Immunopolysaccharides. Part I. Preliminary Studies of a Polysaccharide from Azotobacter chroococcum, *containing a Uronic Acid.*

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The acidic capsular polysaccharide of the soil organism *Azotobacter chroococcum* (Cooper, Daker, and Stacey, *Biochem. J.*, 1938, **32**, 1752) which gives serological cross reactions with certain pneumococcal specific antisera contains principally D-glucose and D-galactose units with a small proportion of D-glucuronic acid residue. From a neutral part of the methylated polysaccharide there were identified 2 : 3 : 6-tri-*O*-methyl-D-glucopyranose, 2 : 4 : 6-tri-*O*-methyl-D-glucopyranose, 2 : 3-di-*O*-methyl-D-glucopyranose, and 2 : 4 : 6-tri-*O*-methyl-D-galactopyranose. From an acidic portion of the hydrolysate there were isolated 2 : 4 : 6-tri-*O*-methylglucose, 2 : 3-di-*O*-methylglucose and 2 : 3-di-*O*-methyl-D-glucuronic acid. Comments on the structure are made. Possession of the aldobiuronic acid component accounts for the cross serological activity.

FOLLOWING the description by the senior author with other colleagues (Cooper, Daker, and Stacey (*Biochem. J.*, 1938, **32**, 1752) of an acidic polysaccharide produced by *Azotobacter chroococcum*, frequent attempts have been made to elucidate its structure. Although methylated derivatives could readily be made, these on methanolysis gave a very complicated mixture of methylated glycosides which even a most elaborate series of high-vacuum fractionations failed to separate satisfactorily. Chromatographic techniques now make it possible to attack the problem afresh.

The prolific production of capsular material, long recognised as a characteristic of *Azotobacter* species, may be an important factor in the development of humic substances in certain soils. From the recognition of glucose and uronic acids as constituents of the polysaccharide, Cooper, Daker, and Stacey (*loc. cit.*) placed it in the same class of compound as the immunologically specific polysaccharides of various pneumococcal types. Some while ago Professor M. Heidelberger kindly carried out some precipitin experiments for us with the *Azotobacter* polysaccharide and with a polysaccharide from *Rhizobium radicicolum*, with various pneumococcal specific antisera. He showed that both polysaccharides separately gave cross precipitin reactions in high dilution (1 : 100,000 after 4 hours at room temperature) with Type III anti-*Pneumococcus* horse serum and also with a mixture of anti-*Pneumococcus* sera of Types VI and XIV. It has been shown (Schlüchterer and Stacey, *J.*, 1945, 776) that part of the structure for the *Rhizobium* polysaccharide bore a close resemblance to that of the Type-III *Pneumococcus*-specific polysaccharide inasmuch as they both contained cellobiuronic acid residues in their respective repeating units. (In Schlüchterer and Stacey's paper the linkages in the oxycellulose formula are incorrectly shown as α instead of β.) The significance of the cellobiuronic acid unit in relating chemical constitution to serological specificity was demonstrated by Heidelberger and Hobby (*Proc. Nat. Acad. Sci.*, 1948, **28**, 516) in work with Types III and VIII anti-*Pneumococcus* horse sera and various oxycelluloses.

The nature of the uronic acid component of the *Azotobacter* polysaccharide is therefore of interest. It formed, however, a minor component only of the polysaccharide and indeed highly viscous polysaccharides have since been isolated from various other strains of *Azotobacter chroococcum* in which no uronic acid component could be detected.

The sample of material selected for the present study was part of the original sample described by Cooper, Daker, and Stacey (*loc. cit.*), and the crude material was purified by repeated precipitation from aqueous solution by ethyl alcohol. It appeared to be inhomogeneous and was obtained after being dried, as a water-soluble white fibrous solid, nitrogen-free and having a low negative optical activity. The free acid, obtained by repeated precipitation from acid solution, contained only traces of inorganic material. Paper chromatography of an acid hydrolysate revealed the presence of glucose, galactose and

glucuronic acid, the amount of the latter being estimated as *ca.* 4%. An unidentified sugar was also present. The galactose component had not previously been identified.

