472. *Structure of the Capsular Polysaccharide of* Aerobacter aerogenes (*N.C.T.C.* 418).

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The capsular acidic polysaccharide of *Aerobacter aerogenes* (N.C.T.C. 418) has been shown to contain D-glucose, D-mannose, glucuronic acid, and probably mannuronic acid. Partial acidic hydrolysis of the polysaccharide gave *inter alia* two aldobiuronic acids which were assigned the structures 4-*O*-α-D-glucuronosyl-D-mannose and 4-*O*-α-D-mannuronosyl-D-glucose. Hydrolysis of the methylated polysaccharide gave 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, 2·3%; 2 : 3 : 4-tri-*O*-methylglucuronic acid, 2·6%; tri-*O*-methylhexoses, 90% and di-*O*-methylhexoses (mainly 2 : 6-di-*O*-methyl-D-mannose), 5·1%. Two of the tri-*O*-methylhexoses were shown to be 2 : 3 : 6-tri-*O*-methyl-D-glucose and 2 : 4 : 6-tri-*O*-methyl-D-glucose.

Few detailed chemical studies have been made on the capsular polysaccharides elaborated by the many serologically distinguishable strains [1] of the *Aerobacter–Klebsiella* group. The capsular polysaccharides corresponding to *Klebsiella* types 1, 2, and 3 have been examined by Heidelberger, Goebel, and Avery,[2] Goebel and Avery,[3] and Goebel [4] who found them to be glucose-containing polyuronides. More recently Wilkinson, Dudman, and Aspinall [5] have shown that *Klebsiella* type 54 elaborated a polyuronide consisting of D-glucose (50%), L-fucose (10%) and an unidentified uronic acid (29%). Dudman and Wilkinson [6] also slowed that the polysaccharides of *Klebsiella* types 8, 26, 29, and 57 contained varying proportions of galactose, mannose, and uronic acid; types 8 and 26 contained glucose in addition. The present communication deals with a structural study of the capsular acidic polysaccharide produced by the Hinshelwood strain of *Aerobacter aerogenes* obtained from the Oxford University Physical Chemistry Laboratory and later found to be identical with N.C.T.C. 418.

A large-scale culture (500 l.) of the Hinshelwood strain, in a synthetic medium containing mineral salts and glucose as the sole source of carbon, gave enough acidic polysaccharide for most of the structural determination. The major step in the purification of the polysaccharide involved the use of " Cetavlon " [7] since this precipitated a mixture of nucleic acid and acidic polysaccharide and left in solution small amounts of neutral polysaccharide. Paper chromatography of a hydrolysate of the neutral polysaccharide indicated that its major constituent was glucose with small amounts of galactose. The cetyltrimethylammonium salts of the nucleic acid and the acidic polysaccharide mixture were separated by differential solubility in 0·25M-sodium chloride.

A purified sample of the acidic polysaccharide (Na salt) had $[\alpha]_D^{21}$ +97·3° and was electrophoretically pure except for a small amount (3·5%) of nucleic acid impurity. Paper chromatography and ionophoresis [8] of a hydrolysate showed glucose, mannose (*ca.* 3 : 1), glucuronic acid, and aldobiuronic acids. The acidic polysaccharide had a uronic acid content of 16·5% when analysed by the McCready, Swensen, and Maclay [9] method modified to enable determinations to be carried out on 20 mg. samples. (Control determinations on samples of crystalline glucurone gave reproducible recoveries of 101·2%.) Confirmation of the presence of carboxylic acid groupings was obtained from the infrared spectrum [10]

[1] Edwards and Fife, *J. Infect. Diseases*, 1952, **91**, 92.
[2] Heidelberger, Goebel, and Avery, *J. Exp. Med.*, 1925, **42**, 701.
[3] Goebel and Avery, *ibid.*, 1927, **46**, 601.
[4] Goebel, *J. Biol. Chem.*, 1927, **74**, 619.
[5] Wilkinson, Dudman, and Aspinall, *Biochem. J.*, 1955, **59**, 446.
[6] Dudman and Wilkinson, *Biochem. J.*, 1956, **62**, 289.
[7] Jones, *Biochim. Biophys. Acta*, 1953, **10**, 607.
[8] Foster, *J.*, 1953, 982.
[9] McCready, Swensen, and Maclay, *Ind. Eng. Chem.*, 1946, **18**, 290.
[10] Barker, Bourne, and Whiffen, " Methods of Biochemical Analysis," Interscience, New York, Vol. 3, 1956, p. 213.

of the acidic polysaccharide. The spectrum of the sodium salt showed strong absorption at 1612 cm.$^{-1}$ and 1410 cm.$^{-1}$ characteristic of the carboxylate ion. No amide bands (C=O, 1650 cm.$^{-1}$ and $-$NH, 1550 cm.$^{-1}$) such as those given by proteins or analogous to those displayed by an *N*-acetyl group could be detected. The absence of absorption at 1735 cm.$^{-1}$ in the spectrum of the sodium salt also indicated the absence of *O*-acetyl groups. However, absorption did appear in the spectrum of the free acid attributable to the C=O of the un-ionised $-CO_2H$ group. No attempt was made to interpret the region 700—1000 cm.$^{-1}$ since the α- and β-configurations of the hexose units cannot be deduced in the presence of uronic acids which show interfering absorption in this region.

