[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, QUEEN'S UNIVERSITY]

The Structure of the Type VIII Pneumococcus Specific Polysaccharide[1a,b]

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Acid hydrolysis of the specific polysaccharide of the Type VIII pneumococcus (S VIII) gave D-glucouronic acid (1 part), D-glucose (2 parts) and D-galactose (1 part). Partial acid hydrolysis afforded three acidic oligosaccharides: (a) an aldobiouronic acid, 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose; (b) an aldotriouronic acid, *O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose; (c) an aldotetraouronic acid, *O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-galactopyranose; and (d) a neutral disaccharide, 4-*O*-α-D-glucopyranosyl-(1 → 4)-D-galactopyranose. Hydrolysis of the methylated S VIII polysaccharide gave 2,3-di-*O*-methyl-D-glucuronic acid (1 part), 2,3,6-tri-*O*-methyl-D-glucose (2 parts) and 2,3,6-tri-*O*-methyl-D-galactose (1 part). Esterification of the S VIII acidic polysaccharide with diazomethane followed by reduction of the product with sodium borohydride in aqueous solution yielded a neutral polysaccharide which on hydrolysis gave D-glucose (3 parts) and D-galactose (1 part). Methylation and hydrolysis of this latter polysaccharide gave 2,3,6-tri-*O*-methyl-D-glucose (3 parts) and 2,3,6-tri-*O*-methyl-D-galactose (1 part). Periodate oxidation of the S VIII polysaccharide showed a consumption of 1.03 moles of metaperiodate per hexose unit. It is considered that the S VIII polysaccharide is essentially a linear chain, high molecular weight polymer with the repeating unit -*O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-galactopyranosyl-(1 → 4)-.

I. Introduction

The cross precipitin reactions observed between the S III and the S VIII polysaccharides and their corresponding antisera[2-4] can be related to the occurrence in these two polysaccharides of a common structural unit of 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose.[5] Similarly the cross reactions observed between the S III antiserum and the polysaccharides from *Azotobacter chroococcum*,[6] *Rhizobium radicicolum*,[7] gum arabic,[8,9] Friedlander's bacillus[10] and oxidized cotton cellulose,[5] all of which possess closely related aldobiouronic acid units, illustrates the remarkable correlation which exists between the fine structure of the carbohydrate and its serological specificity.

The structure of the S III polysaccharide is known to be that of a linear chain of 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose units joined together by 1 → 3 linkages having the β-configuration.[10-12] The aldobiouronic acids isolated from the partial acid hydrolysis of both the S III and S VIII polysaccharides proved to be identical, both being convertible to the one crystalline hepta-*O*-acetyl methyl ester derivative.[13] The physical and chemical properties of the purified polysaccharide used in this investigation agree very closely with those previously recorded for the S VIII polysaccharide.[2,3,13]

On the basis of immunological evidence using quantitative precipitin and quantitative agglutination techniques, Heidelberger, Kabat and Mayer[4] concluded that the S VIII polysaccharide was probably a linear polymer having 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose units interposed between other anhydrohexose units and that it had a minimum molecular weight of 140,000. This theory is in agreement with the experimental findings described in this paper.

II. Experimental

Paper chromatography was performed by the descending method[14] on Whatman No. 1 filter paper using several solvent systems: (a) ethyl acetate–acetic acid–water (9:2:2 v./v.); (b) ethyl acetate–acetic acid–formic acid–water (18:8:3:6 v./v.); (c) butan-1-ol–ethanol–water (4:1:2 v./v.); (d) butan-1-ol–pyridine–water (10:3:3 v./v.). Sugars were detected on the chromatogram by spraying with a solution of *p*-anisidine hydrochloride[15] or with a solution of silver nitrate in acetone followed by ethanolic sodium hydroxide solution.[16]

The rate of movement of the sugars on the chromatogram is quoted relative to that of tetra-*O*-methylglucopyranose (R_G), to galactose (R_{Gal}) or to rhamnose (R_{Rh}).

Solutions were concentrated under reduced pressure. Melting points are uncorrected. Optical rotations were determined at 20° in water, unless otherwise stated.

Isolation and Purification of the S VIII Polysaccharide.—The polysaccharide, which had been prepared by a method specifically designed to cause as little breakdown of the molecule as possible during its isolation,[17] was supplied in the form of a white fibrous solid which dissolved in water to give a highly viscous acidic solution, $[\alpha]_D$ +115° (*c* 0.4). The polysaccharide (7 g.) was dissolved in Fehling solution A (alkaline tartrate) (*ca.* 150 ml.), and Fehling solution B ($CuSO_4$) was added dropwise to the stirred solution until no more precipitation of the polysaccharide complex was obtained. The complex was removed by filtration under gravity on a sintered glass filter and washed well with cold water. The copper was removed from the complex by the addition of *N* hydrochloric acid (*ca.* 60 ml.) to the material suspended in water (1 l.) kept at 0°. The viscous solution was centrifuged, and the clear solution was poured into ethanol (4 vols.) with vigorous stirring. The precipitated polysaccharide was collected on the filter, washed with

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ethanol and dried *in vacuo* to give a fine white powder (6.2 g.).

The sodium salt of the S VIII polysaccharide was examined by electrophoresis using Whatman fiber-glass as the support and an electrolyte of crystalline borax (38 g.) in 0.12 *N* sodium hydroxide solution (1 l.). The paper was uniformly wetted with the electrolyte, and a 10% solution of the polysaccharide dissolved in the prepared electrolyte, was spotted on the base line.[18] After electrophoresis the carbohydrates were located by first spraying the paper with a 3% (w./v.) solution of α-naphthol in methanol followed by spraying it with concentrated sulfuric acid. The S VIII polysaccharide was thus located as a single discrete spot.

Electrophoresis of the S VIII polysaccharide (400 mg.) as described above, followed by extraction of the appropriate part of the paper with water, and deionization (resins), afforded the electrophoretically pure polysaccharide which had $[\alpha]_D$ +124° and equivalent weight 692. Hydrolysis of this polysaccharide with formic acid, followed by paper chromatographic examination of the hydrolyzate, showed it to be composed of glucuronic acid (1 part), galactose (1 part) and glucose (2 parts). The electrophoretically pure polysaccharide is chemically and physically indistinguishable from the starting material.

The polysaccharide could not be further fractionated by precipitation of its cetyltrimethylammonium salt and subsequent regeneration of the free polysaccharide.[19,20a,b] The S VIII polysaccharide obtained *via* its copper complex was deionized by passing its solution first through a bed of Amberlite ion-exchange resin IR 120(H) and then through a bed of IR 4B(OH).[21] The eluate was freeze-dried to give the ash-free polysaccharide which had $[\alpha]_D$ +121° (*c* 0.4) and equivalent weight of 703 (≡25.2% hexuronic anhydride), found by titration with standard alkali.

