338. *The Structure of Alginic Acid. Part II.**

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The methyl derivative of alginic acid has been hydrolysed with formic acid. The products were converted into the methyl esters of methylated methylmannuronoside by means of methanolic hydrogen chloride and diazomethane, and lithium aluminium hydride then yielded the corresponding methylmannosides. The methylated mannoses were separated chromatographically, 2 : 3-dimethyl mannose, identified by oxidation to a derivative of *meso*dimethoxysuccinic acid with periodic acid, being the main component. Traces of 2 : 3 : 4-trimethyl mannose, monomethyl mannose, and dimethyl glucose were also present. The results confirm the view that the main structural feature of the alginic acid molecule is a chain of 1 : 4-linked β-D-mannuronic acid residues, the chain length of the alginic acid used in these experiments being *ca.* 100.

Alginic acid is found, usually as a salt, in certain seaweeds and is readily isolated by soaking the dried and finely ground seaweed in dilute acid, after which the alginic acid can be extracted with aqueous sodium carbonate. The acid itself is insoluble in water but its sodium salt forms highly viscous solutions. The viscosity of these solutions varies greatly from sample to sample and the differences are thought to be due to the varying chain length of the polysaccharide.

Alginic acid has been shown to contain D-mannuronic acid residues (Nelson and Cretcher, *J. Amer. Chem. Soc.*, 1929, **51**, 1914; 1932, **54**, 3409; Bird and Haas, *Biochem. J.*, 1931, **25**, 403), although direct hydrolysis results in very poor yields. More recently Hirst, Jones, and Jones (*J.*, 1939, 1880) prepared a degraded alginic acid by treatment with methyl-alcoholic hydrogen chloride and methylated it by the thallium method. Hydrolysis of the methylated derivative was difficult but nitric acid caused simultaneous hydrolysis and oxidative degradation to *meso*dimethoxysuccinic acid, indicating that the methyl groups were attached either to $C_{(2)}$ and $C_{(3)}$ or to $C_{(4)}$ and $C_{(5)}$. Proof that the methyl groups were at $C_{(2)}$ and $C_{(3)}$ was obtained by treatment of the methylated alginic acid with methyl-alcoholic hydrogen chloride, which gave the methyl ester of 2 : 3-dimethyl methyl-D-mannuronoside. These results have been confirmed by the present investigation which was undertaken with the idea of working with a less degraded sample of alginic acid and thereby obtaining information concerning the structure of a larger portion of the alginic acid molecule.

Alginic acid was prepared from *Laminaria digitata* fronds and directly methylated in an atmosphere of nitrogen by methyl sulphate and aqueous potassium hydroxide at room temperature. After sixteen treatments the product had a methoxyl content of 25·8% (yield, 90%). The methylated material was converted into the methyl ester, and viscosity determinations were carried out. If the validity of the Staudinger hypothesis and the applicability of the constant for methylated cellulose used in the calculation can be assumed, the results indicated a molecular weight of about 3000 (Staudinger and Reinecke, *Annalen*, 1938, **535**, 47) but this figure may be much too low.

Hydrolysis of the potassium salt of methylated alginic acid was achieved by using 98% formic acid. Chromatographic investigation of the resulting mixture of methylated mannuronic acids was unsatisfactory owing to trailing, and no convenient spray could be found. In view of this difficulty the mixture was heated with methanolic hydrogen chloride, and the solution was neutralised with diazomethane. The mixed methylated methylmannurosides so obtained were reduced by lithium aluminium hydride (method of Lythgoe and Trippett, *J.*, 1950, 1983) to the corresponding mannoside derivatives, which were readily separated on a cellulose column after aqueous hydrolysis to the methyl mannoses. This hydrolysis was incomplete, as shown by the presence of methylmannosides in the fractions from the

* Part I, *J.*, 1939, 1880.

