699. *Immunopolysaccharides. Part X.* The Structure of the Immunologically Specific Polysaccharide of* Pneumococcus *Type XIV.*

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Pneumococcus Type XIV polysaccharide has been shown to contain D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose residues. Partial acidic hydrolysis of the polysaccharide gave, *inter alia*, two disaccharides which were assigned the probable structures 4-*O*-β-D-glucosyl-2-acetamido-2-deoxy-D-glucose and 3-*O*-β-(2-acetamido-2-deoxy)-D-glucosyl-D-galactose. Hydrolysis of the methylated polysaccharide gave 3-*O*-methyl-D-glucosamine (1 part) and neutral sugars (2 parts) consisting of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose <4%, 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose and 2 : 3 : 6-tri-*O*-methyl-D-glucose 69%, 2 : 4 : 6-tri-*O*-methyl-D-galactose 23%, and di-*O*-methylhexose 4%.

From a chemical investigation of *Pneumococcus* Type XIV polysaccharide, Goebel, Beeson, and Hoagland [1] concluded that it was a polymer consisting of units of 2-acetamido-2-deoxy-D-glucose and D-galactose in the ratio 1 : 3. Preliminary studies by three of us [2] indicated that the substance contained D-glucose as an additional constituent which was liberated under more drastic conditions of acid hydrolysis (2N-sulphuric acid at 100° for 4 hr.) than those used by Goebel, Beeson, and Hoagland [1] (N-acid at 100° for 2·5 hr.). Recent immunological studies [3–6] on the cross-reactivity of antiserum to Type XIV *Pneumococcus* made it appear certain that *Pneumococcus* Type XIV polysaccharide would contain non-reducing end groups of D-galactose together with D-galactose residues linked β-1 : 3 or β-1 : 6, or involved in β-1 : 3 : 6-branch points. The present investigation was undertaken to test these predictions by purely chemical methods and to elucidate the principal structural features of *Pneumococcus* Type XIV polysaccharide.

Purified Type XIV polysaccharide on acid hydrolysis afforded galactose, glucose, and glucosamine together with glutamic acid. Similar hydrolysis of a sample of the original Type XIV polysaccharide studied by Goebel and his co-workers [1] gave the same three sugars but no glutamic acid. The absence of glutamic acid can probably be ascribed to the use of nitrous acid in the purification procedure of these workers.[1] Moreover, the

* Part IX, *J.*, 1958, 1895.

[1] Goebel, Beeson, and Hoagland, *J. Biol. Chem.*, 1939, **129**, 455.
[2] Heidelberger, Barker, and Stacey, *Science*, 1954, **120**, 781.
[3] Heidelberger, Dische, Neely, and Wolfrom, *J. Amer. Chem. Soc.*, 1955, **77**, 3511.
[4] Heidelberger, *ibid.*, p. 4308.
[5] Heidelberger, Barker, and Björklund, *ibid.*, 1958, **80**, 113.
[6] Rebers, Barker, Heidelberger, Dische, and Evans, *ibid.*, p. 1135.

substance (probably a peptide) which gave glutamic acid on hydrolysis could be removed from purified Type XIV polysaccharide by prolonged dialysis or by precipitation with Type XIV antiserum which had previously been absorbed with the somatic C-polysaccharide of *Pneumococcus*, and recovery of the type-specific polysaccharide from the washed precipitate. Finally, dialysed purified Type XIV polysaccharide moved as a single homogeneous component, except for a small nucleic acid impurity, when separated in borate buffer pH 9·2 in a Tiselius electrophoresis apparatus.

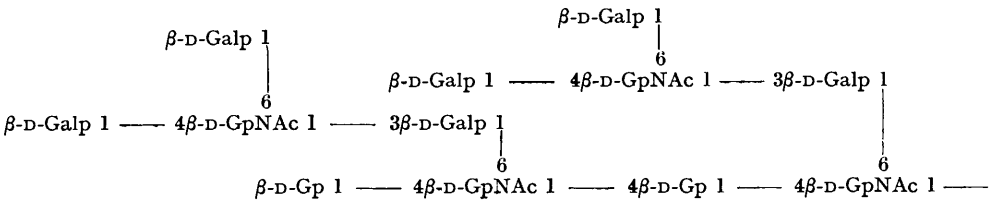
Type XIV polysaccharide was submitted to partial acidic hydrolysis and after *N*-acetylation the mixture was fractionated on a charcoal–Celite column.[7] The monosaccharide components were characterised as crystalline α-D-galactopyranose and 2-acetamido-2-deoxy-α-D-glucose. The glucose present was isolated as its crystalline β-penta-acetate and as *N*-*p*-nitrophenyl-β-D-glucopyranosylamine.