Anal. Calcd. for $[(C_6H_{10}O_5)_3.(C_6H_8O_6)]_x$ (polymer with a repeating unit of 3 hexose units and 1 hexuronic acid unit): C, 43.5; H, 5.72. Found: C, 43.6; H, 5.56; N, nil; P, nil.

The polysaccharide gave no color with iodine but gave a strong positive test for hexuronic acid with the naphthoresorcinol reagent.[22] Attempted autohydrolysis and attempted hydrolysis with 0.01 *N* sulfuric acid of 1% solutions of the polysaccharide at 100° for 8 hr. failed to liberate any sugars detectable on the chromatogram.

Component Sugars of the S VIII Polysaccharide.—The polysaccharide (1 g.) was heated with 90% formic acid (100 ml.) at 100° for 22 hr. The formic acid was removed by distillation and the residue further hydrolyzed by heating it with *N* sulfuric acid (15 ml.) for 2 hr. at 100°. After neutralization ($BaCO_3$) and filtration, a portion of the hydrolyzate was concentrated and examined on paper chromatograms. In solvent system d, detection with *p*-anisidine revealed two spots corresponding in position and color to galactose and glucose (visual ratio 1:2), and a component giving a red colored spot remained near the base line. In solvent system a, spots corresponding to galactose and glucose were again obtained together with a red spot (R_{Gal} 0.96) corresponding in position to a hexuronic acid. The main bulk of the hydrolyzate was passed first through a column of IR 120(H) resin (1.5 × 6 cm.) and then through a column of IR 4B(OH) resin (1.5 × 5 cm.). Concentration of the eluate gave the neutral sugars as a sirup (560 mg.) which was fractionated by partition chromatography on large sheets of filter paper using solvent system d as the mobile phase. Development was allowed to proceed for 5 days with intermediate drying of the papers at 24-hr. intervals. Extraction of appropriate sections of the paper afforded crystalline D-galactose, m.p. 165°, $[\alpha]_D$ +80° (*c* 1.1), which gave a 1-methyl-1-phenylhydrazone derivative, m.p. 186°, and D-glucose, m.p. 145°, $[\alpha]_D$ +50° (*c* 0.9) (mixed m.p.'s showed no depression).

The IR 4B column above was eluted with 10% formic acid and the formic acid then removed from the aqueous eluate by continuous extraction with ether. Examination of this latter eluate on paper chromatograms in solvent system a and detection with *p*-anisidine revealed one main spot (R_{Rh} 1.33) corresponding in position to authentic glucurono-6 → 3-lactone, and a faint spot (R_{Gal} 0.96) corresponding to glucuronic acid (galacturonic acid does not form a lactone and mannuronolactone has R_{Rh} 0.95). Concentration of this aqueous eluate to dryness and dissolution of the residue in glacial acetic acid on cooling afforded D-glucurono-6 → 3-lactone, m.p. and mixed m.p. 178°, $[\alpha]_D$ +18° (*c* 0.8).

Quantitative Estimation of the Component Sugars of S VIII.—Samples of the polysaccharide (*ca.* 30–40 mg.) were hydrolyzed for 20 hr. with 96% formic acid (3 ml.) in sealed tubes placed in a boiling water-bath. A weighed amount of L-rhamnose (*ca.* 15 mg.) was added and the formic acid removed by distillation. The residue was then hydrolyzed with *N* sulfuric acid (2 ml.) for a further 2 hr. at 100°. The acid was neutralized ($BaCO_3$), filtered, the filtrate concentrated to about 2 ml. and ethanol (6 ml.) was added. After standing at 5° for 8 hr. the precipitated barium D-glucuronate was removed on the centrifuge and the supernatant solution was concentrated to a small bulk and applied evenly to the base line of a paper chromatogram. The chromatogram was developed in solvent system d during 6 days with intermediate drying. The separated sugars were quantitatively extracted,[23] and the amounts of the sugars present were estimated colorimetrically by the benzidine method.[24] The results indicate that the galactose and the glucose are present in the ratio 1:2. Found: D-galactose, 21.4, 20.0, 24.0; D-glucose, 40.5, 42.1, 40.3 (based on the recovery of L-rhamnose).

The uronic anhydride content of the polysaccharide was determined by quantitative decarboxylation with 12% hydrochloric acid, and determination of the carbon dioxide produced, by an iodometric procedure.[25] Found: hexuronic anhydride, 27.8%, 26.8%, 27.8%.

Reduction of the S VIII Polysaccharide.—The freeze-dried ash-free S VIII polysaccharide (4 g.) was moistened with methanol and a solution of diazomethane in ether was added. When the initial vigorous liberation of nitrogen was over (10 min.) the excess diazomethane together with the solvents were removed by distillation. The residue was dissolved in water (150 ml.) to give a mobile solution which was added over 1 hr. to a solution of sodium borohydride (2 g.) in water (50 ml.) with vigorous stirring.[26] Stirring was continued overnight, and the excess borohydride was destroyed by acidification with 2 *N* sulfuric acid. The reaction mixture was poured with stirring into ethanol (4 vol.) and the precipitated polysaccharide was collected on the centrifuge. The above operations were repeated on this material after it had been deionized (resins) and freeze-dried again, to give a final product (3.2 g.) which was readily soluble in water to give a neutral mobile solution, $[\alpha]_D$ +130° (*c* 0.5). Attempted decarboxylation[25] yielded negligible amounts of carbon dioxide, and the naphthoresorcinol test[22] for uronic acid was negative.

The neutral polysaccharide (40 mg.) was hydrolyzed with 96% formic acid (4 ml.) in a sealed tube at 100° for 10 hr. and, after removal of the formic acid, hydrolysis was continued with *N* sulfuric acid (2 ml.) for 2 hr. at 100°. Examination of the neutralized ($BaCO_3$) hydrolyzate by paper chromatography in solvent system a revealed spots due to galactose and glucose, but no spot corresponding to glucuronic acid or its lactone could be detected. Prolonged development in solvent system d separated the two component sugars. Measurement of the intensities of the two spots produced with *p*-anisidine, using a photoelectric transmission densitometer,[27,28] indicated the galactose and the glucose to be present in the ratio 1:3.

Methylation of the S VIII Acidic Polysaccharide.—In order to prevent possible alkaline breakdown of the polysaccharide[29,30] during its methylation, the reducing end group was reduced to hexitol end group before methylation.