The mixture of saccharides produced by partial hydrolysis of the acidic polysaccharide was fractionated (without prior neutralisation) on a charcoal–Celite column.[11] The major fraction (A) eluted with water was found to contain mainly glucose and mannose when analysed by paper chromatography. Further elution with water, and gradient elution[12] with aqueous ethanol (0 ⟶ 20%), gave fractions B and C containing the barium and the sodium salt respectively of glucuronic acid together with traces of another hexuronic acid, and fraction D, an aldobiuronic acid mixture.

The components of fraction A were separated by preparative paper chromatography and the glucose was characterised as crystalline *N*-*p*-nitrophenyl-β-D-glucopyranosylamine dihydrate,[13] and the mannose as *N*-*p*-nitrophenyl-β-D-mannopyranosylamine dihydrate.[13] The two *p*-nitroanilides showed a large difference in their solubility in methanol, and in fact a mixture of glucose and mannose may be separated readily by conversion into the *p*-nitroanilides.

The two hexuronic acid fractions B and C were converted into their methyl ester methyl hexuronosides. Reduction with lithium aluminium hydride[14–16] and acidic hydrolysis of the resulting methyl hexosides gave glucose and small amounts of mannose. This indicated the presence of both glucuronic and mannuronic acid in the acidic polysaccharide.

Paper chromatography indicated the presence of two aldobiuronic acids in fraction D and these were separated on sheets of Whatman No. 1 paper. An acidic hydrolysate of the component (1) with lower R_F value was analysed and showed the production of glucose and a component suspected to be mannuronic acid. Similar treatment of the faster moving component (2) gave mannose and glucuronic acid. Each aldobiuronic acid was converted into its methyl ester methyl glycoside and reduced with lithium aluminium hydride.[14–16] Chromatography of the hydrolysates of the resulting methyl biosides showed the presence of equal amounts of glucose and mannose in each case. This evidence, together with the acid stability of the aldobiuronic acids, indicated that component 1 was a glucuronosyl-mannose and component 2 a mannuronosyl-glucose. Periodate oxidation of the methyl biosides obtained by reduction from fraction D consumed 3 moles of periodate and produced 1·1 moles of formic acid per 356 g. of methyl biosides. No formaldehyde could be detected and a minute amount (*ca.* 2·5%) of mannose was produced on acid hydrolysis of the periodate-oxidised methyl biosides. This evidence indicated that no furanoside ring was present, that only a minute amount of the methyl biosides could be linked through the 3 position, and that the major portion were linked 1 : 4 or 1 : 2.

In order to decide between these possibilities the same methyl bioside fraction was methylated[17] three times with methyl sulphate–sodium hydroxide and once with the Purdie reagents. Acidic hydrolysis of the product gave approximately equal amounts of components having the mobilities of tetra-*O*-methylmannose (or -glucose) and tri-*O*-methylhexoses respectively. When these products were eluted from paper and submitted

[11] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[12] Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826.
[13] Perkow, Kuhner, and Weygard, *Chem. Ber.*, 1951, **84**, 594.
[14] Lythgoe and Trippete, *J.*, 1950, 1983.
[15] Akher and Smith, *Nature*, 1950, **166**, 1037.
[16] Whistler and Hough, *J. Amer. Chem. Soc.*, 1954, **76**, 1670.
[17] Albon, Bell, Blanchard, Gross, and Rundell, *J.*, 1953, 24.

to paper ionophoresis [8] in borate buffer of pH 10, no components could be detected having the mobilities of the 3 : 4 : 6-tri-*O*-methyl ether of either mannose or glucose. This indicated the absence of 1 : 2-linkages in the methyl biosides and hence in the aldobiuronic acids. From the methylation and periodate data it therefore appears that the aldobiuronic acids are 1 : 4-linked.

The high specific rotation (+97·3°) of the acidic polysaccharide indicated a high percentage of α-linkages. Several observations suggested that α-linkages were present in the aldobiuronic acids. Both the aldobiuronic acid mixture (fraction D) and the mixture of methyl biosides derived therefrom were resistant to emulsin; this indicated the absence of β-linkages. The action of α-glucamylase on the methyl biosides, reputedly [18] specific for α-1 : 4-glucose residues, gave small amounts of glucose and mannose.

The acidic polysaccharide gave precipitates in varying amounts with several types of anti-*Pneumococcus* horse sera; the strongest reactions were given with antisera to Types II and XV. Only the structure [19] of the Type II *Pneumococcus* polysaccharide is known. Since Type II *Pneumococcus* polysaccharide only contains D-glucose, D-glucuronic acid, and L-rhamnose, the strong cross-reaction must be due to a similarity of linkage of the glucose residues (which are involved in 1 : 4 : 6-branch points), the glucuronic acid residues, or both. It has already been demonstrated [20, 21, 22] that the cross-reaction of anti-*Pneumococcus* Type II serum with certain dextrans, amylopectins, etc., is probably due to glucose in 1 : 4 : 6-branch points, and that with gum arabic to the similar disposition of D-glucuronic acid residues. It is significant that the *Aerobacter aerogenes* polysaccharide failed to cross-react with Type I anti-serum; the specific polysaccharide of Type I contains galacturonic acid.[23]

On periodate oxidation the acidic polysaccharide consumed 0·98, 1·15, 1·22, and 1·27 moles of periodate per 162 g. of polysaccharide after oxidation for 48, 72, 96, and 112 hr. respectively. The values for formic acid after the same times expressed as moles per 162 g. of polysaccharide were 0·55, 0·65, 0·71, and 0·74. Recent work by Eddington, Hirst, and Percival [24] on the periodate oxidation of methyl ethers of glucuronic acid suggests that owing to the possibility of over-oxidation considerable caution should be exercised in interpreting periodate data of polysaccharides containing uronic acids. Over-oxidation is the probable explanation of the high ratio of acid produced to periodate consumed by the acidic polysaccharide. From the work of Eddington *et al.*[24] it appears that such over-oxidation is to be expected where the uronic acids are present as non-reducing terminal units or where they are linked through the 2-position. We can, however, deduce that the maximum number of residues in the acidic polysaccharide which could be linked 1 : 6 or be present as non-reducing terminal units is 63%. This leaves a minimum of 37% of the residues which are linked 1 : 3 or are involved in branching. Acid hydrolysis of the periodate-oxidised acidic polysaccharide revealed appreciable amounts of glucose and mannose, indicating that only these hexose moieties could be included in those residues resistant to periodate oxidation and so in the figure of 37%.

