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A Study of the Constitution of Alginic Acid by Partial Acid Hydrolysis

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Alginate from *Laminaria digitata* was hydrolysed in 1 M oxalic acid at 100°. Approximately 30 % of the sample was dissolved, and the hydrolysis of this fraction of the sample followed 1st order kinetics. The insoluble material had a number average degree of polymerization of 20–30 and was separated into two fractions by precipitation at pH 2.85. The soluble fraction at this pH contained 80–90 % mannuronic acid residues, the insoluble fraction 80–90 % guluronic acid residues.

In 1955 Fischer and Dörfel [1] established the presence of L-guluronic acid residues in alginate, and this was confirmed by Whistler and Kirby [2] and Drummond, Hirst and Percival.[3] In a recent publication, Hirst and Rees [4] isolated 2,3-di-*O*-methyl-D-mannose and 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose from methylated alginic acid after reduction and hydrolysis. Thus, the alginic acid molecule seems to be a linear polymer of D-mannuronic and L-guluronic acid residues linked together by 1,4-linkages, probably β- and α-, respectively. No evidence of other forms of linkage or of branching was obtained.

Little is known, however, about the distribution of the two different uronic acid residues among the polymer molecules, or about the sequence of residues along the polymer chain. Fractionation [5–7] has shown that ordinary alginate samples are chemically heterogeneous. Vincent [8] hydrolysed alginic acid with strong sulphuric acid and separated the oligouronides by paper chromatography. He isolated di- and oligo-uronides which contained both uronic acids. However, the possibility of reversion during the hydrolysis and the difficult separation of oligouronides by paper chromatography made it desirable to obtain further evidence of the existence of guluronic and mannuronic acid residues in the same molecule. This was obtained by Hirst, Percival and Wold,[9] who isolated 4-*O*-β-D-mannosyl-L-gulose from reduced alginic acid. They also isolated a mannobiose from the reduced polymer. Yoshikawa and Kiyohara [10] isolated a tri-uronide from enzymatic digests of alginate and identified

it as *O*-(α-L-gulo-4-enepyranuronosyl)-(1→4)-*O*-(β-D-mannuronosyl)-(1→4)-L-guluronic acid.

Thus, it is unequivocally proved that the two different uronic acid residues at least to some extent are linked together in the same molecule. The present work is an attempt to obtain more detailed knowledge about the sequence of uronic acid residues in the alginate molecule by partial acid hydrolysis.

RESULTS

a) *Hydrolysis of alginic acid with 1 M oxalic acid.* Alginate from *Laminaria digitata* was subjected to hydrolysis with 1 M oxalic acid on a water bath at 100°. Alginic acid is insoluble in strong oxalic acid and the hydrolysis was therefore a heterogeneous reaction. The reaction was followed by removing samples of the heterogeneous mixture, centrifuging, and determining the amount of material present in solution and the degree of polymerization (P) of the soluble and the insoluble fraction. The results for the soluble fractions are given in Fig. 1. The amount of material in the hydrolysate increases rapidly and, after 5 h of hydrolysis, about 25 % of the alginate is in solution. The shape of the curve indicates, however, that the amount of soluble material approaches a limit of about 28 % of the alginate.

The insoluble fraction, remaining after 10 h of hydrolysis, was suspended in water and dissolved by addition of alkali. Oxalic acid was added to the solution to 1 M concentration and the hydrolysis continued at 100° for 10 h. Samples were taken at intervals for analysis. The curve giving the amount of material in solution was of the same shape as that given in Fig. 1, and approached in this case a limit of 19 % of the material, corresponding to 13.7 % of the original alginate sample. The dissolution of the material and the formation of reducing end groups in the solution are shown in Fig. 2.