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To the sodium salt of the S VIII polysaccharide (2.1 g.) in water (50 ml.) was added a solution of sodium borohydride (15 mg.) in water (10 ml.) and the mixture was stirred for 1 hr. The excess sodium borohydride was destroyed by acidification of the reaction mixture with acetic acid. To the above solution of the polysaccharide was added, simultaneously and dropwise, sodium hydroxide (14 ml., 30% w./v.) and methyl sulfate (10 ml.) with vigorous stirring and ice-cooling, at a rate such that the mixture remained slightly alkaline. After 5 hr., the reaction mixture was allowed to warm to room temperature and sodium hydroxide (280 ml., 30% w./v.) and methyl sulfate (95 ml.) were added dropwise over 6 hr. and the stirring continued overnight. The same quantities of reagents were added over 5 hr., and the stirring was continued for a further 40 hr. The reaction mixture was heated on a boiling water-bath for 30 min. to destroy the excess methyl sulfate, and the volatile products were removed by distillation. The ice-cooled reaction mixture was brought to *p*H 2 by the cautious addition of 2 *N* sulfuric acid; the partially methylated polysaccharide was precipitated and was removed on a sintered glass filter. The product thus separated was methylated again using the above conditions, sufficient acetone being added to keep the polysaccharide in solution. The product isolated on acidification of the reaction mixture was further methylated by refluxing it with methyl iodide (40 ml.) containing methanol (1 ml.), while silver oxide (6 g.) was added portionwise over 6 hr. The final product obtained on chloroform extraction (1.71 g.) had $[\alpha]_D$ +68° (*c* 0.98 in acetone).

Anal. Calcd. for $(C_{36}H_{62}O_{21})_x$: OMe, 44.8. Found: OMe, 44.1.

Fission of the Methylated S VIII Polysaccharide.—A portion of the methylated S VIII polysaccharide (0.691 g.) was hydrolyzed by heating with 90% formic acid (100 ml.) for 10 hr. at 100°, and after removal of the formic acid by distillation, hydrolysis was continued by heating with *N* sulfuric acid (15 ml.) for a further 4 hr. After neutralization ($BaCO_3$) the hydrolyzate was filtered, and the filtrate was passed first down a column of IR 120(H) ion-exchange resin followed by IR 4B(OH) to give an eluate which on concentration gave the neutral methylated sugars as a sirup (A, 0.498 g.). The IR 4B resin above was eluted with *N* sulfuric acid, the eluate was neutralized ($Ba(OH)_2$) and the filtered solution was passed through a small bed of IR 120(H) resin to give an acid eluate, which on concentration, afforded the acidic methylated sugar (B, 0.123 g.).

Examination of the Neutral Methylated Sugars (A).—Paper chromatographic examination of this fraction in the solvent system d revealed two spots, having R_G 0.83 and 0.91 in the visual ratio 1:2. The mobility of the two components and the colors they gave with *p*-anisidine corresponded to those of 2,3,6-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose. Spraying with a 2% solution of dimethylaniline in glacial acetic acid containing 5% trichloroacetic acid[15] gave two purple spots corresponding to those revealed with the *p*-anisidine spray. No spots corresponding to tetra-*O*- or di-*O*-methyl sugars were detected. The sirup was moistened with a little methanol and set aside. The crystals which formed slowly were collected and recrystallized from ethanol (A1, 0.201 g.). The residual sirup when examined on paper chromatograms showed an increased intensity of the slow moving component (R_G 0.83). This sirup was fractionated on a cellulose column (3 × 24 cm.)[31] using light petroleum (b.p. 100–120°): butan-1-ol (7:3 v./v.) as the mobile phase. Evaporation of appropriate parts of the eluate gave two sirupy fractions which were purified by dissolution in water, acetone and then ether with intermediate filtration and evaporation, and dried.

Fraction 1 was chromatographically identical with (A1), and on seeding the whole fraction crystallized. After recrystallization from ethanol–ether mixture, the crystals were proved identical with (A1) causing no depression of m.p. and the two lots were combined.

Fraction 2 had R_G 0.83 in solvent system d, and was chromatographically pure (A2, 0.135 g.).

Characterization of (A1) as 2,3,6-Tri-*O*-methyl-D-glucose.—The crystals had m.p. 122° undepressed on admixture with authentic 2,3,6-tri-*O*-methyl-D-glucose and $[\alpha]_D$ +73° (*c* 1.0).

Anal. Calcd. for $C_9H_{18}O_6$: OMe, 41.9. Found: OMe, 40.2.

The bis-*p*-nitrobenzoate,[32] prepared in the usual way, had m.p. and mixed m.p. 188°, $[\alpha]_D$ −34° (*c* 0.8 in chloroform).

Characterization of (A2) as 2,3,6-Tri-*O*-methyl-D-galactose.—The sirup failed to crystallize. It had $[\alpha]_D$ +85° (*c* 0.9).

Anal. Calcd. for $C_9H_{18}O_6$: OMe, 41.9. Found: OMe, 40.6.

The sirup was dissolved in 1% methanolic hydrogen chloride (*c* 1.0) at 20° and the change in the specific optical rotation followed: $[\alpha]_D$ +46° (initial value), +20° (3 hr.), +2° (6 hr.), −10° (11 hr.), −28° (24 hr. constant value). This change in the specific optical rotation is that expected of 2,3,6-tri-*O*-methyl-D-galactose. The original sirup (A2, 50 mg.) was oxidized with bromine in the usual manner to give 2,3,6-tri-*O*-methyl-D-galactono-γ-lactone, 38 mg., m.p. and mixed m.p. 97°, $[\alpha]_D$ −37° (*c* 0.5).

Anal. Calcd. for $C_6H_{16}O_6$: OMe, 42.4. Found: OMe, 41.7.

Characterization of (B) as 2,3-Di-*O*-methyl-D-glucuronic Acid.—The sirup had $[\alpha]_D$ +36° (*c* 1.0).

Anal. Calcd. for $C_8H_{14}O_7$: OMe, 28.0. Found: OMe, 27.1.

Paper chromatographic examination of the sirup B in solvent system a revealed a single red spot with the *p*-anisidine spray R_G 0.64 which had the same rate of movement as authentic 2,3-di-*O*-methyl-D-glucuronic acid.

