674. *The Structure of the Extracellular Polysaccharide of* Aerobacter aerogenes A3 (S1) (*Klebsiella Type* 54).

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Preliminary studies have shown that the extracellular slime polysaccharide of *Aerobacter aerogenes* A3 (S1) possesses a highly branched molecular structure containing residues of D-glucose, L-fucose, and glucuronic acid. Cellobiose has been identified amongst the products of partial acid hydrolysis. A number of methylated sugars, including 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, 2 : 3-di-*O*-methyl-D-glucose, 3 : 5-di-*O*-methyl-L-fucose, and 2-*O*-methyl-L-fucose, have been characterised from the products of hydrolysis of the methylated polysaccharide.

In a previous study [1] it was shown that the extracellular slime polysaccharide of *Aerobacter aerogenes* A3 (S1) (Klebsiella Type 54), isolated as the free acid, was composed of three sugar residues, D-glucose (46%), L-fucose (10%), and an unidentified uronic acid (27%), together with traces of galactose (*ca.* 2%). It was also shown that the composition of the polysaccharide was independent of the carbon source, when the bacterium was grown in the presence of a variety of carbohydrates as the sole carbon and energy source. A start has now been made in the determination of the molecular structure.

The nature of the uronic acid residue was established as glucuronic acid by the following observations: (*a*) glucurone was detected by paper chromatography when the polysaccharide was hydrolysed with 4N-hydrochloric acid; (*b*) glucose was the only hexose detected in significant amount when the polysaccharide was hydrolysed after reduction of the acidic residues (as methyl esters) with potassium borohydride; and (*c*) estimation of the glucose and fucose formed on hydrolysis of the polysaccharide, before and after reduction of the uronic acid to hexose residues, showed that the ratio (by weight) of glucose to fucose increased from 4·0 : 1 to 5·8 : 1. Although the value obtained for the ratio of glucose to fucose given on direct hydrolysis of the polysaccharide was slightly different from that obtained in the previous investigation,[1] it is clear that treatment of the uronic acid residues (as methyl ester) with potassium borohydride has resulted in the formation of further glucose residues; it is probable, however, that reduction with this reagent (cf. sodium borohydride [2]) was not complete.

Chromatographic examination of the products of graded hydrolysis of the polysaccharide showed that some of the glucose was released readily and that shortly afterwards cellobiose could also be detected. On prolonged heating, fucose, traces of galactose, and more glucose were formed. On a larger scale the products of partial acid hydrolysis were separated chromatographically, and cellobiose and D-glucose were identified as crystalline derivatives. The partially degraded polysaccharide yielded L-fucose and more D-glucose on more vigorous hydrolysis, together with a mixture of acidic oligosaccharides, all of which gave glucose, fucose, and glucurone after drastic hydrolysis.

The polysaccharide was methylated by Fear and Menzies's method.[3] The methylated sugars obtained on hydrolysis of the methylated polysaccharide were fractionated chromatographically on cellulose,[4] and the following were characterised as crystalline derivatives: 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, 2 : 3-di-*O*-methyl-D-glucose, 3 : 5-di-*O*-methyl-L-fucose, and 2-*O*-methyl-L-fucose. Evidence was also obtained for the presence of a trace of 2 : 3 : 4-tri-*O*-methyl-D-glucose, a mixture of tri-*O*-methyl-D-glucoses (including the 2 : 3 : 6-isomer, as shown by the downward change in rotation in methanolic hydrogen chloride), a mixture of 2 : 6- and 3 : 6-di-*O*-methyl-D-glucoses, and a mixture of mono-*O*-methylglucoses (including the 2- and the 3-methyl ether). In addition, a complex acidic fraction was obtained from this hydrolysis, but attempts to isolate individual components failed. After hydrolysis under drastic conditions, chromatography showed tri-, di-, and

[1] Wilkinson, Dudman, and Aspinall, *Biochem. J.*, 1955, **59**, 446.
[2] Wolfrom and Anno, *J. Amer. Chem. Soc.*, 1952, **74**, 5583.
[3] Fear and Menzies, *J.*, 1926, 937.
[4] Hough, Jones, and Wadman, *J.*, 1949, 2511.

mono-*O*-methyl-uronic acids to be present; furthermore, after reduction of the acidic fraction with lithium aluminium hydride, hydrolysis yielded sugars travelling on the chromatogram at the same rate as tri-, di-, and mono-*O*-methylglucoses.

