208. *The Structure of Alginic Acid. Part V.*[1] *Isolation and Unambiguous Characterization of Some Hydrolysis Products of the Methylated Polysaccharide.*

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2,3-Di-*O*-methyl-D-mannose and 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose have been isolated from methylated alginic acid after hydrolysis and reduction. Both methyl sugars have been characterized unambiguously for the first time from this source.

Evidence relating to the types of glycosidic linkage present in alginic acid has recently been obtained by partial hydrolysis of the reduced polysaccharide,[1] by alkaline degradation,[2] and by oxidation and hydrolysis of the periodate-oxidized polysaccharide.[3,4] These different approaches agree in pointing to the presence of 1,4-linked D-mannuronic acid and L-guluronic acid units, although the possibility has been raised that linkages of other types might occur in addition.[1,3]

Potentially the most useful method for obtaining information about the structural units present in polysaccharides is methylation analysis, but this has met with relatively little

[1] Part IV, Hirst, Percival, and Wold, *J.*, 1964, 1493.
[2] Whistler and BeMiller, *J. Amer. Chem. Soc.*, 1960, **82**, 457.
[3] Drummond, Hirst, and Percival, *J.*, 1962, 1208.
[4] Lucas and Stewart, *J. Amer. Chem. Soc.*, 1940, **62**, 1792.

success so far in the case of alginic acid. On neither of the two occasions when this method was used [5,6] was it possible to identify unequivocally any methylated sugar after hydrolysis of the methylated polysaccharide. The positive evidence obtained was that the methylated sugar(s) could be degraded to di-*O*-methylerythraric acid. The isolation of this compound, together with the fact [5,7] that D-mannuronic acid was a component of alginic acid, was taken to show that 2,3-di-*O*-methyl-D-mannuronic acid units had been present in the methylated polysaccharide. That this inference may not have been valid became apparent with the subsequent isolation of L-guluronic acid from alginic acid.[8] The di-*O*-methylerythraric acid could therefore have been derived from the 2,3-di-*O*-methyl ether of either mannuronic acid or guluronic acid, or from a mixture of both. We now report a more successful methylation study of alginic acid, and offer explanations for some of the results of the earlier workers.

Alginic acid from *Laminaria cloustoni* stipes was converted into its methylated derivative by repeated treatment with dimethyl sulphate and potassium hydroxide until the theoretical degree of etherification had been achieved. Hydrolysis of this product with hot 50% formic acid gave a mixture of methylated uronic acids which was converted into the corresponding mixture of methyl ester methyl glycosides, and then reduced with lithium aluminium hydride. Further hydrolysis gave a mixture which was subsequently shown to contain 2,3-di-*O*-methyl-D-mannose, 2,3-di-*O*-methyl-L-gulose, and 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose. Mono-*O*-methyl- and tri-*O*-methyl sugars were barely detectable on paper chromatograms, suggesting that there is no branching in this sample of alginic acid. The mixture of 2,3-di-*O*-methylmannose and 2,3-di-*O*-methylgulose travelled as a single compact spot on paper chromatography in several solvent systems, and separation of the mixture on a cellulose column was therefore not attempted. Instead, advantage was taken of the greater ease with which the gulose derivative forms a 1,6-anhydride; by alternately heating an acid solution of the mixture and extracting with chloroform, all of the gulose derivative was eventually removed from the aqueous layer. Neutralization and concentration gave a syrup from which crystalline 2,3-di-*O*-methyl-D-mannonophenylhydrazide was readily prepared.

The material which extracted into chloroform included some mannose derivatives in addition to 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose. This was shown by paper chromatography of the products of demethylation. However, the amount of these contaminants was small (5—10% of the mixture) and the anhydrogulose derivative was readily purified by conversion into the crystalline *p*-nitrobenzoate, followed by repeated recrystallization. That the compound was indeed a derivative of L-gulose was shown by demethylation [9] and preparation of crystalline 1,6-anhydro-2,3,4-tri-*O*-benzoyl-β-L-gulopyranose, identical in all respects except the sign of optical rotation with the anhydro-D-gulose derivative which had been prepared and characterized by Stewart and Richtmyer [10] and a sample kindly given by Dr. N. K. Richtmyer to Dr. E. E. Percival. The *p*-nitrobenzoate was shown to be derived from an anhydrodi-*O*-methylhexose by analysis and by the proton magnetic resonance spectrum. The sequence ring-opening by acetolysis, deacylation, periodate oxidation, bromine oxidation, and treatment with methanolic hydrogen chloride gave the crystalline methyl ester of di-*O*-methylerythraric acid, thus proving the location of the two methyl groups at positions 2 and 3. The 1,6-anhydropyranose structure is inferred because this is the type of anhydride that is formed when gulose itself is heated with acid.[10] Further confirmation for the structures of the 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose and its *p*-nitrobenzoate was the satisfactory