The sirup B (60 mg.) was refluxed with 1% methanolic hydrogen chloride (15 ml.) for 6 hr. and was neutralized (Ag_2CO_3). The methanolic solution was concentrated to dryness and the solution of the residue in dioxane–ether mixture (1:1 v./v., 8 ml.) was added dropwise with stirring to a solution of lithium aluminum hydride (0.1 g.)[33] in dioxane–ether mixture (1:1 v./v., 8 ml.). After stirring overnight, the excess lithium aluminum hydride was destroyed by the addition of ethyl acetate followed by water. The reaction mixture was brought to *p*H 8 by the addition of *N* sulfuric acid and the mixture filtered. The methyl-2,3-di-*O*-methyl-D-glucoside was extracted from the filtrate with chloroform. The concentrated chloroform extract was hydrolyzed by heating with 2 *N* hydrochloric acid (10 ml.) for 4 hr., neutralized (Ag_2CO_3), filtered and the filtrate evaporated to dryness. Extraction of the residue with hot ethyl acetate and concentration of the extract to a small bulk gave crystals of 2,3-di-*O*-methyl-D-glucose which had m.p. and mixed m.p. 109°, $[\alpha]_D$ +48° (*c* 0.7 in acetone).

Anal. Calcd. for $C_8H_{16}O_6$: OMe, 29.8. Found: OMe, 29.2.

Paper chromatographic examination of the above chloroform extract showed a single spot (R_G 0.73 in solvent system d, and R_G 0.71 in c), which had the same mobility as authentic 2,3-di-*O*-methyl-D-glucose.

Methylation of the Reduced S VIII Polysaccharide.—The neutral, reduced polysaccharide (3.4 g.) was methylated using sodium hydroxide and methyl sulfate as in the case of the acidic S VIII polysaccharide. The partially methylated product separated out during the final heating of the reaction mixture and the material was collected at this point. A product having OMe 36.6% was thus obtained, and its methoxyl content was raised[34] by dissolving the material (3.9 g.) in anisole (150 ml.) and freezing the solution using a Dry Ice-bath. Sodium (2 g.) in liquid ammonia (80 ml.) was added and the mixture allowed to warm to room temperature with frequent shaking. The ammonia was removed by distillation and the residue was refluxed with methyl iodide (48 ml.) for 8 hr. The reaction mixture was freed from excess methyl iodide and the distillation continued until about 10 ml. of anisole had been collected. The anisole solution of the polysaccharide remaining was again frozen, and lithium (2 g.) in liquid ammonia (100 ml.) was added and the above operations repeated. The reaction mixture was concentrated to near dryness, water being added at intervals during the distillation, until no more anisole

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remained in the flask. The aqueous solution was extracted continuously with chloroform, and the extract in 50% ethanol was passed down a small bed of ion-exchange resin MB 3 (Rohm & Haas Co., Philadelphia 5, Pa.) and concentrated to a light golden sirup (3.9 g.), $[\alpha]_D$ +63° (*c* 1.2 in chloroform).

Anal. Calcd. for $(C_9H_{16}O_5)_x$: OMe, 45.6. Found: OMe, 44.1.

Fractionation and Fission of the Methylated Reduced (Neutral) S VIII Polysaccharide.—The methylated polysaccharide (3.8 g.) was dissolved in chloroform (25 ml.), and petroleum ether (b.p. 100–120°) was added portionwise, the precipitated fractions being removed before carrying out further precipitation.

FRACTIONATION OF THE METHYLATED REDUCED S VIII POLYSACCHARIDE

Fraction	Petroleum ether added, ml.	Weight, g.	$[\alpha]_D$ (in $CHCl_3$)
1	50	0.102	+69°
2	70	0.010	+66
3	100	2.10	+65
4	140	1.34	+65
5	Residue	0.280	+59

Fractions 1, 2, 3 and 4 were combined and hydrolyzed as described for the methylated acidic S VIII polysaccharide. On paper chromatographic examination of the hydrolyzate in solvent system d, spots corresponding to 2,3,6-tri-*O*-methylgalactose and 2,3,6-tri-*O*-methylglucose in the visual ratio 1:3 were detected. No spots due to tetra-*O*, di-*O*- or mono-*O*-methylhexoses could be detected. On standing, the concentrated hydrolyzate gave crystals of 2,3,6-tri-*O*-methyl-D-glucose which were removed and characterized as described previously. Fractionation of the residual sirup upon a cellulose column gave more 2,3,6-tri-*O*-methyl-D-glucose which was combined with the first crop of crystals (2.3 g.) and 2,3,6-tri-*O*-methyl-D-galactose (0.785 g.) which was characterized as its crystalline aldono-γ-lactone, m.p. and mixed m.p. 97°, $[\alpha]_D$ −37° (*c* 0.8).

Periodate Oxidation of the Reduced Mixture of Tri-*O*-methylhexoses.—A portion (10 mg.) of the mixed tri-*O*-methylhexoses, obtained on hydrolysis of the fully methylated reduced S VIII polysaccharide, in water (2 ml.) contained in a 5-ml. volumetric flask, was reduced by the addition of sodium borohydride (8 mg.). After standing at room temperature for 2 hr., the excess borohydride was destroyed by the dropwise addition of 3 *N* sulfuric acid, and the acid solution was allowed to stand for 1 hr. to ensure complete removal of the reagent. Saturated *p*-hydroxybenzaldehyde solution (1 ml.)[35] and 0.3 *M* sodium metaperiodate solution (1 ml.) were added to the contents of the flask and the volume adjusted. with water, to 5 ml. The reaction mixture was kept in the dark at 20°, and 1-ml. samples were withdrawn at intervals and pipetted into centrifuge tubes containing 20% lead dithionite solution (1 ml.)[36] to precipitate the periodate as insoluble lead periodate which was removed on the centrifuge. One ml. of the clear supernatant liquid was added to a 0.2% solution of chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) (10 ml.) in sulfuric acid (60% v./v.) and the mixed reagents were allowed to stand for 30 min. The lead sulfate was removed by centrifugation and the supernatant liquid was transferred to glass stoppered tubes and heated on a vigorously boiling water-bath for 40 min. The absorption of the solution at 570 mμ was read on a Hilger spectrophotometer. A standard graph of absorption at 570 mμ against formaldehyde concentration was prepared using erythritol as a standard. No formaldehyde was liberated during the periodate oxidation of the above mixed tri-*O*-methylhexitols.

Periodate oxidation of 2,3,4-tri-*O*- and 1,3,4-tri-*O*-methylhexitols prepared by the borohydride reduction of the corresponding 2,3,4-tri-*O*- and 3,4,6-tri-*O*-methylhexoses under the conditions described above were found to liberate exactly 1 mole of HCHO per mole of tri-*O*-methylhexitol (complete after 8 min.). Periodate oxidation of the 1,4,5-tri-*O*- and 1,3,5-tri-*O*-methylhexitols prepared by reduction of the 2,3,6-tri-*O*- and 2,4,6-tri-*O*-methylhexoses failed to liberate any formaldehyde.

