203. *The Capsular Polysaccharide of* Rhizobium Radicicolum.

By (Miss) E. Schlüchterer and M. Stacey.

On acid hydrolysis the methylated derivative of the capsular polysaccharide synthesised by *Rhizobium radicicolum* (Clover Bartel "A" strain) yields approximately equimolecular parts of 2 : 3 : 6-trimethyl glucose, 2 : 3-dimethyl glucose, and 2 : 3-dimethyl glucuronic acid. Part of the last two constituents were shown to originate from a cellobiuronic acid derivative the presence of which relates the *Rhizobium* polysaccharide immunologically to the Type III pneumococcus specific polysaccharide. A structure is suggested for the repeating unit in the *Rhizobium* polysaccharide.

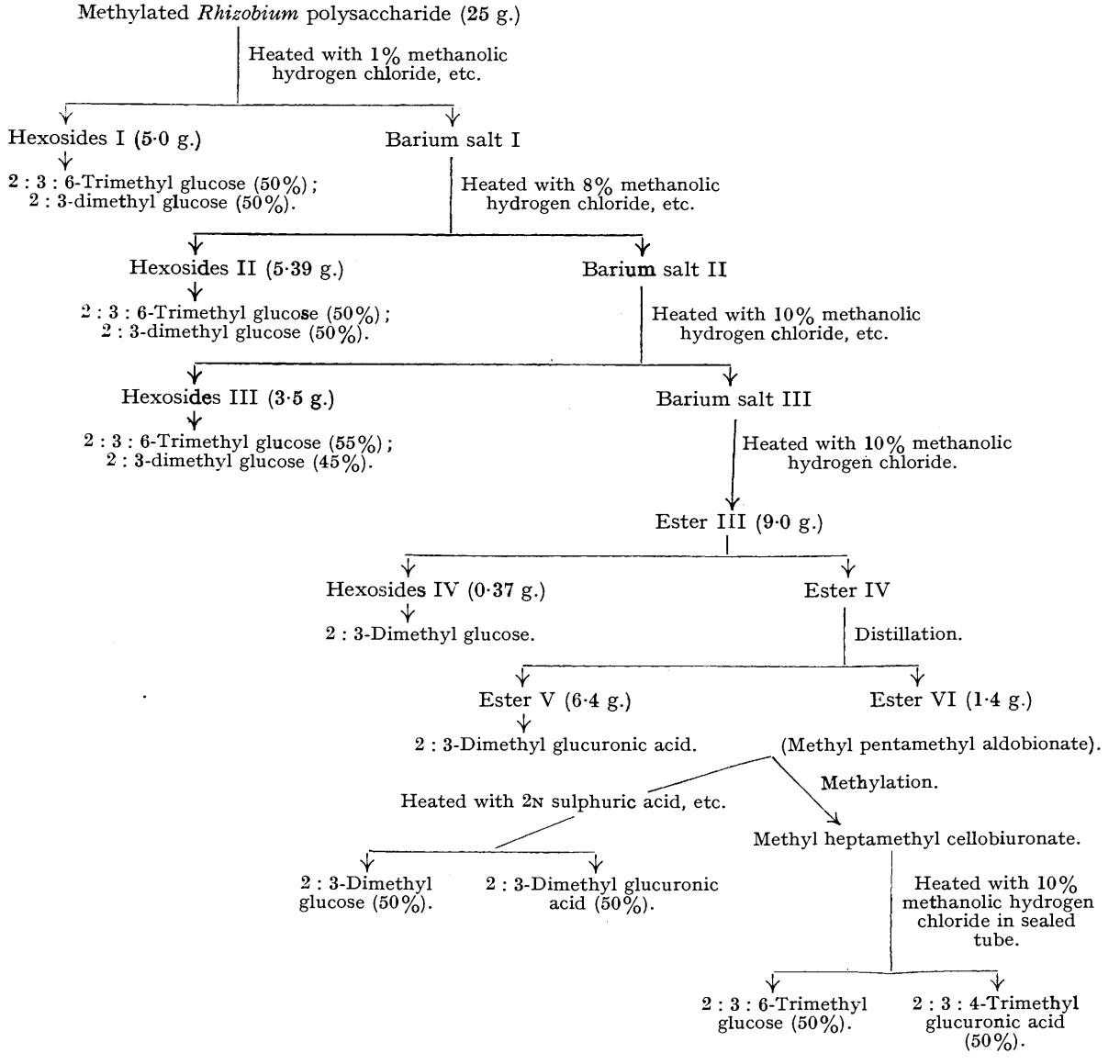
Certain strains of *Rhizobium radicicolum*, the nitrogen-fixing organism living in symbiosis with the *Leguminosæ*, are characterised by their remarkable power to produce large amounts of a mucilaginous capsular substance (Hopkins, Peterson, and Fred, *J. Amer. Chem. Soc.*, 1930, **52**, 3659; 1931, **53**, 306; Cooper, Daker, and Stacey, *Biochem. J.*, 1932, 1752; Bray, Schlüchterer, and Stacey, *Biochem. J.*, 1944, 154). The function of the capsule

in *Rhizobium* is incompletely understood. It may act as a food reserve material for the plant, it appears to form the infection threads through which the organism ramifies through the cortical cells of the plant and it may act as a defence mechanism for the organism against soil protozoa (Dr. H. G. Thornton, F.R.S., private communication). The latter protective function would be analagous to that of the specific polysaccharides of the pneumococcus and other pathogens. The *Rhizobium* capsule is composed largely of polysaccharide material in combination with small amounts of protein and belongs to the class of muco-polysaccharides (Stacey, *Chem. and Ind.*, 1943, **62**, 110). It was shown (Cooper, Daker, and Stacey, *loc. cit.*) that the polysaccharide consisted of glucose and glucuronic acid residues only and thus resembled the specific polysaccharides of Types II and III pneumococcus. More recently Dr. M. Heidelberger has shown (private communication) that the polysaccharide from the Clover Bartel " A " strain of *Rhizobium* described herein gives a precipitin reaction in high dilution not only with Type III pneumococcus anti-serum, but also with mixed anti-sera from other pneumococcus types.