column. The following fractions were obtained : Fraction (*a*) was a mixture of 2 : 3 : 4-trimethyl mannose with 2 : 3-dimethyl methylmannoside, containing 6·85% of the trimethyl sugar as calculated from the reducing power to alkaline hypoiodite solution. This fraction, on two subsequent hydrolyses, was shown chromatographically to yield only 2 : 3 : 4-tri- and 2 : 3-di-methyl mannoses, the amount of trimethyl mannose being equivalent to 0·98% of the total hydrolysate obtained from the methylated alginic acid. The presence of 2 : 3 : 4-trimethyl mannose was confirmed by demethylation, and by periodate oxidation, after chromatographic separation. Demethylation was achieved by treatment in a sealed tube with concentrated hydrobromic acid (Hough, Jones, and Wadman, *J.*, 1950, 1702) and chromatographic examination of the resulting sugars. No free sugar other than mannose was detected. Oxidation with aqueous periodic acid for twenty hours was carried out and the solution after addition of potassium chloride was decanted. This solution, on addition of solutions of phenylhydrazine hydrochloride and potassium ferricyanide, gave the characteristic port wine colour due to formaldehyde, indicating that no methyl group was present on $C_{(6)}$. Two small fractions (*b*) and (*c*) consisted of di- and mono-methyl methylmannosides respectively. Fraction (*d*) was a dimethyl sugar, later identified as a glucose derivative. Fraction (*e*) was a mixture of the preceding and following fractions. Fraction (*f*) was composed of 2 : 3-dimethyl mannose, identified as the corresponding lactone and by its oxidation to *meso*dimethoxysuccinic acid; this was the main fraction. Fraction (*g*) was small, consisting of monomethyl mannose. Analysis of the various fractions indicated that the hydrolysate had the following percentage composition : 2 : 3 : 4-trimethyl mannose, 1; dimethyl glucose, 6; 2 : 3-dimethyl mannose, 88; monomethyl mannose, 4·5.

These results coupled with the high yields obtained at each stage and with the high negative rotations of derivatives of the alginic acid confirm the view that alginic acid is composed mainly, or perhaps entirely, of β-D-mannuronic acid residues linked between $C_{(1)}$ and $C_{(4)}$. The origin of the dimethyl glucose mentioned above is as yet unexplained. It was thought that it might be due to epimerisation in the alkaline conditions of the reduction with lithium aluminium hydride, but a control experiment on 2 : 3-dimethyl mannose negatived this possibility and indicated merely that a small degree of demethylation took place which might account for much of the monomethyl mannose found in the present series of experiments. Further work will be necessary to decide whether the glucose is due to impurity or originates from some part of the structure of alginic acid.

The proportion of trimethyl mannuronic acid in the products of hydrolysis indicates the presence of one end group for every hundred mannuronic acid residues. This particular sample of alginic acid appears therefore to have a chain length of about 100. The viscosity of this sample was comparatively low and further investigations are clearly desirable with alginic acid of high viscosity as starting material, but the experimental difficulties will be extremely formidable. The present results are nevertheless of value in that they show that the type of structure previously proved only for highly degraded alginic acid may be extended to alginic acids of much higher molecular weight.

Experimental

Methylation of Alginic Acid.—The sample was prepared from *Laminaria digitata* fronds in the Institute of Seaweed Research Laboratory by soaking the dried ground weed overnight in 0·2N-acid, followed by extraction with sodium carbonate solution (3%). The alginic acid (purity 98%) was recovered by conversion into the calcium salt and treatment of the latter with 2N-hydrochloric acid, followed by separation, washing, and drying. Alginic acid (40 g.) was dissolved in aqueous potassium hydroxide (50%; 900 c.c.), and methyl sulphate (450 c.c.) was added with vigorous stirring during 5 days, in an atmosphere of nitrogen at room temperature, The solution was treated with further alkali (300 c.c.) and methyl sulphate (150 c.c.), dialysed. and concentrated *in vacuo* at 35°/15 mm., giving methylated potassium alginate. After four methylations the product had OMe, 11·8%. After sixteen methylations the methoxyl content was 25·8% (Calc. for $C_8H_{11}O_6K$: OMe, 25·6%). The yield was 50 g., and $[\alpha]_D^{15}$ −118° (*c*, 1·0 in water).

Preparation of the Methyl Ester.—The potassium salt (20 g.) was dissolved in water (200 c.c.) and treated with ten times the amount of hydrochloric acid required to form the free acid, and

the solution was dialysed until free from chloride ions, neutralised with silver oxide, and centrifuged. The silver salt was obtained by precipitation with acetone, separated, and dried. Attempts to convert this salt into the methyl ester by (*a*) boiling methyl iodide and silver oxide and (*b*) treatment with methanolic hydrogen chloride and diazomethane were unsuccessful. More success was achieved by the following method. The silver salt (5 g.) was dissolved in water (1 l.), and 2N-nitric acid added (400 c.c.). The solution was then dialysed for 3 days. Further nitric acid (100 c.c.) was added and dialysis continued for 7 days, whereafter the solution was treated with Amberlite I.R. 100 resin for 5 days and then concentrated in the presence of silver oxide. The residue was heated under reflux for 3 days with methyl iodide (200 c.c.), and the solution was then diluted with methanol, centrifuged, and evaporated to dryness. The solid residue was treated with methanolic hydrogen chloride (1%; 25 c.c.) for 12 hours at 0°, then neutralised with ethereal diazomethane, and the solution was evaporated to dryness. The last-mentioned treatment was repeated and the residue was extracted with chloroform which was evaporated to a thin syrup and poured into light petroleum (b. p. 60—80°), giving a precipitate which was separated and dried (2·5 g.) (Found: OMe, 41·8. Calc. for $C_9H_{14}O_6$: OMe, 42·6%). The product had $[\alpha]_D^{15}$ −87° (*c*, 8·0 in chloroform), −126° (*c*, 0·9 in *m*-cresol), $\eta_{sp.}^{20}/c$, 3·9 (*c*, 0·9 in *m*-cresol).

