

## Quantitative Determination of the Uronic Acid Composition of Alginates

ARNE HAUG and BJØRN LARSEN

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

A method for quantitative determination of the uronic acid composition of alginates is described. The method is based on hydrolysis of the alginate with 80 % sulphuric acid at 20°C, followed by treatment with 2 N acid at 100°C, and separation of the uronic acids in the hydrolysate by chromatography on an anion exchange column. The amounts of the different uronic acids are determined by the orcinol reaction.

The loss of uronic acids in the different steps in the procedure has been investigated and a factor correcting for the different rate of breakdown of guluronic and mannuronic acid is introduced.

Until 1955 D-mannuronic acid was supposed to be the sole constituent of alginic acid. In that year, however, Fischer and Dörfel<sup>1</sup> discovered the presence of L-guluronic acid in hydrolysates of alginates. The presence of L-guluronic acid has later been confirmed by other authors<sup>2,3</sup>.

Fischer and Dörfel described a method for quantitative determination of the uronic acid composition of alginates<sup>1,4</sup>. The method was based on hydrolysis with 80 % sulphuric acid at room temperature, followed by boiling in 2 N acid, separation of the uronic acids by paper chromatography and determination of the amounts by using the tetrazolium chloride reaction and extracting the coloured compounds from the paper. The uronic acid composition of a number of seaweed species was investigated.

Recent works have shown that there exists a close correlation between properties and composition of alginates<sup>5-8</sup>, and that algae used as raw material for commercial alginate production may have very different uronic acid composition. It has therefore become of importance for the practical application of alginates to have a convenient routine method for the determination of the proportion of the two uronic acid residues in the alginates.

The method of Fischer and Dörfel has not proved satisfactory for routine determinations. The chromatographic separation requires very careful handling to give a separation good enough for quantitative work, and the colour reaction needs rigorously standardized conditions to give reproducible results.

The present communication describes a method suited for routine determination of the composition of alginate samples. All methods for chemical hydrolysis of polyuronides so far investigated lead to a considerable breakdown of uronic acids, and the rate of breakdown of guluronic and mannuronic acid has been shown to be different<sup>2</sup>. The loss of material during the procedure has therefore been investigated, and attempts have been made to correct the results for the different rate of breakdown of the two uronic acids.

### EXPERIMENTAL

*Standard procedure.* The preparation of alginate from seaweed samples has been described earlier<sup>9</sup>. Sodium alginate (50 mg) is mixed with 0.5 ml of 80 %  $\text{H}_2\text{SO}_4$  while the mixture is cooled in an ice bath. The mixture is left for 18 h in a thermostat at 20°C. The acid is diluted to obtain an acid strength of 2 N by adding 6.5 ml of water while the mixture is cooled in an ice bath. The mixture is then heated in a boiling water bath in sealed glass tubes for 5 h. Neutralization is carried out by adding a slight excess of  $\text{CaCO}_3$  to the hydrolysate after cooling. The bulky precipitate is filtered off and washed with water until about twice the original volume of hydrolysate has passed through the filter. The column chromatography is carried out on Dowex 1  $\times$  8 anion exchange resin (200—400 mesh). The resin was previously activated with 2 N NaOH and transformed to acetate form by washing with 2 N acetic acid and finally washed thoroughly with 0.5 N acetic acid. The column is 20 cm  $\times$  2 cm and is conveniently made of plexiglas tube, fitted with a screw cap bottom piece. A Teflon gasket is usually necessary. A 1.5 mm nylon tube is forced through a 1.0 mm hole in the bottom piece. Before the hydrolysate is applied to the column, all lactones are transformed to uronic acids by adding sufficient alkali to keep the solution at a pH of about 8 for half an hour. The elution of the column is carried out with acetic acid in a linear gradient from 0.5 N to 2 N. The flow rate is 0.3 ml per minute. Approximately 1 liter of acid is passed through the column in each run, and fractions of 10 ml are collected. The same column may be used for 10 to 20 runs.