The present investigation was undertaken to determine whether there is a structural relationship between this polysaccharide and the pneumococcus capsular polysaccharides.

Methods.

The polysaccharide was grown in artificial culture by the procedure described by Bray, Schlüchterer, and Stacey (*loc. cit.*) from a Clover Bartel " A " strain provided by Dr. Hugh Nichol of the Rothamstead Experi-



mental Station. It was purified by repeated filtration in dilute aqueous solution through kieselguhr and was obtained by alcoholic precipitation as a fibrous white powder which gave solutions of high viscosity in water. When isolated by evaporating its aqueous solution in a vacuum desiccator the polysaccharide had the appearance of a typical gum. Methylation was smoothly effected by the agency of sodium hydroxide and methyl sulphate, the product being separated by dialysis.

The methylated polysaccharide was a white powder (OMe, 39·0%, $[\alpha]_D^{20°}$ +12° in chloroform) and was shown by fractional precipitation methods to be reasonably homogeneous. It was subjected to graded hydrolysis by being heated with methanolic hydrogen chloride of varying strength. At each acid concentration level, the hydrolysate was separated into fractions each of which consisted of two main products, namely a mixture of methyl hexosides and the barium salt of a partly methylated uronic acid derivative.

The separation is briefly outlined in the accompanying table. The losses in this separation were due in part to the manipulation losses during the involved fractionations and in part to the rather drastic hydrolysis conditions in the later stages which caused some degradation and production of methyl lævulinate (cf. Haworth and co-workers, J., 1939, 1885). The only constituent units identified were 2 : 3-dimethyl glucuronic acid (VII), 2 : 3-dimethyl glucopyranose (VI), and 2 : 3 : 6-trimethyl glucopyranose (V) and it was estimated that these were present in the methylated polysaccharide in approximately equimolecular proportions.

In this fractionation the partially methylated glucosides were identified (*a*) by being further methylated and subsequently hydrolysed to crystalline 2 : 3 : 4 : 6-tetramethyl glucose, (*b*) by being hydrolysed to the corresponding crystalline methylated hexoses and (*c*) by formation of the anilides of these hexoses.

2 : 3-Dimethyl glucuronic acid (VII) was identified (*a*) by its conversion to the characteristic 2 : 3 : 4-trimethyl glucuronamide, (*b*) by its oxidation with aqueous bromine to 2 : 3-dimethyl saccharolactone (crystalline amide) and (*c*) by its oxidation with nitric acid to *d*-dimethoxy-succinic acid (crystalline amide).

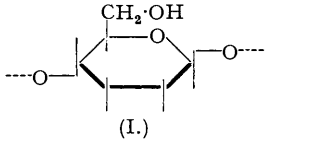
The stability of the polysaccharide and its methylated derivative to acid hydrolysis rendered unlikely the presence of furanose forms of (V), (VI), or (VII) as constituent units.

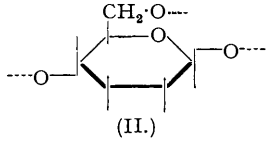
The methyl pentamethyl aldobionate was identified as being derived from a *tetramethyl cellobiuronic acid*, to which the structure (IV) was assigned for the following reasons : (*a*) On acid hydrolysis the partly methylated aldobionic acid split into equivalent parts of 2 : 3-dimethyl glucopyranose and 2 : 3-dimethyl glucuronic acid. (*b*) Further methylation with silver oxide and methyl iodide gave a product having the methoxyl content and equivalent weight required by a fully methylated aldohexobionic acid. (*c*) This methyl ester of heptamethyl aldobionic acid was hydrolysed to its constituent units which were identified as approx. equivalent parts of 2 : 3-dimethyl glucuronic acid and 2 : 3 : 4 : 6-tetramethyl glucopyranose.

Discussion.

By acid hydrolysis the methylated polysaccharide was resolved into its constituent units which were recognised as 2 : 3 : 6-trimethyl glucose (one part), 2 : 3-dimethyl glucose (one part), and 2 : 3-dimethyl glucuronic acid (one part). A portion of the last two constituents was shown to be derived from the tetramethyl cellobiuronic acid (IV) the characterisation of which appears to indicate that cellobiuronic acid units form part of the structure of the original polysaccharide. These units are of high immunological significance (see below).

It is a striking fact that no fully methylated derivatives could be found among the hydrolysis products of the methylated polysaccharide. This indicates that " end " residues do not form part of its structure which cannot therefore be of the terminated linear type such as is present in starch or cellulose. Since the di- and trimethyl glucoses and the dimethyl glucuronic acid are present in equimolecular amounts the minimum repeating unit is that of a trisaccharide composed of two methylated glucopyranose units (I) and (II) and a methylated glucuronic acid unit (III) linked in such a manner that (I) gives rise to 2 : 3 : 6-trimethyl glucose (V), (II) gives rise to 2 : 3-dimethyl glucose (VI), and (III) gives 2 : 3-dimethyl glucuronic acid (VII).


(I.)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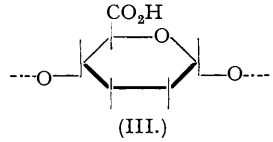
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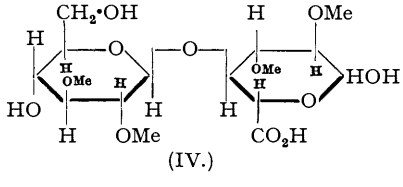
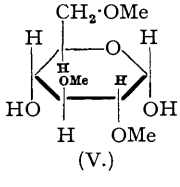

(IV.)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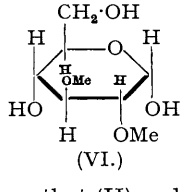
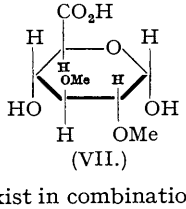

(VI.)