Two disaccharides (III and IV) constituted the major part of the disaccharide fractions. Two other disaccharides (I and II) detected occurred in insufficient amounts for characterisation. On acid hydrolysis, disaccharide III gave galactose and glucosamine, while disaccharide IV gave glucose and glucosamine. Analysis of the acid hydrolysate of the disaccharide alcohol, obtained from III by reduction with sodium borohydride, showed that the only reducing sugar present was glucosamine. Similar analysis of disaccharide IV alcohol hydrolysate revealed glucose as the only reducing sugar. This indicated that III was a 2-acetamido-2-deoxyglucosylgalactose and that IV was a glucosyl-2-acetamido-2-deoxyglucose. On periodate oxidation, disaccharide IV alcohol consumed 2·8 moles of periodate and produced 1·35 moles of formic acid and 0·85 mole of formaldehyde per mole of disaccharide alcohol. This was in accord with the formulation of disaccharide IV as 4-*O*-D-glucopyranosyl-2-acetamido-2-deoxy-D-glucose (theoretical for its alcohol, 3 mols. of periodate consumed and 1 mol. of formic acid and 1 mol. of formaldehyde produced). This structure is also in agreement with its low mobility (M_G 0·16) on paper ionophoresis [8] in borate buffer, pH 10. The large amount of periodate consumed (6·6 mols.) by disaccharide III alcohol and the large amounts of formic acid (4·1 mols.) and formaldehyde (2·4 mols.) produced suggested that over-oxidation was occurring, possibly owing to production of a malondialdehyde derivative from a 1 : 3- or 1 : 4-linked disaccharide alcohol. The alkali-lability of disaccharide III and its high mobility in borate buffer of pH 10 (M_G 0·57) were in favour of the structure 3-*O*-(2-acetamido-2-deoxy)-D-glucosyl-D-galactose. The structures assigned to both these disaccharides receive further confirmation from the study of methylated Type XIV polysaccharide reported below.

Exhaustive methylation of Type XIV polysaccharide with sodium and methyl iodide in liquid ammonia [9] afforded a methyl ether (I), OMe 37·1% (a methylated polymer consisting of two hexose units for every *N*-acetylglucosamine unit requires OMe, 38·8%). Another sample (II) obtained by methylation with sodium hydroxide and methyl sulphate had OMe, 34·4%. Methanolysis and hydrolysis of II followed by passage down a column of Dowex 50(H^+) gave a neutral methyl-sugar fraction and basic methyl-hexosamine fraction. The latter consisted of a single component which was identical with 3-*O*-methyl-D-glucosamine both in its behaviour when chromatographed and in the products of oxidation by ninhydrin.[10] Further confirmation was obtained by periodate oxidation of the alcohol produced by reduction with sodium borohydride: this consumed 1·8 mols. of periodate and produced 0·56 mol. of formic acid and 1·1 mol. of formaldehyde compared with the theoretical figures of 2, 1, and 1 respectively for 2-acetamido-2-deoxy-3-*O*-methyl-D-glucitol. The neutral methyl-sugars obtained were fractionated on a cellulose column. One of these fractions on treatment with aniline in ethanol afforded 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine. Owing to the small amounts available,

[7] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[8] Foster, *J.*, 1953, 982.
[9] Freudenberg and Boppel, *Ber.*, 1938, **71**, 2505.
[10] Gardell, Heijenskjold, and Rochnorlund, *Acta Chem. Scand.*, 1950, **4**, 970.