Localization of the uronic acids is carried out by spotting a drop of each fraction on a filter paper strip. After drying, the filter paper strip is dipped in a solution containing 2.5 % aniline-trichloroacetate in glacial acetic acid, dried at room temperature and heated for 3 to 5 min at 100°C. The paper is examined in ultraviolet light. Apart from the first fractions which usually contain small amounts of neutral sugars, the fractions showing positive reaction with the staining reagent fall into two groups, the first containing guluronic and the second mannuronic acid<sup>10</sup>. The fractions in each group are combined and the volume determined. Care must be taken to include all fractions which contain uronic acids. The amount of uronic acid in each of the two groups is determined by the orcinol method<sup>11</sup>.

*Materials.* Alginates prepared from four different seaweed samples were used in this investigation: *Ascophyllum nodosum*, Flak 6/8—58, *Laminaria hyperborea* stipes, Hustad 26/2—59, *Laminaria digitata*, Espevær 10/6—52 and 8/1—53. The uronic acid composition of the samples is given in Table 7. In addition the uronic acid composition of a few laboratory preparations and commercial samples has been investigated. Guluronic and mannuronic acid were prepared by chromatography of the lactones on cellulose columns as described by Fischer and Dörfel<sup>4</sup>. Attempts to crystallize guluronic acid and its lactone were unsuccessful, and the concentration of guluronic acid in the solutions used for standard curves was determined in the following way: The aqueous solution of guluronic acid was treated with alkali to transform any lactone present to salt, and the acid liberated by running the solution through a column of Dowex 50 W ion exchange resin. The solution was immediately titrated with 0.1 N sodium hydroxide.

*Hydrolysis.* Three methods of hydrolysis were investigated: (1) Sodium alginate (50 mg) was treated with 80 % sulphuric acid as in the standard procedure. After dilution to 2 N acid, more 2 N  $\text{H}_2\text{SO}_4$  was added until a volume of 100 ml was obtained. The hydrolysis was carried out on a boiling water bath in a boiling flask fitted with a reflux condenser. (2) Sodium alginate (50 mg) was hydrolysed with 100 ml 1 N  $\text{H}_2\text{SO}_4$  on the water bath

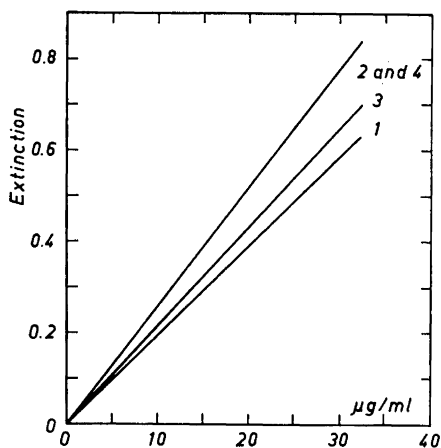


Fig. 1. The orcinol reaction. 1 = Gularonic acid. 2 = Mannuronic acid. 3 = Galacturonic acid. 4 = Glucuronic acid.

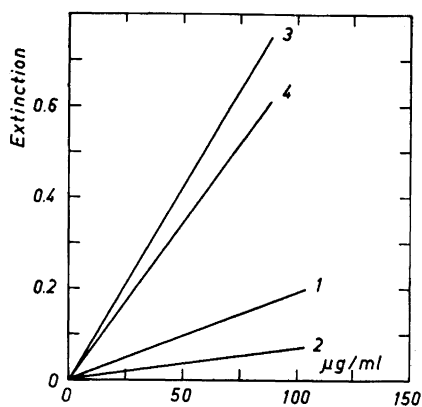


Fig. 2. The carbazole reaction. Symbols as in Fig. 1.

without pretreatment with concentrated acid. (3) Sodium alginate (50 mg) was hydrolysed with 100 ml 1 N oxalic acid on the water bath. In all cases, samples of 10 ml were taken out at fixed intervals for analysis.

*Colour reactions.* Apart from the orcinol reaction used in the standard procedure, the phenol sulphuric acid method<sup>12</sup> and the carbazole method<sup>13</sup> have been employed. In the phenol sulphuric acid reaction 0.5 ml of a 3% solution of phenol was used. The colour was measured in a Beckman B spectrophotometer. In the orcinol reaction 1.5 ml samples were used, in the phenol-sulphuric acid reaction 2 ml and in the carbazole reaction 1 ml, according to the standard procedures.

*Reducing power* was determined by the method of Hagedorn and Jensen, modified to a semi-micro method by Fujita and Iwatake (described in Ref.<sup>14</sup>).

