ACTA CHEMICA SCANDINAVICA 17 (1963) 1653–1662

The Solubility of Alginate at Low pH

ARNE HAUG and BJØRN LARSEN

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

The solubility at low pH is significantly different for alginate fractions prepared from *Laminaria digitata*, *L. hyperborea* stipes and *Ascophyllum nodosum*. The solubility differences between the alginate fractions from the two former algae may be due to different uronic acid composition. The alginate fraction from *A. nodosum* has been shown to contain appreciable amounts of the polysaccharide ascophyllan, and the different solubility of alginate fractions of *L. digitata* and *A. nodosum* is supposed to be due to the presence of ascophyllan in the latter. The results indicate that ascophyllan may be bound to the alginate to some extent.

Analytical examination has revealed the presence of xylose, fucose, sulphate and glucuronic acid in hydrolysates of fractions containing ascophyllan.

During the course of an investigation of the alkaline extract of the brown alga *Ascophyllum nodosum*, the electrophoretic mobility of the components of the alginate fraction at low pH was determined [1,2]. It is well known that alginic acid is insoluble in water, and below pH 2.3 even a degraded product was only partly soluble. It was found desirable to carry out a further investigation of the solubility of alginates of different types at low pH and different values of intrinsic viscosity, and some results of this investigation are reported here.

The preparation of alginate involves usually a preextraction of the dried seaweed with dilute acid, extraction of the alginate with a sodium carbonate solution, and isolation of the alginate by precipitation with a suitable precipitating agent, *e.g.* ethanol, calcium salts or acids. While one of the two latter precipitating agents are used in industry, precipitation with ethanol is often conveniently used in the laboratory. In this work the fraction precipitated with ethanol is investigated, and, for the sake of convenience, designated as the alginate fraction.

EXPERIMENTAL

The preparation of the alginate fraction is described elsewhere [3].

The following three degradation methods were used: (1) Degradation with acid: Samples of alginate fractions (0.5 g) were treated with 5 ml 1.0 N HCl at various temperatures and periods of time. The acid was filtered off and the amounts of carbohydrates in

the acid determined by the phenol-sulphuric acid method [4]. The residue was dissolved in a 0.1 N NaCl solution with a slight excess of alkali to give a 1 % solution. (2) Degradation at pH 7: Alginate fractions were dissolved in phosphate buffer at pH 7 and heated to a temperature between 60 and 90°C. (3) Enzymic degradation: Extracellular alginase from a marine bacterium [5,6] was concentrated by adsorption on a calcium phosphate gel. Alginate fractions were dissolved in 0.045 M phosphate buffer at pH 7.5, NaCl added to give a 3 % solution, and a suitable amount of concentrated enzyme thoroughly mixed with the alginate solution. The mixture was kept at constant temperature (20 or 30°C). When the desired viscosity was obtained, the enzyme action was stopped by adding HCl in amounts sufficient to obtain a pH between 3 and 4.

The following three methods were used to investigate the solubility at low pH: (1) Extraction of the alginate fraction with HCl at pH 2. A 100 mg sample was extracted with 10 ml solution overnight and the amount of carbohydrate material in the extract determined by the phenol-sulphuric acid method. Standard curves were determined by using material isolated from acid extracts by precipitation with alcohol. (2) Dialysing a 1 % solution of alginate fraction against 0.01 M HCl containing 0.05 M NaCl. The outer liquid was changed at least four times and the proportion between the outer and inner volume was > 20. A cellulose dialysing tube was used. The pH of the solution inside the bag was measured in a Beckman Zeromatic pH meter. The mixture was centrifuged, and the amounts in the soluble and insoluble fraction determined by the phenol-sulphuric acid method. The amounts present in the dialysate were determined by the same method. Standard curves corresponding to the composition of the alginate were used. (3) Equal volumes of a 0.5 % alginate solution in 0.1 N NaCl and a solution containing a certain amount of HCl were mixed. The pH value obtained was determined, the mixture left for 30 min and centrifuged. The distribution of alginate between the centrifugate and precipitate was determined by the phenol-sulphuric acid method.