[5] Hirst, Jones, and Jones, *J.*, 1939, 1880.
[6] Chanda, Hirst, Percival, and Ross, *J.*, 1952, 1833.
[7] Nelson and Cretcher, *J. Amer. Chem. Soc.*, 1929, **51**, 1914; 1932, **54**, 3409; Bird and Haas, *Biochem. J.*, 1931, **25**, 403.
[8] Fischer and Dörfel, *Z. physiol. Chem.*, 1955, **302**, 186.
[9] Bonner, Bourne, and McNally, *J.*, 1960, 2929.
[10] Stewart and Richtmyer, *J. Amer. Chem. Soc.*, 1955, **77**, 1021.

assignment of the peaks and coupling constants in their proton magnetic resonance spectra and in that of the tri-*O*-benzoate (see Experimental section).

The isolation of the 2,3-di-*O*-methyl derivatives of both mannose and gulose, raises again the question whether one sugar is an artefact derived from the other at some stage during the extraction or subsequent treatment. Evidence has previously been presented [3] that such a transformation does not occur during the alkaline extraction of alginic acid, and Fischer and Dörfel [8] found no evidence for the acid-catalysed interconversion of mannuronic and guluronic acids. These results make the formation of one of these acids by the inadvertent chemical modification of the other seem unlikely, but as a further check we have treated mannurone with the sequence of reagents methanolic hydrogen chloride, aqueous formic acid, methanolic hydrogen chloride, lithium aluminium hydride, and sulphuric acid under conditions approximating to those that were used for the depolymerization and reduction of the methylated polysaccharide. No evidence was found for the formation of gulose by paper chromatography, and we therefore conclude that both 2,3-dimethylmannose and 2,3-di-*O*-methylgulose represent genuine structural features in alginic acid.

2,3-Di-*O*-methyl-D-mannose and 2,3-di-*O*-methyl-L-gulose were found to be inseparable in the solvent systems used for chromatography by Chanda *et al.*,[6] as also were the parent sugars, mannose and gulose, which would be obtained from them by demethylation. These facts explain how the gulose derivative escaped the notice of these earlier workers. The presence of 2,3-di-*O*-methyl-L-gulose in the di-*O*-methylmannose fraction which was isolated by them after column chromatography would no doubt make the formation of a crystalline derivative difficult, and might account for the failure to prepare such, despite repeated attempts.[6,11] In retrospect, the " methyl 2,3-di-*O*-methylmannoside " which was isolated after cellulose column chromatography, and which resisted complete hydrolysis by acid, is more likely to have been 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose, which cannot of course be completely hydrolysed by aqueous acid to the free sugar, but only equilibriated with it. The optical rotation (−83·7°) quoted for the substance is rather far removed from the value (+20°) for the most stable methyl glycoside of 2,3-di-*O*-methylmannose [12] and much closer to the value found in the present work for the anhydrodi-*O*-methylgulose derivative (−97°).

In conclusion, it seems likely that the 2,3-di-*O*-methyl-D-mannose and 2,3-di-*O*-methyl-L-gulose derivatives which have been firmly characterized in the present work, arise from 1,4-β-linked mannuronic acid and 1,4-linked (possibly α) guluronic acid, which are therefore the major structural units in this sample of alginic acid—although it should be noted that such methylation evidence fails to distinguish between 1,4- and 1,5-linkages. The fine structural details of alginic acid, including the sequence of units and the possible occurrence of small amounts of non-1,4-linked residues cannot be discussed on the basis of the present results. However, it does appear that if other linkages occur they are not so placed as to give rise to branching in the molecule—the mono- and tri-methyl sugars which would have arisen as a result would have been readily detected by paper chromatography.