and the fact that most of the fractions obtained were mixtures, a further batch of neutral sugars was isolated by a similar procedure from the product (I) of sodium–methyl iodide methylation. Quantitative analysis [11] of the mixture of neutral methyl-sugars showed that it consisted of: 2 : 3 : 4 : 6-tetra-*O*-methylglucose, <4%; 2 : 3 : 4 : 6-tetra-*O*-methylgalactose and 2 : 3 : 6-tri-*O*-methylglucose, 69%; 2 : 4 : 6-tri-*O*-methylgalactose, 23%; and di-*O*-methylhexose, 4%. Three of these were isolated as the following crystalline derivatives: 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, 2 : 3 : 6-tri-*O*-methyl-β-D-glucose 1 : 4-di-*O*-*p*-nitrobenzoate, and 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine. From the weights of methyl-sugars isolated and the quantitative estimation it was concluded that for every six sugar residues, two were non-reducing end groups of D-galactose, one was a D-galactose residue linked through positions 1 and 3, one was a D-glucose residue linked through positions 1 and 4, and two were 1 : 4 : 6-linked 2-acetamido-2-deoxy-D-glucose residues engaged in branching. A small number of glucose residues (<1 in 25) were non-reducing end groups and the di-*O*-methylhexose fraction could be attributed to either incomplete methylation or to hexose residues involved in branching. In the arrangement shown below all the linkages are assigned the β-configuration on the basis of (*a*) the low optical rotation of both the polysaccharide itself (+9·4°) [1] and its methyl ether (+18°) and (*b*) the absence of Type 2a absorption [12] in the infrared spectrum of the polysaccharide.



Type XIV polysaccharide was submitted to periodate oxidation initially at room temperature and later at 25°. For every six sugar residues of the type postulated above it consumed 4·9 mols. of periodate and produced 2·05 mols. of formic acid compared with the theoretical values of 5 and 2 respectively. The periodate-oxidised polysaccharide precipitated less than one-half of the antibody in a Type XIV antipneumococcal horse serum.[5] Acidic hydrolysis of the periodate-oxidised polysaccharide yielded galactose and glucosamine but no glucose. Partial hydrolysis, as would be predicted, yielded *inter alia* disaccharide III but no disaccharide IV.

Experimental

Purification of Pneumococcus *Type XIV Polysaccharide.*—The crude polysaccharide was kindly supplied by E. R. Squibb and Sons Ltd., of New Brunswick, New Jersey. A typical purification procedure (Sevag [13]) involved removal of the protein present in the crude polysaccharide (4 g.) by shaking its aqueous solution (300 ml.) several times with chloroform–pentyl alcohol (9 : 1) (4 × 30 ml.). The aqueous phase was then passed through a column of Nalcite (H^+ form) which was further washed with water (50 ml.). This operation was performed in the refrigerator. The polysaccharide in the eluate was precipitated with propan-2-ol (equal volume) after addition of 5% lithium chloride (10 ml.). The precipitate was taken up in water (235 ml.), and the solution centrifuged and again precipitated with propan-2-ol (220 ml.), washed with alcohol and acetone, and dried (yield, 2·2 g.).

Part (10 mg.) of the purified polysaccharide was hydrolysed with 2N-sulphuric acid (1 ml.) at 100° for 4 hr. and the neutralised hydrolysate separated by paper ionophoresis [8] in borate buffer (pH 10). Spraying with aniline hydrogen phthalate [15] revealed the presence of

[11] Hirst, Hough, and Jones, *J.*, 1949, 928.
[12] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.
[13] Sevag, *Biochem. Z.*, 1934, **273**, 419.
[14] Partridge, *Nature*, 1949, **164**, 443.
[15] Roseman and Ludowieg, *J. Amer. Chem. Soc.*, 1954, **76**, 301.

components having mobilities identical with those of glucose, galactose, and glucosamine together with an unidentified component. Paper ionophoresis in citrate–phosphate buffer of pH 6·2 and spraying with ninhydrin showed a component with the mobility of glucosamine and showed that the unidentified component had a mobility expected of a disaccharide containing one hexosamine residue. In addition an acidic component with the mobility of glutamic acid was detected.

Anti-type XIV *Pneumococcus* serum (15 ml.) was incubated with some of the purified polysaccharide (2 mg.) at 0° for 24 hr. and the specific precipitate obtained washed thoroughly with 0·9% saline solution. The specific polysaccharide was extracted from the precipitate with 5% trichloroacetic acid [3] and then hydrolysed as above. Paper-ionophoretic analysis (as above) showed the presence of all the components previously detected. However, precipitation of the purified polysaccharide (3 mg.) with C-absorbed Type XIV antiserum (11 ml.) and recovery from the washed precipitate gave a glutamic acid-free polysaccharide. A similar glutamic acid-free polysaccharide was obtained on prolonged dialysis of the purified polysaccharide against saline solution.