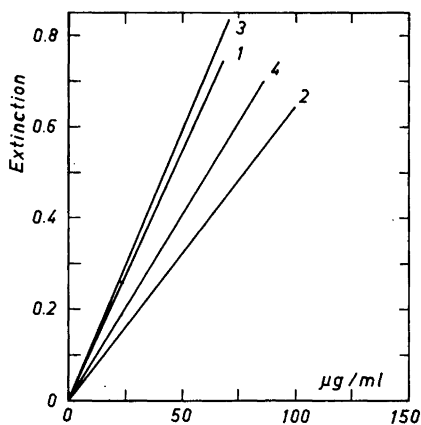


Fig. 3. The phenol-sulphuric acid reaction. Symbols as in Fig. 1.

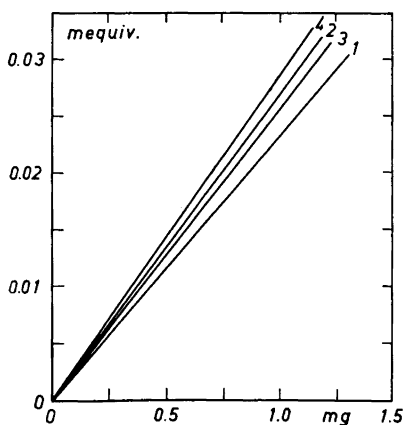


Fig. 4. Reducing power. Symbols as in Fig. 1.

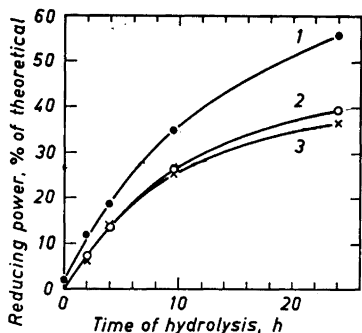


Fig. 5. Hydrolysis with 1 N oxalic acid. Alginate prepared from: 1 = *A. nodosum*, Flak, 6/8-58. 2 = *L. digitata*, Espevær, 10/6-52. 3 = *L. hyperborea* stipes, Hustad, 26/2-59.

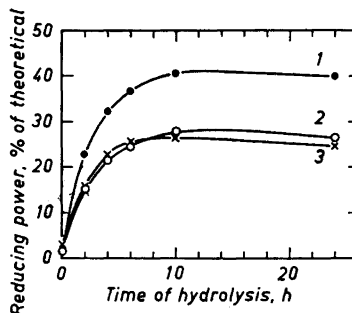


Fig. 6. Hydrolysis with 1 N sulphuric acid. Symbols as in Fig. 5.

## RESULTS

Standard curves for the three colour reactions and the reducing power determination were determined for guluronic, mannuronic, galacturonic and glucuronic acid and the results are given in Figs. 1-4. The standard curves for the colour reactions refer to the concentration of uronic acids in the samples used in the reaction, given in  $\mu\text{g}$  per ml.

Three different methods of hydrolysis were investigated; (a) hydrolysis with 1.0 N oxalic acid, (b) with 1.0 N sulphuric acid and (c) with 2.0 N sulphuric acid after pretreatment with 80 % sulphuric acid at room temperature. The rate of hydrolysis has been investigated by measuring the reducing power of the hydrolysates, and the results are given in Figs. 5-7 for three

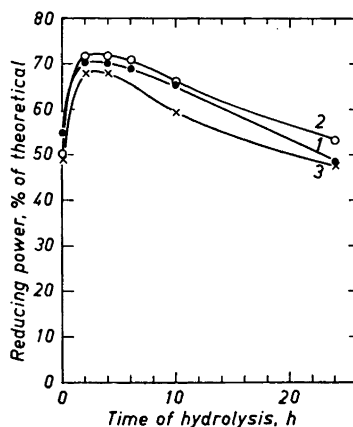


Fig. 7. Hydrolysis with 2 N  $\text{H}_2\text{SO}_4$  after pretreatment with 80 %  $\text{H}_2\text{SO}_4$  at  $20^\circ\text{C}$ . Symbols as in Fig. 5.

Table 1. Pretreatment with 80 % sulphuric acid at different temperatures. Reducing power and colour with phenolsulphuric acid as per cent of theoretical before and after hydrolysis with 2 N sulphuric acid for 5 h at 100°C.