Experimental

The paper chromatography solvents used for general purposes were butanol–ethanol–water (4 : 1 : 5) and butanol–acetic acid–water (4 : 1 : 5). For the separation of mannose and gulose, ethyl methyl ketone–acetic acid–water (9 : 1 : 1) saturated with boric acid was used. *p*-Anisidine hydrochloride spray [13] was used to detect reducing carbohydrates, and a modification of the periodate–benzidine reagents [14] for non-reducing carbohydrates. Thin layer chromatography

[11] S. K. Chanda, Research Notebooks, Edinburgh University, 1949—1951.
[12] Smith, *J. Amer. Chem. Soc.*, 1948, **70**, 3249.
[13] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[14] Gordon, Thornburg, and Werum, *Analyt. Chem.*, 1956, **28**, 849.

was carried out on microscope slides (N. G. Richardson, unpublished method) coated with Silica Gel G containing calcium sulphate as binder, with benzene–ethanol (2 : 1) as solvent and anisaldehyde–sulphuric acid spray.[15] Melting points are corrected. Evaporations to dryness were carried out under diminished pressure.

Methylation of Alginic Acid.—The polysaccharide, which had been extracted from *Laminaria cloustoni* stipes, was kindly given by Mr. R. H. McDowell of Alginate Industries Ltd. The sodium salt (40 g.) was methylated repeatedly, using essentially the same method as described earlier [6] to give methylated potassium alginate (27·5 g. Found: OCH_3, 25·0%).

Hydrolysis and Reduction.—Conversion of the polysaccharide (10 g.) into the acid form by passage through Amberlite IR-120 resin, followed by esterification by suspending the freeze-dried material in methanol and treating with ethereal diazomethane gave a product (isolated by evaporation to dryness) which was completely insoluble in tetrahydrofuran. Preliminary hydrolysis of the polysaccharide was therefore necessary before reduction could be effected. After evaporation of the tetrahydrofuran, the residue dissolved readily when formic acid (150 ml.) and water (150 ml.) were added. The solution was heated on a boiling-water bath (8 hr.), evaporated to dryness, and after the residue had been dried thoroughly in a vacuum oven at 60°, 3% methanolic hydrogen chloride (150 ml.) was added and boiled under reflux for 6 hr. The solution was neutralized (sodium bicarbonate; similar results were obtained when silver carbonate was used), filtered, and concentrated to dryness. Reduction was effected by treatment with lithium aluminium hydride (4 g.) in tetrahydrofuran (200 ml.) for 16 hr. at room temperature and then 4 hr. under reflux. Excess of hydride was destroyed by the addition of ethyl acetate, and the minimum quantity of water was added to precipitate lithium aluminate. The solution was filtered and evaporated to dryness, and the residue heated with N-sulphuric acid (300 ml.) to achieve complete hydrolysis. After neutralisation (calcium carbonate) and de-ionization (Amberlite IR-120 and IR-45 resins), the solution was evaporated to a golden brown syrup (Fraction A; 5·8 g.). Paper chromatography showed a single round intense spot with the same mobility as 2,3-di-*O*-methylmannose, with no other component detectable by the *p*-anisidine hydrochloride spray.

Non-interconversion of Mannuronic and Guluronic Acids under these Conditions.—D-Mannurone (m. p. 188—190°; 20 mg.) was heated under reflux with 3% methanolic hydrogen chloride (10 ml.) for 24 hr. and after neutralization with silver carbonate the solution was concentrated to dryness. The residue was heated under reflux, with 50% aqueous formic acid (10 ml.) for 24 hr., and the solution was again concentrated to dryness. The treatment with methanolic hydrogen chloride was repeated, and the mixture of glycoside esters obtained by evaporation to dryness was reduced with excess of lithium aluminium hydride in tetrahydrofuran (10 ml.) using essentially the same method for the reduction, work-up, and hydrolysis as described above. Paper chromatography showed the presence of mannose only.