The reduced mixed tri-*O*-methylhexoses obtained from the polysaccharide were examined on paper chromatograms in solvent system d. Detection with a spray reagent of 1% aqueous sodium metaperiodate (4 parts), mixed prior to use with a 1% solution of potassium permanganate in 2% aqueous sodium carbonate solution (1 part),[37] revealed the sugars initially as a single pink spot against a white background (R_G 0.69) later observed as a white spot against a brown background. The *p*-anisidine spray failed to detect any sugars.

Partial Hydrolysis of the S VIII Acidic Polysaccharide.—The polysaccharide (6 g.) dissolved in 0.15 *N* sulfuric acid (300 ml.) was kept at 95° for 10 hr.. neutralized ($BaCO_3$), filtered and the filtrate passed down columns of ion-exchange resins IR 120(H) (3 × 10 cm.) followed by IR 4B(OH) (3 × 14 cm.). The acid effluent was neutralized ($Ba(OH)_2$), concentrated to about 30 ml. and ethanol (70 ml.) was added to precipitate the barium uronates which were collected on the centrifuge (C, 2.4 g.). The ethanol supernatant was passed through a small bed of ion-exchange resin MB 3 and the neutral eluate concentrated to a sirup (D, 1.08 g.). The IR 4B resin above, which retained the low molecular weight acid oligosaccharides, was eluted[38–40] with 0.3 *N* formic acid at the rate of 0.5 ml. per min., and the eluate was collected on a fraction cutter. Appropriate fractions were combined; tubes containing two components owing to slight overlap were combined and further fractionated on sheets of Whatman 3MM filter paper in solvent system b. Formic acid was removed from the fractions by continuous extraction with ether. The following fractions were obtained: U1, hexuronic acid, 0.084 g., R_{Gal} 0.96 (in solvent b); U2, aldobiouronic acid, 0.650 g., R_{Gal} 0.46; U_3, aldotriouronic acid, 0.255 g., R_{Gal} 0.10; and U4, aldotetraouronic acid, 0.030 g., R_{Gal} 0.05.

Characterization of U1 as D-Glucuronic Acid.—The acid was converted to crystalline D-glucurono-6 → 3-lactone, m.p. and mixed m.p. 178°, $[\alpha]_D$ +18° (*c* 0.5).

Characterization of U2 as 4-*O*-(β-D-Glucopyranosyluronic Acid)-D-glucopyranose.—The chromatographically pure acid had $[\alpha]_D$ +7° (*c* 2.0) and equivalent weight 359 by titration with standard alkali ($C_{12}H_{20}O_{12}$ requires equiv. wt. 356). Hydrolysis with formic acid followed by paper chromatographic examination of the hydrolyzate showed the presence of glucuronic acid and glucose.

Conversion of U2 to its Hepta-*O*-acetyl-methyl Ester.[13]—The acid (150 mg.) in methanol (9 ml.) was esterified by the addition of a solution of diazomethane in ether. After removal of the solvents the residue was dissolved in acetic anhydride containing 5% anhydrous zinc chloride (12 ml.), and the reaction mixture was kept at 60° for 90 min. The residue remaining after removal of the acetic anhydride was extracted with warm methanol (2 × 10 ml.). Water (20 ml.) was added to the extract and, after standing for 6 min. at 0°, the precipitated derivative was collected. Recrystallization from ethanol afforded a product (130 mg.) which had m.p. 251° and $[\alpha]_D$ +42° (*c* 0.7 in chloroform) (literature[13] m.p. 250°, $[\alpha]_D$ +41.7°).

Anal. Calcd. for $C_{27}H_{36}O_{19}$: C, 48.7; H, 5.55; OMe, 4.7. Found: C, 48.64; H, 5.31; OMe, 4.94.

Reduction of U2 to Cellobiitol.[41]—The aldobiouronic acid (150 mg.) was esterified with diazomethane, and the ester dissolved in water (10 ml.) was added dropwise with stirring to a solution of sodium borohydride (50 mg.) in water (6 ml.). Reaction was allowed to proceed at room temperature for 5 hr., and acetic acid was added to destroy the excess borohydride. Removal of the ionic material on ion-exchange resins gave a non-reducing eluate which was concentrated to dryness, extracted with hot methanol (3 × 8 ml.) and the extract concentrated to about 0.5 ml. On keeping, crystals of cellobiitol were obtained (120 mg.) which after three recrystallizations from methanol had $[\alpha]_D$ −7.8° (*c* 5.0) and m.p. 143°, undepressed on admixture with au-

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(41) M. Abdel-Akher, J. K. Hamilton and F. Smith, *ibid.*, **73**, 4691 (1951).

thentic cellobiitol prepared by the borohydride reduction of cellobiose.

Characterization of U3 as *O*-β-D-Glucopyranosyluronic Acid-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose.—The acid had $[\alpha]_D$ +12° (*c* 2.0) and equivalent weight 522 (calcd. for $C_{18}H_{28}O_{17}$: equiv. wt., 518). The acid was chromatographically pure, and determination of its reducing power by the alkaline hypoiodite method[42,43] gave a molecular weight of 528. Hydrolysis of the aldotriouronic acid with formic acid followed by paper chromatographic examination showed it to contain glucuronic acid and glucose.

Methylation of U3.—The aldotriouronic acid (100 mg.) was methylated using sodium hydroxide (105 ml., 30 w./v.) and methyl sulfate (24 ml.) in the usual way. The product was extracted with chloroform and further methylated using methyl iodide (8 ml.) and silver oxide (1 g.) to give a final product (92 mg.) which had $[\alpha]_D$ +11.5° (*c* 1.0 in $CHCl_3$).

Anal. Calcd. for $C_{29}H_{52}O_{17}$: OMe, 50.8. Found: OMe, 49.1.

The methylated aldotriouronic acid (80 mg.) was hydrolyzed with formic acid (as for the S VIII polysaccharide) and the hydrolysate fractionated on a sheet of filter paper in solvent system d. The acidic component remained near the base line and after elution from the paper was passed through a little IR 120(H) resin and concentrated to dryness (19 mg.). This acid sugar was chromatographically pure giving a single red spot with the *p*-anisidine spray having R_G 0.84 in solvent system a and moved to the same position as authentic 2,3,4-tri-*O*-methylglucuronic acid. It had $[\alpha]_D$ +40° (*c* 0.5). The neutral methylated sugar gave a single spot R_G 0.91 in solvent system d and on keeping gave crystals of 2,3,6-tri-*O*-methyl-D-glucose (41 mg.) which had $[\alpha]_D$ +72° (*c* 0.8), m.p. and mixed m.p. 122°.