For the analytical examination of the various fractions the following methods were used: The uronic acid composition was determined as described earlier [7]. The amounts of decarboxylating material were determined as described by Anderson [8]. Fucose was determined according to Dische [9]. Sulphate was determined by combustion in oxygen, oxidation with H_2O_2, precipitation as barium sulphate and complexometric titration of excess barium with phtalein purple as indicator. Xylose was determined by the orcinol reaction,[10] using a heating time of 40 min. The results are corrected for the amount of uronic acid found by decarboxylation.

The viscosity was measured in Ubbelohde viscometers, and the intrinsic viscosity found by using empirical curves relating the relative viscosity determined in a certain viscometer with the intrinsic viscosity determined by extrapolation [11].

The electrophoretic analyses of the fractions were carried out as described in the preceding paper [2].

RESULTS

The results of acid extraction of alginate fractions are given in columns 1 and 2 of Table 1. Column 1 gives the results of one extraction and column 2 of four successive extractions. A marked difference between the *Ascophyllum* samples and the *Laminaria* samples was observed. The difference between the laboratory preparations and a commercial sample of *Ascophyllum* alginate is most probably due to different methods of preparation.

The influence of intrinsic viscosity on the solubility at low pH was investigated by dialysing degraded samples of alginate fractions against a sodium chloride solution at pH 2. The results for samples from *Ascophyllum* and *Laminaria digitata* are shown in Figs. 1 and 2. Alginate fractions from the two algae show a very marked difference in solubility at the same value of intrinsic viscosity. Three different methods of degradation were used: enzymic degradation, thermal degradation at pH 7 and degradation in 1 N acid. For the alginate fraction from *L. digitata* the results of the three different degradation methods

Table 1. Percentage of alginate fraction soluble at low pH.

	Extraction pH 2		Dialysis pH 2		Precipitation
	1. extr.	Σ 4 extr.	$[\eta] = 7$	$[\eta] = 1$	pH 2.6, $[\eta]=10$
A. nodosum, Være Aug. 1959	13.2	19.0	27	60	100
» » , Være 2/3	14.2	20.0	29	60	100
» » , Flakk 15/3	14.8	22.0	32.4	61	100
L. digitata, Espevær 10/6	0.4	0.6	6.9	27	53
L. digitata, Svellingen Jan. 1960	1.3	1.6	4.7	28	40
L.hyperborea, stipes, Hustad 26/2	0.8	1.2	3.7	5.1	7
L.hyperborea, fronds, Reine 30/1	0.8	1.2	13.9	36.3	61.7
Protanal HF (*L.digitata*)	0.9	1.3	4.1	26.0	46
Manucol SS (*L.hyperborea* stipes)	1.2	1.9	2.1	6.3	14.5
Scotia Marine Products (*A.nodosum*)	6.5	8.2	16.5	41.6	98
Kelco SS (*Macrocystis pyrifera*)	2.3	3.2	4.2	26.2	38

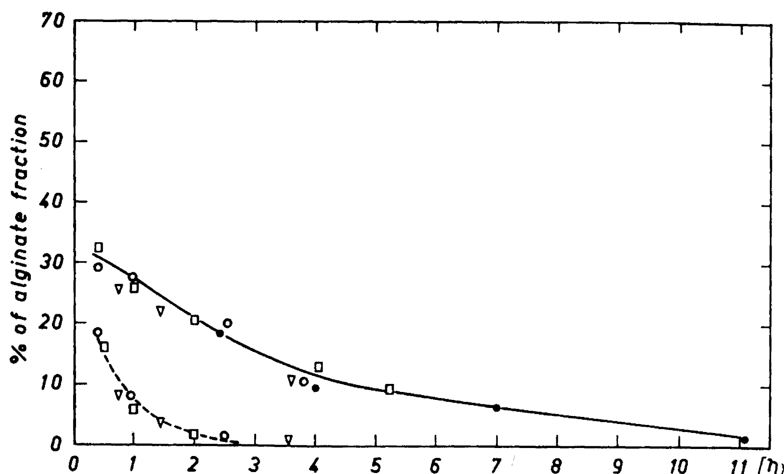
give the same results, while the acid degradation of the preparations from *Ascophyllum* leads to a considerably smaller non-dialysable, soluble fraction than the two other degradation methods. Correspondingly, the amounts of carbohydrate material dissolved in the 1 N acid is much higher for the *Ascophyllum* than for the *Laminaria* samples.