The polysaccharide was readily methylated satisfactorily by the usual techniques; the methylated polysaccharide was an acid which was carefully fractionated by solvent-precipitation from chloroform solution. An essentially homogeneous fraction (85% yield) was selected for methanolysis by boiling 5% methanolic hydrogen chloride for eight hours. By solvent extraction the hydrolysate was separated into a mixture of methyl glycosides and the barium salt of a partly methylated uronic acid derivative. Further complete methylation of the mixture of glycosides followed by isolation of the resulting tetra-*O*-methylhexoses and fractional crystallisation of their phenylglycosylamines showed it to contain D-glucopyranose and D-galactopyranose residues in the approximate ratio of 3 : 1.

The glycoside mixture was subjected to a complex series of fractional distillations in a high vacuum. No tetramethylhexose fraction could be detected. The properties of the various fractions and examination of them by paper chromatography indicated that this original mixture consisted of methyl tri-*O*-methylhexosides and methyl di-*O*-methylhexosides. Further methylation and examination of the tetra-*O*-methylhexoses produced, showed that both the tri- and the di-*O*-methylhexosides contained derivatives of both D-glucose and D-galactose. Preparation of crystalline aniline derivatives of the free sugars prepared from the mixture of tri-*O*-methylhexosides indicated the presence of 2 : 4 : 6-tri-*O*-methyl-D-glucopyranose and 2 : 4 : 6-tri-*O*-methyl-D-galactopyranose.

Various fractions having essentially similar properties were mixed, hydrolysed, and, initially in collaboration with Dr. J. K. N. Jones, were separated on cellulose columns according to the original method described by Hough, Jones, and Wadman (*J.*, 1949, 2511). The mixed methyl trimethylhexosides were found to consist of 2 : 3 : 6-tri-*O*-methyl-D-glucopyranose (40%), 2 : 4 : 6-tri-*O*-methyl-D-glucopyranose (15%), and 2 : 4 : 6-tri-*O*-methyl-D-galactopyranose (15%), together with unidentified material.

The fractions with lower methoxyl content contained small amounts of the same three trimethyl sugars together with two dimethylhexoses one of which was identified as 2 : 3-di-*O*-methyl-D-glucopyranose.

The barium salt, which gave a strong naphtharesorcinol test for uronic acid, had the properties of an oligosaccharide derivative and was further hydrolysed with 2N-sulphuric acid. After neutralisation and removal of barium ions with a resin the mixture of free sugars was fractionated on a cellulose column. There were separated 2 : 4 : 6-tri-*O*-methyl-D-glucose (30%), 2 : 3-di-*O*-methyl-D-glucose (30%), and a di-*O*-methyl-D-glucuronic acid containing the 2 : 3-di-*O*-methyl derivative. At this stage of the work it is not possible to estimate the molecular structure of the polysaccharide but several important facts have been established. The absence of a fully methylated "end" group and the significant content of dimethylhexoses indicate that the molecule is a highly branched structure similar to that of the serologically related *Rhizobium radicicolum* polysaccharide. The main chain of the polysaccharide is built up from both glucose and galactose residues in the proportion of 3 : 1. From the direction of rotational change on hydrolysis of the polysaccharide and on methanolysis of its methyl ether and from the infra-red spectrum of the parent polysaccharide (Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171) it is clear that the linkages were mainly of the β-type. The galactose residues and a proportion of the glucose residues are linked through the 1 and the 3 position. Other glucose residues are attached by both the 1 ⟶ 4 and the 1 ⟶ 6 linkages. The order of attachment of the hexose residues has not yet been established but it is clear that glucose units form the main branch points in the molecule.

The most striking finding is that only glucose residues are isolated from the glucuronic acid-containing oligosaccharide. In this portion of the molecule the proved 1 ⟶ 3 linkages of the glucose units and the 1 ⟶ 4 linkages in the glucuronic acid residue, together with the glucose residues with undoubted β-linkages in the molecule show a very close structural relation to that of the Type-III *Pneumococcus* specific polysaccharide molecule (Reeves and Goebel, *J. Biol. Chem.*, 1941, **131**, 511). This structural similarity undoubtedly accounts for the Type III cross serological relationships already mentioned.