Since the acidic polysaccharide prepared in 500 l. of medium was then exhausted an attempt was made to prepare more polysaccharide. However, with both our culture and a fresh slope sent from Oxford University Physical Chemistry Laboratory only minute amounts of the polysaccharide were produced. Fortunately, Professor Sir Cyril Hinshelwood had many years previously sent a culture of this strain to the National Collection of Type Cultures (there designated N.C.T.C. 418). With this culture success was immediately obtained.

[18] Barker, Bourne, and Fleetwood, *J.*, 1957, 4865.
[19] Butler and Stacey, *J.*, 1955, 1537.
[20] Heidelberger and Aisenberg, *Proc. Nat. Acad. Sci.*, 1953, **39**, 453.
[21] Heidelberger, Aisenberg, and Hassid, *J. Exp. Med.*, 1954, **99**, 343.
[22] Heidelberger and Adams, *ibid.*, 1956, **103**, 189.
[23] Heidelberger, *ibid.*, 1936, **64**, 559.
[24] Eddington, Hirst, and Percival, *J.*, 1955, 2281.

By using the N.C.T.C. strain and the medium employed by Duguid and Wilkinson [25] more acidic polysaccharide (3·4 g.) was isolated from the capsules of the organisms. The polysaccharide thus obtained appeared to be identical with the previous polysaccharide on the basis of paper chromatographic and ionophoretic examination of its hydrolysate.

The acidic polysaccharide was methylated with sodium and methyl iodide in liquid ammonia and after four methylations, each involving four alternate additions of the methylating reagents, a methyl ether was obtained having $[\alpha]_D^{16}$ +65·6° in $CHCl_3$ and OMe, 40%.

The infrared spectra of the methylated polysaccharide, as isolated, showed no absorption at 1730—1760 cm.$^{-1}$ (C=O stretching), indicating the absence of CO_2Me and un-ionised CO_2H groups. However an absorption peak at 1635 cm.$^{-1}$ indicated the presence of a carbonyl group of CO_2Na, CO_2NH_4 or $CO{\cdot}NH_2$, and/or water. The free acid form of the methylated polysaccharide showed a peak at 1730—1735 cm.$^{-1}$ and hence the presence of free CO_2H groups. This evidence clearly shows that none of the carboxylic acid groups was esterified during methylation and most were isolated in the form of an ionizable salt. Finally, from the intensity of the OH stretching band the estimated amount of free hydroxyl groups in the methylated polysaccharide could not be more than 5%.

The methylated polysaccharide was subjected to methanolysis and hydrolysis, and the hydrolysate separated into neutral and acidic fractions on an anion-exchange resin [26] (Deacidite FF, micro-bead, carbonate form). The neutral methyl sugars were further separated into di-*O*-methyl-, tri-*O*-methyl-, and tetra-*O*-methyl-hexose fractions, by using paper chromatograms.

The syrupy tetra-*O*-methylhexose fraction showed $[\alpha]_D^{20}$ +66° [cf. 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, +84° (equil.); 2 : 3 : 4 : 6-tetra-*O*-methyl-D-mannose, +1·2° (equil.)]. The component behaved in an identical manner to 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose on paper chromatography and its infrared spectrum was identical with that of an authentic sample of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose eluted from the paper under similar conditions. This evidence showed that some of the glucose residues were present as non-reducing end groups in the acidic polysaccharide, and the amount of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose isolated indicated such residues constituted *ca.* 1 in 44 sugar units.

The di-*O*-methyl fraction showed $[\alpha]_D^{20}$ +9·1° in H_2O. On demethylation [27] it gave, *inter alia*, mainly mannose with very small amounts of glucose. Paper ionophoresis [8] revealed the presence of a major component (M_G 0·092) with minute traces of two other components having M_G values identical with those of 2 : 3- and 2 : 4-di-*O*-methylglucose. The major component (M_G 0·092) was shown, by comparison with authentic samples, to differ from 2 : 4-, 2 : 3-, 3 : 4-, 3 : 6-, and 4 : 6-di-*O*-methylglucose and 2 : 3-, 3 : 4-, and 4 : 6-di-*O*-methylmannose. This di-*O*-methyl fraction was reduced with sodium borohydride, and the resulting di-*O*-methylhexitol subjected to periodate oxidation. It consumed 1·9 moles of periodate and produced 0·9 mole of formic acid per mole; no formaldehyde was detected. These periodate data strongly indicate a 2 : 6-di-*O*-methylhexitol.