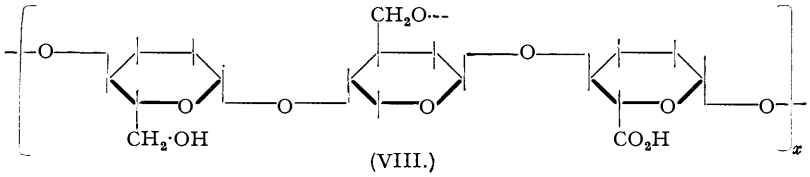

(VII.)

The characterisation of the tetramethyl aldobionic acid (IV) proves that (II) and (III) exist in combination by means of 1 : 4-β-linkages, but the method of hydrolysis did not give a means of assaying the amount of (IV). From the ash contents of the mineral salts of the polysaccharide and the acid equivalents of the methylated oligosaccharides obtained on partial hydrolysis, it was clear that all the carboxyl groups in the glucuronic acid residues were free, a property which appears to be a characteristic of most polyuronic acids.

Based on the isolation of a cellobiuronic acid derivative and on the directional increases in rotation in the positive sense on acid hydrolysis of the polysaccharide and its methylated derivative it appears that the main linkages are of the β-type, but the presence of some α-linkages is not excluded.

Although the evidence provided by the investigation is insufficient to give a detailed configuration of the polysaccharide and despite the fact that opportunity has not been found to make molecular weight determinations, it is possible to give an indication of the type of structure and to relate it to that of the Type III pneumococcus specific polysaccharide.

Thus, from the isolation of (IV) it is clear that in the minimum trisaccharide repeating unit (II) must be interposed between (I) and (III) giving a structure of the type shown in (VIII). The manner in which this

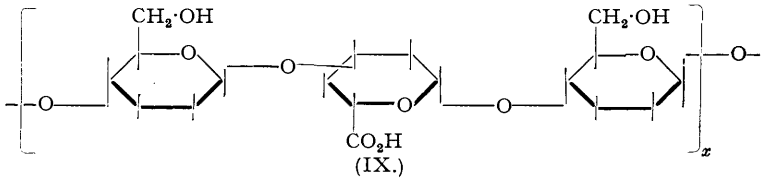


(VIII.)

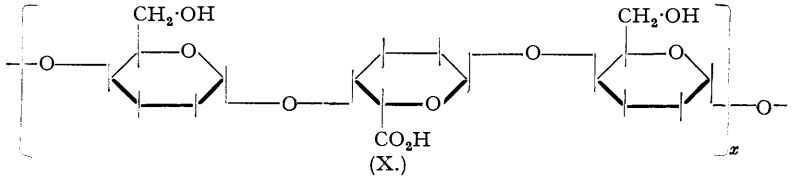
repeating unit is combined in the polysaccharide is not yet apparent though a likely possibility is that by linkages engaging C_1 of (III) and C_4 of (I) it forms part of a chain structure. In this chain, C_6 of (II) must be further engaged in polymeric cross-linkage probably with similar chains, finally building up a highly complex laminated molecule.

In its numerous linkages of the 1 : 6-β-type the structure bears some relationship to that of cellulose and more closely to that of some types of oxycellulose. In this connection it is of particular interest to note the work of Heidelberger and Hobby (*Proc. Nat. Acad. Sci.*, 1942, **28**, 516) who have connected the serological relationships which exist between the pneumococcus specific polysaccharides (Types III and VIII) and certain oxycelluloses (Zackel and Kenyon, *J. Amer. Chem. Soc.*, 1942, **64**, 121), with a close structural similarity particularly characterised by cellobiuronic acid constituents. Thus, an oxycellulose with 21% of its primary alcohol groups oxidised to carboxyls and having a glucose : glucuronic acid ratio of 1 : 1, precipitated more specifically with Type III pneumococcus antiserum; while an oxy-cellulose with 16% of its primary alcoholic groups oxidised to carboxyls and having a glucose : glucuronic acid ratio of 2 : 1 precipitated more specifically with Type VIII pneumococcus antiserum. These observations were in keeping with the facts that Type III pneumococcus specific polysaccharide has a glucose : glucuronic acid ratio of 1 : 1 while the Type VIII polysaccharide has a glucose : glucuronic acid ratio of 2 : 1.

The Type III polysaccharide was shown by Reeves and Goebel (*J. Biol. Chem.*, 1941, **139**, 511) to possess the structure shown in (IX) while the related oxycellulose would presumably have a structure of the type shown in (X).



(IX.)



(X.)

Heidelberger and Hobby (*loc. cit.*) consider that their results emphasise the existence of a strict correlation between chemical constitution and immunological specificity and show that predictions as to serological reactivity may be made when the structure of the repeating unit responsible for the reactivity is known. In this case the responsible unit is cellobiuronic acid (cf. W. F. Goebel, *J. Exp. Med.*, 1939, **69**, 33) and the presence of this same unit undoubtedly accounts for the cross-specificity shown by the reaction between the *Rhizobium* polysaccharide and Type III pneumococcus antiserum. The lower degree of cross specificity in this reaction when compared with the reaction between the Type III polysaccharide and its homologous antiserum is accounted for by the differences herewith shown in the finer detailed structure of (VIII) and (IX).

Experimental.

The acidic polysaccharide, an aqueous solution of which had been repeatedly filtered through a pad of kieselguhr, was obtained on acid alcoholic precipitation as a white fibrous mass having $[\alpha]_D^{20°}$ −17° in water (*c*, 1·0); ash, 2%; uronic anhydride, 18% (this value is lower than that found by isolation of the methylated uronic acid derivative). For the purpose of methylation a material was used which had been precipitated once only and had ash, 38·4%.