Experimental

Properties of the Polysaccharide.—The polysaccharide was part of the material prepared on a gelatine medium by the method of Cooper, Daker, and Stacey (*loc. cit.*), and it contained a high proportion of ash and a protein constituent (total N, 1·0%). It was purified by repeated precipitation from dilute hydrochloric acid solution by addition of alcohol and when dried it formed an asbetos-like mass which was essentially free from ash and nitrogen and had $[\alpha]_D^{15}$ −10°.

Infra-red Absorption Spectra.—The infra-red spectra of both the *Azotobacter* polysaccharide and the *Rhizobium radicicolum* polysaccharide were investigated in the range 1700—725 cm.$^{-1}$. The absence of absorption at 840 cm.$^{-1}$ in the case of both polysaccharides and the presence of peaks of moderate intensity at 890 cm.$^{-1}$ and 892 cm.$^{-1}$ in the case of the *Azotobacter* and *Rhizobium* polysaccharides, respectively, indicate that in each case the glucose units in the polysaccharides, are in the β-configuration.

The presence of absorption peaks at 1595 and 1583 cm.$^{-1}$ in the spectrum of the *Rhizobium* polysaccharide confirmed the presence of the carboxylate ion arising from the glucuronic acid residues. Relatively weak absorption was displayed in the same region by the *Azotobacter* polysaccharide showing that the proportion of uronic acid residues in this polysaccharide was very small.

Hydrolysis and Chromatographic Behaviour.—A sample of the polysaccharide (40 mg.) was hydrolysed at 100° for 2 hr. with 2N-sulphuric acid (10 c.c.). After being neutralised with barium carbonate the hydrolysate was analysed (*a*) by separation for 1—3 days in butyl alcohol (40%)–ethyl alcohol (10%)–water (49%)–ammonia (1%) by ordinary paper chromatography and the components present detected by spraying with aniline hydrogen phthalate, (*b*) by ionophoresis in a borate buffer (pH 10·6) for 1—2 hr. at 900 v and the components detected by spraying with aniline hydrogen phthalate. The sugars identified were glucose (major portion), galactose (minor portion), glucuronic acid (trace), and mannose (trace).

In addition an unidentified sugar was present in significant amount. It stained yellowish-brown with aniline hydrogen phthalate and had a high R_F value (>0·5) and a negligible *Mg* value. It would appear to be a dideoxy-sugar and it is still under investigation.

Methylation Procedure.—The polysaccharide (15 g.) (ash, 5%; moisture, 24%) in water (100 c.c.) and 30% aqueous sodium hydroxide (80 c.c.), was stirred at room temperature for 2½ hr. during the dropwise addition of 30% sodium hydroxide (10 × 40 c.c.) and dimethyl sulphate (11 × 16 c.c.). After a further 3 hours' stirring, the mixture was heated at 100° for 10 min., cooled in ice, and neutralised (5N-sulphuric acid). When reheated to 100°, the separated sodium sulphate redissolved, and the insoluble methyl ether was filtered off. Partially methylated material remaining in the filtrate was recovered by cooling, removing the sodium sulphate, and concentrating the liquor to dryness at 30°, the residue being added to the bulk of the methylated product and further methylated at 16°. Four more methylations were applied. The crude product, separated as the sodium salt, was purified by suspending it in water (200 c.c.), acidifying it (Congo-red) with sulphuric acid, and shaking it with equal volumes of chloroform–acetone (5 : 1). The resulting solution was washed, dried, and concentrated, and the acidic methylated product precipitated by addition of excess of light petroleum (b. p. 40—60°). A sample so isolated after four methylations had: ash, 1·2; OMe (corr.), 37·0%: after six methylations the product, after being dried in a vacuum, formed a white powder (6·94 g.): ash, 0·4; OMe (corr.), 40·0%. Further methylation did not increase the methoxyl content.