Reducing power Pretreatment at	<i>A. nodosum</i> 6/8		<i>L. digitata</i> 10/6		<i>L. hyperborea</i> stipes, 26/2	
	Before	After	Before	After	Before	After
15°	41	66	42	61	36	55.5
20	50.5	66	53	65	45.5	56
25	60.5	65.5	61.5	64	54	61.5
30	62	59	63.5	64.5	58	62.5
35	66	56	68	60	65	58.5
Phenol-sulphuric acid						
15°	105	86	99	74	104	64
20	100	80	95	78	98	66
25	101	75	96	71	96	66
30	81	73	87.5	73	87	60
35	85	71	84.5	68	84	64

different alginate samples. The results are given as per cent of the theoretical value. The calculation of the theoretical value is based on the uronic acid composition of the hydrolysates, determined by the standard procedure. As shown by the figures, the highest reducing power was obtained by using pretreatment with strong sulphuric acid, followed by hydrolysis in dilute acid for a relatively short time.

The effect of different temperatures during the pretreatment with strong acid was investigated and the results obtained are shown in Table 1. Whilst high temperature during the pretreatment gives high values of the reducing power before hydrolysis with the dilute acid, the reducing power after 5 h hydrolysis with 2 N acid does not vary markedly with the temperature during the pretreatment, thus 20°C has been chosen for the standard procedure.

The loss of material which occurs throughout the procedure has been investigated by means of the phenol-sulphuric acid reaction, the orcinol reaction and reducing power determinations. The results are given in Table 2. It should be noted that the low value for unhydrolysed alginate by the orcinol reaction is not due to loss of material, but is caused by the fact that a complete reaction with orcinol is not obtained with the undegraded polymer.

Table 2. Recovery of material in the different stages of the standard procedure. Alginate from *L. digitata*, Espevær, 8/1—53. The results are given as per cent of the theoretical value for starting material.

	Phenol-sulphuric acid reaction	Orcinol	Reducing power
Before hydrolysis	102	54	0
After hydrolysis	84	63	73
After neutralization	75	56.5	64.5
Totale eluate from column	45.5	41.5	
Guluronic & mannuronic acid	40.0	39.5	40.5

Table 3. Loss of uronic acids in the neutralization with calcium carbonate. 100 mg alginate from *L. digitata* 8/1.

	Guluronic acid	Mannuronic acid	Total	M/G
Standard filtrate	12.9 mg	22.5 mg	35.4 mg	1.75
Second washing	0.86 »	1.8 »	2.66 »	2.09
Total	13.76 »	24.3 »	38.06 »	1.77

The loss in the different steps in the procedure has been investigated further, in order to establish if the loss of the two uronic acids is of the same magnitude, and, if that is not the case, to attempt to correct for the difference in recovery of the two components.

The loss occurring in the neutralization is due to incomplete removal of all uronic acids from the calcium sulphate precipitate. A sample of alginate from *L. digitata*, 8/1, was hydrolysed by the standard procedure and neutralized. After filtration and washing according to the standard procedure the uronic acid composition of the hydrolysate was determined. The residue on the filter was washed thoroughly with water and the washwater concentrated by evaporation and the uronic acid composition determined. The result is given in Table 3.

The loss occurring during hydrolysis was investigated by subjecting pure uronic acids to the same treatment as the alginate samples. After pretreatment with 80 % sulphuric acid at 20°C for 18 h, the uronic acids were heated in 2 N sulphuric acid on the water bath. The results are given in Figs. 8 and 9. As in the case of alginate hydrolysates (Table 2) the colour obtained with orcinol was considerably lower, compared to the theoretical value, than the reducing power and the colour with phenol-sulphuric acid. The results indicate

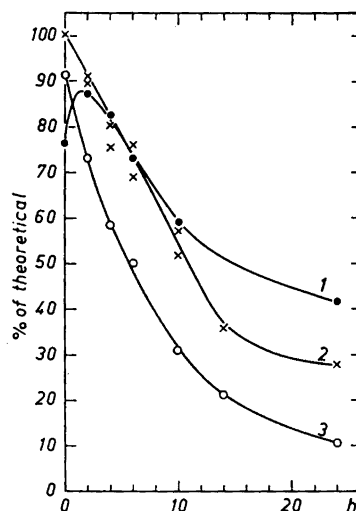


Fig. 8. Treatment of guluronic acid with 2 N  $\text{H}_2\text{SO}_4$  at 100°C after pretreatment with 80 %  $\text{H}_2\text{SO}_4$  at 20°C. 1 = Reducing power. 2 = Phenol-sulphuric acid reaction. 3 = Orcinol reaction.