The low optical rotation of the di-*O*-methylhexose fraction {$[\alpha]_D^{20}$ +9·1° compared with $[\alpha]_D$ +63·3° (equil.) for 2 : 6-di-*O*-methyl D-glucose [28]} strongly suggests that the major di-*O*-methylhexose is 2 : 6-di-*O*-methyl-D-mannose. Such moieties would arise from the branch points in the acidic polysaccharide and would constitute (from the amount isolated) *ca.* one in twenty of the sugar residues. The presence of 2 : 6-di-*O*-methylmannose accounts for at least some of the mannose residues that were resistant to periodate oxidation in the acidic polysaccharide.

[25] Duguid and Wilkinson, *J. Gen. Microbiol.*, 1953, **9**, 174.
[26] Machell, *J.*, 1957, 3389.
[27] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[28] Freudenberg and Hüll, *Ber.*, 1941, **74**, 237.

The methylated uronic acid fraction showed only one reducing component on paper chromatography and this had an R_F value and staining properties identical with those of 2 : 3 : 4-tri-*O*-methylglucuronic acid. However, after reduction with lithium aluminium hydride, hydrolysis, and paper chromatography, one spot was detected having an R_F value and staining properties identical with those of 2 : 3 : 4-tri-*O*-methylglucose and another had those of the 2 : 6-di-*O*-methylmannose reported above. The infrared spectrum of the 2 : 3 : 4-tri-*O*-methylglucose isolated from a paper chromatogram was identical with that of an authentic specimen eluted from the paper in a similar way. This evidence indicated the presence of non-reducing end groups of glucuronic acid and the amount of tri-*O*-methylglucuronic acid isolated corresponded to *ca.* 1 in every 40 sugar units.

The detection of 2 : 6-di-*O*-methylmannose in the hydrolysate of the reduced methylated uronic acid fraction suggested the presence of small amounts of the methyl glycoside of a methylated aldobiuronic acid. This inference was supported by the fact that a 2 : 6-di-*O*-methyl-sugar could not result from the reduction of a di-*O*-methylhexuronic acid and also by the evidence that the unreduced methylated uronic acid fraction on rehydrolysis, followed by paper chromatography and ionophoresis of the hydrolysate, showed, in addition to 2 : 3 : 4-tri-*O*-methylglucuronic acid, a dimethyl component having an M_G value identical with that of 2 : 6-di-*O*-methylmannose. It is therefore inferred that some at least of the glucuronic acid end groups are linked to mannose residues engaged in branching.

The tri-*O*-methylhexose fraction was found on ionophoresis to contain neither 3 : 4 : 6-tri-*O*-methylglucose nor 3 : 4 : 6-tri-*O*-methylmannose, thus ruling out the possibility of 1 : 2-linkages. The mixture of tri-*O*-methylhexitols obtained on reduction of this fraction consumed 0·96 mole of periodate per mole and produced no formic acid. This evidence precluded the presence of large amounts of 2 : 4 : 6-tri-*O*-methylhexoses and favoured the presence of either 2 : 3 : 6- or 2 : 3 : 4-tri-*O*-methyl ethers.

Partial separation of the tri-*O*-methylhexose fraction was effected on paper chromatograms into A (slower components) and B (faster components). A showed $[\alpha]_D^{20}$ +36·4° (in H_2O) and on demethylation [27] gave, *inter alia*, both glucose and mannose. When treated with aniline in methanol, A gave a very small yield of crystalline 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-glucosylamine. B showed $[\alpha]_D^{20}$ +45° (in H_2O) and on demethylation [27] gave, *inter alia*, mainly glucose with comparatively small amounts of mannose. In order to decide whether any 2 : 3 : 6-tri-*O*-methylglucose was present in the mixture it was kept in 2% dry methanolic hydrogen chloride at room temperature. It showed $[\alpha]_D^{22}$ +50·7° (10 min.) ⟶ +7·2° (24 hr., equil.). Paper chromatographic comparison of solutions of B before and after the treatment with methanolic hydrogen chloride showed almost complete disappearance of the faster region of the spot (R_F identical with that of 2 : 3 : 6-tri-*O*-methyl-D-glucose). It is known [29] that under these conditions 2 : 3 : 6-tri-*O*-methyl-D-mannose does not form a furanoside. It is concluded that the major component of B is 2 : 3 : 6-tri-*O*-methyl-D-glucose.

It is concluded that the polysaccharide has a branched structure in which the average repeating unit is about 40 sugar units and contains D-glucose and D-mannose residues with some of the former present as non-reducing end groups. The D-glucose residues are linked mainly 1 : 4 but a few are linked 1 : 3. Non-reducing end groups (*ca.* 1 in 40) of glucuronic acid are linked α-1 : 4 to D-mannose residues, which in turn are linked to the remaining sugar units through the 3-position. The linkage of the remaining mannose residues in the repeating unit has still to be determined but is through the 4- or the 6-position. The first batch of acidic polysaccharide showed a higher uronic acid content (16·5%) than the second (2·6%), probably because of the use of different culture media. In the first batch, mannuronic acid residues were detected and these were attached α-1 : 4 to D-glucose residues.

[29] Rebers and Smith, *J. Amer. Chem. Soc.*, 1954, **76**, 6097.