In column 3 and 4 of Table 1 the percentage of the alginate fraction which was soluble and not dialysable at pH 2 at two values of intrinsic viscosity is given for several commercial and laboratory preparations. The solubility decreases in the sequence *Ascophyllum, L. hyperborea* fronds, *L. digitata* and *L. hyperborea* stipes.

The solubility of alginate fractions at different values of pH was investigated by mixing a solution of alginate fraction and dilute acid and determining the amount of alginate precipitated as a function of the pH obtained. The results for samples from *Ascophyllum, L. digitata*, and *L. hyperborea* stipes with three different values of intrinsic viscosity are given in Fig. 3. Enzymic degradation has been used in order to obtain the desired intrinsic viscosity. Alginate fractions from the three species investigated were precipitated at different values of pH, and, in agreement with the results from the dialysis experiments, the *Ascophyllum* sample contained a larger amount of material that was not precipitated at pH below 2. It should also be noted that the alginate sample from *Ascophyllum* with intrinsic viscosity of 2 was less soluble at pH values above 2.1 than the samples with intrinsic viscosity of 5, while for the other samples no significant difference in solubility was observed for samples with intrinsic viscosity 2 and 5.

In column 5, Table 1, the percentage of the sample not precipitated at pH 2.6, is given for some laboratory and commercial samples of alginates.

The alginate fraction from *Ascophyllum nodosum*, harvested at Være, August 1959, was further investigated by extracting a sample three times



Figs. 1 and 2: Dialysis of solutions of alginate fractions with different intrinsic viscosities against pH 2 buffer. Amounts of dialysable material and soluble, non-dialysable material. *Fig. 1.* Alginate fractions prepared from *L. digitata*. Enzymic degradation: ○*L.d.*, Espevær 10/6, □ Protanal HF. Thermal degradation, pH 7: ● *L.d.*, Espevær 10/6. Acid degradation: ▽ *L.d.*, Espevær 10/6. ——: Soluble, non-dialysable fraction. - - - -: Dialysable fraction, or fraction dissolved in strong acid.

with acid at pH 2 and isolating the extracted material by precipitation with alcohol. Electrophoretic examination revealed the presence of three components in all three extracts, with mobilities of 0.45, 1.00 and 1.75 $\times$ 10^{-4} cm^2/sec. volt at pH 2 in 0.05 M sodium chloride solution. In the first extract, the com-

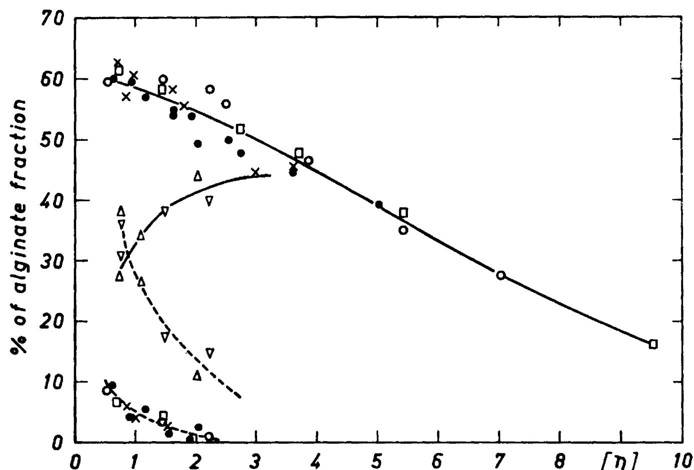


Fig. 2. Alginate fractions prepared from *A. nodosum*. Enzymic degradation: □ *A.n.*, Være 2/3, ● *A.n.*, Være, August. Thermal degradation, pH 7: ○ *A.n.*, Være 2/3, × *A.n.*, Være, August. Acid degradation: △ *A.n.*, Være 2/3, ▽ *A.n.*, Være, August. ——: Soluble, non-dialysable fraction. - - - -: Dialysable fraction, or fraction dissolved in strong acid.