Separation of the Mixture of Methylated Sugars.—Fraction A (5·5 g.) was dissolved in water (100 ml.) and extracted continuously with chloroform in a liquid–liquid extractor, the process being followed by thin-layer chromatography. After 1—2 hr., the aqueous solution was removed and heated on a boiling-water bath with 2N-sulphuric acid (50 ml.) for 15 hr. The acid solution was then extracted with chloroform, and the cycle of heating and extraction was repeated a further six times. In removing the last traces of gulose derivative, a small amount of reducing sugar was also extracted into the chloroform and this small mixed fraction (0·44 g.) was kept separate. The two further fractions obtained (i) by keeping the other chloroform extracts over sodium bicarbonate and then sodium sulphate followed by evaporation to dryness (Fraction B: 4·6 g.), and (ii) by neutralization (calcium carbonate), filtration, and evaporation of the aqueous layer (Fraction C: 0·44 g.)—were more pure as judged by thin layer chromatography.

After bromine oxidation and treatment with phenylhydrazine in ethanol, Fraction C gave 2,3-di-*O*-methyl-D-mannonophenylhydrazide,[16] m. p. and mixed m. p. 170° (slow heating).

Gulose and Dimethylgulose Derivatives from Fraction B.—Fraction B (0·44 g.) was treated with boron trichloride (10 g.) in dichloromethane (15 ml.) at −80° and then at room temperature.[9] The solution was evaporated to dryness and boric acid removed by distillation of methanol from the residue. Partial conversion of the gulose into the 1,6-anhydride was effected by

[15] Stahl and Kaltenbach, *J. Chromatog.*, 1961, **5**, 351.

[16] Hirst and Jones, *J.*, 1948, 1278; Smith, *J. Amer. Chem. Soc.*, 1948, **70**, 3249; Rafique and Smith, *ibid.*, 1950, **72**, 4634; Whistler and Saarnio, *ibid.*, 1957, **79**, 6055.

heating in N-sulphuric acid on a boiling-water bath (16 hr.) and the anhydride isolated by thick paper chromatography and converted into 2,3,4-tri-*O*-benzoyl-β-L-gulopyranose with benzoyl chloride in pyridine. After purification by adsorption on a silica gel column and elution with benzene–ether (9 : 1),[17] followed by two recrystallizations from ethanol, the derivative (0·1 g.) had m. p. 158·5—159°, $[\alpha]_D$ −216° (*c* 1·1 in $CHCl_3$), and an *X*-ray powder photograph identical with that given by the material synthesized by Stewart and Richtmyer.[10]

In addition to gulose and anhydrogulose which were the major components of demethylated fraction B, small amounts of mannose were also detected by paper chromatography—perhaps arising from a contaminating anhydromannose derivative. Purification was effected by treatment with *p*-nitrobenzoyl chloride in pyridine [18] to give crystalline 1,6-anhydro-2,3-di-*O*-methyl-4-*O*-*p*-nitrobenzoyl-β-L-gulopyranose in 60% yield. After repeated crystallization from ethanol, the thin white needles had m. p. 174—174·5°, $[\alpha]_D$ −70·5° (*c* 1·2 in $CHCl_3$) [Found: C, 53·3; H, 5·25; N, 4·7; OCH_3, 17·9. Calc. for $C_{13}H_{13}O_6N(OCH_3)_2$: C, 52·8; H, 5·6; N, 4·1; OCH_3, 18·2%]. Treatment of this compound (0·06 g.) with dry methanol (4 ml.) containing sodium (2 mg.) at room temperature followed by neutralization with solid carbon dioxide and evaporation gave 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose $[\alpha]_D$ −97° (*c* 0·5 in water) which after washing an aqueous solution with benzene, was pure as judged by thin layer chromatography and proton magnetic resonance spectroscopy.