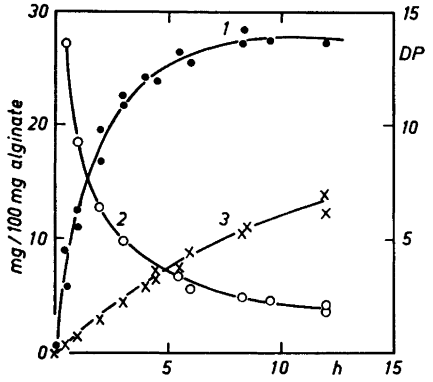

Fig. 1. Hydrolysis of alginic acid in 1 M oxalic acid, 100°C. 1 = Soluble material, 2 = DP, 3 = Reducing power of hydrolysate.

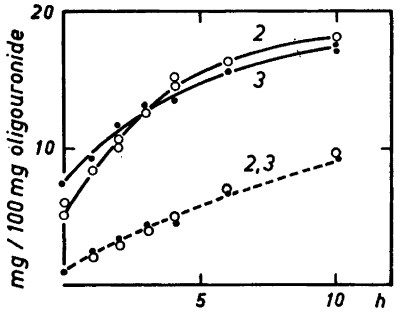

Fig. 2. Further hydrolysis of resistant fractions, 2nd (curves 2) and 3rd (curves 3) hydrolysis.
——— = Soluble material
------- = Reducing power of hydrolysate.

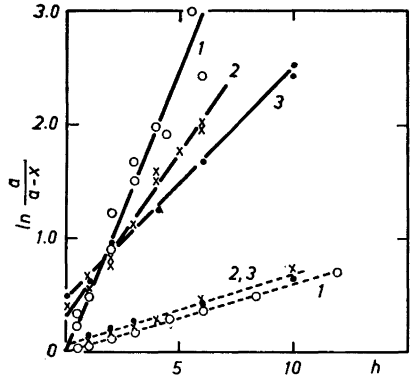

Fig. 3. Rate of solubilization of material and formation of reducing end groups for 1st, 2nd and 3rd hydrolysis.
——— = Rate of solubilization
------- = Rate of formation of reducing end groups.

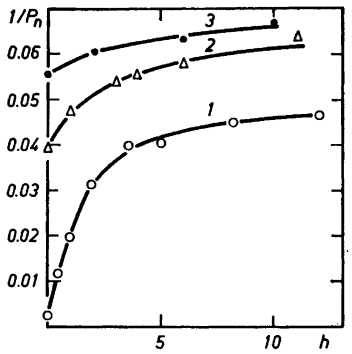

Fig. 4. Decrease of number average degree of polymerization (P_n) of the insoluble fraction of alginate in 1st, 2nd and 3rd hydrolysis.

The insoluble fraction from this hydrolysis was dissolved as described above and subjected to a third hydrolysis for 10 h at 100°. The results are given in Fig. 2. The amount of material passing into solution approached again 19 %, corresponding to 11.1 % of the total alginate sample.

In each case a certain fraction of the material is hydrolysable while the rest of the sample for some reason appears to be protected against hydrolysis. The kinetics of the solubilization of the hydrolysable material and the formation of reducing end groups in the hydrolysate has been examined. By plotting ln $[a/(a-x)]$ against t, where a is the hydrolysis limit and x is the amount of material reacted at the time t, straight lines were obtained, demonstrating that both reactions follow first order kinetics (Fig. 3).

The number average degree of polymerization of the insoluble phase was determined by measuring the amount of carbohydrate material and the reducing power. The results, plotted as $1/P_n$ against the time of hydrolysis, are given in Fig. 4 for the three hydrolysis steps investigated. It should be noted that for a first order, random degradation, $1/P_n$, when plotted against time, gives a straight line, provided $P_n > 10$. For the first step, the intrinsic viscosity of the insoluble material was determined. The intrinsic viscosity of alginate is proportional to the weight average degree of polymerization (P_w),[11] and the result given in Fig. 5 demonstrates that this quantity also decreases rapidly in the period when the hydrolysable material is dissolved.

b) *Examination of the insoluble fraction after the hydrolysis.* The results described above demonstrate that the alginate sample investigated consists of two fractions: one which is hydrolysed under the given condition, and another which, to some extent, is protected against hydrolysis. This fraction is in the following designated "the resistant fraction". The two fractions were examined separately.