Fractionation of the Methylated Polysaccharide.—The methylated polysaccharide (6·8 g.) was dissolved in chloroform (100 c.c.)–acetone (25 c.c.) and fractionally precipitated by the gradual addition of light petroleum (b. p. 40—60°). The precipitates were isolated on the centrifuge, washed with petroleum, and dried in a vacuum at 60°. Fractions were obtained as follows:

Fraction	Wt. (g.)	Ash (%)	OMe (%)	$[\alpha]_D^{21}$	n_D^{25}
F1	0·22	—	—	—	—
F2	3·905	0·3	38·5	−24°	3·78
F3	0·739	nil	38·8	−25	1·80
F4	0·308	nil	39·5	−25	0·79
F5	1·114	nil	40·5	+27	0·35
F6	0·099	nil	—	—	—

[All rotations and viscosities were measured at 0·9% concentration in chloroform–acetone (8 : 1).]

Fractions F1, which was mainly inorganic, and F6, a syrup obtained by evaporation of the final liquors, were not investigated. F2 separated very rapidly as a gel, which hardened when ground with light petroleum (b. p. 40—60°). Attempts to re-fractionate F2 succeeded in

separating only traces of material with reduced viscosity, the bulk remaining unchanged. Fractions F2 and F3 gave identical hydrolysis curves and thus were reasonably homogeneous. They were therefore combined for methanolysis with similar fractions from subsequent methylations and fractionations: F4 was not further investigated.

Fraction F5 was relatively unstable to acid: when boiled with 0·25% dry methanolic hydrogen chloride, its rotation changed: $[\alpha]_D$ +27° (initial), +66° (15 min.), +66° (30 min.), +62° (45 min.), +61° (90 min.), +54° (210 min.), +49° (330 min.), +44° (510 min., equilibrium value). It was not further investigated.

Esterification of the Methylated Polysaccharide.—Fraction F2 (80·3 mg.), dissolved in dry methanol (10 ml.), was treated with an excess of ethereal diazomethane, slight effervescence occurring. After removal of the solvents, the residue was dissolved in chloroform–acetone (8 : 1) and precipitated by addition of light petroleum, forming a white powder {ash, 0·6; OMe (corr.), 39·3%; $[\alpha]_D^{20}$ −25°; $n_{sp.}^{25}$ 1·82}.

Methanolysis of the Methylated Polysaccharide.—Preliminary experiments showed that the most convenient concentration of methanolic hydrogen chloride was 5%. The methylated polysaccharide (10·0 g.) was boiled under reflux with 5% dry methanolic hydrogen chloride (500 c.c.). The polysaccharide was treated in batches in order to compare the methanolysis curves of various fractions: typical rotations were as follows: $[\alpha]_D$ +6° (1 hr.), +26·5° (2 hr.), +47·5° (3·5 hr.), +56·5° (4·5 hr.), +65·5° (6 hr.), +70·5° (7 hr.), +77·5° (9 hr.), +81·5° (11 hr.), +88° (14 hr.), +90·5° (17 hr.), +92° (19 hr.), +94° (22 hr.), +94·5° (24 hr.), +95° (27 hr., equilibrium value). The combined mixtures, after neutralisation with silver carbonate, were filtered, the silver residues well washed with warm methanol, and the combined filtrate and washings concentrated to a syrup. This syrup, which did not reduce Fehling's solution, was digested for 3½ hr. at 50—60° with saturated aqueous barium hydroxide (200 c.c.), the solution neutralised with carbon dioxide and filtered, and the barium carbonate exhaustively extracted with methanol. The combined aqueous and methanolic solutions were evaporated at 40—50° to a syrup which after being dried in a vacuum (P_2O_5) was dissolved in dry methanol (20 c.c.), and dry ether (50 c.c.) added. Addition of dry light petroleum precipitated a barium salt which was isolated on the centrifuge, twice reprecipitated from the same solvents, and finally triturated with ether. "Barium salt (A1)" formed a white powder (0·911 g.) giving a positive naphtharesorcinol test. The equiv. weight was estimated by dissolving it in an excess of ethanolic hydrogen chloride, and back titrating potentiometrically [Found: OMe, 29·9; Ba (sulphated ash), 5·8%; equiv., 496].