The polysaccharide in dilutions of 1 : 100,000 reacted after 4 hours at room temperature with Type III and with mixed Types VI and XIV antipneumococcus horse sera (private communication from Dr. M. Heidelberger).

Methylation of the Polysaccharide.—The material (5 g.) was dissolved in distilled water (20 c.c.), allowed to swell for about three hours, and then 50 c.c. of a 30% sodium hydroxide solution were added. This solution was allowed to stand for 15 hours, stirred for one hour in the cold, and over a period of 7 hours, acetone (150 c.c.), dimethyl sulphate (90 c.c.) and 30% sodium hydroxide (130 c.c.) were added in amounts of 1/10th at a time, keeping the temperature at 25°. The solution was then cooled in ice and slowly neutralised with 5N sulphuric acid. It was then dialysed against tap water until it was free from sulphate. After concentration to a small bulk in a vacuum at 40° the solution was methylated a second time under the above conditions. After 3 methylations acidification of the methylation mixture gave a gum-like product which was isolated by filtration through cloth.

The gum was dissolved in acetone, dried over anhydrous magnesium sulphate, the solution filtered and concentrated to a small bulk. Attempted precipitation from ligroin (b. p. 40—60°) was unsuccessful since the methylate still came down as an intractable gum. The gum was therefore dissolved in little acetone, methylated with silver oxide and methyl iodide, and the product extracted with acetone, the combined extracts then being evaporated giving a mobile syrup. This was poured into a large excess of ligroin with continuous stirring. There was obtained a fine yellow powder which was separated and dried in a vacuum. The product (3·6 g.) had ash, 2·4; OMe, 32·9%. After six methylations a fine white powder (2·7 g.) was obtained by precipitation from ligroin and had OMe, 38·6%. Further methylations failed to give any significant increase in methoxyl content.

Fractionation of methylated polysaccharide. The methylated polysaccharide prepared as above (37 g.) was dissolved in chloroform (300 c.c.) giving a thin syrup, and by fractional addition of ligroin, four fractions were obtained. They were triturated with ligroin, dried thoroughly at 60° in a vacuum and had the following constants :

Fraction.	Yield (g.).	$[\alpha]_D^{20°}$ in chloroform.	% OMe.	% Ash.	$\eta^{20°}$ in *m*-cresol.
I	4·9	11·6	38·6	0·06	0·13
II	20·8	12·3	38·6	0·17	0·16
III	6·7	13·0	37·7	0·12	0·11
IV	2·1	12·9	37·0	0·20	0·09

These data showed that the methylated polysaccharide was reasonably homogeneous.

Hydrolysis of the methylated polysaccharide. Various strengths of methanolic hydrogen chloride were first tried in order to find the minimum concentration of hydrogen chloride which would effect hydrolysis. This was found to be 1%.

The methylated polysaccharide (25 g.) was dissolved in 1% methanolic hydrogen chloride (1500 c.c.) and heated under reflux at 70—75° when the following readings were obtained : $[\alpha]_D^{20°}$ +8° (1 hr.), +15° (2 hrs.), +18° (3 hrs.), +20° (4 hrs.), +24° (6 hrs.), +29° (9 hrs.), +32° (14 hrs.), +35° (19 hrs.), +37° (21 hrs.), +40° (24 hrs.), +43° (30 hrs.) (equilibrium value). The solution did not reduce boiling Fehling's solution and it was neutralised with silver carbonate. The filtered liquid was concentrated under diminished pressure at 40—50° to a syrup which was digested with saturated barium hydroxide solution (750 c.c.) at 50—60° for 3 hours. The solution, neutralised with carbon dioxide, was filtered and concentrated in a vacuum at 40—50° and the dry residue dissolved in dry methanol. Ether was added until a faint precipitate just appeared and then ligroin was added to precipitate a barium salt. After this had settled, the clear supernatant liquid was decanted, the residue redissolved in dry methanol and the barium salt reprecipitated as before. This process was repeated and the combined supernatant liquids were taken down to a syrup " Hexosides I "; OMe, 40·0%; $[\alpha]_D^{20°}$ +68·3° in chloroform (c, 0·85).

Investigation of " Hexosides I."—Fraction " Hexosides I " (3·46 g.) was transferred to a Widmer flask and the following fractions were obtained on distillation in a high vacuum :

Fraction.	Weight (g.).	B. p. at 0·02 mm.	$n_D^{17·5°}$.	% OMe.	Physical state.
A I	0·42	120°	1·4565	52·01	Mobile colourless syrup.
A II	0·47	160—170	1·4592	48·01	Mobile colourless syrup.
A III	0·38	170—175	1·4622	51·3	Mobile colourless syrup.
A IV (smaller flask)	0·27	158—170	1·4670	44·7	Fairly mobile colourless syrup.
A V	0·21	170	1·4695	38·1	Fairly mobile syrup.
A VI	1·69	Residue	—	32·1	Dark brittle mass.

The refractive indices and the methoxyl contents of fractions A II, A III, and A IV indicated that they might contain some tri- and some di-methyl methylhexosides. They were therefore combined and redistilled. One fraction [A II(a)] (0·68 g.), b. p. 116—123°/0·024 mm., was obtained having $n_D^{17°}$ 1·4575; OMe, 49·7%. The residue [A II(R)] in the distilling flask (0·4 g.) had OMe, 42·06%; its further treatment will be described later.

Identification of fraction A I and A II(a). Fraction, A II(a), (0·1 g.), on hydrolysis with N hydrochloric acid at 100° showed : $[\alpha]_D^{20°}$ +100° (initial), +102° (15 minutes), +105° (30 mins.), +104° (60 mins.), +102° (2 hrs.), +99° (3 hrs.), +93° (5 hrs.), +92° (7 hrs.), +88° (9 hrs.), +85° (13 hrs.). The solution was neutralised with barium carbonate, filtered and concentrated in a vacuum at 40° almost to dryness. The residue was extracted with chloroform, the chloroform extracts dried over anhydrous magnesium sulphate and, after filtering, concentrated to a syrup. After solution in ether and removal of the solvent, it crystallised (0·077 g.). It was recrystallised several times from ether and had m. p. 117° alone and in admixture with an authentic specimen of 2 : 3 : 6-trimethyl glucose; $[\alpha]_D^{20·5°}$ +90·3° → +70·5° in water (c, 0·52).