Reduction of U3 to Cellotriitol.—The aldotriouronic acid (60 mg.) was converted to its methyl ester and reduced with aqueous sodium borohydride (as described for the conversion of U2 to cellotriitol), to give crystalline cellotriitol (48 mg.) which had $[\alpha]_D$ −5.5° (*c* 0.5) and m.p. 160° undepressed on admixture with authentic cellotriitol prepared by the borohydride reduction of cellotriose. The derivative co-chromatographed with authentic cellotriitol[44] having R_{Gal} 0.16 in solvent system a.

Characterization of U4 as *O*-β-D-Glucopyranosyluronic Acid-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-galactopyranose.—The barium uronates (C), obtained on the addition of ethanol to the acidic solution passing through the ion-exchange resins after partial hydrolysis of the S VIII polysaccharide, were examined on paper chromatograms in solvent system b. A component (R_{Gal} 0.05) corresponding in position to an aldotetraouronic acid was observed to be present. Separation of the uronates on sheets of Whatman 3MM filter paper in solvent system b during 5 days gave the chromatographically pure tetraouronic acid (280 mg.). It was identical in physical and chemical properties with U4 and the two parts were combined and examined together. No spot due to an aldopentaouronic acid could be detected on the chromatograms of the partially hydrolyzed S VIII polysaccharide.

The aldotetraouronic acid had $[\alpha]_D$ +65° (*c* 1.0), and equivalent weight 675 (required for an aldotetraouronic acid: equiv. wt., 670); hypoiodite oxidation[42,43] gave a molecular weight 698. Hydrolysis of U4 (8 mg.) with formic acid followed by paper chromatographic examination of the mixture of sugars showed it to contain glucuronic acid, galactose and glucose. Measurement of the spot intensities[27,28] indicated the galactose and glucose to be present in the ratio 1:2. The aldotetraouronic acid (10 mg.) was reduced directly with sodium borohydride (3 mg.) in water (5 ml.), and after deionization (resins) the reduced product was hydrolyzed with formic acid. Paper chromatographic examination of the hydrolyzate in solvent system d followed by detection with the *p*-anisidine spray revealed only glucose and a spot remaining near the base line (glucuronic acid). Detection with the silver nitrate spray showed in addition to glucose a spot on the chromatogram behaving in a manner similar to authentic dulcitol. The aldotetraouronic acid (U4, 120 mg.) was methylated as described for U3 to give a product (110 mg.) which had $[\alpha]_D$ +15° (*c* 1 in chloroform).

Anal. Calcd. for $C_{38}H_{67}O_{22}$: OMe, 49.7. Found: OMe, 48.8.

Hydrolysis of the methylated aldotetraouronic acid with formic acid and examination of the hydrolyzate on paper chromatograms in solvent d showed two spots with the *p*-anisidine spray, R_G 0.83 and 0.91 in the visual ratio 1:2, together with a third component which remained near the base line (tri-*O*-methylglucuronic acid). The components were separated on a sheet of Whatman 3MM filter paper in solvent system d to give: 1. 2,3,4-Tri-*O*-methyl-D-glucuronic acid, chromatographically pure. It gave a single red spot with the *p*-anisidine spray, had R_G 0.84 in solvent system a moving to the same position as an authentic specimen and had $[\alpha]_D$ +40° (*c* 0.4). The acid (19 mg.) in methanol (2 ml.) was neutralized with diazomethane and concentrated to dryness. The residue in water (8 ml.) was reduced by the addition of sodium borohydride (10 mg.), and the excess reagent was destroyed by the addition of *N* sulfuric acid. 0.3 *M* sodium metaperiodate (0.3 ml.) was added and the mixture kept at 40° for 4 hr. The reaction mixture was extracted with chloroform (4 × 8 ml.), the extract dried (anhyd. Na_2SO_4) and allowed to evaporate on a watch glass. Crystals of 2,3,4-tri-*O*-methyl-L-xylose, m.p. 89°, were obtained, which on examination on paper chromatograms in solvent system d gave a single red spot with the *p*-anisidine spray, R_G 0.96 corresponding in position with authentic 2,3,4-tri-*O*-methylxylose.

2. 2,3,6-Tri-*O*-methyl-D-galactose whose specific optical rotation in 1% methanolic hydrogen chloride fell from $[\alpha]_D$ +40° to −27° (28 hr.). The sugar (19 mg.) did not crystallize. It had R_G 0.83 in solvent system d and moved to the same position as authentic 2,3,6-tri-*O*-methylgalactose.

3. 2,3,6-Tri-*O*-methyl-D-glucose (32 mg.) which on keeping gave crystals, m.p. and mixed m.p. 121°, $[\alpha]_D$ +72°.

Partial Hydrolysis of the Aldotetraouronic Acid U4.—The acid (95 mg.) was partially hydrolyzed by heating it with 0.1 *N* sulfuric acid (10 ml.) for 4 hr. at 95°. Neutralization ($BaCO_3$) and examination of the filtered hydrolyzate on paper chromatograms in solvent system d showed the presence of a small amount of the aldotriouronic acid (U3) (R_{Gal} 0.10), a neutral disaccharide, R_{Gal} 0.61 ($[\alpha]_D$ +140), a large proportion of an aldobiouronic acid (U2) (R_{Gal} 0.46) and galactose and glucose in the visual ratio 3:1. The component (17 mg.) with R_{Gal} 0.46 was separated on a paper chromatogram and on reduction of its methyl ester with sodium borohydride gave crystals of cellobiitol, m.p. and mixed m.p. 143°.

Examination of the Neutral Sugars (D) Obtained from the Partial Acid Hydrolysis of the S VIII Polysaccharide.—The sirup D was examined on paper chromatograms in solvent system d, and detection with the *p*-anisidine spray showed galactose and glucose in the visual ratio 2:1 together with a disaccharide, R_{Gal} 0.62, and a trace amount of a spot corresponding in position with a trisaccharide, R_{Gal} 0.33 (trace). The sirup (D, 980 mg.) dissolved in water (50 ml.) was passed down a column of charcoal-Celite (1:1 w./w.; 3 × 28 cm.)[45] and the column eluted with water until all the monosaccharides had been removed, whereupon the column was eluted with 5% ethanol solution in water. Appropriate portions of the eluate were combined and concentrated to give the disaccharide (121 mg.) and a trisaccharide (8 mg.).

Characterization of the Neutral Disaccharide as 4-*O*-(α-D-Glucopyranosyl)-D-galactopyranose.—The disaccharide was chromatographically pure and had $[\alpha]_D$ +140° (*c* 1.1). Determination of its reducing power by alkaline hypoiodite gave a molecular weight of 349 (required 342). Hydrolysis with 2 *N* sulfuric acid for 8 hr. at 100° and paper chromatographic analysis of the hydrolysate showed the disaccharide to be composed of glucose and galactose in the ratio of 1:1. The disaccharide (5 mg.) was reduced with sodium borohydride (3 mg.) in water (2 ml.) at room temperature for 6 hr.; after removal of the ionic material (resins) the solution was hydrolyzed with sulfuric acid and examined on paper chromatograms in solvent system d. Detection with the *p*-anisidine spray showed only glucose, while detection with the silver nitrate spray showed glucose to be present together with a second component which corresponded in position to dulcitol and which produced a dark spot on the

(42) S. K. Chanda, E. L. Hirst, J. K. N. Jones and E. G. V. Percival, *J. Chem. Soc.*, 1289 (1950).