The liquors and washings from the separation of the barium salt were concentrated to a syrup, "Glycosides A1" (9·030 g.), which gave a negative naphtharesorcinol test {Found: OMe, 29·9%; $[\alpha]_D^{20}$ +76·5°, *c*, 1·9 in $CHCl_3$}.

Methylation of "Glycosides A1".—"Glycosides A1" (0·486 g.) was methylated four times with methyl iodide and silver oxide, and the resulting syrup (0·503 g.) distilled in a high vacuum, as follows:

Fraction	Wt. (g.)	B. p./0·005 mm.	n_D^{19}	OMe (%)	Physical state
GT1	0·275	62—69°	1·4440	62·0	Highly mobile syrup
GT2	0·039	Bath temp. 200	1·4568	—	Mobile syrup
GT3	0·189	Still residue	—	—	Dark resin

Fraction GT1 (0·25 g.), which showed $[\alpha]_D^{20}$ +94° in chloroform (*c*, 1·0), was hydrolysed at 100° with 1·5N-sulphuric acid (20 c.c.): $[\alpha]_D^{18}$ +106·5° (initial) ⟶ +80° (19·5 hr., equilibrium value). The strongly-reducing hydrolysate was neutralised (litmus) with sodium hydroxide, evaporated to dryness at 40°, and the dry residue extracted with chloroform. The filtered extract was concentrated to a syrup which crystallised; repeated recrystallisation from light petroleum (b. p. 60—80°) afforded needles (0·058 g.) of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, m. p. and mixed m. p. 80—86°, $[\alpha]_D^{26}$ +84° (*c*, 1·0 in H_2O) (Found: OMe, 52·3. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%). The sugar (0·1 g.) was slowly distilled with aniline (0·04 g.), in dry ethanol (2 c.c.) and dry benzene (0·5 c.c.) during 7 hr. After removal of solvents crystalline tetra-*O*-methyl-*N*-phenylglucopyranosylamine was obtained, having m. p. and mixed m. p. 131—133°.

The mother-liquors from which the crystalline hexose had been isolated were concentrated to a syrup (0·126 g.) which was similarly converted into the aniline derivative. The resulting syrup was dissolved in a little hot ethanol, needles (23 mg.) separating on cooling. They were tetra-*O*-methyl-*N*-phenyl-D-galactopyranosylamine, m. p. and mixed m. p. 191°, $[\alpha]_D^{17}$ −68° ⟶ +41° (*c*, 0·4 in $COMe_2$). Concentration of the ethanolic mother-liquors afforded a residue which when crystallised from light petroleum yielded needles (10 mg.) of the above aniline derivative of tetra-*O*-methyl-D-glucose.

Distillation of "Glycosides A1."—"Glycosides A1" (8·536 g.) was distilled in a high vacuum from a Widmer flask, as follows :

Fraction	Wt. (g.)	B. p./0·005 mm.	n_D^{18}	OMe (%)	$[\alpha]_D^{20°}$ in $CHCl_3$	Physical state
M1	3·700	90—94°	1·4587	51·6	+88° (*c*, 1·1)	Mobile syrup
M2	0·769	100—120	1·4619	49·5	+95° (*c*, 2·2)	Syrup
M3	3·930	—	—	—	—	Very viscous brown syrup

The first drop of distillate had n_D^{18} 1·4579, indicating the absence of any tetra-*O*-methyl-hexosides.

Fraction M3 was further hydrolysed by boiling 10% methanolic hydrogen chloride as follows : $[\alpha]_D$ +76·5° (1·0 hr.), +80·5° (3·0 hr.), +87·5° (7·5 hr.), +88·5° (10·5 hr.), +90·5° (13·5 hr., equilibrium value). The hydrolysate, non-reducing and giving a negative naphtharesorcinol test, was neutralised with silver carbonate and worked up in the usual way to yield a syrup, "Glycosides A2," having OMe, 37·2%. Treatment with barium hydroxide failed to yield a barium salt, thereby confirming the absence of any uronic acid residues.