Homogeneity of Type XIV Pneumococcus *Polysaccharide.*—Dialysed purified polysaccharide (130 mg.) in borate buffer (pH 9·2; 15 ml.) was dialysed against more borate buffer (pH 9·2; 4 l.) for 3 days. The centrifuged polysaccharide solution and isotonic borate buffer were placed in the medium cell of a Tiselius electrophoresis apparatus, and the current (5·5 mA) was applied. Except for a small fast-moving impurity, the Type XIV polysaccharide moved as a single homogeneous component for 2 hr. The fast-moving impurity was trapped and was found to be ribonucleic acid from (*a*) its ultraviolet absorption peak at 259 mμ and (*b*) the production of ribose on acid hydrolysis.

Infrared Spectrum of Type XIV Pneumococcus *Polysaccharide.*—The infrared spectrum of the polysaccharide was measured as a mull in liquid paraffin with a single-beam Grubb–Parsons spectrometer. It showed absorption peaks at 1642 and 1551 cm.$^{-1}$ (C=O stretching and N–H bending of an *N*-acetyl group). In the region 720—1000 cm.$^{-1}$ the absence of absorption [12] around 840 cm.$^{-1}$ (type 2a) and the presence of a peak at 890 cm.$^{-1}$ (type 2b) indicated that the majority of the linkages in the polysaccharide were of the β-type.

Partial Acidic Hydrolysis of the Polysaccharide.—The polysaccharide (4·8 g.) was hydrolysed with N-hydrochloric acid (150 ml.) at 100° for 2·5 hr. After neutralisation with silver carbonate the solution was concentrated by freeze-drying (to 100 ml.) and the hexosamine-containing saccharides were *N*-acetylated by Roseman and Ludovieg's method [15] with acetic anhydride (2 ml.) and Amberlite IRA-400 (CO_3^{2-}) (15 ml.) in 10% aqueous methanol. Any unacetylated hexosamine-containing saccharides were removed by passage down a column of Amberlite IR-120(H^+). The resulting mixture of saccharides was fractionated [7] on a charcoal–Celite column (*l* 34 cm.; diam. 6 cm.). Gradient elution (0 ⟶ 25% aqueous ethanol; 15 l.) gave fraction A (1·275 g.) (glucose, galactose, and traces of ribose), B (0·344 g.) (2-acetamido-2-deoxyglucose and traces of ribose), C (0·057 g.) (a mixture of disaccharides I and II), D (0·156 g.) (disaccharide III), E (0·202 g.) (disaccharide IV with small amounts of disaccharide III and a trisaccharide), F (0·263 g.) (trisaccharides and higher saccharides).

Characterisation of monosaccharides. Crystallisation of A from methanol gave α-D-galactopyranose (0·835 g.), m. p. and mixed m. p. 164—165°, $[\alpha]_D^{17}$ +126° (3 min.) ⟶ +80·8° equil. (*c* 0·39 in H_2O) (Found: C, 40·0; H, 6·6. Calc. for $C_6H_{12}O_6$: C, 40·0; H, 6·7%). The mother-liquors were separated on sheets of Whatman No. 3 paper irrigated with the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1). Part (0·055 g.) of the glucose component eluted from the paper (0·180 g.) was refluxed for 15 min. with *p*-nitroaniline (12 mg.) in acidified methanol (5 ml.) [16] to give *N*-*p*-nitrophenyl-β-D-glucopyranosylamine (34 mg.), m. p. and mixed m. p. 182—183°, $[\alpha]_D^{17}$ −193° (5 min.) ⟶ −204° equil. (*c* 1·22 in dry pyridine) (Found: C, 45·5; H, 5·6. Calc. for $C_{12}H_{16}O_7N_2,H_2O$: C, 45·3; H, 5·7%). The glucose component was further characterised as β-D-glucose penta-acetate, m. p. and mixed m. p. 134—134·5°.