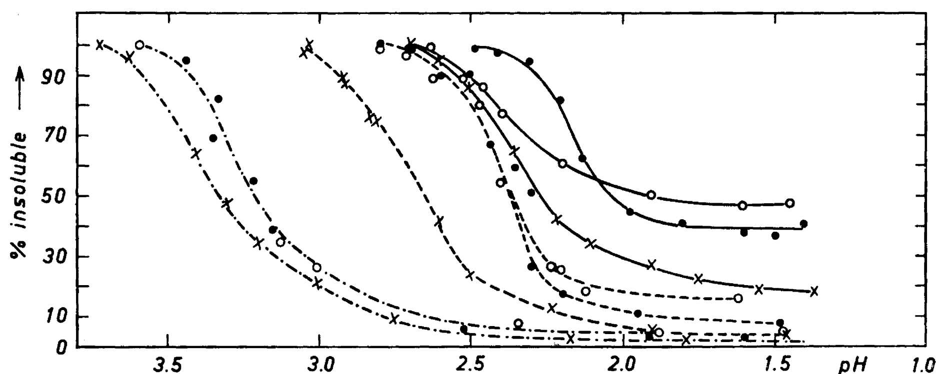


Fig. 3. Precipitation of alginate fractions with acid. —— *A. nodosum*, Være 2/3, - - - - - *L. digitata*, Espevær 10/6; —.—.—. *L. hyperborea* stipes, Hustad 26/2. × $[\eta] \approx 10$; ● $[\eta] \approx 5$; ○ $[\eta] \approx 2$.

pound with mobility 1.0×10^{-4} was dominating, while in the second and third extract the compound with mobility 0.45×10^{-4} was the main component. In all cases only small amounts of the compound with mobility 1.75×10^{-4} was present. The three fractions were analysed for decarboxylating matter, fucose, xylose and sulphate, and the uronic acid composition determined. The results are given in Table 2.

The residue from the acid extraction was dissolved in phosphate buffer and subjected to enzymic degradation. Samples taken at various values of intrinsic viscosity were fractionated by dialysis at pH 2. The results are given in Fig. 4. At intrinsic viscosities of 5 and 2, the soluble and insoluble fractions were prepared and investigated separately, and for the sake of convenience described as fraction S and I with indexes 5 and 2 respectively. The intrinsic viscosity of the fractions S_5 and I_5 was found to be 4.5 and 5.2, respectively, and of fractions S_2 and I_2 1.1 and 2.2. The fractions S_2 and I_2 were further enzymatically degraded and fractionated by dialyses at pH 2. The results are given in Fig. 5.

Table 2. Composition of fractions of alginate from *Ascophyllum nodosum*, Være Aug. 1959 as g per 100 g dry matter.

Fraction	SO_3Na	Fucose *	Xylose *	Na-uronate *	Sum	Uronic acid composition Man.	Gul.	Gluc.
1st acid extract	10.8	19.1	18.6	41.4	89.9	1.0 :	1 :	0.6
2nd acid extract	4.8	10.1	7.7	71.0	93.6	1.4 :	1 :	0.1
3rd acid extract	3.0	10.0	2.0	78.0	93.0	1.9 :	1 :	0.1
Residuum	1.0	1.9	—	91.0	93.4	2.4 :	1	**
Soluble pH $2[\eta] \approx 2$	2.4	4.0	—	90.5	96.9	2.1 :	1	**
Insoluble pH $2[\eta] \approx 2$	0	0.6	—	96.3	96.9	2.8 :	1	**
Non-dialysable material after enzymic degradation	19.5	25.0	26.0	32.0	102.5	1.0 :	**	2.4

* Calculated as the anhydro unit.
** Not detected.