Distillation of "Glycosides A2." This syrup (3·722 g.) was distilled from a Widmer flask as follows :

Fraction	Wt. (g.)	B. p./0·005 mm.	n_D^{19}	OMe (%)	$[\alpha]_D^{20}$ in $CHCl_3$	Physical state
M3*a*	0·951	104—120°	1·4596	50·0	+99° (*c*, 2·1)	Syrup
M4	0·973	124—125	1·4685	44·8	+93° (*c*, 1·0)	Viscous syrup
M5	1·690	residue	—	—	—	Brown resin

Redistillation of fraction M5 produced one fraction (0·295 g.), b. p. 120—180°/0·007 mm., n_D^{18} 1·482 (Found : OMe, 36·1%), which formed a gel on cooling. Since these properties did not correspond with those of a monosaccharide derivative, the distillate was recombined with the residue and boiled under reflux with 10% methanolic hydrogen chloride for 52 hr., the solution being too dark for optical measurements. From the hydrolysate syrupy "Glycosides A3" (1·422 g.; OMe, 36·0%) was obtained in the usual way and distilled :

Fraction	Wt. (g.)	B. p./0·005 mm.	n_D^{21}	OMe (%)	$[\alpha]_D^{20}$ in $CHCl_3$	Physical state
M6	0·212	106—110°	1·4612	44·8	+77° (*c*, 1·1)	Mobile syrup
M7	0·287	111—118	1·4740	43·5	+84·5° (*c*, 1·0)	Syrup
M8	0·145	131—*ca.* 200	1·472	40·0	+88° (*c*, 1·0)	Slightly yellow viscous syrup
M9	0·712	residue	—	36·5	—	Dark resin

The residue was not investigated.

Preliminary Chromatographic Examination of Fractions M1—M9.—Small portions of these glycoside fractions were hydrolysed for 30 min. with boiling 2·5N-sulphuric acid, the hydrolysate being neutralised with sodium hydroxide and evaporated to dryness. The residue was extracted with chloroform, evaporation of each extract yielding a reducing syrup which was dissolved in a few drops of water. A little of each solution was placed at the top of a Whatman No. 1 filter-paper chromatogram (45 × 10 cm.) and allowed to dry, and the paper irrigated for 12 hr. with the organic phase of an acetic acid–*n*-butanol–water mixture (1 : 4 : 5). After being dried and developed in the usual way with ammoniacal silver nitrate, each fraction was found to give two spots, corresponding to a trimethyl- and a dimethyl-hexose, their relative intensities varying roughly according to the methoxyl content of the original hexoside fraction. No evidence was obtained of any spot corresponding to a tetra-*O*-methylhexose. Applied to known methylated hexoses, the above procedure clearly separated di-, tri-, and tetra-methylglucose, but failed to give adequate separation between the isomeric trimethylglucoses. Fractions M2 and M3 gave identical chromatograms, and were combined for further examination.

Hydrolysis of Fraction M1. Fraction M1 (1·01 g.) was hydrolysed with 1·5N-sulphuric acid (50 c.c.) at 100° : $[\alpha]_D^{18}$ +103° (0·25 hr.) ⟶ +72° (17 hr., equilibrium value). The strongly-reducing hydrolysate was neutralised with sodium hydroxide and worked up as previously described, yielding syrup (0·939 g.; OMe, 41·5%). A portion (0·205 g.) was converted into the aniline derivative (50 mg.), identified as the derivative of 2 : 4 : 6-tri-*O*-methyl-D-glucose.

Methylation of Fraction M1.—Fraction M1 (0·977 g.) was methylated four times with methyl iodide and silver oxide, and the resulting syrup (1·033 g.; OMe, 58·6%) distilled in a high vacuum as follows :

Fraction	Wt. (g.)	B. p./0·02 mm.	$n_D^{18·5}$	(OMe %)	$[\alpha]_D^{18}$ in $CHCl_3$	Physical state
M1T1	0·864	64°	1·4438	62·4	+94° (*c*, 1·0)	Highly mobile syrup
M1T2	0·100	130	1·4469	56·4	+98° (*c*, 1·0)	Mobile syrup
M1T3	0·059	residue	—	—	—	Brown resin

M1T2 and M1T3 were not investigated.