Crystallisation of B from alcohol–ether gave 2-acetamido-2-deoxy-α-D-glucose (0·135 g.), m. p. and mixed m. p. 195—196°, $[\alpha]_D^{17}$ +56° (6 min.) ⟶ +42° equil. (*c* 0·32 in H_2O) (Found: C, 43·4; H, 6·9; N, 6·0. Calc. for $C_8H_{15}O_6N$: C, 43·5; H, 6·8; N, 6·3%).

Characterisation of disaccharides. Chromatographically pure disaccharides III and IV, obtained from fractions D and E, showed M_G 0·57 and 0·16 respectively when separated by paper ionophoresis [8] in borate buffer of pH 10.

[16] Weygand, Perkow, and Kuhner, *Chem. Ber.*, 1951, **84**, 594.

Disaccharides III and IV had $R_{Glucose}$ 0·40 and 0·47 respectively in butan-1-ol–ethanol–water–ammonia (40 : 10 : 49 : 1), $R_{Glucose}$ 0·36 and 0·39 in butan-1-ol–acetic acid–water (40 : 10 : 50), and $R_{Glucose}$ 0·68 and 0·82 in pentyl alcohol–pyridine–water (1 : 1 : 1). Both disaccharides were detected by aniline hydrogen phthalate (III, brown; IV, orange-brown) and the Elson–Morgan reagent.

Disaccharides III and IV (3 mg.) were hydrolysed separately with 2N-sulphuric acid (0·5 ml.) for 7·5 hr. at 100°. Paper-ionophoretic analysis of disaccharide III hydrolysate showed galactose and glucosamine while disaccharide IV hydrolysate contained glucose and glucosamine. Portions of III and IV (30 mg.) in water (3 ml.) were reduced separately with sodium borohydride (40 mg.) in water (0·5 ml.) for 5 hr. at room temperature and part (1 mg.) of the de-ionised product from both was submitted to hydrolysis with 2N-sulphuric acid (0·5 ml.) for 5 hr. at 100°. Paper-ionophoretic analysis of the reducing sugars present in disaccharide III alcohol hydrolysate showed glucosamine only and in disaccharide IV alcohol hydrolysate showed glucose only.

Disaccharide III alcohol (8·6 mg.) and disaccharide IV alcohol (12·1 mg.) were each oxidised separately at 17° with 0·02M-sodium periodate (50 ml.). The periodate consumption expressed in moles per mole of disaccharide alcohol after 26 hr. was: III, 6·6 and IV, 2·8. After 26 hr., 4·1 moles of formic acid and 2·4 moles of formaldehyde had been produced per mole of disaccharide III alcohol, and 1·35 moles of formic acid and 0·85 mole of formaldehyde per mole of disaccharide IV alcohol.

Methylation of the Polysaccharide.—The polysaccharide (3·15 g.) was treated with sodium and methyl iodide in liquid ammonia at −55° by a modification of the methods employed by Freudenberg and Boppel [9] and by Hodge, Karjala, and Hilbert.[17] After four additions of the methylating reagents (reaction times: sodium, 4 hr.; methyl iodide, 0·5 hr.) had been made, the ammonia was allowed to evaporate with the exclusion of moisture, the last traces being removed at 75°/15 mm. After dialysis and freeze-drying, the methylation procedure was repeated. The product was suspended in chloroform and centrifuged and the residue (0·23 g.; OMe, 5·6%) discarded. Addition of light petroleum (b. p. 40—60°) to the supernatant liquid precipitated methylated polysaccharide (1·25 g.; OMe, 32%) and a further fraction (0·68 g.; OMe, 35%) was recovered from the mother-liquors. These fractions were recombined and the methylation procedure repeated. That portion of the product soluble in chloroform–light petroleum (1·79 g.; methylated polysaccharide I) had OMe, 37·1%, and $[\alpha]_D^{12}$ +18° (*c* 0·61 in $CHCl_3$).

A second sample of the polysaccharide (1·9 g.) was methylated four times with methyl sulphate and sodium hydroxide,[18] and the crude product (1·64 g.) fractionated with chloroform–light petroleum to methylated polysaccharide II (0·517 g.; OMe, 34·4%) and another fraction (0·873 g.; OMe, 28·0%).