The combined fractions, A I and A II(a) were hydrolysed by being heated with N hydrochloric acid as above : $[\alpha]_D^{20°}$ +109° (initial) → +78° (15 hrs.). After the usual procedure, crystalline 2 : 3 : 6-trimethyl glucose was obtained in quantitative yield and crystallised several times from ether (Found : C, 48·8; H, 8·35; OMe, 42·1. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·5; OMe, 41·8%).

Treatment of residue from the distillation of Hexosides I. The dark resin-like residue (fraction A VI) (1·69 g.) was hydrolysed by being heated with 10% methanolic hydrogen chloride for 30 hours. After the usual procedure and the formation of the barium salt, a material (0·92 g.) (OMe, 40·9%), possibly " Hexosides," was isolated. The barium salt (0·25 g.) was added to the barium salt obtained from a further 10% methanolic hydrogen chloride hydrolysis (see later).

The " Hexosides " were combined with fraction A II(R) and distilled in a high vacuum, when the following fractions were obtained :

Fraction.	Weight (g.).	B. p. at 0·02 mm.	$n_D^{18°}$.	% OMe.	Physical state.
A I (b)	0·114	132—137°	1·4592	48·1	Mobile syrup.
A II (b)	0·119	140—150	1·4600	45·3	Mobile syrup.
		Transferred to smaller flask.			
A III (b)	0·328	138—143	1·5710	42·5	Viscid syrup.
A IV (b)	0·493	Residue	—	39·4	Dark brittle resin.

The residue (0·49 g.) was again rehydrolysed by being heated with 10% methanolic hydrogen chloride for 50 hours and after the usual procedure 0·303 g. of hexosides (OMe, 35·9%) was obtained. No barium salt could be detected. The methoxyl content (35·9%) of the hexosides indicated that the residue consisted mainly of dimethyl methylhexosides.

The loss in this hydrolysis and in subsequent hydrolyses using 10% methanolic hydrogen chloride was due in part (1—3%) to the formation of methyl lævulinate, $n_D^{17°}$ 1·422 (Found : OMe, 23·0. Calc. for $C_6H_{10}O_3$: OMe, 23·8%). Since there appeared to be no way of avoiding the loss (cf. Haworth and co-workers, J., 1939, 1885) this degradation product was distilled off with the solvent from the glucoside and ester mixtures.

Identification of dimethyl methylhexoside fractions. Fraction A V (0·204 g.) was hydrolysed by being heated with N hydrochloric acid until equilibrium was reached : $[\alpha]_D^{20°}$ +85° (15 minutes), +94° (1 hr.), +76° (2½ hrs.), +59° (8 hrs.), +44° (12 hrs.). The solution was neutralised with barium carbonate, filtered and the filtrate concentrated in a vacuum to dryness. The dry residue was extracted with acetone and the acetone extracts were concentrated to a syrup which showed $[\alpha]_D^{20·5°}$ +66° → +58° in water (c, 0·78).

Fraction A III (b) (0·328 g.) was hydrolysed in the same way and the free sugar obtained was combined with that from Fraction A V above and called " Fraction D."

Anilide formation. The dimethyl hexose Fraction D (0·34 g.) was converted quantitatively in the usual way to an anilide which came down in the form of colourless crystals, m. p. 134° alone or in admixture with an authentic specimen of 2 : 3-*dimethyl glucose anilide* (Found : C, 59·2; H, 7·4; N, 4·7; OMe, 21·5. $C_{14}H_{21}O_5N$ requires C, 59·4; H, 7·4; N, 5·0; OMe, 21·9%).

Methylation of Hexosides I.—These hexosides (0·37 g.) were methylated four times with methyl iodide and silver oxide. The resulting syrup (0·348 g.) was then distilled in a high vacuum when the following fractions were obtained :

Fraction.	Weight (g.).	B. p. at 0·03 mm.	$n_D^{23·5°}$.	Physical state.
I	0·094	95—100°	1·4430	Mobile colourless syrup.
II	0·175	100—115	1·4450	Mobile colourless syrup.
III	0·084	Residue	1·4450	Brown syrup.

Fractions I and II were combined (0·169 g.) and hydrolysed by being heated with 6% hydrochloric acid. The following readings were taken : $[\alpha]_D^{20°}$ +61·4° (initial), +56·7° (30 minutes), +54·3° (60 mins.), +51·9° (120 mins.), +40·1° (240 mins.). The solution was neutralised with barium carbonate and the product, isolated in the usual way, was 2 : 3 : 4 : 6-tetramethyl glucopyranose, m. p. 92—93°, $[\alpha]_D^{18°}$ +100° → +82·5° in water (c, 0·34).

That Hexosides I consisted of glucose only, was also indicated when the porous porcelains on which the tetramethyl glucose crystals had been drained were extracted with chloroform and the resulting syrup dissolved in absolute alcohol (20 c.c.). After addition of freshly distilled aniline (0·1 c.c.), the solution was heated under reflux for 5—6 hours. After removal of the solvent, crystals separated out; these were recrystallised from hot absolute alcohol. They had m. p. 135—136° alone and in admixture with an authentic specimen of 2 : 3 : 4 : 6-tetramethyl glucopyranose anilide.

The foregoing investigations showed that the Hexosides I consist of glucose only and that about 50% is 2 : 3 : 6-trimethyl methylglucoside and about 50% 2 : 3-dimethyl methylglucoside.

" *Barium Salt I.*"—The barium salt obtained from the 1% methanolic hydrogen chloride hydrolysis had the following constants : ash, 28·7; Ba, 16·9; OMe, 25·8%; $[\alpha]_D^{16·5°}$ +19·4° in chloroform (c, 0·72).