Experimental

Isolation of Acidic Polysaccharide.—Aqueous solutions containing glucose (194 l.; 50 g./l.), $(NH_4)_2SO_4$ (97 l.; 5 g./l.), $MgSO_4,7H_2O$ (9 l.; 1 g./l.) together with a phosphate buffer (194 l.; pH 7—7·2) made from Na_2HPO_4 (17·3 g./l.) and KH_2PO_4 (2·7 g./l.) were sterilised separately at 10 lb./sq. in. for 5 min., mixed, and then steamed for 20 min. at 100°. After cooling, the medium was inoculated with a culture (6 l.) of *Aerobacter aerogenes* (Oxford University Physical Chemistry Laboratory strain) prepared by growing the organism for 18 hr. on a medium with the same composition as that described above. This large-scale culture was kindly grown for us at the Antibiotics Research Station, Clevedon, where it was incubated for 48 hr. at 37° with aeration. The cells were harvested by means of a Sharples Super Centrifuge, frozen, and then disrupted with the aid of glass beads. The cell debris was removed by centrifugation, and the nucleic acid and acidic polysaccharide in the extract were precipitated by addition of excess of Cetavlon. The precipitate was dissolved in M-sodium chloride, then diluted with water until 0·25M with respect to sodium chloride. The resulting nucleic acid precipitate was centrifuged off and the crude acidic polysaccharide precipitated from the supernatant liquid by addition of alcohol (3 vol.). The precipitate was removed in the centrifuge and dried with alcohol and ether (yield, 13 g.).

Cultures from the above strain two years later had become " rough " and produced only minute amounts of acidic polysaccharide. Subcultures from a slope of the same strain sent many years previously to the National Collection of Type Cultures (there designated N.C.T.C. 418) were used to produce two further 20 l. batches, yielding 3·4 g. of acidic polysaccharide. A similar procedure was adopted but the medium used was obtained by mixing aseptically aqueous solutions, each 5 l., containing (i) Na_2HPO_4 (200 g.) and KH_2PO_4 (60 g.), (ii) $(NH_4)_2SO_4$ (6 g.), K_2SO_4 (20 g.), $MgSO_4,7H_2O$ (4 g.) and $FeSO_4,7H_2O$ (0·2 g.), (iii) NaCl (20 g.) and $CaCl_2$ (0·2 g.), (iv) glucose (200 g.) which were sterilised separately at 10 lb./sq. in. for 10 min.

Further purification of the acidic polysaccharide was effected by deproteinising it (Sevag [30] method) several times with chloroform–pentyl alcohol (9 : 1), reprecipitating it with Cetavlon, and fractionating it, as above, with sodium chloride.

One fraction, so purified, had $[\alpha]_D^{20}$ +97·3° (*c* 0·339 in N-sodium hydroxide) and analysis showed: C, 35·9; H, 6·5; N, 1·4; P, 0·5%. The optical density at 260 mμ corresponded to a maximum nucleic acid impurity of 3·5%. Uronic determination, by a modification of the McCready, Swensen, and Maclay method,[9] showed a uronic acid content of 16·5%. The fraction gave 1·69% of ash. On analysis of the fraction in a Tiselius electrophoresis apparatus, using a 0·05M-borate buffer (pH 9·2), only one major component could be detected and corresponded to the polysaccharide. A very small faster-moving component corresponded to the nucleic acid impurity.

Infrared Spectra of Acidic Polysaccharide.—The infrared absorption spectra of the acidic polysaccharide were determined as the sodium salt and free acid at two concentrations (0·375 mg./cm.^{2} and 1·5 mg./cm.^{2}) in potassium chloride discs. The polysaccharide was obtained in the free acid form by precipitation of the polysaccharide (50 mg.) from acid solution (pH 1·2) with alcohol (3 vol.), dialysis of an aqueous solution of the precipitate, and freeze-drying.

Periodate Oxidation of Acidic Polysaccharide.—The polysaccharide was subjected to periodate oxidation by the procedure of Jeanes and Wilham [31] which incorporates Fleury and Lange's method [32] for the determination of the quantity of periodate consumed, and that of Halsall, Hirst, and Jones,[33] with certain modifications, for the determination of formic acid produced. The number of moles of sodium periodate consumed per 162 g. of polysaccharide was: 48 hr., 0·98; 72 hr., 1·15; 96 hr., 1·22; 112 hr., 1·27. The corresponding figures for formic acid produced were: 48 hr., 0·55; 72 hr., 0·65; 96 hr., 0·71; 112 hr., 0·74.

The solution (50 ml.) remaining from the oxidation of the polysaccharide was dialysed for 4 days against running tap-water and then freeze-dried. This material was then hydrolysed with 2N-sulphuric acid (1 c.c.) for 3 hr. at 100°. After neutralisation with barium carbonate the hydrolysate was submitted to paper chromatography with butan-1-ol–acetic acid–water (4 : 1 : 5) and found to contain glucose and mannose but no uronic acids.

[30] Sevag, *Biochem. Z.*, 1934, **273**, 419.
[31] Jeanes and Wilham, *J. Amer. Chem. Soc.*, 1950, **72**, 2655.
[32] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107, 196.
[33] Halsall, Hirst, and Jones, *J.*, 1947, 1427.

Immunological Specificity of Acidic Polysaccharide (with Professor M. HEIDELBERGER).—The polysaccharide (0·06 mg. in 0·04 c.c. of physiological saline) cross-reacted with the following anti-*Pneumococcus* serums after being kept at 0° for 8 days.

