1** (1962), Academic, p. 498.
17. Larsen, B. and Haug, A. *Acta Chem. Scand.* **15** (1961) 1397.
18. Dische, Z. *Methods Carbohydrate Chem.* **1** (1962), Academic, p. 501.
19. Whistler, R. L. and BeMiller, J. N. *Advan. Carbohydrate Chem.* **13** (1958) 289.
20. Weissbach, A. and Hurwitz, J. *J. Biol. Chem.* **234** (1959) 705.
21. Haug, A., Larsen, B. and Smidsrød, O. *Acta Chem. Scand.* **17** (1963) 1466.
22. Kylin, H. *Z. physiol. Chem.* **83** (1913) 171; **94** (1915) 357.
23. Bird, G. M. and Haas, P. *Biochem. J.* **25** (1931) 403. Percival, E. G. V. and Ross, A. G. *J. Chem. Soc.* **1950** 717.
24. Quillet, M. *Colloq. Intern. Centre Natl. Sci. (Paris)* 1961 No. 103,
25. Brown, A. H. *Arch. Biochem.* **11** (1946) 269.
26. Svennerholm, L. *Biochim. Biophys. Acta* **24** (1957) 604.
27. Werner, I. and Odin, L. *Acta Soc. Med. Upsalien.* **57** (1952) 230.
28. Rondle, C. J. M. and Morgan, W. T. J. *Biochem. J.* **61** (1955) 586.
29. Fiske, C. H. and Subbarow, Y. *J. Biol. Chem.* **66** (1925) 375.
30. Haug, A. and Larsen, B. *Acta Chem. Scand.* **16** (1962) 1908.

Received September 30, 1965.