The previously unknown sugar 3 : 5-di-*O*-methyl-L-fucose was obtained crystalline, and its structure was established from the following observations: (*a*) demethylation showed the sugar to be a derivative of fucose; (*b*) the derived di-*O*-methyl-L-fuconolactone underwent hydrolysis at a rate characteristic of γ-lactones, and the sign of the optical rotation, according to Hudson's lactone rule,[5] was only consistent with that of a 1 : 4-lactone; (*c*) the consumption of 1 mol. of periodate by the sugar suggested that the sugar was the 3 : 5-dimethyl ether rather than the 2 : 5-dimethyl ether of L-fucose, and the absence of a methoxyl group at $C_{(2)}$ was confirmed, as the derived di-*O*-methyl-L-fuconamide gave a positive Weerman test.

It is now clear that the molecule is highly branched and that the following sugar residues are definitely present:

$$\text{Gp 1}\cdots,\quad \cdots\text{4 Gp 1}\cdots,\quad \cdots\text{2 Fucf 1}\cdots,\quad \cdots\text{4(or 5) Fuc 1}\cdots \begin{matrix}\\ \\ 3\\ \vdots\\ \vdots\end{matrix}$$

(Gp = D-glucopyranose, Fucf = L-fucofuranose)

The isolation of cellobiose on graded hydrolysis of the polysaccharide shows that adjacent β-1 : 4-linked D-glucopyranose residues are present. It is not clear, however, which of the dimethyl and monoethyl ethers of D-glucose have structural significance as arising from branching points and which have arisen from incomplete methylation of the polysaccharide and/or demethylation during hydrolysis. On the other hand, it is certain that some of the L-fucose residues are branching points in the molecule as 2-*O*-methyl-L-fucose cannot have been formed from the incomplete methylation of 3 : 5-di-*O*-methyl-L-fucose, the only other methyl-L-fucose isolated. The release of 1 mole of formic acid per 3 sugar residues on periodate oxidation of the polysaccharide suggests the presence of a high proportion of non-reducing terminal groups and/or 1 : 6-linked D-glucose residues. In view of the small amount of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose isolated on hydrolysis of the methylated polysaccharide and in the absence of more than traces of 2 : 3 : 4-tri-*O*-methyl-D-glucose, it seems probable that some of the glucuronic acid residues may occupy terminal positions in the molecule.

Experimental

Paper partition chromatography was carried out on Whatman No. 1 filter paper with the solvent systems: (*A*) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3; v/v; top layer); (*B*) butan-1-ol–ethanol–water (4 : 1 : 5; v/v; top layer); (*C*) butan-1-ol–acetic acid–water (4 : 1 : 5; v/v; top layer); (*D*) butan-1-ol saturated with 5% aqueous formic acid.

Isolation and Examination of the Extracellular Polysaccharide.—The organism was grown in a buffered medium (pH 7·3) containing D-glucose as sole carbon source, and the extracellular polysaccharide was isolated as the free acid as described by Wilkinson, Dudman, and Aspinall.[1] The polysaccharide had an equivalent of 683 (by titration), corresponding to 25·8% uronic anhydride (compare 29·0% uronic anhydride previously found). The uronic anhydride content was also determined by Kaye and Kent's method [6] after the polysaccharide had been converted into the corresponding methyl ester by heating with methanolic hydrogen chloride, a value of 25·5% being obtained. Direct reaction of the polysaccharide with the same reagents showed that ester and lactone groups were absent and no methoxyl content was detected. When the polysaccharide was hydrolysed and the hydrolysate was examined chromatographically, the three sugars described previously (glucose, fucose, and galactose) were detected, but after hydrolysis with 4N-hydrochloric acid glucurone was also found.