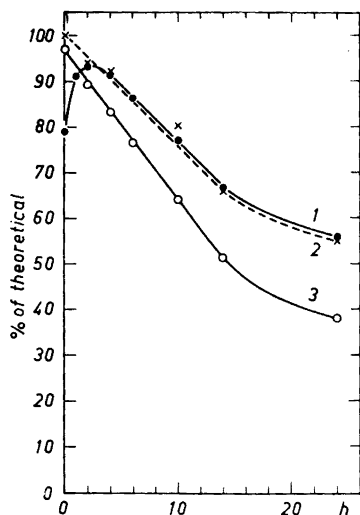


Fig. 9. Treatment of mannanuronic acid with 2 N  $\text{H}_2\text{SO}_4$  at  $100^\circ\text{C}$  after pretreatment with 80 %  $\text{H}_2\text{SO}_4$  at  $20^\circ\text{C}$ . Symbols as in Fig. 8.

that the breakdown of uronic acids leads to formation of products which have reducing power and give colour formation with phenol-sulphuric acid but which give little or no colour with orcinol.

This point was further investigated by treating galacturonic and glucuronic acid with sulphuric acid as described above. After 24 h at  $100^\circ\text{C}$  the reducing power, orcinol colour and phenolsulphuric acid colour were determined and the solution passed through a column of anion exchange resin. The same quantities were determined in the eluate from the column. The results are given in Table 4 as a percentage of the theoretical value.

The separation of uronic acids on a column of anion exchange resin is described in an earlier work<sup>10</sup>. In order to study further the behaviour of an alginate hydrolysate on the column, the elution was followed by the orcinol and phenol-sulphuric acid reactions. A sample of alginate (50 mg) prepared from *L. digitata*, 8/1, was hydrolysed according to the standard procedure,

Table 4. Analysis of "hydrolysates" of uronic acid before and after treatment with anion exchange resin, given as per cent of theoretical value.

	Treatment with anion exchange resin	
	Before	After
Glucuronic acid		
Reducing power	57	39.9
Phenol-sulphuric acid	55	41.7
Orcinol	37.5	35.6
Galacturonic acid		
Reducing power	53	17.0
Phenol-sulphuric acid	35	21.8
Orcinol	18.5	18.5

Table 5. Chromatography of hydrolysate of alginate prepared from *L. digitata*, Espevær 8/1. Sample 50 mg.

Tube No.	Volume	Phenolsulphuric acid	Orcinol	Identity
1—3	22	0.25 mg	0.08 mg	
4—5	14.5	0.68 »	0.14 »	Neutral sugars
6—82	430	0.63 »	0.18 »	
83—104	86	6.80 »	6.45 »	Guluronic acid
105—119	69.5	0.41 »	0.20 »	
120—131	96.5	11.00 »	10.80 »	Mannuronic acid
132—140	59.5	0.39 »	0.22 »	
141—164	150	0.35 »	0.27 »	
		20.51	18.44	

Table 6. Reproducibility of the determination and correction for different rate of breakdown in the hydrolysis. Alginate prepared from *L. digitata*, Espevær, 8/1. Proportion between mannuronic and guluronic acid (M/G).

	M/G	Average	Correction factor (Figs. 8 & 9)	Corrected result
Standard procedure (5 h, 100°, 2 N H <sub>2</sub> SO <sub>4</sub> )	1.75			
	1.67			
	1.74	1.75	53/80	1.16
	1.80			
10 h, 100°, 2 N H <sub>2</sub> SO <sub>4</sub> )	1.76			
	2.56			
	2.48	2.52	31/64	1.22
24 h, 100°, 2 N H <sub>2</sub> SO <sub>4</sub> )	4.20			
	4.77	4.48	10.5/37.5	1.25

Table 7. Uronic acid composition of some alginate samples, given as the proportion between mannuronic and guluronic acid residues (M/G).