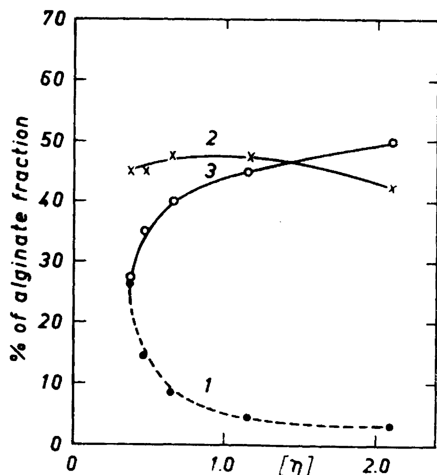


Fig. 4. Dialysis of solutions of alginate fractions with different intrinsic viscosities against pH 2 buffer. Amounts of dialysable material (1), soluble non-dialysable material (2), and insoluble material (3).
Alginate fraction prepared from *A. nodosum*, Være August 1959, and previously extracted three times with acid. Enzymic degradation.

Precipitation experiments were carried out with the fractions described above, and the results are given in Fig. 6. It should be noted that the fraction I_2 was precipitated at a higher pH value than fraction I_5.

Electrophoretic examination of the fractions soluble at pH 2 (S_2 and S_5) showed the presence of two components with mobilities 0.45 and 1.0×10^{-4}

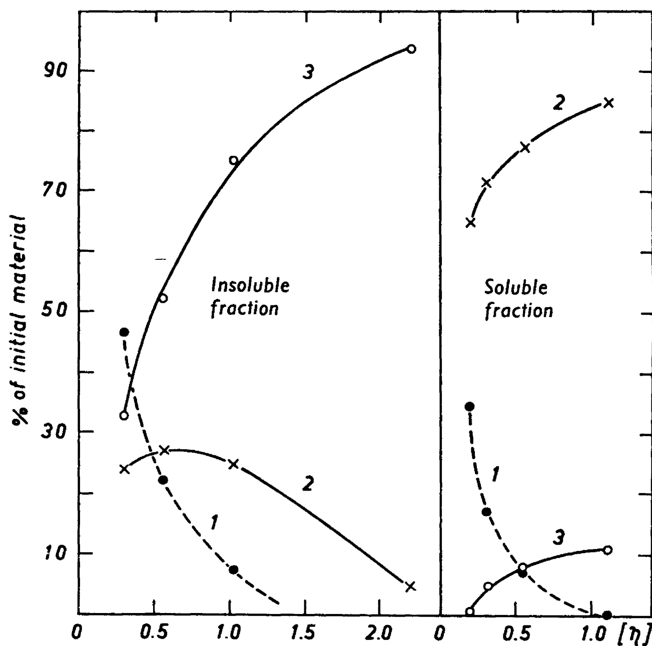


Fig. 5. Soluble and insoluble fractions prepared from experiment given in Fig. 4 subjected to further enzymic degradation and fractionated by dialysis against pH 2 buffer.

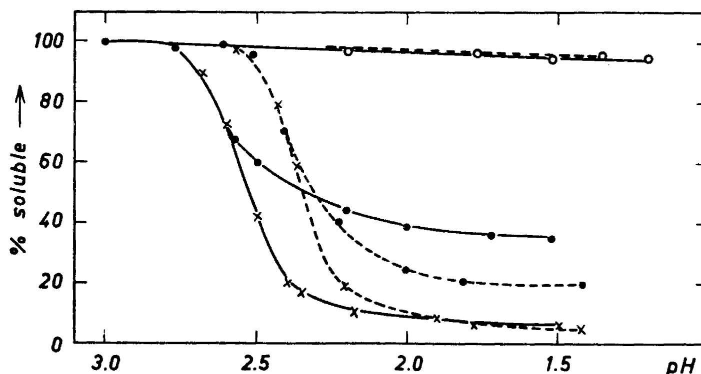


Fig. 6. Precipitation of alginate fractions from *A. nodosum*, Være August 1959. Alginate fractions previously extracted three times with acid. Fractions prepared by dialysis against pH 2 buffer.

