Immunopolysaccharides. Part II. Structure of a* Betacoccus arabinosaceous *Dextran.*

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A dextran elaborated by *Betacoccus arabinosaceous* (Birmingham strain) is shown to be a branched polyglucosan, in which the principal glucosidic linkages are α-1 : 6, and the branch points involve positions 1 and 3. The average chain length is 6—7 glucose residues.

Previous workers have established that there is one group of dextrans consisting of long, and virtually unbranched, chains of α-1 : 6-D-glucopyranose units, while a second group is comprised of highly branched dextrans, in which short chains of the above type are joined by 1 : 4-branches [see the review by Stacey and Ricketts (*Fortschr. Chem. Org. Naturstoffe*, 1951, **8**, 28)]. As we have previously reported briefly (Stacey, I.U.P.A.C. Congr., New York, Sept., 1951; Barker, Bourne, Bruce, and Stacey, *Chem. and Ind.*, 1952, 1156), yet a third type of dextran has been discovered in which 1 : 3-glucosidic linkages constitute the branch points. A detailed account of structural studies on this dextran is now presented.

The dextran, elaborated by *Betacoccus arabinosaceous* (Birmingham strain), was isolated from a sucrose medium in which the potassium chloride, *p*-aminobenzoic acid, peptone, and ferrous sulphate, used by Stacey and Swift (*J.*, 1948, 1555), were replaced by a yeast extract. A purified sample of the dextran had $[\alpha]_D^{21}$ +202° in N-sodium hydroxide; it was virtually free from minerals, nitrogenous impurities, and lower saccharides. Paper-chromatographic analysis of a hydrolysate showed glucose (later crystallised), and traces of two other components, which were possibly fructose and hydroxymethylfurfuraldehyde.

* Part I, *J.*, 1954, 1925.

No structural significance is necessarily attached to the detection of this ketose, which may have arisen from an impurity of levan (levans are known to be synthesised by certain dextran-producing organisms), or may have resulted from epimerisation of glucose. The extent of the conversion of the dextran into glucose was shown by cuprimetric titration to be 96%.

The dextran was methylated with sodium and methyl iodide in liquid ammonia at −55°, by a modification of the methods employed by Freudenberg and Boppel (*Ber.*, 1938, **71**, 2505) and Hodge, Karjala, and Hilbert (*J. Amer. Chem. Soc.*, 1951, **73**, 3312). The product (62% yield) had OMe 43·3% (a tri-*O*-methylglucosan requires OMe, 45·6%), ash 0·5%, and $[\alpha]_D^{21}$ +214° (in $CHCl_3$). A paper chromatogram of a hydrolysate of the trimethyl ether revealed two components, having R_F values and staining properties identical with those of 2 : 3 : 4 : 6-tetra- and 2 : 3 : 4-tri-*O*-methylglucose, together with a third component (R_G 0·64, red stain), which was neither 2 : 3-di-*O*-methylglucose (R_G 0·69, brown stain) nor 3 : 4-di-*O*-methylglucose (R_G 0·65, brown stain). This third component (M_G 0·05) could again be differentiated from 2 : 3-di-*O*-methylglucose (M_G 0·12) and 3 : 4-di-*O*-methylglucose (M_G 0·31) by ionophoresis in borate buffer (Foster, *Chem. and Ind.*, 1952, 828); its very low M_G value made it virtually certain that it was the 2 : 4-derivative (cf. Foster, *J.*, 1953, 982), a conclusion which was confirmed subsequently when it was shown that it could not be distinguished, by chromatography or ionophoresis, from a sample of 2 : 4-di-*O*-methylglucose kindly supplied by Drs. D. J. Bell and D. J. Manners. A quantitative assay on filter paper, essentially as described by Hirst, Hough, and Jones (*J.*, 1949, 928), showed that the hydrolysate of the tri-*O*-methyldextran contained (as molecular proportions): 2 : 4-di-*O*-methylglucose, 26; 2 : 3 : 4-tri-*O*-methylglucose, 57; 2 : 3 : 4 : 6-tetra-*O*-methylglucose, 17. This proportion of the tetramethyl ether corresponded to an average chain length of *ca.* 6 glucose units.

Confirmation of the identity of the methylglucoses was obtained on larger-scale fractionation of the hydrolysate on a silica gel column (Bell, *J.*, 1944, 473). The 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose was characterised as the crystalline α-anomer and as the aniline derivative; the trimethyl ether by conversion into *N*-phenyl-2 : 3 : 4-tri-*O*-methyl-D-glucosylamine; and the 2 : 4-di-*O*-methyl-D-glucose by crystallisation from ethyl acetate and as the aniline derivative.