Samples	M/G
<i>A. nodosum</i> , Flak, 6/8—58	1.82
<i>L. hyperborea</i> stipes, Hustad, 26/2—59	0.37
» » fronds, Reine, 30/1—57	1.28
<i>L. digitata</i> , Espevær, 8/1—53	1.16
» » » , 10/6—52	1.58
Manucol SS/LD ( <i>L. hyperborea</i> stipes)	0.46
» SA/LM ( <i>A. nodosum</i> )	1.10
Protanal LF ( <i>L. digitata</i> )	1.43
Kelco SS ( <i>Macrocystis pyrifera</i> )	1.56

neutralized and added to the column. The results are given in Table 5 as mg substance in each fraction. For the fractions not identified as guluronic or mannuronic acid the calculation is based on an extinction corresponding to a mixture of guluronic and mannuronic acid in the same proportion as in the hydrolysate.



The reproducibility of the method was investigated by determining the uronic acid composition of five samples of the alginate prepared from *L. digitata*, 8/1, by the standard procedure. In addition two samples were hydrolysed for 10 h and two samples for 24 h and the results corrected for different rate of breakdown of the two uronic acids during hydrolysis according to the curves for the orcinol reaction in Figs. 8 and 9. The results are given in Table 6.

The uronic acid composition of some laboratory preparations and commercial samples is given in Table 7, determined by the standard procedure. The results are corrected for the different rate of breakdown of the two uronic acids during hydrolysis by multiplying the proportion between the mannuronic and the guluronic acid in the hydrolysates with 0.66.

#### DISCUSSION

The different uronic acids give very different colour reaction with carbazole (Fig. 2) and our results are in agreement with earlier observations by Hoffman, Linker and Meyer<sup>15</sup>. The colour development in the phenol-sulphuric acid reaction is also different for the different uronic acids (Fig. 3) and significant differences are also found for the orcinol reaction (Fig. 1)<sup>15</sup>. The colour reaction may thus be used for a preliminary identification of the uronic acids.

As shown in Table 2, the phenol sulphuric acid reaction gives a colour corresponding to approximately 100 % of the theoretical value when carried out on an alginate solution not subjected to hydrolysis. On account of the marked difference in colour formation with this reagent for guluronic and mannuronic acid a rough estimation of the proportion between the two uronic acids may be obtained in this way. A more exact determination is not possible because of the small amounts of neutral sugars usually present in the alginate samples which interfere in the determination. The possibility of using the more specific carbazole reaction for this purpose is limited by the fact that small amounts of glucuronic acid is present in some alginate preparations, and that the sensitivity of the reaction is very much higher for this compound than for mannuronic and guluronic acid. It is also possible that a slight epimerization of mannuronic acid to glucuronic acid may occur in the acid medium in the reaction<sup>16</sup>. The orcinol reaction gives only a colour corresponding to approximately 50 % of the theoretical value when carried out on unhydrolysed alginate.

The results from the hydrolysis experiments (Figs. 5—7) indicate a considerable breakdown in the hydrolysis with sulphuric acid, while hydrolysis with oxalic acid does not give a sufficient yield in a reasonable time. While pretreatment with 80 % sulphuric acid gives the best results for our purpose, it should be pointed out that the experiments with pure uronic acids (curves 8 and 9) indicate a marked formation of reversion products in the strong acid treatment. This method should therefore be avoided if oligosaccharides produced by partial hydrolysis is to be studied for structural investigations.

The results in Table 2 show that loss of material occurs during the hydrolysis, in the neutralization and on the column, and that a small proportion of

the material eluted from the column is not guluronic or mannuronic acid. Considering the results from the orcinol reaction, the most important loss occurs in the hydrolysis (37 %) while 15 % is lost on the column. The results from the phenol-sulphuric acid reaction, however, indicate a loss of about 15 % in the hydrolysis and 30 % on the column. This is in agreement with the results from the experiments with pure uronic acids (Figs. 8 and 9 and Table 4) which clearly indicate that breakdown products are formed which give colour reaction with phenol-sulphuric acid but not with orcinol, and which are absorbed on the anion exchange resin. These products may be identical with the brown-coloured compounds which are irreversibly absorbed on the top of the anion exchange resin column. The results from Table 4 show that the compounds giving the orcinol reaction are quantitatively eluted from the column, thus indicating that these compounds are unchanged uronic acids. This has been confirmed by paper electrophoresis<sup>17</sup> and paper chromatography<sup>4</sup>. This suggests that the orcinol curves in Figs. 8 and 9 can be used for estimating the breakdown of uronic acids. According to these curves, between 20 and 50 % of the uronic acids are destroyed during the standard hydrolysis, depending on the proportion of the two uronic acids in the alginate.