Esterification of barium salt I. The barium salt (1·1 g.) was dissolved in 1% methanolic hydrogen chloride (100 c.c.) and heated under reflux at 70—75° for 7 hours. After neutralisation with silver carbonate, the filtered solution was concentrated in a vacuum and the dry residue extracted with chloroform. On removal of the solvent the syrup was again extracted with chloroform removal of which gave " Ester I " (0·97 g.) (equiv., 509). On distillation in a high vacuum the following fractions were obtained :

Fraction.	Weight (g.).	B. p. at 0·01 mm.	$n_D^{18°}$.	% OMe.	Physical state.
E I	0·13	168—180°	1·4700	39·8	Colourless syrup.
E II	0·05	180—210·	1·4750	41·0	Colourless syrup.
E III	0·64	Residue	—	41·9	Dark and brittle.

Fractions E I and E II were methylated further, but the product could not be satisfactorily distilled, and it was apparent that Ester I was a partly methylated oligosaccharide.

Hydrolysis of barium salt I. " Barium salt I " (30 g.) was hydrolysed by being heated with 8% methanolic hydrogen chloride (1500 c.c.) and the following readings were obtained : $[\alpha]_D^{20°}$ +66° (3 hours), +81° (8 hrs.), +86° (12 hrs.), +98° (16 hrs.). The solution was non-reducing to boiling Fehling's solution and after the usual procedure of neutralisation with silver carbonate and concentration of the filtrate, the " Barium salt II " was formed by digestion with saturated barium hydroxide solution. Separation of hexosides and Barium salt II by the precipitation method described above gave acetone-soluble " Hexosides II " (5·25 g.) having OMe, 44·36%; $[\alpha]_D^{19·5°}$ +85·8° in chloroform (c, 0·27). Further methylation of Hexosides II gave in quantitative yield a syrup, b. p. 105—110° at 0·03 mm., $n_D^{16°}$ 1·4440, which on hydrolysis gave 2 : 3 : 4 : 6-tetramethyl glucopyranose, m. p. 92° (anilide m. p. 135—136°), also in quantitative yield. Hexosides II thus consists of glucose derivatives only.

Distillation of " Hexosides II."—Hexosides II (2·86 g.) were distilled in a high vacuum and the following fractions were obtained :

Fractions.	Weight (g.).	B. p. at 0·02 mm.	$n_D^{18°}$.	% OMe.	Physical state.
B I	0·735	110—118°	1·4575	50·4	Mobile colourless syrup.
B II	0·530	118—122	1·4595	50·2	Mobile colourless syrup.
B III	0·471	145	1·4705	40·3	Viscid colourless syrup.
B IV	0·275	165—169	1·4762	38·9	Viscid colourless syrup.
B V	0·758	Residue	—	28·5	Dark and brittle glass.

The above data appeared to indicate that fractions B I and B II represented trimethyl methylglucosides, and fractions B III and B IV dimethyl methylglucosides; they were examined as follows :

Hydrolysis of fraction B I. A portion of fraction B I (100 mg.) was hydrolysed by being heated with N hydrochloric acid and the following changes were observed : $[\alpha]_D^{20°}$ +92° (initial), +93° (30 mins.), +93° (60 mins.), +98° (180 mins.), +96° (6 hrs.), +87° (12 hrs.), +84° (14 hrs.). After the usual procedure a crystalline substance (76 mg.) was obtained which, after repeated recrystallisation from ether, had m. p. 117° alone and in admixture with an authentic specimen of 2 : 3 : 6-trimethyl glucopyranose. This showed that the trimethyl fractions of " Hexosides II " consisted of 2 : 3 : 6-trimethyl methylglucopyranoside (approx. 50%).

Hydrolysis of fraction B III. A portion of fraction B III (100 mg.) was hydrolysed with N hydrochloric acid and the

following readings were taken: $[\alpha]_D^{20°}$ +98° (initial), +94·5° (30 mins.), +90·9° (90 mins.), +81·8° (3 hrs.), +81·8° (5 hrs.), +78° (10 hrs.). After the usual procedure and extraction with acetone, the free Hexose D II (87·4 mg.) was obtained.

Formation of anilide of dimethyl hexose of "Hexosides II." The Hexose D II (87·4 mg.) was dissolved in absolute alcohol and after addition of 0·46 c.c. of freshly distilled aniline was heated under reflux for 5—6 hours. The solvent was removed and the syrupy residue crystallised completely and was triturated with ether. The crystals were kept at 0° for 48 hours and filtered off. After recrystallisation they had m. p. 134° alone and in admixture with 2 : 3-dimethyl glucose anilide.

Fractions B III and B IV crystallised on inoculation with 2 : 3-dimethyl α-methylglucoside. The crystallised material was drained for several days on porous porcelain until quite dry. It had m. p. and mixed m. p. 82—83° with 2 : 3-dimethyl α-methylglucoside.

Hydrolysis of residue B V of "Hexosides II" distillation. The dark brittle residue (0·75 g.) was hydrolysed with 2N sulphuric acid. A small precipitate separated out at once and the following readings were obtained: $[\alpha]_D^{20°}$ +93° (30 mins.), +80° (60 mins.), +78° (90 mins.), +75° (2½ hrs.), +72° (3½ hrs.). The solution was neutralised with barium carbonate and the filtered solution taken down to dryness in a vacuum. The dry residue was extracted several times with ethanol and the alcoholic extracts were concentrated in a vacuum to a syrup (0·617 g.) having OMe, 30·1%; it reduced boiling Fehling's solution, and contained no barium.

The free sugar (0·6 g.) was converted into the methylglucoside, which when distilled in a high vacuum gave one fraction (0·29 g.) having b. p. 145—150° at 0·02 mm.; $n_D^{20·5°}$ 1·4690; OMe, 43·2%. This indicated that the residue B V also consisted mainly of dimethyl methylglucoside. It was inoculated with a crystal of 2 : 3-dimethyl α-methylglucopyranoside and it slowly crystallised. The residue of this distillation (0·189 g.) was a hard brown mass which was later added to Barium Salt II.