Hydrolysis of Dimethyl Potassium Alginate.—A solution of the polysaccharide (5 g.) in formic acid (500 c.c.; 98%) to which some charcoal was added was heated on a water bath. $[\alpha]_D^{15}$ changed from −118° to +14° in 8 hours. The solution was concentrated *in vacuo* to a syrup which was dissolved in water (200 c.c.). The solution was heated on a boiling-water bath for 4 hours, and concentrated to a syrup. Dissolution in water and concentration were repeated several times to remove traces of formic acid. Potassium ions were removed by treatment in water with Amberlite I.R. 100 resin overnight, the resin removed by filtration, and the solution concentrated to a syrup which was extracted with acetone. Evaporation of the solvent yielded a glassy solid (4·2 g.).

This material was examined chromatographically with *n*-butanol–acetic acid–water (4 : 1 : 5) as solvent and aniline oxalate as spray. Three spots were identified with R_G values, 0·65, 0·59, and 0·46, respectively, but the last spot which represented the bulk of the material showed a long trail almost to the starting line. An attempt to separate the mixture on a cellulose column with *n*-butanol–acetic acid (4 : 1) partly saturated with water was unsatisfactory. Various other solvent mixtures of different proportions of butanol, light petroleum, and acetic acid were tried without success.

Conversion into Ester before Separation.—As the trailing on the paper chromatogram was thought to be due to lactonisation and that this might be avoided by esterification, methylated alginic acid was dissolved in acetone–methanol (10 : 1), and the solution cooled in ice–salt and neutralised with diazomethane. The solution was left in the refrigerator in the presence of excess of diazomethane and then concentrated to a thin syrup. Examination of this syrup on the chromatogram showed improved definition of the spots but these were very faint and close together. Various spraying agents were tried without great success, the most satisfactory being aqueous aniline oxalate and dimethyl-*p*-phenylenediamine in trichloroacetic acid.

Reduction with Lithium Aluminium Hydride.—The potassium salt of methylated alginic acid (3·81 g.) was hydrolysed with formic acid as described previously, and the metallic ions were removed by two treatments with Amberlite I.R. 100 resin, the mixture of methylated mannuronic acids being finally obtained as a syrup (1·46 g.). The syrup was boiled with methanolic hydrogen chloride (50 c.c.; 1%) for 7 hours and the solution neutralised with diazomethane and concentrated *in vacuo* to a brown glass (1·47 g.). The glass was treated with gently boiling dry ethereal lithium aluminium hydride (2—3 g. in 50 c.c.) for 4 hours; then, after cooling, the excess of reagent was carefully destroyed with water, and the whole acidified with sulphuric acid (2N) and neutralised with barium carbonate. Aluminium was precipitated as the hydroxide by barium hydroxide, and barium and lithium were removed as carbonates (carbon dioxide), and the clear solution after filtration was concentrated *in vacuo*, giving a solid residue which was extracted with boiling chloroform. This yielded a syrup, which (2·90 g.) was converted into the methyl glycoside-ester and treated again as above to ensure complete reduction to mannose derivatives. The chloroform-insoluble solid was treated in the same way, giving a total yield of 2·70 g. The methylmannosides were hydrolysed with 2N-sulphuric acid (75 c.c.; 5·5 hours), and the free sugars (2·08 g.) were isolated in the usual way.

Chromatographic Analysis.—Paper chromatographic analysis of the above free sugars showed the presence of trimethyl mannose, R_G 0·75 (weak), dimethyl mannose, R_G 0·54 (very strong), and monomethyl mannose, R_G 0·34 (weak).