Identification of the Methyl-sugars obtained from the Methylated Polysaccharide.—(a) 3-O-*Methyl-*D-*glucosamine.* Methylated polysaccharide II (0·5 g.) was refluxed with 5% methanolic hydrogen chloride (50 ml.) for 9 hr. After neutralisation with silver carbonate and removal of methanol the resulting methyl glycosides were hydrolysed with aqueous 3·8% hydrochloric acid (50 ml.) at 100° for 7·5 hr. After neutralisation, the product was passed down a column of Dowex 50 (H^+) and the neutral sugars (0·34 g.) were eluted with water. Washing with 3·8% hydrochloric acid eluted the amino-sugar fraction which consisted of a single component having paper chromatographic and staining properties identical with those of 3-*O*-methyl-D-glucosamine. Portions (5 mg.) both of the amino-sugar fraction and of authentic 3-*O*-methyl-D-glucosamine were oxidised separately with ninhydrin. Paper-chromatographic separation showed that they gave identical products, one of which had the properties reported for 2-*O*-methylarabinose [R_F 0·40 in butanol–ethanol–water (40 : 10 : 50); Lederer and Lederer [19] quote R_F 0·38]. Paper ionophoresis in borate buffer of pH 10 also showed identical products.

A further portion (20 mg.) of the amino-sugar fraction was treated with acetic anhydride (0·5 ml.) and Amberlite IRA-400 (CO_3^{2-}; 25 ml.) in aqueous 10% methanol (25 ml.) at 0° for 1·5 hr. After further purification by passage down an Amberlite IR-120(H^+) column, the product was reduced with sodium borohydride (100 mg.). The resulting 2-acetamido-2-deoxy-3-*O*-methyl-D-glucitol (14 mg.) was oxidised with 0·04M-sodium periodate (12 ml.) at 17°.

[17] Hodge, Karjala, and Hilbert, *J. Amer. Chem. Soc.*, 1951, **73**, 3312.
[18] Haworth, *J.*, 1915, **107**, 8.
[19] Lederer and Lederer, " Chromatography," Elsevier Ltd., Amsterdam, p. 164.

After 24 hr., 1·8 moles of periodate had been consumed and 0·56 mole of formic acid and 1·1 mole of formaldehyde had been produced per mole of 2-acetamido-2-deoxy-3-*O*-methyl-D-glucitol.

(b) *Neutral sugars.* The neutral sugars (0·27 g.) were fractionated on a cellulose column washed with butanol–ethanol–water–ammonia (40 : 10 : 49 : 1). Paper-chromatographic analysis of the fractions indicated that I (56 mg.) contained 2 : 3 : 4 : 6-tetra-*O*-methylgalactose and 2 : 3 : 6-tri-*O*-methylglucose, II (99 mg.) contained the same two sugars together with 2 : 4 : 6-tri-*O*-methylgalactose, III (62 mg.) was mainly 2 : 4 : 6-tri-*O*-methylgalactose, and IV (45 mg.) was a mixture of di- and mono-methyl-sugars. Treatment of I with aniline in ethanol gave 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine (24 mg.), m. p. and mixed m. p. 192° (Found: C, 61·7; H, 8·0; N, 4·6. Calc. for $C_{16}H_{25}O_5N$: C, 62·2; H, 8·0; N, 4·4%).

More neutral sugars were obtained as follows: methylated polysaccharide I (1·6 g.) was refluxed with 5% methanolic hydrogen chloride (170 ml.) to constant optical rotation. The resulting methyl glycosides were hydrolysed with N-hydrochloric acid (170 ml.) for 10 hr. at 100°, and the solution freed from ions and concentrated to a syrup (1·59 g.). The methyl sugars were then passed down a column of Amberlite IR-120 (H^+) (200 ml.). The neutral sugars (1·07 g.) were not absorbed and washing the column with 2·5N-hydrochloric acid (500 ml.) eluted the basic methyl sugar fraction (0·52 g.). A portion (5—7 mg.) of the neutral sugar fraction was analysed quantitatively by paper chromatographic separation in butanol–ethanol–water–ammonia (40 : 10 : 49 : 1), elution from the paper and estimation of the components by hypoiodite oxidation according to the method of Hirst, Hough, and Jones.[11] The percentage molecular composition was: 2 : 3 : 4 : 6-tetra-*O*-methylglucose, <4%; 2 : 3 : 4 : 6-tetra-*O*-methylgalactose and 2 : 3 : 6-tri-*O*-methylglucose, 69%; 2 : 4 : 6-tri-*O*-methylgalactose, 23%; and di-*O*-methylhexose, 4%. The remainder (1·06 g.) of the neutral sugars were fractionated on a cellulose column as above, to give A (78 mg.) (mainly 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose), B (564 mg.) (2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose and 2 : 3 : 6-tri-*O*-methyl-D-glucose), C (107 mg.) (same sugars as B together with some 2 : 4 : 6-tri-*O*-methyl-D-galactose), D (197 mg.) (2 : 4 : 6-tri-*O*-methyl-D-galactose), and E (43 mg.) (mainly a di-*O*-methylhexose).