Properties of "Barium Salt II." The reprecipitated Barium Salt II had ash, 33·7; Ba, 20·7; OMe, 30·0%.

A sample (0·27 g.) of this salt was esterified in the usual way and the resulting "Ester II" was methylated four times with methyl iodide and silver oxide. After four methylations it had OMe, 42·6% and since this value did not correspond to a fully methylated methyl aldobionate or to a fully methylated methyl hexuronate, two more methylations were carried out. The ester (190 mg.) then had OMe, 45·3% and was distilled in a high vacuum.

Fraction.	Weight (g.).	B. p. at 0·015 mm.	$n_D^{18°}$.	Physical state.
F I	0·032	140—175°	1·4550	Mobile colourless syrup.
F II	0·036	175—195	1·4678	Viscid colourless syrup.
Residue	0·120	—	—	Viscid syrup.

Thus Ester II was still mainly of an oligosaccharide nature.

Hydrolysis of "Barium Salt II." Barium Salt II (20 g.) was hydrolysed further, using a 10% methanolic hydrogen chloride solution (1000 c.c.). The solution was non-reducing to boiling Fehling's solution and was neutralised with silver carbonate. Digestion with saturated barium hydroxide solution and subsequent precipitation gave, together with a Barium Salt III, "Hexosides III" (3·44 g.) having: OMe, 33·0%; $[\alpha]_D^{20·5°}$ +57·3° in chloroform (c, 0·73).

Investigation of "Hexosides III." By further methylation followed by hydrolysis, Hexosides III were quantitatively converted into 2 : 3 : 4 : 6-tetramethyl glucopyranose.

Distillation of "Hexosides III." "Hexosides III" (2 g.) were distilled in a high vacuum and the following fractions were obtained:

Fraction.	Weight (g.).	B. p. at 0·02 mm.	$n_D^{15°}$.	% OMe.	Physical state.
C I	0·509	125—133°	1·4612	48·5	Colourless syrup.
C II	0·248	158—163	1·4650	45·1	Colourless syrup.
C III	1·357	Residue	—	27·9	Hard and brittle.

The residue C III (1·35 g.) was dissolved in 2N sulphuric acid and hydrolysed until equilibrium was obtained. A small flocculent precipitate separated out at the commencement of the hydrolysis. The following changes were observed: $[\alpha]_D^{20°}$ +65° (30 mins.), +62° (2½ hrs.), +64° (4½ hrs.), +70° (7 hrs.), +82° (9 hrs.). After neutralisation with barium carbonate and filtration, the solution was concentrated to dryness in a vacuum, and the dry residue was extracted with boiling ethanol. The combined alcoholic extracts gave a free hexose (0·71 g.). Extraction of the dry residue with hot water and subsequent treatment gave a barium salt (0·130 g.) which was added to Barium Salt III.

The free hexose (0·7 g.) had OMe, 25·3% and was converted into the methylglucosides and gave on distillation in high vacuum:

Fraction.	Weight (g.).	B. p. at 0·01 mm.	$n_D^{20·5°}$.	% OMe.	Physical state.
CR I	0·205	135—139°	1·4650	46·5	Colourless syrup.
CR II	0·185	150—158	1·4695	38·3	Viscid colourless syrup.
CR III	0·302	Residue	—	—	Very hard dark mass.

Identification of fraction C I. Fraction C I from "Hexosides III" (0·112 g.) was hydrolysed by being heated with N hydrochloric acid until equilibrium was obtained: $[\alpha]_D^{20°}$ +96° (initial), +98° (30 mins.), +94·6° (90 mins.), +89·2° (4 hrs.), +85·7° (5½ hrs.), +78·5° (7 hrs.). After the usual procedure a syrup (0·96 g.) was obtained which crystallised to a hard mass. After repeated crystalllisation from hot ether, the crystals had m. p. 117° alone and in admixture with an authentic specimen of 2 : 3 : 6-trimethyl glucopyranose. Fractions C II and CR I similarly were hydrolysed to give this substance in *ca.* 90% yield.

Fraction CR II was inoculated with a crystal of the authentic 2 : 3-dimethyl α-methylglucopyranoside and it readily crystallised. It thus was clear that "Hexosides III" also was composed of glucose derivatives only. The proportion of tri- to di-methyl methylglucoside is about equal.

Properties of "Ester III."—A sample of "Barium Salt III" (2·75 g.) was esterified by being heated under reflux for 7 hours with 1% methanolic hydrogen chloride (300 c.c.). The resulting "Ester III" was a dark viscous syrup (1·43 g.) which was insoluble in ether (OMe, 36·6%; equiv., 286). A small amount of this ester (370 mg.) was methylated three times with methyl iodide and silver oxide, giving a syrup (OMe, 49·0%) which could not be satisfactorily distilled, indicating that this "Ester III" too was possibly of an oligosaccharide nature. It was further hydrolysed.

10% *Methyl-alcoholic hydrogen chloride hydrolysis of "Ester III."* "Ester III" (9 g.) was hydrolysed with 10% methanolic hydrogen chloride (1000 c.c.) for 24 hours until equilibrium had been obtained; $[\alpha]_D^{20°}$ +104°→120° (24 hrs.). The barium salt was prepared in the usual way and a small amount of "Hexosides IV" (0·373 g.) was isolated. Esterification of the barium salt was carried out as before and an ether soluble "Ester IV" (8·0 g.) was obtained. By the usual procedure "Hexosides IV" were shown to consist entirely of 2 : 3-dimethyl methylglucoside.