Type	II	VII	IX	X	XII	XV	XVIII	XIX	XX
Reaction ...	++++	+	++	+	±	++++	+	++	+±

Partial Hydrolysis of Acidic Polysaccharide.—The polysaccharide (2·5 g.) was hydrolysed with 2N-sulphuric acid (75 c.c.) under nitrogen for 3 hr. at 100°. The hydrolysate (without neutralisation) was added to a charcoal–Celite column (l, 24 cm.; diam. 3 cm.) and fractionated by Whistler and Durso's method.[11] The column was washed with water, and the effluent collected (19 × 50 c.c. fractions), neutralised with barium carbonate, centrifuged, and concentrated *in vacuo*. Thereafter the column was washed by gradient elution with aqueous ethanol (0 ⟶ 20%; 3 l.), and the fractions (50 c.c.) were neutralised with sodium hydroxide. After paper chromatography with the solvent used above the fractions were grouped as follows: A (1—13) contained 0·66 g. of a glucose, mannose, ribose (*ca.* 3 : 1 : trace) mixture; B (14—19) and C (20—40) contained 25 mg. and 55 mg. respectively of the barium and sodium salt of glucuronic acid together with traces of another hexuronic acid; D (45—54) contained two components (400 mg.), suspected of being aldobiuronic acids, which were separable after lengthy irrigation in the above solvent.

(i) *Fraction A.* A portion of this fraction, after methanol-extraction, was separated into bands on 8 sheets of Whatman No. 1 paper (46 × 57 cm.) and irrigated with the above solvent for 5 days. Elution with water of the appropriate strips gave glucose (0·18 g.) and mannose (0·08 g.). The glucose fraction was purified by repeated extraction with methanol, and the resulting syrup (80 mg.) refluxed for 15 min. with an aliquot part (2 c.c.) of a solution containing *p*-nitroaniline (9 g.), methanol (200 c.c.), and concentrated hydrochloric acid (0·14 c.c.). The mixture was filtered while hot and on cooling gave a solid which after two recrystallisations (yield, 30 mg.) showed $[\alpha]_D^{19}$ −194° (3 min.) ⟶ −201° (equil.) (*c* 1·0 in pyridine) and had m. p. 184° undepressed on admixture with *N*-*p*-nitrophenyl-β-D-glucopyranosylamine,$2H_2O$. The mannose fraction was freed from impurities as above and the resulting syrup (40 mg.) refluxed for 15 min. with *p*-nitroaniline solution (2 c.c.). Deep yellow crystals, which separated from the hot solution, were washed with hot methanol and dried (yield, 8 mg.). The crystals showed $[\alpha]_D^{19}$ −402° (3 min.) ⟶ −332° (equil.) (*c* 0·1 in dry pyridine) and had m. p. 219° undepressed on admixture with *N*-*p*-nitrophenyl-β-D-mannopyranosylamine,$2H_2O$.

(ii) *Fractions B and C.* Fraction B (25 mg.) was refluxed with 2% methanolic hydrogen chloride (1 c.c.) for 6 hr. Part of the hydrogen chloride was removed *in vacuo* by repeated distillation with methanol, and the remainder in a vacuum-desiccator over solid potassium hydroxide. The residue was dissolved in tetrahydrofuran (4 c.c.)–ether (1 c.c.) and reduced with excess of lithium aluminium hydride (*ca.* 100 mg.), added in small amounts during 1 hr., at room temperature. After a further 1 hour's stirring, the reaction was completed at 80° (0·5 hr.). The excess of lithium aluminium hydride was decomposed with water, the suspension was filtered, the residue was extracted twice with water, and the combined filtrates were de-ionised with Amberlite IR-120 and IR-4B resins. The resulting methyl hexoside was hydrolysed with 2N-sulphuric acid (0·5 c.c.) at 100° for 3 hr. Paper chromatography of the neutralised hydrolysate showed the presence of glucose with traces of mannose. Fraction C, submitted to the same reduction and hydrolysis, gave a solution which contained glucose with small amounts of mannose.

(iii) *Fraction D.* A portion (60 mg.) of fraction D was separated on two sheets of Whatman No. 1 paper (46 × 57 cm.) irrigated with the butanol–acetic acid solvent for 10 days. After extraction of the appropriate strips with water and concentration *in vacuo* the yields obtained were slower-moving aldobiuronic acid (0·020 g.) (component 1) and faster-moving aldobiuronic acid (0·026 g.) (designated component 2).

Portions (5 mg.) of component 1, component 2, and the original mixture fraction D were each hydrolysed with 2N-sulphuric acid (0·5 c.c.) at 100° for 3 hr. Paper chromatography of the neutralised hydrolysates showed: component 1 hydrolysate contained glucose, mannuronic acid (?), and unhydrolysed component 1; component 2 hydrolysate contained mannose, glucuronic acid, and unhydrolysed component 2; fraction D hydrolysate contained glucose, mannose (*ca.* 1 : 3), glucuronic acid, mannuronic acid (?), and unhydrolysed aldobiuronic acids.

The methyl ester methyl glycosides of component 1 (15 mg.), component 2 (20 mg.), and

the original mixed fraction D (20 mg.; 300 mg.) were each reduced with lithium aluminium hydride by the same procedure used for fraction B. All the products were non-reducing. Part of the methyl biosides was retained for further examination (see below) while the remainder was hydrolysed with 2N-sulphuric acid at 100° for 3 hr. Paper chromatography of the hydrolysates of reduced component 1, reduced component 2, and the reduced mixed fraction D showed that each contained glucose and mannose in approximately equal quantities.

The aldobiuronic mixture, fraction D (4 mg.), was incubated at 30° for 4 days with emulsin (4 mg. in 0·25 c.c.). A control containing cellobiose (4 mg.) in place of fraction D was incubated simultaneously. Aliquot portions were withdrawn at intervals of 24, 48, 72, and 96 hr. and examined by paper chromatography in the butanol–acetic acid solvent. Cellobiose was progressively broken down to glucose, but the aldobiuronic acid fraction remained unaffected. The methyl biosides obtained by reduction of fraction D were also stable to emulsin. The same methyl biosides (4 mg.) were incubated at 30° for 4 days with α-glucamylase (4 mg. in 0·25 c.c.). A control solution containing maltose (4 mg.) was incubated simultaneously. Paper chromatography showed the progressive formation of glucose from maltose, and small amounts of glucose and mannose from the methyl biosides.