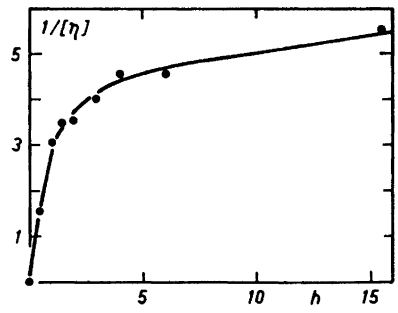

Fig. 5. Decrease of intrinsic viscosity of the insoluble fraction of alginate by 1 M oxalic acid hydrolysis.

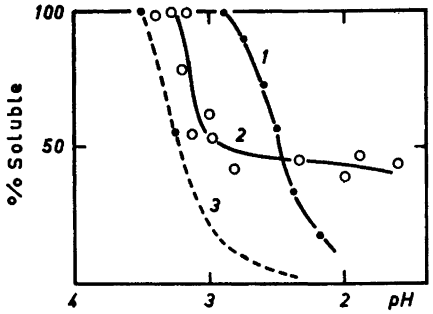

Fig. 6. Solubility of alginate in acidic solutions.
1 = Alginate from *L. digitata*, Tarva 29/8, 61 % mannuronic acid, $[\eta] = 5$.
2 = Hydrolysis resistant fraction of the same sample, DP $\approx$ 20.
3 = Alginate from *L. hyperborea* stipes, Hustad 26/2, 28 % mannuronic acid, $[\eta] = 5$.

After a suitable time of hydrolysis, the resistant fraction was removed by centrifuging, washed and suspended in water. Alkali was added to the suspension until all the alginate was dissolved. The alginate was then precipitated with ethanol in the presence of 0.5 % sodium chloride (2 volumes ethanol to 1 volume solution), washed with ethanol and ether and dried. The time of hydrolysis was 10, 20, and two times 20 h. In the latter case the resistant fraction was removed after 20 h of hydrolysis, dissolved in water by neutralization and the hydrolysis continued for another 20 h after addition of oxalic acid.

The resistant fraction of the alginate after two times 20 h of hydrolysis, prepared as described above, was dissolved in 0.1 M sodium chloride to make a 0.5 % solution and mixed with equal volumes of dilute acid solutions of various strengths. After the pH had been determined, the mixtures were centrifuged. The amount of soluble and insoluble material was determined and these results, together with those from similar experiments carried out with the whole, undegraded alginate, are given in Fig. 6. The results show that the degraded, resistant fraction is less soluble at approximately pH 3 than the undegraded alginate. Earlier investigations in this laboratory [7,12] have shown that the solubility of alginate samples at low pH depends on the uronic acid composition of the samples. The precipitation curve of an alginate sample with a high guluronic acid content is shown, for comparison, in the figure, thus demonstrating that the first part of the precipitation curve of the resistant fraction corresponds approximately to that of a guluronic-rich alginate.

The precipitation curve of the resistant fraction indicates that a fractionation into two components may be possible. A solution of the resistant fraction (0.5 %) in 0.1 M sodium chloride was mixed with an equal volume of dilute acid at a strength sufficient to bring the pH of the mixture to 2.85 ± 0.05.

Table 1. Hydrolysis of alginic acid and fractionation of the resistant fraction by precipitation at pH 2.85. Alginate sample containing 61 % mannuronic acid.

Fractions	Hydrolysis, h	Amount of fraction % of total alginate	Composition of fraction, % mannuronic acid	P_n
Hydrolysable	10	27.0		
Resistant, soluble pH 2.85	10	48.0	80	23
,, , insoluble pH 2.85	10	22.8	18.5	28
Hydrolysable	20	30.0	69	
Resistant, soluble pH 2.85	20	43.0	90	21
,, , insoluble pH 2.85	20	20.8	15	25
Hydrolysable	2 × 20	42.0	71.5	
Resistant, soluble pH 2.85	2 × 20	30.5	90	13
,, , insoluble pH 2.85	2 × 20	20.0	8.5	16

The mixture was centrifuged, the fractions prepared by neutralization and precipitation with ethanol and the uronic acid composition of the fractions determined. The results are given in Table 1. The uronic acid compositions of the soluble and insoluble fraction of the resistant part of the alginate were remarkably different. The result is particularly significant as no intermediate fraction with a less extreme uronic acid composition has been obtained. This means that the resistant part of the alginate consists of molecules which either contain mainly mannuronic or mainly guluronic acid residues.