The main bulk of the syrup (2·08 g.) was separated on a cellulose column (90 × 3 cm.) (Hough, Jones, and Wadman, *J.*, 1949, 2511), with light petroleum (b. p. 100—120°)–*n*-butanol (6 : 4) saturated with water as solvent. Fractions were collected in the usual way and after evaporation of the solvent the syrups were dissolved in water and treated with charcoal. After filtration the solvent was removed, the products were taken up in acetone, and on removal of the acetone the following fractions were obtained.

Fraction	Substance	R_G	Wt. (g.)
A	2 : 3 : 4-Trimethyl mannose and 2 : 3-dimethyl methyl-mannoside	0·77	0·338
B	2 : 3-Dimethyl methylmannoside	—	0·046
C	Monomethyl methylmannoside	—	0·022
D	A dimethyl sugar	0·59	0·058
E	Mixture of D and F	0·54 & 0·59	0·204
F	2 : 3-Dimethyl mannose	0·54	1·151
G (water wash)	Monomethyl mannose	0·34	0·087

Fraction A (0·338 g.). This crystallised partly after being dried in a vacuum-desiccator, and had m. p. 68—71°, $[\alpha]_D^{15}$ −83·7° (*c*, 1·59 in water). 15·1 Mg. of the fraction on treatment with alkaline hypoiodite consumed 0·94 c.c. of 0·01N-iodine, indicating that only 6·9% of free reducing sugar, calculated as trimethyl mannose, was present. A portion of the fraction (0·259 g.) was rehydrolysed with sulphuric acid (2N; 10 c.c.; 3·5 hours) and chromatographic analysis of the product showed a considerable amount of 2 : 3-dimethyl mannose. The material was separated again on a cellulose column, but the new trimethyl fraction still contained a dimethyl mannoside as confirmed by a third hydrolysis (2N-H_2SO_4; 5·5 hours). Hypoiodite oxidation of the second hydrolysate indicated that 8·07% of free reducing sugar was present, equivalent to 1·09% of the original mixture of methylated mannoses (15·0 mg. consumed 1·09 c.c. of 0·01N-iodine). The difficulty of hydrolysis of 2 : 3-dimethyl methylmannoside is apparent from these results (compare Smith, *J. Amer. Chem. Soc.*, 1948, **70**, 3249, who found that methylmannoside required 25 hours at 95°).

A fourth hydrolysis was carried out with sulphuric acid (10 c.c.; 2N) for 7·5 hours, and the sugars were recovered in the usual way (0·128 g.). As the amount of trimethyl sugar was small, a separation of the tri- and di-methyl mannoses was effected on a wide strip of filter paper by heavy spotting of the solution along the starting line, two strips at the side being spotted separately as controls in a manner similar to that used for quantitative chromatography. After running for one night with *n*-butanol–ethanol–water solvent the side strips were developed with aniline oxalate solution, and the area of the centre strip containing the trimethyl sugar was cut out and extracted with boiling acetone from which the trimethyl mannose (8 mg.) was recovered by removal of the solvent *in vacuo*. Qualitative chromatography showed the presence of only one sugar in the syrup. This syrup was divided into two portions, one of which was subjected to demethylation with hydrobromic acid as described for Fraction D. The syrup thus obtained showed chromatographically the following sugars: 2 : 3-dimethyl mannose, R_G 0·54; monomethyl mannose, R_G 0·33; and free mannose, the presence of which was confirmed by comparison with controls. The other portion of the syrup was dissolved in water (2 c.c.) and oxidised with periodic acid (2 c.c.; 0·5M) for 40 hours, at the end of which the bulk of excess of periodate was removed as potassium periodate by addition of potassium chloride; the presence of formaldehyde was shown by the addition to the clear decanted solution of fresh solutions of phenylhydrazine hydrochloride and potassium ferricyanide, followed by two drops of concentrated hydrochloric acid: the characteristic port wine colour was then obtained. Of the trimethyl mannoses, only 2 : 3 : 4-trimethyl mannose would give formaldehyde on periodate oxidation.

Fraction B (0·046 g.). Rehydrolysis of this fraction showed that it consisted almost entirely of 2 : 3 dimethyl methylmannoside.

Fraction C (0·022 g.). Rehydrolysis of this fra ion showed that it consisted mainly of monomethyl methylmannoside.