● Before fractionation; × Fraction insoluble at pH 2; ○ Fraction soluble at pH 2.
—— $[\eta] = 2$; - - - - - - $[\eta] = 5$.

at pH 2 in 0.05 N sodium chloride. The component with mobility 0.45×10^{-4} was dominating. The insoluble samples (I_2 and I_5) were degraded further to obtain an intrinsic viscosity of 0.5 (Fig. 5) and the soluble degradation products examined electrophoretically. Only one component with the mobility of 0.28×10^{-4} was observed in the degradation products from I_2, while in addition to this component, small amounts of the component with mobility 1.0×10^{-4} were present in the degradation products of fraction I_5.

The results of analysis of the fractions I_2 and S_2 are given in Table 2.

It was observed that when alginate fractions were subjected to enzymic degradation, the samples from *Ascophyllum* contained a certain amount of material that was not dialysable even after prolonged treatment with enzyme. Approximately 25 % of the total fraction was not dialysable, and when examined electrophoretically at pH 2 showed the presence of two components with mobilities of 0.98 and 1.68×10^{-4}, with the former predominating. The results of the analysis of the non-dialysable fraction are given in Table 2. When alginate fractions from *L. digitata* were treated in the same manner, more than 95 % of the material was dialysable after prolonged enzymic degradation.

DISCUSSION

The insolubility in water is a well known property of alginic acid, and already Standford [12] has recognized that alginic acid could be precipitated from a sodium alginate solution by addition of acid. Very little is known, however, about the various factors influencing this precipitation, such as the conditions during the precipitation, *e.g.* alginate concentration, ionic strength etc., and the properties of the alginate, *e.g.* intrinsic viscosity, uronic acid composition etc. In the present investigation standardized conditions have been used for the precipitation, and only the influence of the different properties of the alginates on the solubility in acid media has been studied.

According to Preiss and Ashwell [13] enzymic degradation of alginate is accompanied by the introduction of a double bond between C_4 and C_5 in the uronic acid residue. Degradation with the enzyme used in this work led to the formation of compounds giving a colour reaction with thiobarbituric acid,[14] thus demonstrating that the same mechanism is operative in our case. It is supposed that this does not significantly alter the properties of the alginates within the range of viscosity examined in this work.

When acid is added to an alginate solution of the standard concentration and ionic strength used in this work, most of the material is precipitated within a rather narrow pH range, about 0.5 pH units. Part of the alginate fraction, however, is not precipitated even at a much lower pH (Fig. 3). In this work, the amount of material soluble at pH 2 has been used to characterize this property of the sample, and in the following discussion this fraction is described as the acid soluble material.

The influence of the intrinsic viscosity of the alginate on the solubility in acid medium is shown in Figs. 1, 2, and 3. The amounts of acid soluble material increases in all cases with decreasing intrinsic viscosity. The influence of the intrinsic viscosity on the pH range of precipitation is, however, markedly different for samples prepared from *Ascophyllum* and *L. digitata*, as may be seen by regarding the curves for precipitation with intrinsic viscosity 2 and 5 in Fig. 3. Samples from *L. hyperborea* stipes behave, in this respect, as samples from *L. digitata*.