Reprecipitation of the fractions by acid did not lead to a significant change in the uronic acid composition. It should, however, be pointed out that the method used for the determination of the uronic acid composition of alginate samples is not very accurate when the amount of one of the uronic acids is less than 10 % of the total.

c) *Examination of the hydrolysable fractions.* So far, only a preliminary investigation of the hydrolysable fractions has been carried out. Paper chromatography of the soluble phase after hydrolysis of the alginate for 10 h revealed the presence of guluronic acid, mannuronic acid, two components with a mobility corresponding to diuronides, and some slower moving compounds. The uronic acid composition of the hydrolysate was determined after further hydrolysis by the standard method used for determination of the uronic acid composition. The result is given in Table 1.

A sample of alginic acid was hydrolysed with 1 M oxalic acid for 10 h. The soluble and the insoluble phase were separated and the insoluble part hydrolysed for a further 10 h with 1 M oxalic acid. The procedure was repeated until four successive hydrolysates were prepared. These were subjected to paper chromatography in pyridine, ethyl acetate, acetic acid, water (5:5:1:3). The relative amounts of the two diuronides were estimated by visual examination of the two spots and the results are given in Table 2.

Table 2. Diuronides after successive hydrolysis with oxalic acid. Mobilities are given relative to that of galactose.

Hydrolysate	R_{Gal} 0.40	R_{Gal} 0.47
1	× × ×	×
2	× ×	× ×
3	—	× × ×
4	—	× × ×

A very marked difference in the relative amounts of the diuronides in the successive hydrolysates is shown in Table 2. This difference must indicate a difference in the uronic acid sequence in the parts of the alginate hydrolysed in the four successive hydrolyses. The examination of the resistant fraction after two successive hydrolyses (Table 1) revealed the presence of two types of oligouronides, containing either mainly guluronic or mainly mannuronic acids. It should, therefore, be expected that the diuronides formed in the third and fourth hydrolysis are mainly dimannuronic and diguluronic acids. The results in Table 2, therefore, indicate that the fraction of the alginate which is originally hydrolysable is characterized by a large proportion of alternating mannuronic and guluronic acid residues.

DISCUSSION

The most conspicuous of the results described above is the fractionation of the resistant fraction after the acid hydrolysis (Table 1). After one hydrolysis the resistant fraction constitutes 60—70 % of the total alginate sample and has a number average degree of polymerization of 20—30. These oligouronides are of two different types, containing either more than 80 % mannuronic acid or more than 80 % guluronic acid. After two successive hydrolyses, the yield of the resistant fraction was 50 % with a number average degree of polymerization of approximately 15. In this case the oligouronides contained either more than 90 % mannuronic acid or more than 90 % guluronic acid. Therefore, the distribution of the two uronic acid residues along the alginate chain must be non-random, with long sequences of only one type of uronic acid residues.

The curves shown in Fig. 1 indicate that the amount of material which passes into solution approaches a limit. The slight degradation which takes place during the hydrolysis tends to accentuate this limit. The dissolution of the hydrolysable material follows 1st order kinetics with a reasonable degree of accuracy up to 80—90 % yield when the observed limit is used in the calculation. This observation strongly supports the distinction between a hydrolysable and a non-hydrolysable part of the alginate molecule. A possible explanation of the difference in hydrolysability is that the hydrolysable and the resistant fractions of the alginate sample have a different degree of crystallinity. It is well known, from cellulose chemistry,[13] that crystalline regions are hydrolysed at a much lower rate than more amorphous parts. The preliminary investigation of the diuronides in the hydrolysate indicated a high proportion of alternating guluronic and mannuronic acid residues in the hydrolysable fraction of the

alginate, and this may be the cause of the lower degree of crystallinity in this fraction of the sample.