Examination of Ester IV.—This was distilled in a high vacuum giving a main fraction "Ester V" (6·4 g.), b. p. 140—145° at 0·02 mm., a colourless liquid, $[\alpha]_D^{20°}$ +80° in water (c, 0·9), $n_D^{21°}$ 1·4685 (Found: OMe, 48·6. Calc. for $C_{10}H_{18}O_7$: OMe, 49·6%). The residue of the distillation was a viscid syrup, "Ester VI." "Ester V" was identified as 2 : 3-dimethyl methylglucuronoside methyl ester as follows:

Investigation of "Ester V." Further Methylation.—A small portion of this ester (0·45 g.) was methylated twice with methyl iodide and silver oxide and then distilled in a high vacuum.

Fraction.	Weight (g.).	B. p. at 0·018 mm.	$n_D^{20·5°}$.	% OMe.	Physical state.
H I	0·0855	135°	1·4550	—	Mobile colourless syrup.
H II	0·2673	210	1·4685	43·5	Colourless syrup.
H III	0·100	Residue	—	—	Dark and brittle.

Fraction H I was dissolved in a few c.c. of dry methanol and ammonia was passed into the solution at 0° for some minutes. The solution was kept at 0° for two days. After removal of the solvent an amide crystallised out. It was recrystallised several times from alcohol–ether–ligroin. The crystals had m. p. 183° alone and in admixture with an authentic specimen of 1 : 2 : 3 : 4-tetramethyl glucuronic acid amide (Found: C, 48·0; H, 7·7; N, 6·05; OMe, 48·6. Calc. for $C_{10}H_{19}O_6N$: C, 48·1; H, 7·6; N, 5·6; OMe, 49·8%).

Fractions H II and H III were methylated further with silver oxide and methyl iodide and the products on treatment with ammonia gave further crops (0·25 g. and 0·45 g.) of the above amide.

Hydrolysis of Ester V. A sample (0·62 g.) was dissolved in N hydrochloric acid (50 c.c.) and heated at 95°. The rotation changed as follows: $[\alpha]_D^{20°}$ +84° (initial), +87° (30 mins.), +77° (2 hrs.), +68° (4 hrs.), +59° (7 hrs.), +54° (9 hrs.) (equilibrium value). The solution was neutralised with barium carbonate and then evaporated to dryness. No hexose derivatives could be extracted from the barium salt. The free acid, a syrup (0·55 g.), was extracted in the usual way and had $[\alpha]_D^{21°}$ +40° in water (c, 1·0) (OMe, 27·4%).

Oxidation with bromine. The syrup (0·5 g.) was dissolved in water (10 c.c.) and bromine (1 c.c.) added. The liquid was kept at 40° for 48 hours and then no longer reduced Fehling's solution. The bromine was removed by aeration and the solution neutralised with silver carbonate, filtered before and after treatment with hydrogen sulphide and then evaporated to a syrup under diminished pressure. This syrup was esterified by being boiled for 6 hours with 2% methanolic hydrogen chloride (50 c.c.). The hydrogen chloride was removed by means of silver carbonate and the filtered solution saturated with dry ammonia. After being kept for 24 hours a little of the methanol was removed in a vacuum desiccator, a crystalline amide (0·38 g.) separating. After recrystallisation from ethanol the substance had m. p. 156° alone and in admixture with 2 : 3-dimethyl glucosaccharamide described by Smith (J., 1940, 1044).

Oxidation of 2 : 3-Dimethyl Methylglucuronoside Methyl Ester with Nitric Acid.—A sample of Ester V (2·0 g.) was oxidised with nitric acid (*d*, 1·42) by essentially the method described by Smith (*loc. cit.*). The main product after esterification was a syrup (1·1 g.), b. p. 98°/0·02 mm., $[\alpha]_D^{20°}$ +56° in water (c, 1·0), consisting mainly of methyl *d*-dimethoxysuccinate since on treatment with methanolic ammonia it gave a quantitative yield of *d*-dimethoxysuccinamide, m.p. 287° (decomp.) alone or in admixture with an authentic specimen, $[\alpha]_D^{20°}$ +88° in water (c, 1·1).

The residual syrup (0·3 g.) was treated with methanolic ammonia in the usual way and there was obtained a mixture of amides from which a small amount (0·1 g.) of 2 : 3-dimethyl glucosaccharamide, m. p. 186°, was obtained.

Examination of Ester VI.—It had $[\alpha]_D^{18°}$ −20° in water, OMe, 42·6%, equiv., 430. A hexamethyl aldobionate, $C_{18}H_{32}O_{12}$, requires OMe, 43·3; equiv., 440. On hydrolysis with 2N sulphuric acid ($[\alpha]_D^{21°}$ −20° → +40° after 12 hrs.) a mixture of reducing sugars was obtained. After boiling this mixture with 2% methanolic hydrogen chloride the products were separated into a mixture of glucosides (0·2 g.) and the ester of a dimethyl hexuronic acid (0·2 g.). By the methods described above these were readily identified as 2 : 3-dimethyl methylglucoside and 2 : 3-dimethyl methylglucuronate.

Methylation of Ester VI. A sample (0·6 g.) was methylated six times with silver oxide and methyl iodide and a syrup (0·6 g.) was obtained having $[\alpha]_D^{20°}$ −30° (c, 1·1) (changing to +42° on hydrolysis with 2N sulphuric acid) (Found: OMe, 51·0; equiv., 460. Calc. for heptamethyl methyl cellobiuronate, $C_{20}H_{36}O_{12}$: OMe, 53·0%; equiv., 468).

The syrup was hydrolysed by being heated with 10% methanolic hydrogen chloride in a sealed tube at 130° for 2 hours. The hydrolysis products were identified, by the methods described above, as 2 : 3 : 4 : 6-tetramethyl methylglucoside (0·25 g.) and 2 : 3-dimethylglucuronic acid methyl ester (0·20 g.).

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THE A. E. HILLS LABORATORIES, THE UNIVERSITY, EDGBASTON, BIRMINGHAM, 15. [*Received, July 2nd,* 1945.]