Methyl biosides (27·8 mg.) obtained by reduction of fraction D were oxidised with 0·15M-sodium periodate (25 c.c.). The consumption of periodate and production of formic acid (expressed as moles per 356 g. of methyl biosides) were 3 and 1·1 respectively. No formaldehyde was detected. Acid hydrolysis of the remaining periodate-oxidised methylbiosides and semiquantitative paper chromatography showed that only *ca.* 2·5% of mannose was produced.

The methyl bioside(s) (40 mg.) obtained by reduction of fraction D were methylated [17] in dioxan (4 c.c.) by alternate additions of dimethyl sulphate (3·6 c.c.) and 30% sodium hydroxide (7·2 c.c.) in ten equal amounts. After the resulting mixture had been stirred for 3 days at room temperature, water (5 c.c.) was added and the mixture heated at 100° for 0·5 hr. The methylated product was isolated by extraction with chloroform (10 × 5 c.c.), and the syrup (27 mg.) was obtained by concentrating the dried extract and then submitted to a Purdie methylation. The syrup (22 mg.) was hydrolysed with 2N-sulphuric acid (1 c.c.) at 100° for 3 hr. Paper chromatography showed two components (equal amounts) with the R_F values of a tetra- and a tri-*O*-methylhexose respectively. Paper ionophoresis [8] of the hydrolysate in borate buffer of pH 10 showed the absence of both 3 : 4 : 6-tri-*O*-methylglucose and 3 : 4 : 6-tri-*O*-methylmannose.

Note: All the above work was carried out on the batch of polysaccharide produced by the Oxford University Physical Chemistry Laboratory strain of *Aerobacter aerogenes*. The methylation studies described below were carried out on the polysaccharide obtained from the morphologically identical N.C.T.C. 418 strain.

Methylation of Acidic Polysaccharide.—The polysaccharide (3·4 g.) was treated with sodium and methyl iodide in liquid ammonia at −70° according to Freudenberg and Boppel's method.[34] After four additions of the methylating reagents (reaction times: sodium 4 hr.; methyl iodide, 1 hr.) had been made, the ammonia was allowed to evaporate, with exclusion of moisture, the last traces being removed *in vacuo* at 90°. After dialysis, the freeze-dried product (3·4 g.) had OMe, 26·4%. Two repetitions of the methylation procedure gave products 2·4 g. and 1·79 g. which had OMe, 30·1% and OMe, 34·5% respectively. The crude methyl ether obtained after a final remethylation (1·35 g.) was dissolved in dry chloroform (300 c.c.) and light petroleum (40—60°; 275 c.c.) added. The insoluble material (0·075 g.) which was centrifuged off, suspended in water, and freeze-dried had OMe, 14·1%. The chloroform–petroleum-soluble fraction (1·28 g.) had OMe, 40%, and N, 1·13%. This methyl ether showed $[\alpha]_D^{16}$ +65·5° (*c* 0·35 in dry $CHCl_3$).

The infrared absorption spectra of the methylated acidic polysaccharide was measured as it was isolated and also in the free acid form. To obtain the latter, the methylated polysaccharide (10 mg.) was dissolved in methanol (0·5 c.c.) and treated with two drops of 5N-hydrochloric acid; the addition of methanol was necessary to bring the methyl ether into solution. The solution was dialysed for 3 days against running water and finally deionised with Amberlite IR-120 (2 c.c.).

Isolation of the Methyl Sugars from the Methylated Polysaccharide.—The methyl ether (1·25 g.) was refluxed with 4% methanolic hydrogen chloride (50 c.c.) for 6 hr. The solution was neutralised with silver carbonate, filtered, and evaporated *in vacuo* to a syrup. This syrup was

[34] Freudenberg and Boppel, *Ber.*, 1938, **71**, 2505.

hydrolysed with 2N-sulphuric acid (25 c.c.) for 3 hr. at 100°. The hydrolysate was neutralised with barium carbonate, filtered, and stirred with Deacidite FF (micro-bead; carbonate form) for 24 hr. The slurry was then poured into a column and washed with water (700 c.c.) to elute neutral methyl sugars (0·78 g.) and with N-ammonium carbonate (500 c.c.) to elute the methylated uronic acid fraction (0·056 g.). The latter was recovered by removing most of the ammonium carbonate by sublimation *in vacuo* at 65—70° and the remainder by pouring the solution down a column of Amberlite IR-120 (10 ml.).

Methylated uronic acid fraction. This fraction was separated on a Whatman No. 1 paper (46 × 57 cm.) irrigated with the butanol–acetic acid solvent and the strip bearing the component having an R_F value and staining properties identical with 2 : 3 : 4-tri-*O*-methylglucuronic acid eluted and concentrated (yield 18·4 mg.). On reduction of its methyl ester methyl glycoside with lithium aluminium hydride in ether (as above) and hydrolysis of the resulting methyl glycoside two components were detected on a paper chromatogram irrigated with butan-1-ol–ethanol–water (5 : 1 : 4). The two components were eluted. The faster-moving component had an R_F value and infrared spectrum identical with those of 2 : 3 : 4-tri-*O*-methyl-D-glucose. The slower-moving component had an R_F value and an M_G value (0·09) identical with those of the main di-*O*-methylhexose detected in the neutral sugar fraction (for characterisation see below). Examination of a hydrolysate (2N-sulphuric acid for 5 hr. at 100°) of the methylated uronic fraction (1 mg.) before reduction showed the same di-*O*-methylhexose together with 2 : 3 : 4-tri-*O*-methylglucuronic acid.