In the discussion we have so far not considered the distribution of the hydrolysable and the resistant parts of the alginate among the alginate molecules. The hydrolysable parts could either occur as separate molecules or form parts of the same molecules as the resistant fraction. The results shown in Figs. 3 and 4 showed that both the number and weight average molecular weight of the insoluble material decrease rapidly during the hydrolysis. This demonstrates that the resistant fraction is formed by splitting of long molecules. This splitting is most probably connected with the removal of the hydrolysable fraction, and it seems reasonable from the evidence presented above that the resistant parts are distributed along the chain molecule and separated by the hydrolysable parts of the molecule.

When the resistant fraction from the first acid hydrolysis is dissolved and reprecipitated with acid, a part of the former resistant fragments becomes hydrolysable. This has been observed in this laboratory by Szejtli.[14] Only a moderate decrease of the degree of polymerization of the insoluble material was observed during the hydrolysis of the resistant fragments (Fig. 3), indicating that in this case the hydrolysable material is removed from the chain ends. Most probably, the fact that a certain amount of the original resistant fragments becomes hydrolysable after reprecipitation is caused by incomplete crystallization.

According to the results discussed above we can roughly depict the alginate molecule as in Fig. 7, where two alternatives are given: one where resistant

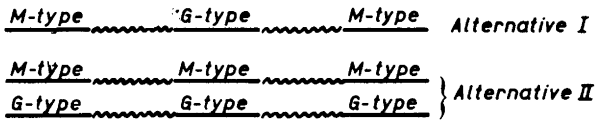

Fig. 7. ——— = Resistant part. ~~~~ = Hydrolysable part.

parts with mainly mannuronic acid residues and parts with mainly guluronic acid residues occur in the same molecule (alternative I) and another where the two types of resistant parts occur in separate molecules (alternative II). The acid precipitation curves (Fig. 6) seem to favour alternative I. After removal of the hydrolysable parts, the guluronic-rich resistant fragments are less soluble in dilute acid than the undegraded alginate molecules. The guluronic-rich parts of the molecule must be prevented from precipitation by being bound to more soluble structures, *i.e.* either the hydrolysable parts of the molecule or the mannuronic-rich type of resistant fragment. The precipitation curve of a guluronic acid rich alginate is shown in Fig. 6. Hydrolysis in 1 M oxalic acid at 100° of this sample showed that 23 % of the material was hydrolysable. If the hydrolysable parts were sufficient to prevent precipitation, one would expect this sample to be more soluble than the guluronic-rich fragments. However, this is not the case, and it is reasonable to assume that the guluronic acid rich fragments are made more soluble in the alginate molecule because

they are bound to the more soluble mannuronic acid rich fragments in the same chain molecule.

The results given in Fig. 3 show that the rate of formation of reducing end groups is the same in all the three successive steps of hydrolysis. The rate of dissolution of the material, however, is markedly different, indicating that the average degree of polymerization of the material passing into solution is highest in the first hydrolysis and decreases further from the second to the third step. A more detailed analysis of the kinetics of the hydrolysis will be published later.

EXPERIMENTAL

Degradation of alginic acid. 5 parts of sodium alginate were suspended in 100 parts of 1 M oxalic acid and stirred vigorously for 10 min. The viscous suspension was heated with reflux on a boiling water bath. A stream of nitrogen was passed through the suspension. Samples of 2—3 ml were removed at intervals with a quick delivery pipette. The samples were centrifuged for 20 min at 20000 *g*.

Analysis. The amount of alginate present in solution was determined by the phenol-sulphuric acid reaction [15] and the reducing power by the Nelson method.[16] The reducing power is calculated as mg uronic acid per 100 mg alginic acid. The extinction of the two uronic acids in the phenol-sulphuric acid reaction is different and the results are calculated by correcting the extinction according to the uronic acid composition of the sample. The uronic acid composition was determined as described earlier.[17] The intrinsic viscosity was determined by using empirical curves [18] as described elsewhere.

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