Both the precipitation range and the amount of acid soluble material is markedly different for the three species mentioned above. It is known that the uronic acid composition of alginate from *L. hyperborea* stipes differs markedly from that of *L. digitata* and *Ascophyllum nodosum*, as the former contains a much higher proportion of guluronic acid [7]. The samples used in this work for the determination of the precipitation range have been analysed,[7] and the proportion between mannuronic and guluronic acid found to be 0.37, 1.58 and 1.62 for the samples from *L. hyperborea* stipes, *L. digitata* and *A. nodosum*, respectively. It is also known that the dissociation constant of the two uronic acids, and of alginates with different uronic acid composition, is significantly different [15]. The different precipitation range can, however, not be explained only as a difference in dissociation constants, as the amount of material precipitated is markedly different for the same degree of neutralization. In order to precipitate 10 % of a sample of alginate from *L. hyperborea* stipes ($[\eta] \approx 10$), about 60 % of the carboxyl groups must be transferred to acid form, while a corresponding precipitation of a sample from *L. digitata* is first obtained when about 80 % of the carboxyl groups are in acid form. The solubility of the two types of polymers is thus different at the same degree of neutralization and this difference in solubility may be due to the different uronic acid composition.

The different behaviour in acid media for alginates from *L. digitata* and *L. hyperborea* stipes may thus be explained partly by the difference in dissociation constant for guluronic and mannuronic acid and partly by a postulated difference in solubility of polymers with different uronic acid composition. A similar explanation is, however, not possible for the difference between samples from *L. digitata* and *Ascophyllum nodosum*. The uronic acid composi-

tion is not significantly different, while both the range of precipitation and the amount of acid soluble material is markedly different.

Approximately 60 % of the alginate fraction from *Ascophyllum nodosum* is soluble and not dialysable at pH 2, when the intrinsic viscosity has been reduced to 1 by thermal or enzymic degradation. The difference between the two fractions is not mainly due to a difference in molecular weight, as is shown by degrading the two fractions separately and comparing the solubility of the fractions at identical values of intrinsic viscosity (Fig. 5). The soluble fraction was shown to contain two components with mobilities 0.45 and 1.0×10^{-4} cm^2/sec.volt at pH 2. According to the preceding paper the component with mobility 1.0×10^{-4} at pH 2 is a new compound which has been given the name of ascophyllan. The identity of the component with mobility 0.45×10^{-4} is discussed in the preceding paper.

Approximately 25 % of the alginate fraction from *L. digitata* is soluble at the same condition ($[\eta] = 1$), and an electrophoretic examination revealed the presence of only one component with mobility 0.45×10^{-4} at pH 2. The difference between the soluble parts of the alginate fraction from the two species may thus be due to the presence of ascophyllan in the alginate fraction from *Ascophyllum*. So far no exact quantitative determination of the amounts of ascophyllan is undertaken. The approximate amount of this compound is, however, indicated by the fact that about 25 % of the total alginate fraction from *Ascophyllum* were found to be non-dialysable after extensive enzymic degradation, and only ascophyllan and traces of fucoidin (mobility 1.75×10^{-4} at pH 2) could be observed electrophoretically.

The range of precipitation is significantly different for alginate fractions from *Ascophyllum nodosum* and *L. digitata* when the intrinsic viscosity is 5 and 10. When the intrinsic viscosity is reduced to 2, however, the precipitation range of the *Ascophyllum* alginate is changed towards higher values of pH, and no significant difference between the first part of the precipitation curves can be observed for samples from *L. digitata* and *Ascophyllum nodosum* at this value of the intrinsic viscosity (Fig. 3). The observation might indicate that at higher values of intrinsic viscosity part of the ascophyllan is bound to the alginate, and thus inhibits the precipitation with acid. Further degradation makes the fragments containing ascophyllan soluble, while the rest of the alginate contains less ascophyllan and thus is precipitated at a higher pH value. According to this hypothesis the precipitation range of the insoluble fractions prepared by dialysis at pH 2 should depend on the intrinsic viscosity of the alginate when the fractionation was carried out. When the insoluble fraction is prepared at an intrinsic viscosity of 5, it should be expected to contain a certain amount of ascophyllan bound to the alginate, while when the fractionation is carried out at an intrinsic viscosity of 2, the fragments containing ascophyllan should mainly be left in the soluble fraction. It should thus be expected that the insoluble fraction with the lowest intrinsic viscosity should be precipitated at higher values, and this is in agreement with the results given in Fig. 6. Accordingly, small amounts of ascophyllan was found to be present in the insoluble sample with the highest intrinsic viscosity, while this compound could not be detected in the insoluble fraction with the lowest intrinsic viscosity.