Degradation of the Dimethylgulose Derivative to Methyl Dimethylerythrarate.—1,6-Anhydro-2,3-di-*O*-methyl-4-*O*-*p*-nitrobenzoyl-β-L-gulopyranose (0·105 g.) was dissolved in a mixture of acetic anhydride (5 ml.) and concentrated sulphuric acid (0·05 ml.) and set aside at room temperature for 20 hr. The solution was poured into water (100 ml.) and treated with solid sodium bicarbonate until it was neutral and effervescence ceased, and then extracted with chloroform. The chloroform solution was dried (sodium sulphate) and evaporated to a syrup, which was dissolved in dry methanol (6 ml.) containing sodium (2 mg.). Thin layer chromatography showed that the deacylation reaction was complete in 10 minutes and that the sole product had the mobility of dimethylgulose, with no anhydride detectable. Excess of solid carbon dioxide was added and the solution was concentrated to dryness. The residue was treated with 0·25M-periodic acid solution (10 ml.) at room temperature (white crystals, presumably of methyl *p*-nitrobenzoate did not dissolve), and after 2 days the reaction was stopped by addition of barium hydroxide solution to neutrality. The filtrate and washings were concentrated (10 ml.) and oxidized at room temperature for 2 days with bromine (2 ml.) in the presence of barium carbonate (2 g.). After aeration, filtration, and evaporation, the dimethylerythraric acid was esterified by boiling the residue under reflux with 3% methanolic hydrogen chloride (10 ml.) for 16 hr. The solution was neutralized (sodium bicarbonate), filtered, and concentrated to dryness. Extraction of the solid residue with chloroform gave methyl dimethylerythrarate [5,6] which, after purification in a vacuum sublimation apparatus (water-pump) and recrystallization from ether–light petroleum had m. p. and mixed m. p. 67° (0·015 g.).

Proton Magnetic Resonance Spectra of 1,6-*Anhydrogulose Derivatives.*—The three spectra [in $CDCl_3$ solution, using a Perkin-Elmer R10 (60 Mc./sec.) nuclear magnetic resonance spectrometer with tetramethylsilane as an internal standard] could be satisfactorily interpreted (compare ref. 19) on the premise that all three compounds (1,6-anhydro-tri-*O*-benzoyl-β-L-gulopyranose; 1,6-anhydro-2,3-di-*O*-methyl-4-*O*-*p*-nitrobenzoyl-β-L-gulopyranose, and 1,6-anhydro-2,3-di-*O*-methyl-β-L-gulopyranose) exist in the Reeves C1 conformation. Thus, in the spectrum of the *p*-nitrobenzoate, $H_{(1)}$ appeared as a doublet at τ 4·39 ($J_{1,2}$ = 2·1 c./sec.), $H_{(4)}$ as a quartet at τ 4·56 ($J_{3,4}$ = 9·9 c./sec.; $J_{4,5}$ = 4·3 c./sec.), $H_{(5)}$ as a triplet at τ 5·29 ($J_{4,5} = J_{5,6}$ = 4·3 c./sec.), $H_{(6')}$ as a doublet at τ 5·90 ($J_{6,6'}$ = 8·2 c./sec.), and $H_{(2)}$, $H_{(3)}$, and $H_{(6)}$ occurred together as a multiplet centred on τ 6·2; the spectrum of the tri-*O*-benzoate, as well as the $H_{(5)}$ triplet at τ 5·13 ($J_{4,5} \simeq J_{5,6} \simeq$ 4·3 c./sec.) and the $H_{(6')}$ doublet at τ 5·81 ($J_{6,6'}$ = 8·2 c./sec.) (see also below) clearly showed $H_{(6)}$ as a quartet at τ 6·22 ($J_{6,6'}$ = 8·2 c./sec.; $J_{5,6}$ = 4·4 c./sec.) in agreement with these assignments. Further evidence in favour of these assignments was obtained by comparing the spectra of the di-*O*-methyl compound and the tri-*O*-benzoate; only the peaks which had been assigned to $H_{(2)}$, $H_{(3)}$, and $H_{(4)}$ were shifted downfield in the spectrum of the benzoate (appearing together as a multiplet centred on τ 4·3)—as would be expected since the environment of these protons would be the most affected in going from one

[17] Jeanloz and Jeanloz, *J. Amer. Chem. Soc.*, 1958, **80**, 5692.
[18] Rebers and Smith, *J. Amer. Chem. Soc.*, 1954, **76**, 6097.
[19] Hall and Hough, *Proc. Chem. Soc.*, 1962, 382.

compound to the other. The most important observation in relation to the structure determination of the *p*-nitrobenzoate was that on deacylation the only significant change in the ring proton spectrum was that the $H_{(4)}$ quartet (see above) was moved upfield to around τ 6—7. This confirms the location of the ester group at position 4, and therefore that of the methyl ether groups at positions 2 and 3. The internally consistent assignments of the peaks and coupling constants to the various ring protons may be regarded as supporting evidence for the 1,6-anhydrogulopyranose structure in these compounds.

The value found for the ratio of aromatic : methyl : sugar ring protons was in excellent agreement with the suggested structure in each case.

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