The mixture of saccharides produced by partial hydrolysis of the dextran was fractionated on a charcoal column (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677). *iso*Maltose, identified as its crystalline β-octa-acetate, constituted the major portion (at least 90%) of the disaccharide fraction. It was accompanied by a second disaccharide, which behaved similarly to nigerose and laminaribiose, but differently from maltose, when submitted to paper chromatography either as the free sugar or as its benzylamine derivative (Bayly and Bourne, *Nature*, 1953, **171**, 385). Yet again, the *iso*maltotriose, which formed the bulk of the trisaccharide fraction, was accompanied by another trisaccharide having an R_F value consistent with the presence of a 1 : 6- and a 1 : 3-linkage (Bayly and Bourne, *loc. cit.*).

The dextran consumed 1·67 mol. of periodate, and produced 0·74 mol. of formic acid, per anhydroglucose unit, when oxidised for 93 hr. at 29°. On the assumption that only 1 : 3-branches were present, the former figure indicated a chain length of 6 glucose residues (in good agreement with the methylation evidence), while the latter corresponded to a chain length of only 4 units. This low conversion into formic acid (0·74 mol. instead of 0·83 mol.) may well be attributable to the known inaccuracy of the determination in the presence of oxidised polysaccharides, and possibly also to traces of levan and protein. Hydrolysis of the periodate-oxidised dextran gave, *inter alia*, a component with an R_F value equal to that of glucose, again in conformity with the presence of 1 : 3-branch links. The proportion of glucose in the mixture corresponded to a chain-length of *ca.* 7 units.

Finally, the infra-red absorption spectrum of the dextran was consistent with the structure now assigned (cf. Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171). There were absorption peaks at 917 and 768 $cm.^{-1}$ (α-1 : 6-linkages), at 794 $cm.^{-1}$ (α-1 : 3-linkages), and at 841 $cm.^{-1}$ (this band is shown by α-, but not β-, anomers in the D-glucopyranose series).

Thus, we reach our conclusion that the branch points of the dextran entail principally 1 : 3-linkages from four independent observations, based on methylation, partial hydrolysis to oligosaccharides, oxidation with periodate, and infra-red analysis. It should be noted that the strain of *Betacoccus arabinosaceous* used for the production of the dextran was derived, through many sub-cultures, over a period of about five years, from that isolated by Stacey and Swift (*loc. cit.*) which was claimed to elaborate a dextran with the more usual 1 : 4-branches. It is, of course, possible that a mutation, or a strain selection, had occurred during the interval; alternatively, it may be that the earlier authors were misled by the fact that the aniline derivative of their di-*O*-methyl-D-glucose was somewhat impure, and that its melting point was unfortunately close to that of *N*-phenyl-2 : 3-di-*O*-methyl-D-glucosylamine.

During the course of this investigation, other workers have reported the behaviour of dextrans during periodate oxidations, and have concluded that periodate-resistant units (probably linked through position 3) are present in certain types (cf. Lohmar, *J. Amer. Chem. Soc.*, 1952, **74**, 4974; Abdel-Akher, Hamilton, Montgomery, and Smith, *ibid.*, p. 4970; Jeanes and Wilham, *ibid.*, p. 5339). In addition, it has been shown that some dextrans contain more than one component, which situation is perhaps analogous to the case of starch (Burket and Melvin, *Science*, 1952, **115**, 516; Lohmar, *loc. cit.*; Jeanes and Wilham, *loc. cit.*).

Experimental

Purification of the Crude Dextran.—The dextran (kindly supplied by Dextran Ltd.) had been isolated by precipitation with excess of acetone from culture media (containing per l. : extract from 200 g. of yeast; $Na_2NH_4PO_4$, 5 g.; KH_2PO_4, 1 g.; $MgSO_4,7H_2O$, 0·5 g.; sucrose, 100 g.) of *Betacoccus arabinosaceous* (Birmingham strain), after incubation for 2—3 days at 25°. The crude polysaccharide (40 g.; moisture, *ca.* 12%) was dispersed by shaking it in water (1350 c.c.) for 15 hr. 0·1M-Phosphate buffer (200 c.c.; pH, 6·8) was added, and the whole diluted with water to 1750 c.c. The dispersion was boiled for 45 min., then cooled, and the insoluble material (0·59 g.) collected in a Sharples supercentrifuge. Protein was removed from the concentrated solution (800 c.c.) by the method of Sevag, Lackman, and Smolens (*J. Biol. Chem.*, 1938, **124**, 425), involving three partitions with equal volumes of chloroform–amyl alcohol (3·5 : 1). The polysaccharide was precipitated from the aqueous solution (concentrated to 750 c.c.) with ethanol (3 l.). After three such precipitations, it was dissolved in water (400 c.c.), containing toluene and a trace of sodium ethyl mercurithiosalicylate, and dialysed at 0° for 72 hr., before being freeze-dried. The dextran (23·2 g.) was ground in ether to a powder, and, after being dried *in vacuo* over phosphoric oxide at 60°, had ash, 0·3; N, 0·2%; $[\alpha]_D^{21}$ +202° (*c*, 0·82 in N-sodium hydroxide).