For the alginate used in Table 2, this corresponds to a loss of about 35 % of the total amount of uronic acids, in good agreement with the loss during hydrolysis observed with the orcinol reaction.

While no significant loss of substance giving orcinol reaction could be observed by treating the "hydrolysates" of pure uronic acids with anion exchange resins (Table 4), about 15 % is lost when hydrolysates of alginates is chromatographed on anion exchange resin (Table 2). This loss may be due to unhydrolysed fragments of alginate or reversion products being absorbed on the column and not eluted with the acid strength used in our procedure.

Table 3 shows that no significant selective absorption of one of the uronic acids occurs on the calcium sulphate precipitate, and the loss of material during neutralization is thus of no importance for the determination of the proportion between the two uronic acids.

Analysis of the eluate from the column (Table 5) shows that 93.5 % of the substances eluted is mannuronic and guluronic acid if the calculation is based on the orcinol reaction and 87 % if based on the phenol-sulphuric acid reaction. This difference is probably due to small amounts of neutral sugars eluted in the first fractions giving a higher extinction in the phenol-sulphuric acid reaction than in the orcinol reaction.

According to Table 2, the recovery of uronic acids in the standard procedure is approximately 40 %. Of the 60 % lost in the procedure 43 to 44 % are due to loss during neutralization and breakdown in the hydrolysis. If the proportion between the two uronic acids in the 15 % of the material lost on the column is not very different from the proportion in the whole alginate, it should be possible to estimate the uronic acid composition of the alginate sample by correcting the results obtained by the standard procedure for the different rate of breakdown of guluronic and mannuronic acid. This correction by means of the orcinol curves in Figs. 8 and 9 has been tested by increasing the time of hydrolysis with 2 N acid to 10 and 24 hours. While the composition of the hydrolysates is very markedly altered by increasing the time of

hydrolysis, the corrected results show a satisfactory agreement. It seems safe to conclude that by using our standard procedure and multiplying the proportion between mannuronic and guluronic acid by 0.66 g good estimate of the uronic acid composition of the alginate sample is obtained.

## REFERENCES

1. Fischer, F. G. and Dörfel, H. *Z. physiol. Chem., Hoppe-Seyler's* **302** (1955) 186.
2. Whistler, R. L. and Kirby, K. W. *Z. physiol. Chem., Hoppe-Seyler's* **314** (1959) 46.
3. Hirst, E. L. *Proc. Chem. Soc.* **1958** 177.
4. Fischer, F. G. and Dörfel, H. *Z. physiol. Chem., Hoppe-Seyler's* **301** (1955) 224.
5. Haug, A. *Acta Chem. Scand.* **13** (1959) 1250.
6. Haug, A. *Acta Chem. Scand.* **15** (1961) 950.
7. Haug, A. *Acta Chem. Scand.* **15** (1961) 1794.
8. Haug, A. and Larsen, B. *Proc. 4th Intern. Seaweed Symp.*, Biarritz 1962. *In press.*
9. Haug, A. *Acta Chem. Scand.* **13** (1959) 601.
10. Larsen, B. and Haug, A. *Acta Chem. Scand.* **15** (1961) 1397.
11. Brown, H. H. *Arch. Biochem.* **11** (1946) 269.
12. Dubois, M., Gilles, K. A., Hamilton, J. K., Ribers, P. A. and Smith, F. *Anal. Chem.* **28** (1956) 350.
13. Dische, Z. in Glick, D. *Methods of Biochemical Analysis* **2** (1954).
14. Bamann, R. and Myrbäck, K. *Die Methoden der Fermentforschung*, I. 1074, Leipzig 1941.
15. Hoffmann, Ph., Linker, A. and Meyer, K. *Science* **124** (1956) 1552.
16. Berner, E. and Sandlie, S. *Chem. & Ind. (London)* **1952** 1221.
17. Haug, A. and Larsen, B. *Acta Chem. Scand.* **15** (1961) 1395.

Received April 16, 1962.