Hydrolysis of the Polysaccharide after Reduction with Potassium Borohydride.—The polysaccharide (18·3 mg.) was heated in a sealed tube with 98% formic acid (2 c.c.) at 100° for 24 hr., the formic acid was removed *in vacuo*, L-rhamnose (5·45 mg.) was added, and the mixture was refluxed with methanolic 1% hydrogen chloride (5 c.c.) for 4 hr. After neutralisation with silver carbonate the product was dissolved in water (2 c.c.), and the solution was

[5] Hudson, *J. Amer. Chem. Soc.*, 1910, **32**, 338.
[6] Kaye and Kent, *J.*, 1953, 79.

added dropwise to a solution of potassium borohydride (20 mg.) in water (2 c.c.). After 20 min. the excess of borohydride was destroyed by dilute acetic acid, and the solution was de-ionised with Amberlite resins IR-120(H) and IR-4B(OH). The reduction product was hydrolysed with N-hydrochloric acid (4 c.c.) at 100° for 4 hr. and neutralised, and the ratios of the sugars obtained were determined. A second sample of polysaccharide was treated similarly except that the reduction with potassium borohydride was omitted.

The results showed that the ratio of glucose to fucose was 5·8 : 1 in the former case and 4·0 : 1 when the reduction was omitted. The ratios of fucose to the reference sugar, rhamnose, were the same in both cases.

Graded Hydrolysis of the Polysaccharide.—Chromatographic examination of the products of the hydrolysis of the polysaccharide with hot 0·5N-hydrochloric acid, showed that glucose was released after 5 min. and that after 30 min. a disaccharide travelling on the chromatogram at the same rate as cellobiose was observed. On further heating fucose and traces of galactose were also released.

The polysaccharide (2 g.) was heated at 100° with 0·5N-sulphuric acid (100 c.c.) for 30 min. The cooled solution was neutralised with barium carbonate, and the filtrate was reduced in volume to 10 c.c. and poured into acetone (20 c.c.). The precipitate was redissolved in water, and the solution de-ionised with Amberlite resin IR-120(H) and taken to dryness to give fraction *X* (0·84 g.). The supernatant liquid remaining after the acetone precipitation was taken to dryness to give fraction *Y* (0·70 g.). Most (0·67 g.) of this fraction was separated on filter sheets by using solvent *A* to give small fractions containing sugars travelling on the chromatogram at the same rate as glucose, galactose, and cellobiose, whilst most of the material, fraction *Y*(i) (0·53 g.), remained at the starting line. D-Glucose and cellobiose were identified by conversion into 1 : 2 : 3 : 4 : 6-penta-*O*-acetyl-β-D-glucose, m. p. 126—128° and mixed m. p. 127—130°, and cellobiose α-octa-acetate, m. p. and mixed m. p. 220—222°, respectively.

Chromatographic examination of fractions *X* and *Y*(i) in solvent *C* showed them to contain similar mixtures of acidic oligosaccharides. It was not possible completely to separate the components, but all the fractions examined yielded on further hydrolysis D-glucose, L-fucose (identified as the toluene-*p*-sulphonylhydrazone, m. p. and mixed m. p. 167—170°), galactose (trace), and glucurone. When the acidic fractions were reduced by treatment of the corresponding methyl ester methyl glycosides with potassium borohydride, the hydrolysis products contained glucose, fucose, and galactose (trace).

Methylation of the Polysaccharide.—The polysaccharide (5 g.) was converted into its thallium derivative which was heated with methyl iodide, and the product was further methylated by three treatments with thallous ethoxide and methyl iodide; this gave a methylated polysaccharide (3·7 g.) (Found : OMe, 39·6%), whose methoxyl content could not be raised on further treatments with thallous ethoxide and methyl iodide.

Hydrolysis of the Methylated Polysaccharide and Separation of the Methylated Sugars.—The methylated polysaccharide (3·2 g.) was hydrolysed successively with methanolic 1% hydrogen chloride (200 c.c.) for 17 hr. (constant rotation) and with hydrochloric acid (200 c.c.; 0·5N) for 10·5 hr. (constant rotation). The solution was neutralised with silver carbonate, the silver ions were removed with hydrogen sulphide, and the acidic components were converted into barium salts by treatment with barium carbonate. The solution was evaporated to a dark brown glass (2·8 g.).