Paper chromatography of the purified dextran, using the upper phase of a mixture of *n*-butanol (40%), ethanol (10%), water (49%), and ammonia (1%) as the solvent, and the usual naphtharesorcinol, aniline hydrogen phthalate, and ammoniacal silver nitrate sprays, failed to reveal any oligosaccharides.

Acidic Hydrolysis of the Purified Dextran.—The polysaccharide (20 mg.) was hydrolysed with 1·4N-sulphuric acid (1·4 c.c.) at 100° for 5 hr. in a sealed tube. After removal of ions by the method of Consden, Gordon, and Martin (*Biochem. J.*, 1947, **41**, 590), the neutral hydrolysate was examined by paper chromatography, as described above; the main component had an R_F value equal to that of glucose; traces of compounds which were probably fructose (*ca.* 0·1—0·2%) and hydroxymethylfurfuraldehyde were also detected.

Another sample of the polysaccharide was hydrolysed for 5 hr. with 1·5N-sulphuric acid at 100°, and the glucose produced was determined by cuprimetric titration (Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687). After a small correction (2%) for the loss in reducing power which occurs when glucose itself is treated with acid under these conditions (Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224), the extent of the conversion corresponded to 96%.

After hydrolysis of another sample (0·156 g.) of the dextran, during which $[\alpha]_D$ fell from +201° to +52° (constant), α-D-glucose (30%) was obtained, having m. p. and mixed m. p. 143—145°, $[\alpha]_D^{15}$ +110° (3 min.) ⟶ +53·7° (17 hr., equil.) (*c*, 0·40 in H_2O).

Methylation of the Purified Dextran.—The dextran (10·52 g.) was treated with sodium and methyl iodide in dry redistilled liquid ammonia at −55°, a modification of the methods employed by Freudenberg and Boppel (*loc. cit.*) and Hodge, Karjala, and Hilbert (*loc. cit.*) being used. After four additions of the methylating reagents (reaction times : sodium, 2 hr.;

methyl iodide, 1 hr.) had been made, the ammonia was allowed to evaporate, with exclusion of moisture, the last traces being removed at 75°/20 mm. After dialysis, the freeze-dried product (10·53 g.) had OMe, 28·7%. Repetition of the methylation procedure gave a product (9·83 g.) having OMe, 42·7%. The crude methyl ether obtained after a final remethylation (9·08 g.; ash, 1·1%) was purified by removal of grease in a Soxhlet apparatus by light petroleum (b. p. 60—80°), and subsequent extraction of the residual material with dry chloroform. The cloudy extract was centrifuged, the supernatant liquid evaporated, and the residue ground with ether. The methylated dextran (8·17 g.) showed $[\alpha]_D^{21}$ +214° (*c*, 0·24 in $CHCl_3$) (Found: OMe, 43·3; ash, 0·5. Calc. for $C_9H_{16}O_5$: OMe, 45·6%).

Identification of the Methyl Sugars obtained from the Methylated Dextran.—The polysaccharide ether (0·054 g.), in dry chloroform (1 c.c.), was treated with 8% methanolic hydrogen chloride (1 c.c.) in a sealed tube at 100° for 8 hr., and then, after removal of the solvent, with 4% hydrochloric acid (5·5 c.c.) at 100° for 6 hr., the optical rotation then being constant. The resulting solution of methylglucoses was freed from ions, and concentrated to a syrup (0·056 g.). A paper chromatogram of the hydrolysate, irrigated for 24 hr. in the manner previously described, showed three components after being sprayed with aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443). Two of the components had R_F values and staining properties identical with those of 2 : 3 : 4-tri-*O*-methylglucose and 2 : 3 : 4 : 6-tetra-*O*-methylglucose. The third component (R_G 0·64, red stain) could be differentiated on the same chromatogram from 2 : 3- (R_G 0·69, brown stain) and 3 : 4-di-*O*-methylglucose (R_G 0·65, brown stain), but not from 2 : 4-di-*O*-methylglucose. The same component, after isolation from a paper chromatogram, was submitted to ionophoresis in borate buffer (pH, 10) (Foster, *loc. cit.*); its M_G value and staining reactions were identical with those of 2 : 4-di-*O*-methylglucose (M_G 0·05) isolated from a hydrolysate of methyl 2 : 4-di-*O*-methyl-β-D-glucopyranoside, produced from the methyl ether of tritylated laminarin. 2 : 3-Di-*O*-methylglucose had M_G 0·12, while 3 : 4-di-*O*-methylglucose had M_G 0·31.