Fraction D (0·058 g.). The syrup had OMe, 28·9%, and $[\alpha]_D^{15}$ +35° (*c*, 0·38 in water), suggestive of a dimethyl hexose. A portion of the syrup (5—10 mg.) was demethylated according to the method of Hough, Jones, and Wadman (*loc. cit.*) by treatment for 5 minutes in a sealed tube at 100° with hydrobromic acid (*d* 1·50; 1 c.c.), dilution with water (10 c.c.), neutralisation with silver carbonate, removal of silver ions with hydrogen sulphide, and recovery of the sugars as a syrup. Chromatographic examination showed the presence of glucose, a monomethyl sugar, R_G 0·27, and a little unchanged material. In a control experiment 3-methyl glucose

showed R_G 0·27. 2 : 3-Dimethyl mannose under the same conditions showed mannose, monomethyl mannose R_G 0·32, and the original sugar. These results indicate that this sugar is 2 : 3-dimethyl glucose and comparison with an authentic specimen showed that their R_G values were identical. The fraction was too small for further identification.

Fraction E. The syrup (0·204 g.) had OMe, 29·1% and, by use of a long filter strip (90 cm.), paper chromatography showed it to consist of two sugars, R_G values 0·54 and 0·59 respectively, identical with the sugar from Fraction D and 2 : 3-dimethyl mannose. A quantitative chromatogram was carried out in a similar way, the sugars being separated and their relative percentages determined by treatment with alkaline hypoiodite. Dimethyl glucose consumed 1·03 c.c., and dimethyl mannose 2·68 c.c., of 0·01N-iodine, indicating the presence of 27·7% of dimethyl glucose and 72·3% of dimethyl mannose.

Fraction F. The syrup (1·151 g.) had OMe, 27·4%, $[\alpha]_D^{15}$ −14·0 (*c*, 2·5 in water), and n_D^{15} 1·4830, and paper chromatography showed only one sugar to be present. On hypoiodite oxidation the syrup (2·2 mg.) consumed 2·00 c.c. of 0·01N-iodine, indicating a purity of 94·6%. Oxidation with bromine water converted the sugar into a lactone, $[\alpha]_D^{15}$ +67° falling to 58° after 7 days (equiv., 203). Oxidation of the syrup (31·4 mg.) with sodium metaperiodate (0·25M; 4 c.c.) for 18 hours resulted in the uptake of 1·75 mols. of $NaIO_4$ and the production of 0·85 mol. of formic acid per $C_8H_{16}O_6$ unit.

The syrup (0·178 g.) was treated with periodic acid (0·5M; 12 c.c.) for 24 hours, and the whole aërated for 18 hours to remove formaldehyde. To the solution were added barium chloride (0·6 g.) and excess of barium carbonate, and after filtration barium carbonate (0·5 g.) and bromine (1 c.c.) were added. Oxidation was allowed to continue for 36 hours, whereafter the bromine was removed by aëration. After neutralisation with silver carbonate, filtration, treatment with hydrogen sulphide, and filtration the solution was evaporated *in vacuo* to a solid. This was esterified by boiling methanolic hydrogen chloride (3%; 15 c.c.) for 16 hours, the solution neutralised with silver carbonate, and the solid obtained after filtration and evaporation was extracted with hot chloroform. The resulting extract on evaporation gave a thin syrup which crystallised on being dried overnight *in vacuo*. This was dimethyl *meso*dimethoxysuccinate, m. p. 65—66° not depressed on admixture with an authentic specimen (Found: C, 46·0; H, 7·1. Calc. for $C_8H_{14}O_6$: C, 45·5; H, 6·8%). *X*-Ray powder photographs of the two specimens were compared and the results confirmed the identity of the two samples.

Fraction G. The syrup (0·087 g.) had OMe, 12·0%. 3·4 Mg. consumed 2·47 c.c. of 0·01N-iodine during hypoiodite oxidation, indicating the presence of 70·7% of free reducing sugar as monomethyl hexose. Chromatographic investigation showed no sugars other than monomethyl mannose (R_G 0·34).

Effect of Lithium Aluminium Hydride on 2 : 3-Dimethyl Mannose.—In order to ascertain whether epimerisation of dimethyl mannose can take place during reduction with lithium aluminium hydride, a portion (0·226 g.) of Fraction F was converted into the methylmannoside by boiling methanolic hydrogen chloride (2%; 10 c.c.) during 7 hours. The methylmannoside was isolated as a syrup in the usual way ($[\alpha]_D^{15}$ +21°, n_D^{15} 1·4687). By following exactly the conditions of the original reduction, a syrup was obtained which showed, on chromatographic examination, none of the glucose derivative observed in the earlier experiments, indicating that under these conditions no epimerisation had occurred. The experiment did, however, suggest that the reduction procedure had involved some demethylation as a small amount of monomethyl mannose (R_G 0·34) was indicated on the chromatogram. This demethylation along with under-methylation probably accounts for much of the monomethyl mannose present in Fraction G.

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