The hydrolysate was dissolved in the minimum quantity of water, and the resulting syrup was allowed to soak into the top of a column (60 × 3 cm.) of cellulose. Elution of the column with light petroleum (b. p. 100—120°)–butan-1-ol (7 : 3; later 1 : 1), saturated with water, butan-1-ol partly saturated with water, and water gave nine fractions.

Fraction 1. The syrup (8 mg.) had $[\alpha]_D^{20}$ +87° (*c*, 0·4 in H_2O) and travelled on the chromatogram at the same rate as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. The sugar was identified as this compound by conversion into the aniline derivative, m. p. 123—124° and mixed m. p. 125—127° (with an authentic sample, m. p. 128—129°). In a separate series of experiments 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose crystallised, had m. p. and mixed m. p. 84—86°, and its *X*-ray powder photograph (courtesy of Dr. C. A. Beevers) was identical with that of an authentic specimen.

Fraction 2. The syrup (5 mg.) had $[\alpha]_D^{20}$ +63°, travelled on the chromatogram at the same rate as 2 : 3 : 4-tri-*O*-methyl-D-glucose (R_{Gl} 0·87), and gave glucose on demethylation (Found : OMe, 41·2. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction 3. Chromatographic examination of the syrup (122 mg.) showed the presence of two components travelling at the rates of 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methyl-D-glucose

(R_G 0·81—0·83) (Found: OMe, 41·6. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). Demethylation gave only glucose and the presence of 2 : 3 : 6-tri-*O*-methyl-D-glucose in the mixture was shown by the fall in rotation in methanolic 2% hydrogen chloride at room temperature {$[\alpha]_D^{20}$ +73° ⟶ +35° (26 hr., const.; *c*, 1·8)}.

Fraction 4. The syrup (76 mg.) was chromatographically homogeneous (R_G 0·79—0·80 in solvent *B*), gave a green colour with aniline oxalate, had $[\alpha]_D^{20}$ −72° ⟶ −67° (24 hr., const.; *c*, 1·5 in H_2O), and yielded fucose on demethylation with hydriodic acid (Found: OMe, 32·2. Calc. for a 6-deoxy-di-*O*-methylhexose, $C_8H_{16}O_5$: OMe, 32·3%). The derived 6-deoxy-di-*O*-methylhexonolactone had $[\alpha]_D^{20}$ +66° ⟶ +51° (7 days, const.; *c*, 0·66 in H_2O) and was converted into a 6-deoxy-di-*O*-methylhexonamide, which did not crystallise but yielded hydrazodicarbonamide, m. p. and mixed m. p. 252—254°, when treated with sodium hypochlorite. The original syrup consumed 1·15 moles of periodate per $C_8H_{16}O_5$ unit.

In separate experiments 3 : 5-*di*-O-*methyl*-L-*fucose* was obtained crystalline; it had m. p. 118—121°, $[\alpha]_D^{20}$ −100° ⟶ −69° (24 hr., const.; *c*, 0·42 in H_2O) (Found: OMe, 32·2. $C_8H_{16}O_5$ requires OMe, 32·3%).

Fraction 5. The syrup (45 mg.) travelled on the chromatogram at the same rate as 2 : 3-di-*O*-methyl-D-glucose and gave glucose on demethylation. The sugar had $[\alpha]_D^{20}$ +64° (equil.; *c*, 0·52 in H_2O) (Found: OMe, 29·7. Calc. for $C_8H_{16}O_6$: OMe, 29·7%), and was identified by conversion into 2 : 3-di-*O*-methyl-*N*-phenyl-D-glucosylamine, m. p. and mixed m. p. 129—131°.