Determination of the Methyl Sugars obtained from the Methylated Dextran.—The remainder of the hydrolysate mentioned above was analysed by a method which was essentially that used by Hirst, Hough, and Jones (*loc. cit.*), with minor modifications introduced by Barker, Bourne, and Wilkinson (*J.*, 1950, 3027). As a result of several independent quantitative determinations, based on oxidation with hypoiodite, the calculated composition of the mixture was as given on p. 2396.

Isolation of the Methyl Sugars obtained from the Methylated Dextran.—The methylated dextran (3·00 g.), after methanolysis and hydrolysis, gave a syrup (3·05 g.), which was freed from ions, and then fractionated by Bell's method (*loc. cit.*) on a column of silica gel (Gordon, Martin, and Synge, *Biochem. J.*, 1943, **37**, 79). Three fractions (I, 0·537 g.; II, 1·950 g.; III, 0·379 g.) were obtained; paper-chromatographic analysis and ionophoresis showed them to contain, respectively, 2 : 3 : 4 : 6-tetra-, 2 : 3 : 4-tri-, and 2 : 4-di-*O*-methylglucose.

Fraction I (0·396 g.), crystallised from ether, gave 2 : 3 : 4 : 6-tetra-*O*-methyl-α-D-glucose (0·088 g.), m. p. and mixed m. p. 82—84°, $[\alpha]_D^{15}$ +95·3° (4 min.) ⟶ +83·0° (17 hr., equil.) (*c*, 0·40 in H_2O) (Found: C, 50·7; H, 8·5; OMe, 51·8. Calc. for $C_{10}H_{20}O_6$: C, 50·8; H, 8·5; OMe, 52·5%). The syrup obtained by concentration of the mother-liquors, treated with aniline (0·30 c.c.) in ethanol (10 c.c.) under reflux for 2 hr., gave *N*-phenyl-2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucosylamine (0·040 g.), m. p. 135—136° (cf. Peat, Schlüchterer, and Stacey, *J.*, 1939, 581).

Fraction II (0·100 g.), refluxed with aniline (0·60 c.c.) in ethanol (1·00 c.c.) for 4 hr., gave crystals (0·030 g.), m. p. 132°, raised by two recrystallisations from ether–light petroleum (b. p. 40—60°) to 147—148°, not depressed in admixture with *N*-phenyl-2 : 3 : 4-tri-*O*-methyl-D-glucosylamine. Peat, Schlüchterer, and Stacey (*loc. cit.*) gave m. p. 145—146°.

Fraction III (0·065 g.), crystallised from dry ethyl acetate, gave a small yield of a product, m. p. 125—128°, not depressed in admixture with 2 : 4-di-*O*-methyl-β-D-glucose. The syrup recovered from the mother-liquors was kept in aniline (0·40 c.c.)–ethanol (0·50 c.c.) at 20° for 48 hr., and the crystalline precipitate (0·029 g.) was recrystallised from ether–light petroleum (b. p. 40—60°). The product had m. p. 197—199°, alone and in admixture with *N*-phenyl-2 : 4-di-*O*-methyl-D-glucosylamine, but depressed to 137—140° by the isomeric 3 : 4-dimethyl ether, both samples being supplied by Dr. Bell (Found: C, 59·1; H, 7·2; N, 4·6. Calc. for $C_{14}H_{21}O_5N$: C, 59·3; H, 7·5; N, 4·9%).

Partial Hydrolysis of the Purified Dextran.—The dextran (2·46 g.) was partially hydrolysed with N-sulphuric acid (100 c.c.) at 85° for 5·5 hr., $[\alpha]_D^{19}$ having then fallen to +133°. The acidic solution of saccharides was fractionated on a charcoal column (Whistler and Durso, *loc. cit.*),

which was washed as shown in the Table. Each eluate was neutralised with barium carbonate (when necessary), filtered, and concentrated to a syrup, and the saccharides were extracted with hot methanol (A—D), or hot 50% aqueous methanol (E—I). Thereafter, they were obtained as freeze-dried powders, and analysed by paper chromatography.