Hydrolysis of Fraction M1T1.—This fraction (0·84 g.) was hydrolysed with 1·5N-sulphuric acid at 100°, the following rotations being obtained: $[\alpha]_D^{18}$ +106° ⟶ +87° (17 hr., equilibrium value). When worked up in the usual way, the strongly-reducing hydrolysate afforded a syrup (0·782 g.) which partially crystallised as needles (0·120 g.), m. p. 77—81° alone and in admixture with tetra-*O*-methyl-D-glucopyranose, $[\alpha]_D^{19}$ +87° (*c*, 1·0 in H_2O) (Found: OMe, 52·6. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%). The free sugar (0·110 g.) was converted into the aniline derivative, which formed needles (0·035 g.) (from ethanol), m. p. 133—134° unchanged in admixture with the aniline derivative of tetra-*O*-methyl-D-glucose, $[\alpha]_D^{18}$ +240° (*c*, 1·0 in $COMe_2$) (Found: OMe, 39·8. Calc. for $C_{16}H_{25}O_5N$: OMe, 39·9%).

The mother-liquors from the isolation of the free sugar were concentrated to a syrup (0·503 g.) which was converted into the aniline derivative. Repeatedly crystallised from ethanol, the product formed needles (0·053 g.), m. p. 192—193°, of the aniline derivative of tetra-*O*-methyl-D-galactopyranose, $[\alpha]_D^{17}$ −80° ⟶ +44° (*c*, 0·50 in COMe) (Found: OMe, 39·7. Calc. for $C_{16}H_{25}O_5N$: OMe, 39·9%). No glucose derivatives could be isolated from the mother-liquors.

Hydrolysis of Fractions M2 and M3.—The combined fractions (0·872 g.) were hydrolysed by 1·5N-sulphuric acid at 100°: $[\alpha]_D^{18}$ +104° ⟶ +66·5° (19·5 hr., equilibrium value). The usual procedure yielded a strongly-reducing syrup (0·827 g.; OMe, 35·6%). A portion (0·400 g.) was converted into the aniline derivative, which was a mixture, white needles (21 mg.), m. p. 155° after sintering at 150°, $[\alpha]_D^{18}$ −93° ⟶ +86° (*c*, 0·25 in $COMe_2$).

Methylation of the Combined Fraction M2—M3.—The combined fraction (0·737 g.) was methylated six times with methyl iodide and silver oxide, and the resulting syrup (0·734 g.; OMe, 59·1%) distilled in a high vacuum as follows:

Fraction	Wt. (g.)	B. p./0·005 mm.	n_D^{20}	OMe (%)	$[\alpha]_D^{20}$ in $CHCl_3$	Physical state
M2T1	0·568	67°	1·4430	61·7	+100° (*c*, 1·0)	Highly mobile syrup
M2T2	0·092	100	1·4550	54·3	+95·5° (*c*, 1·0)	Mobile syrup
M2T3	0·047	residue	—	—	—	Brown resin

M2T2 and M2T3 were not investigated.

Fraction M2T1 (0·56 g.) was hydrolysed at 100° with 1·5N-sulphuric acid: $[\alpha]_D^{18}$ +109° ⟶ +79·5° (15·5 hr., equilibrium value). The usual isolation procedure afforded a strongly-reducing syrup (0·471 g.) which partially crystallised. The crystals were separated by trituration with ether and filtration: repeatedly recrystallised from dry ether–dry light petroleum (b. p. 60—80°) they formed needles (0·068 g.) of tetra-*O*-methyl-D-glucopyranose (aniline derivative, m. p. 136°).

The mother-liquors from the isolation of the sugar were evaporated to a syrup (0·228 g.) which was converted into the aniline derivative, identified as that of tetra-*O*-methyl-D-glucopyranose.