Fraction 6. The syrup (164 mg.) was chromatographically homogeneous (R_G 0·54 in solvent *B*) but the methoxyl content (23·4%) suggested the presence of two components. Fractionation gave an acetone-insoluble fraction 6*a* (43 mg.) and an acetone-soluble fraction 6*b* (121 mg.). Fraction 6*a* was obtained crystalline and yielded fucose on demethylation. The sugar had m. p. 142—145° and mixed m. p. (with 2-*O*-methyl-L-fucose, m. p. 147—149°) 144—148°, $[\alpha]_D^{20}$ −71° (initial) ⟶ −81° (24 hr., const.; *c*, 0·33 in H_2O) (Found: OMe, 17·5. Calc. for $C_7H_{14}O_5$: OMe, 17·4%). Fraction 6*b* yielded glucose on demethylation, travelled on the chromatogram at the same rate as 2 : 6- and/or 3 : 6-di-*O*-methyl-D-glucose, and had $[\alpha]_D$ +60° (equil.; *c*, 2·0 in H_2O). Periodate oxidation of the derived methyl glycosides[7] showed that 83% of the fraction was 2 : 6-di-*O*-methyl-D-glucose. The periodate-oxidised methyl glycosides were hydrolysed, and chromatographic examination showed the presence of 3 : 6-di-*O*-methyl-D-glucose.

Fraction 7. The syrup (97 mg.) travelled on the chromatogram at the same rate as 2 : 6- and/or 3 : 6-di-*O*-methyl-D-glucose in solvent *B* but another component was detected by chromatography in solvent *A*. Fractionation of a portion of the syrup in solvent *A* yielded fraction 7*a* (40 mg.) and fraction 7*b* (4 mg.). Periodate oxidation of the derived methyl glycosides showed fraction 7*a* to contain 80% of 2 : 6-di-*O*-methyl-D-glucose, and hydrolysis of the periodate-oxidised methyl glycosides gave 3 : 6-di-*O*-methylglucose. Fraction 7*b* had R_G 0·50 in solvent *B* (yellow colour with aniline oxalate) and gave glucose on demethylation.

Fraction 8. Chromatographic examination of the syrup (190 mg.) in solvent *A* indicated only one component (R_G 0·20—0·22), but on examination in solvent *B* three substances were shown to be present, one of them travelling at the same rate as L-fucose. A portion of the syrup was oxidised by periodate according to Lemieux and Bauer's[8] procedure; chromatographic examination showed the products to be similar to those formed on oxidation of 2- and 3-*O*-methyl-D-glucose. The two faster components were separated from fucose on filter sheets by using solvent *A* to give fraction 8*a* (64 mg.) which yielded glucose on demethylation (Found: OMe, 16·0. Calc. for $C_7H_{14}O_6$: OMe, 16·0%).

Fraction 9. This fraction (1·30 g.) obtained on elution of the cellulose column with water was present as the barium salt and had equivalent wt. 223. Drastic hydrolysis with 2N-sulphuric acid followed by chromatographic examination in solvent *D* showed that three acidic components, R_G 0·84, 0·58, 0·44 (cherry-red coloration with aniline oxalate), and some neutral sugars were produced. A portion was converted into the methyl ester methyl glycoside, which was reduced with lithium aluminium hydride; hydrolysis of the product yielded sugars travelling on the chromatogram (solvent *B*) at the same rate as tri-, di-, and mono-methylglucoses, together with 3 : 5-di-*O*-methylfucose. An attempt to separate the acidic components by elution from Amberlite resin IRA-400 (acetate form) with increasing concentrations of acetic acid resulted in loss of the sugar acids by adsorption and/or decomposition on the resin.

Periodate Oxidation of the Polysaccharide.—The polysaccharide (154·3 mg.) was dissolved

[7] Bell, *J.*, 1948, 992.
[8] Lemieux and Bauer, *Canad. J. Chem.*, 1953, **31**, 811.

in potassium chloride solution (60 c.c.; 0·56M) and the pH of the solution was adjusted to 6·25. Sodium metaperiodate solution (20 c.c.; 0·20M) was added and the solution was shaken in the dark. Aliquot portions (10 c.c.) were removed from time to time, ethylene glycol (1 c.c.) was added, and the mixture was titrated against 0·108N-sodium hydroxide to pH 6·25 in a stream of nitrogen. After 160 hr. the formic acid released corresponded to 0·34 mole per 162 g. of polysaccharide.

The polysaccharide (101·6 mg.) was dissolved in water (50 c.c.), and sodium metaperiodate solution (50 c.c.; 0·40M) was added. Estimation of the periodate consumed showed that a constant value of 1·28 mole per 162 g. of polysaccharide was reached after 71 hr. Hydrolysis of a sample of the periodate-oxidised polysaccharide showed the presence of glucose and fucose.

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