Partial acidic hydrolysis of the dextran.

Eluant	Fraction	Wt. (g.) of product	Probable sugar components (by paper chromatography)
Water, 300 c.c.	A	0·802	Glucose, fructose (<5%)
5% Ethanol (1), 200 c.c.	B	0·234	*iso*Maltose
5% Ethanol (2), 100 c.c.	C	0·017	*iso*Maltose, nigerose, trisaccharide
5% Ethanol (3), 400 c.c.	D	0·187	Trisaccharides
10% Ethanol (1), 100 c.c.	E	0·003	Trisaccharides
10% Ethanol (2), 200 c.c.	F	0·175	Tetrasaccharide
10% Ethanol (3), 100 c.c.	G	0·045	Tetrasaccharide, pentasaccharide
10% Ethanol (4), 400 c.c.	H	0·141	Higher saccharides
35% Ethanol, 400 c.c.	I	0·915	Higher saccharides

Part (0·310 g.) of fraction A was further characterised by conversion into penta-*O*-acetyl-β-D-glucopyranose (0·237 g.), m. p. and mixed m. p. 128—129°. Fraction B had $[\alpha]_D^{19}$ +119° (equil.) (*c*, 0·43 in H_2O); ash, 1·1%. Montgomery, Weakley, and Hilbert (*J. Amer. Chem. Soc.*, 1949, **71**, 1682) report $[\alpha]_D^{25}$ +120° (equil.) (*c*, 1·2 in H_2O) for *iso*maltose. A portion (0·075g .) of this product, treated with acetic anhydride (0·75 c.c.) and fused sodium acetate (0·02 g.) at 100—110° for 1 hr., gave β-*iso*maltose octa-acetate (0·090 g.), m. p. and mixed m. p. 144—145°, $[\alpha]_D^{20}$ +99·6° (*c*, 0·38 in $CHCl_3$) (Found: C, 49·7; H, 5·5. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6%). Fraction C contained an additional disaccharide, which, when submitted to paper chromatography, either as the free sugar or as its benzylamine derivative (Bayly and Bourne, *loc. cit.*), was indistinguishable from nigerose and laminaribiose. Fraction D had the same R_F value as *iso*maltotriose, and, after partial hydrolysis with N-sulphuric acid at 90° for 0·5, 1·0, and 2·0 hr., *iso*maltotriose, *iso*maltose, and glucose were identified by paper chromatography.

Periodate Oxidation of the Purified Dextran.—The procedure adopted was essentially that of Jeanes and Wilham (*J. Amer. Chem. Soc.*, 1950, **72**, 2655), which incorporates Fleury and Lange's method (*J. Pharm. Chim.*, 1933, **17**, 107, 196) for the determination of the quantity of periodate consumed, and that of Halsall, Hirst, and Jones (*J.*, 1947, 1427), with certain modifications, for the determination of the quantity of formic acid produced. The number of moles of sodium periodate consumed per unit of anhydroglucose was: 44 hr., 1·58; 74 hr., 1·65; 93·5 hr., 1·67; and the corresponding figures for formic acid produced were: 0·73, 0·75, 0·74.

Ethylene glycol was added to destroy the excess of periodate, and sodium hydroxide to neutralise the formic acid in a solution of dextran (0·0615 g.), which had been submitted to periodate oxidation for 128 hr., under the same conditions. The oxidised polysaccharide (0·047 g.) was obtained by removal of ions from this solution and concentration under reduced pressure. After hydrolysis with 1·4N-sulphuric acid (5 c.c.) at 100° for 9 hr., the solution was neutralised with barium carbonate, filtered, and concentrated. The syrup (0·048 g.) obtained was dissolved in water (2 c.c.), and a drop of the solution was examined on a paper chromatogram, which, when developed with aniline hydrogen phthalate, showed a component having an R_F value equal to that of glucose, together with a red-staining faster-moving component of unknown identity. The amount of glucose present in the solution was determined by incorporation of a known amount (0·005 g.) of tetra-*O*-methylglucose in a portion (1 c.c.) of the hydrolysate, followed by determination of the molar proportions of the two sugars, after separation by paper chromatography, by the method of Hirst, Hough, and Jones (*loc. cit.*). In this way, it was calculated that 9·8 mg. of glucose had been obtained from 61·5 mg. of dextran, indicating that one glucose unit in every seven of the dextran was linked through position 3.

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