Fraction B (161 mg.) was refluxed with aniline (0·16 ml.) in ethanol (1·5 ml.) for 3 hr. Recrystallisation of the product (51 mg.) from ethanol gave 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m. p. and mixed m. p. 193—194°, $[\alpha]_D^{17}$ +40° (equil.) (*c* 0·3 in EtOH). The concentrated mother-liquors from this anilide formation were heated with Amberlite IR-120(H^+) (10 g.) in water (30 ml.) for 3 hr. at 85°. The syrup (77 mg.) obtained on concentration was heated with *p*-nitrobenzoyl chloride (180 mg.) in dry pyridine (5 ml.) for 0·5 hr. at *ca.* 70°. Recrystallisation of the product from methanol gave 2 : 3 : 6-tri-*O*-methyl-β-D-glucose 1 : 4-di-*p*-nitrobenzoate, m. p. and mixed m. p. 188—189°, $[\alpha]_D^{17}$ −31° (*c* 0·4 in MeOH) (Calc. for $C_{23}H_{24}O_{12}N_2$: N, 5·4. Found: N, 5·3%). Rebers and Smith [20] give m. p. 189—190° and $[\alpha]_D$ −33° in chloroform. Fraction B (343 mg.) was partitioned 28 times between equal volumes of chloroform and water. The chloroform phase contained mainly 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose (210 mg.), and the aqueous phase contained mainly 2 : 3 : 6-tri-*O*-methyl-D-glucose (110 mg.). Treatment of some (17·6 mg.) of the latter with 4% methanolic hydrogen chloride at room temperature caused $[\alpha]_D^{17}$ +50° (4 min.) to become −18° (120 min.) (*c*, 0·22) and complete loss of staining power with aniline hydrogen phthalate.

Fraction D (112 mg.) was refluxed with aniline (0·11 ml.) in ethanol (1 ml.) for 3 hr. Recrystallisation of the product from ethanol gave 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine (46 mg.), m. p. and mixed m. p. 174—176°, $[\alpha]_D^{17}$ −44·1° equil. (*c* 1·12 in EtOH) (Found: C, 60·7; H, 8·0; N, 4·7. Calc. for $C_{15}H_{23}O_5N$: C, 60·6; H, 7·8; N, 4·7%).

Periodate Oxidation of Type XIV Polysaccharide.—The polysaccharide (231 mg.) was oxidised with 0·016M-sodium periodate in the dark for 4 days at room temperature, then for 19 days at 25°. The number of moles of periodate consumed per 1054 gm. of polysaccharide was: 0·75 day, 3·15; 4 days, 3·48; 6 days, 3·9; 8 days, 4·3; 11 days, 4·5; 16 days, 4·7; 23 days, 4·9. The corresponding number of moles of formic acid produced was: 0·75 day, 1·25; 4 days, 1·5; 6 days, 1·7; 8 days, 1·8; 23 days, 2·05.

A portion (37 mg.) of the periodate-oxidised polysaccharide recovered (198 mg.) was hydrolysed with 2·8N-hydrochloric acid (5 ml.) for 7 hr. at 100°. After neutralisation with silver carbonate the hydrolysate was *N*-acetylated.[7] Paper-chromatographic analysis of the resulting mixture showed 2-acetamido-2-deoxyglucose and galactose but no glucose. Using

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

less drastic conditions of hydrolysis (2N-hydrochloric acid for 6 hr. at 100°) we detected the same sugars together with a disaccharide. The latter gave galactose and glucosamine on further acidic hydrolysis and had a mobility identical with that of disaccharide III on ionophoresis in borate buffer of pH 10. No disaccharide IV was detected.

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