Neutral methyl sugars. The mixture of neutral methyl sugars (0·78 g.) was separated on 12 sheets of Whatman No. 3 MM paper (30 × 60 cm.) irrigated with butan-1-ol–ethanol–water (5 : 1 : 4). The three bands obtained on each paper corresponded to the tetra-, tri-, and di-*O*-methylhexose fractions respectively. A test hydrolysis on all of these showed that the tetra-*O*-methylhexose fraction only was contaminated with methyltrimethylglycoside. After rehydrolysis of this fraction with 2N-sulphuric acid (6 c.c.) for 6 hr. at 100° the components were separated again on paper as above. The weights of the fractions finally isolated together with that of the purified tri-*O*-methyluronic acid corresponded to the following molecular composition: di-*O*-methylhexose fraction, 5·1%; tri-*O*-methylhexose fraction, 90%; tetra-*O*-methylhexose fraction, 2·3%; tri-*O*-methylhexuronic acid, 2·6%.

Tetra-O-methylhexose fraction. This fraction showed $[\alpha]_D^{20}$ +66° approx. (*c* 0·18 in H_2O) and on paper chromatography showed a single well-defined component with an R_F value identical with that of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. Its infrared spectrum was also identical with that of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose eluted from the paper under comparable conditions

Tri-O-methylhexose fraction. The fraction had $[\alpha]_D^{20}$ +69° (*c* 0·39 in MeOH) (Found: OMe, 42·1. Calc. for a tri-*O*-methylhexose: OMe, 41·9%). Paper ionophoresis [8] in borate buffer of pH 10 showed the absence of both 3 : 4 : 6-tri-*O*-methylglucose and 3 : 4 : 6-tri-*O*-methylmannose. A portion (30 mg.) of the tri-*O*-methylhexose fraction was treated with aqueous sodium borohydride (30 mg. in 2·5 c.c.) at room temperature for 4 hr. The excess of sodium borohydride was destroyed with glacial acetic acid, and the solution deionised on an Amberlite IR-120 and IR-4B mixed-bed resin. The effluent was concentrated to a syrup (0·0178 g.) and oxidised with periodate under the conditions described above. The tri-*O*-methylhexitol fraction consumed 0·93 mole (6 hr.), 0·96 mole (24 hr.) of periodate per mole but produced no formic acid.

Paper chromatography of the tri-*O*-methylhexose fraction in butan-1-ol–ethanol–water (5 : 1 : 4) disclosed at least two components. Although these showed little relative separation a portion of the tri-*O*-methylhexose fraction was fractionated by paper chromatography into A (slower components) and B (faster components). A showed $[\alpha]_D^{20}$ +36·4° (*c* 0·714 in H_2O) and on demethylation [27] gave, *inter alia*, both glucose and mannose. Part of A (30 mg.) was refluxed with aniline (30 mg.) in ethanol (1 c.c.) for 3 hr. and gave 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-glucosylamine (2 mg.), m. p. and mixed m. p. 161°. B showed $[\alpha]_D^{20}$ +45° (*c* 0·756 in H_2O) and on demethylation [27] gave, *inter alia*, both glucose and mannose, the latter in smaller amount. B was kept in 2% methanolic hydrogen chloride at room temperature and the falling rotation observed until constant. It showed $[\alpha]_D^{22}$ +50·7° (10 min.) ⟶ +7·2° (24 hr.; *c* 0·552). The solution was neutralised with silver carbonate, filtered, and concentrated. A paper chromatogram, bearing spots of equal amounts of B before and after treatment with methanolic hydrogen chloride, was irrigated with butan-1-ol–ethanol–water (5 : 1 : 4). Spraying

with aniline hydrogen phthalate showed the disappearance of the region corresponding to 2 : 3 : 6-tri-*O*-methyl-D-glucose. After spraying with dilute hydrochloric acid and heating, a faster-moving component, presumably the methyl glycoside of this methyl ether, appeared.

Di-O-methylhexose fraction. The fraction had $[\alpha]_D^{20}$ +9·1° (*c* 0·44 in H_2O) and on paper ionophoresis [8] in borate buffer of pH 10 showed the presence of one major component (M_G 0·092) and traces of two other components having M_G values identical with those of 2 : 3-di-*O*-methyl-glucose (or -mannose) and 2 : 4-di-*O*-methyl-glucose (or -mannose). Reference di-*O*-methylglucoses had M_G 0·0 (2 : 4-), 0·135 (2 : 3-), 0·28 (3 : 4-), 0·546 (3 : 6-), 0·185 (4 : 6-) while reference di-*O*-methylmannoses had M_G 0·136 (2 : 3-), 0·514 (3 : 4-), 0·44 (4 : 6-). On demethylation [27] the di-*O*-methylhexose fraction gave, *inter alia*, mannose and glucose. When reduced with sodium borohydride, under the conditions described for the tri-*O*-methylhexose fraction, the resulting di-*O*-methylhexitol fraction consumed 0·74 mole (6 hr.), 0·9 mole (24 hr.) of periodate and produced 1·72 moles (6 hr.), 1·9 moles (24 hr.) of formic acid per mole. No formaldehyde was produced.

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