The chemical analyses of the fractions (Table 2) show that fractions where ascophyllan has been shown to be present by electrophoretic examination contain fucose, xylose, sulphate and glucuronic acid in addition to the components of the alginic acid. The glucuronic acid was identified by paper electrophoresis,[16] paper chromatography of the lactones [17] and chromatography on ion exchange resin [18]. It should be noted that the reliability of the determination of xylose decreases when the proportion between amounts of uronic acids and xylose increases. No fraction containing only ascophyllan has so far been isolated, and the exact composition of this compound has, therefore, not been determined.

From a practical point of view it should be noted that alginate prepared from *Ascophyllum* may be distinguished from alginate from *L. digitata* by determining the solubility at low pH, *e.g.* by precipitation or dialysis at pH 2.0 or 2.6. The same method may also be applied to distinguish between alginate from *L. digitata* and *L. hyperborea* stipes. It should, however, be pointed out that a commercial preparation of alginate is not identical with the alginate fraction investigated in this work. In the commercial process most of the ascophyllan is removed from the alginate, resulting in a decreased solubility at low pH compared with the alginate fraction (Table 1). For alginate fractions from *Ascophyllum* and *L. digitata* we found that the difference in precipitation range was most marked at higher values of the intrinsic viscosity (Fig. 3). Commercial preparations, containing less ascophyllan, have been found to be significantly different in precipitation range at intrinsic viscosity of 10 while the difference is hardly significant at 5.

It has been shown earlier that some of the properties of alginates are determined by the uronic acid composition of the alginate [13,19,20]. The results of the present investigation show that the presence of ascophyllan is also of importance for determining the properties of alginates.

REFERENCES

1. Larsen, B. and Haug, A. *Proc. 4th Intern. Seaweed Symp.*, Biarritz 1961. *In press.*
2. Larsen, B. and Haug, A. *Acta Chem. Scand.* **17** (1963) 1646.
3. Haug, A. *Acta Chem. Scand.* **13** (1959) 601.
4. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. *Anal. Chem.* **28** (1956) 350.
5. Meland, S. M. *Nytt Magasin for Botanikk* **10** (1962) 53.
6. Eimhjellen, K. E., Rosness, P.A. and Hegge, E. *Acta Chem. Scand.* **17** (1963) 901.
7. Haug, A. and Larsen, B. *Acta Chem. Scand.* **16** (1962) 1908.
8. Anderson, D. M. W. *Talanta* **2** (1959) 73.
9. Dische, Z. *Methods of Biochem. Anal.* **2** (1954) 313.
10. Brown, A. H. *Arch. Biochem.* **11** (1946) 269.
11. Haug, A. and Smidsrød, O. *Acta Chem. Scand.* **16** (1962) 1569.
12. Standford, E. C. C. *Eng. Pat.* 142/1881.
13. Preiss, J. and Ashwell, G. *J. Biol. Chem.* **237** (1962) 309.
14. Weissbach, A. and Hurwitz, J. *J. Biol. Chem.* **234** (1959) 705.
15. Haug, A. *Acta Chem. Scand.* **15** (1961) 950.
16. Haug, A. and Larsen, B. *Acta Chem. Scand.* **15** (1961) 1395.
17. Fischer, F. G. and Dörfel, H. *Z. physiol. Chem.* **301** (1955) 224.
18. Larsen, B. and Haug, A. *Acta Chem. Scand.* **15** (1961) 1397.
19. Haug, A. *Acta Chem. Scand.* **13** (1959) 1250.
20. Haug, A. *Acta Chem. Scand.* **15** (1961) 1794.

Received April 6, 1963.