Separation of Hexoside Mixtures on Cellulose Columns.—Samples of the free sugars obtained by hydrolysis of fractions M1 and the combined M2—M3 were combined (total wt., 1·21 g.), dissolved in *n*-butanol–light petroleum (b. p. 80—100°), and separated on cellulose by the method of Hough, Jones, and Wadman (*loc. cit.*), yielding 6 fractions. The first two (0·55 g. and 0·12 g.) consisted mainly of a sugar with R_G value 0·83 together with a trace of a sugar with R_G 0·76. The syrup slowly crystallised, recrystallisation from ether affording 2 : 3 : 6-tri-*O*-methyl-D-glucose, R_G 0·83, m. p. 116° alone and in admixture with an authentic specimen.

Three sugars, R_G values 0·83, 0·76, and 0·68, were present in the remaining four fractions, which were as follows:

Fraction	3	4	5	6
Wt. (g.)	0·08	0·07	0·16	0·28
$[\alpha]_D$ in water	+60°	—	+61°	+70°

Fraction 3 partially crystallised and was identified as 2 : 4 : 6-trimethylglucose. The residue was converted into the aniline derivative in the usual way, the product after recrystallisation from ethanol–ether having m. p. 164°, alone and mixed with the aniline derivative of 2 : 4 : 6-tri-*O*-methyl-D-glucose. The free sugar from this anilide had R_G 0·77, identical with 2 : 4 : 6-tri-*O*-methyl-D-glucose.

The last three fractions did not crystallise: the largest (fraction 6) was converted into the aniline derivative, yielding a product which after recrystallisation from ethanol had m. p. 177°, undepressed on admixture with the authentic aniline derivative of 2 : 4 : 6-tri-*O*-methyl-D-galactose (m. p. 178°). The free sugar had R_G 0·68: 2 : 4 : 6-tri-*O*-methyl-D-galactose has R_G 0·67.

Examination of "Barium Salt A1."—"Barium salt A1" (0·67 g.) was hydrolysed with boiling 2N-sulphuric acid (60 c.c.) for 5 hr. ($[\alpha]_D^{16}$ *ca.* +50°, equilibrium value); the hydrolysate was neutralised with barium carbonate and filtered. The filtrate was treated with Amberlite resin (IR-100) and concentrated to a syrup (0·4 g.) which was found by paper chromatography to contain two sugars. These were separated on a cellulose column, water-saturated *n*-butanol being used as developing solution, whereby three fractions were obtained. The first on concentration afforded crystalline 2 : 4 : 6-tri-*O*-methyl-D-glucose (0·11 g.), m. p. 123° alone and mixed with an authentic sample. The third fraction (0·1 g.) consisted of a dimethylhexose: this was shown to be a glucose derivative by methylation with silver oxide and methyl iodide, the product on hydrolysis affording crystalline 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, m. p. and mixed m. p. 94°, and was identified in a separate experiment as 2 : 3-di-*O*-methyl-D-glucose. The intermediate fraction (0·17 g.) contained largely a mixture of 2 : 4 : 6-tri-*O*-methyl-D-glucose and 2 : 3-di-*O*-methyl-D-glucose. No galactose derivative could be detected in this fraction and the uronic acid derivative was not identified.

Another sample of "Barium salt A1" (0·5 g.) was hydrolysed as above, neutralised with barium carbonate, and evaporated to dryness. The solid was exhaustively extracted in a Soxhlet extractor with chloroform–acetone to remove the methylated sugars. The residue was dissolved in 10 c.c. of water, a few drops of bromine added, and the solution kept at room temperature for 24 hr. After evaporation of the water and extraction of the dry residue with ether there was obtained from the ethereal solution a syrup (50 mg.), which was boiled with dry 1% methyl alcoholic hydrogen chloride (5 c.c.). After removal of the hydrochloric acid with silver carbonate the filtered solution was saturated with ammonia and kept overnight. Removal of the solvent gave a crystalline product (20 mg.), identified by m. p. and mixed m. p. 156° as 2 : 3-di-*O*-methyl-D-glucarodiamide (Smith, *J.*, 1940, 1045).

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