(43) J. K. N. Jones, *ibid.*, 3292 (1950).

(44) We thank Professor M. L. Wolfrom for the gift of a specimen of this compound.

(45) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

chromatogram at a rate corresponding to the authentic hexitol.

The disaccharide (78 mg.) was methylated with sodium hydroxide (15 ml., 30% w./w.) and methyl sulfate (5 ml.) followed by a second methylation using sodium hydroxide (10 ml., 30% w./w.) and methyl sulfate (4 ml.). The product, which was extracted with chloroform, was further methylated with Purdie reagents to give a sirup (70 mg.) having $[\alpha]_D$ +52° (*c* 1.0 in chloroform).

Anal. Calcd. for $C_{20}H_{38}O_{11}$: OMe, 54.6. Found: OMe, 54.2.

The methylated disaccharide (60 mg.) was hydrolyzed with 90% formic acid and the hydrolyzate examined chromatographically. In solvent system d *p*-anisidine revealed two spots, R_G 1.00 and R_G 0.83. The sugars were fractionated on a sheet of filter paper in solvent d and the sugars eluted from appropriate sections of the chromatogram. The fast moving component (R_G 1.00, 20 mg.) had $[\alpha]_D$ +80° (*c* 0.5) and gave an anilide which crystallized from light petroleum in the form of white needles, m.p. 132°. The slower moving component (R_G 0.83, 16 mg.) showed a change in its specific optical rotation from $[\alpha]_D$ +44° → −28° (28 hr.) when it was dissolved at 20° in 1% methanolic hydrogen chloride. The resultant glycoside was hydrolyzed, and the reducing sugar so obtained was oxidized with bromine water to give its aldono-γ-lactone, m.p. and mixed m.p. with authentic 2,3,6-tri-*O*-methyl-D-galactono-γ-lactone, 96°.

Periodate Oxidation of the S VIII Acidic Polysaccharide.—The polysaccharide (*ca.* 100 mg. accurately weighed) was dissolved in water (80 ml.), and the solution was adjusted to *p*H 2.2 by the addition of sulfuric acid; 0.3 *M* sodium metaperiodate solution (5 ml.) was added and the volume made up to 100 ml. A blank solution containing no polysaccharide was prepared similarly. Oxidation was allowed to proceed in the dark at 20°. At intervals, the periodate uptake was estimated by transferring samples (10 ml.), from the oxidation mixture and the blank, to a solution of 2 *N* sulfuric acid (15 ml.) containing 20% potassium iodide solution (4 ml.). The liberated iodine was titrated with 0.02 *N* sodium thiosulfate solution, using starch as an indicator. The molar periodate consumption per mole of anhydrohexose unit was as follows: 0.67 (after 8 hr.), 0.94 (20 hr.), 1.18 (48 hr.), 1.25 (72 hr.), 1.32 (96 hr.), 1.39 (120 hr.) and 1.44 (144 hr.). Extrapolation of the over oxidation part of the curve to zero time gave a value corresponding to a periodate uptake of 1.03 moles per anhydrohexose unit (*i.e.*, 165 g. for a polymer containing 1 hexuronic acid unit per 3 hexose units).

Hydrolysis of the Periodate Oxidized S VIII Polysaccharide.—The S VIII polysaccharide (110 mg.) was oxidized with periodate as described above. After 80 hr., ethylene glycol (5 ml.) was added to destroy the excess periodate, and the reaction mixture was dialyzed against water employing a cellulosic membrane. The concentrated non-dialyzable material was hydrolyzed with 90% formic acid (20 ml.) for 8 hr. at 100°. After removal of the formic acid, hydrolysis was continued with *N* sulfuric acid (8 ml.) for 2 hr. at 100°. Examination of the neutralized ($BaCO_3$) hydrolyzate on paper chromatograms failed to indicate the presence of hexose or hexuronic acid.

III. Discussion

The S VIII polysaccharide used in this investigation was prepared by E. R. Squibb and Sons, New Brunswick, N. J., and was supplied to us through the kindness of Professor M. Heidelberger. The polysaccharide which was free from protein and glucosan was first purified *via* its copper complex to give a material which is considered to be essentially homogeneous. It could not be further fractionated *via* its cetyltrimethylammonium salt, and on electophoresis using a glass fiber support and an electrolyte of borax and sodium hydroxide, the polysaccharide moved as a single sharp band. The polysaccharide recovered in both cases was chemically and physically indistinguishable from the original polysaccharide.

The polysaccharide had a high positive specific optical rotation $[\alpha]_D$ +121°, and its equivalent weight (703) is that expected of a hexose polymer having one hexuronic acid unit in four. The polysaccharide contained no nitrogen, phosphorus or ash and gave no color with iodine. Attempted autohydrolysis and attempted hydrolysis with 0.01 *N* sulfuric acid failed to liberate any reducing sugars, indicating the absence of furanose sugars. Total acid hydrolysis followed by paper chromatographic examination of the hydrolyzate showed the polysaccharide to be composed of galactose, glucose and glucuronic acid. The component sugars were separated chromatographically and identified as D-galactose, D-glucose and D-glucuronic acid. Quantitative estimation of the component sugars showed them to be present in the ratio D-galactose:D-glucose:D-glucuronic acid 1:2:1.

The S VIII acidic polysaccharide was reduced to the corresponding neutral hexose polymer by borohydride reduction of the esterified carboxyl group of the glucuronic acid. The original polysaccharide was freeze-dried in order to produce the largest possible surface area and was treated with a solution of diazomethane in ether. In the short contact time required to neutralize the polysaccharide, no secondary methylation occurred. Reduction of the aqueous solution of the esterified material with sodium borohydride effected an almost quantitative conversion of the glucuronic acid in the polymer to glucose. Acid hydrolysis of this new polysaccharide showed it to contain D-galactose (1 part) and D-glucose (3 parts).

Hydrolysis of the methylated S VIII polysaccharide afforded 2,3-di-*O*-methyl-D-glucuronic acid (1 part), 2,3,6-tri-*O*-methyl-D-galactose (1 part) and 2,3,6-tri-*O*-methyl-D-glucose (2 parts), each identified by the preparation of crystalline derivatives. As no other methyl sugars were detected, the above evidence indicates that the polysaccharide is a high molecular weight, straight chain polymer containing 1 → 4 links only.

The methylated, borohydride reduced S VIII polysaccharide appeared to be homogeneous and on hydrolysis afforded 2,3,6-tri-*O*-methyl-D-galactose (1 part) and 2,3,6-tri-*O*-methyl-D-glucose (3 parts). The absence of any 3,4,6-tri-*O*- or 2,3,4-tri-*O*-methylhexose, and hence the absence of any small proportion of any 1 → 2 or 1 → 6 links in the original polysaccharide, was shown by the fact that no formaldehyde was produced on the periodate oxidation of the reduced ($NaBH_4$) mixture of tri-*O*-methylhexoses.

In a subsidiary experiment it was found that the periodate oxidation of the tri-*O*-methylhexitols obtained on the borohydride reduction of 3,4,6-tri-*O*- and 2,3,4-tri-*O*-methylhexoses produced 1 mole of formaldehyde per mole of tri-*O*-methylhexitol. Periodate oxidation of the reduced 2,3,6-tri-*O*-2,4,6-tri-*O*-methylhexoses failed to liberate any formaldehyde. The proportions of 3,4,6-tri-*O*-methylaldohexose and 2,3,6-tri-*O*-methylaldohexose in a synthetic mixture were determined in this way with an error of less than 1%. The procedure has proved of use in the determination of the proportions of tri-*O*-methylhexoses in the hydroly-

zates of methylated polysaccharides especially when only micro amounts were available.

The evidence obtained from an examination of the hydrolysis products of the methylated neutral polysaccharide above again indicates that the original polysaccharide is a long straight-chain polymer joined throughout by 1 → 4 links.

Partial acid hydrolysis of the S VIII polysaccharide was effected with dilute sulfuric acid. The acid oligosaccharides were absorbed on ion-exchange resins and fractionally eluted with dilute formic acid. The neutral sugars were separated chromatographically to give the monosaccharides glucose and galactose, a disaccharide and a trisaccharide (trace, not examined). One aldobiouronic acid was obtained which was identical with that previously obtained and characterized by Goebel[13] as 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose. The β-linkage originally assigned to the acid was confirmed by reduction of the methyl ester of the acid with sodium borohydride to give crystalline cellobiitol.

One aldotriouronic acid was obtained which on hydrolysis gave D-glucuronic acid (1 part) and D-glucose (2 parts). Hydrolysis of the methylated acid gave 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,6-tri-*O*-methyl-D-glucose. The low positive specific optical rotation, $[\alpha]_D$ +12°, indicated that both links had the β-configuration, a conclusion which was borne out by the reduction of the methyl ester of the acid with sodium borohydride, to give crystalline cellotriitol. The experimental evidence indicates the aldotriouronic acid to be *O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose. One aldotetraouronic acid was obtained which on hydrolysis gave D-glucuronic acid (1 part), D-galactose (1 part) and D-glucose (2 parts). Hydrolysis of a small amount of the directly reduced ($NaBH_4$) acid, followed by paper chromatographic examination of the hydrolyzate, showed the presence of glucuronic acid, glucose and dulcitol. Since no galactose or sorbitol could be detected in the hydrolyzate, the galactose must form the reducing end group of the molecule. Hydrolysis of the methylated acid gave 2,3,4-tri-*O*-methyl-D-glucuronic acid (1 part), 2,3,6-tri-*O*-methyl-D-galactose (1 part) and 2,3,6-tri-*O*-methyl-D-glucose (2 parts). Partial acid hydrolysis of the aldotetraouronic acid gave a small amount of the aldotriouronic acid previously characterized, a small amount of a neutral disaccharide, chromatographically identical with the disaccharide obtained from the partial hydrolysis of the original polysaccharide, and a large proportion of an aldobiouronic acid identified as 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose. The experimental evidence indicates the structure of the aldotetraouronic acid to be *O*-β-D-glucopyranosyluronic acid-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-galactopyranose. The linkage between the glucose and galactose residue is assigned the α-configuration to account for the increase of the specific optical rotation from $[\alpha]_D$ +12° for the aldotriouronic acid to $[\alpha]_D$ +65° for the aldotetraouronic acid. The α-configuration assigned to the terminal link is also supported by the isolation of the neutral disaccharide 4-*O*-(α D-glucopyranosyl)-D-galactopyranose with the high optical rotation of $[\alpha]_D$ +140°, after hydrolysis of the tetrasaccharide. The neutral disaccharide which also was obtained from partial hydrolysis of the S VIII polysaccharide gave, on hydrolysis, glucose and galactose. Hydrolysis of a small amount of the reduced ($NaBH_4$) disaccharide followed by paper chromatographic examination of the hydrolyzate indicated the presence of glucose and dulcitol. This observation shows that galactose forms the reducing part of the molecule. Hydrolysis of the methylated disaccharide gave 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-galactose. The high specific optical rotation ($[\alpha]_D$ +140°) of the disaccharide, considered in conjunction with the above evidence, leads to the sugar being assigned the structure 4-*O*-α-D-glucopyranosyl-D-galactopyranose. No pentaouronic acid could be detected chromatographically, and since the composition of the original polysaccharide would not allow for one if the polymer is to be composed of a single structural repeating unit, the S VIII polysaccharide is believed to be composed of a four-unit repeating block with the structure of the tetraouronic acid. The nature of the link joining the galactose to the glucuronic acid unit in the polymer has not been decided, but the high positive specific rotation of the original polysaccharide indicates that it may be of the α-type. Partial hydrolysis of the neutral ($NaBH_4$ reduced uronic acid free) polysaccharide is at present being carried out in an attempt to isolate the 4-*O*-D-galactopyranosyl-D-glucopyranose disaccharide and to determine its mode of linkage.

The S VIII acidic polysaccharide was oxidized with metaperiodate in a solution adjusted to *p*H 2.2, a *p*H at which hydrolysis of the intermediate formyl esters is a minimum[46,47] and hence "over oxidation" is also at a minimum. Extrapolation of the "over oxidation" part of the periodate curve to zero time gave a value for the periodate uptake corresponding to 1.03 mole of periodate per anhydrohexose unit. An entirely 1 → 4 linked hexapyranose linear polymer should show an uptake of 1.0 mole of periodate per anhydrohexose unit. The experimental result is therefore in agreement with the structure of the polymer put forward as a result of methylation and partial hydrolysis studies. Hydrolysis of the periodate-oxidized S VIII polysaccharide followed by paper chromatographic examination of the hydrolyzate failed to reveal the presence of any sugars. This latter evidence indicates the absence of any 1 → 3 links in the polymer structure.

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