STUDIES ON DEXTRANS AND DEXTRANASES PART X[1]. TYPES AND PERCENTAGES OF SECONDARY LINKAGES IN THE DEXTRANS ELABORATED BY *Leuconostoc mesenteroides* NRRL B-1299*

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ABSTRACT

The water-soluble (dextran *S*) and less water-soluble (dextran *L*) dextrans elaborated by *Leuconostoc mesenteroides* NRRL B-1299 contain α-D-glucopyranose residues linked through positions 1 and 6, 1 and 3, as well as 1, 2, and 6. The approximate number of terminal non-reducing D-glucose residues and those linked through positions 1 and 6, 1 and 3, as well as 1, 2, and 6 in the average repeating-unit of dextran *S* are 5, 4, 1, and 5. The corresponding figures for dextran *L* are 5, 4, 3, and 5.

INTRODUCTION

Two dextran fractions (*S* and *L*) have been isolated[2] from cultures of *Leuconostoc mesenteroides* NRRL B-1299. Dextran *S* is water-soluble and can be extracted from the culture fluid. Dextran *L* can be extracted from the gelatinous "insoluble dextran" sediment and is much less soluble in water than dextran *S*. The results of periodate oxidation[2] and acetolysis[3], and the optical rotations[4] of these materials in cuprammonium solution suggested that, in these polysaccharides, D-glucopyranose residues are linked by α-(1→6) linkages (primary linkages) as well as α-(1→2), α-(1→3), and possibly α-(1→4) linkages (secondary linkages), the ratio of primary to secondary linkages being almost unity. Immunochemical studies[5] had also indicated that many chains in these dextrans might be terminated by α-kojibiosyl units whilst some might be terminated by α-nigerosyl units. The interaction of both dextrans (synthesised *in vitro* by dextransucrase) with concanavalin A has been interpreted to indicate close structural similarities[6]. It has also been suggested[6] that the insoluble dextran acts as a precursor of the water-soluble dextran, and that the gelatinous, insoluble dextran represents a complex between cell-bound dextransucrase and "soluble" dextran which, on completion of synthesis, is released into the supernatant liquid.

We felt that further evidence for structure could be obtained by extending to these unusual dextrans the methods which we had previously applied to dextrans containing α-(1→3) or α-(1→4) branch-linkages. As a preliminary to more-detailed

*Dedicated to Professor M. Stacey, C.B.E., F.R.S., in honour of his 65th birthday.

assignments of structures, we now report our conclusions regarding the types and percentages of secondary linkages in dextrans *S* and *L*.

RESULTS AND DISCUSSION

The dextrans *S* and *L* were shown to be D-glucans. Partial, acidic hydrolysates of each dextran contained components having paper-chromatographic properties identical with those of kojibiose (trace) and the oligosaccharides of the isomaltose series. After fractionation of the partial hydrolysate of dextran *S* on charcoal–Celite, isomaltose was characterised as the β-acetate, whereas isomalto-triose, -tetraose, -pentaose, and -hexaose were characterised by periodate oxidation, hydrolysis with dextranase, and measurement of optical rotations.

Acetolysis. — Products of the acetolysis of both dextrans had, after deacetylation, paper-chromatographic and electrophoretic properties identical with those of isomaltose, kojibiose, and nigerose. Kojibiose and nigerose were characterised more fully after the deacetylated products of the acetolysis of dextran *S* had been fractionated on charcoal–Celite. These results are in agreement[3] with those obtained either with the unfractionated dextran or from chromatographic migration-rates (dextran *S*).

Methylation. — Each dextran was methylated with sodium and methyl iodide in liquid ammonia. Paper chromatography and electrophoresis of the hydrolysates of both methylated dextrans revealed four major components which had properties identical with those of 2,3,4,6-tetra-, 2,3,4-tri-, 2,4,6-tri-, and 3,4-di-*O*-methyl-D-glucoses. G.l.c. of the methanolysates of both methylated dextrans revealed components having retention volumes identical with those of the methyl glycosides of the above *O*-methyl-D-glucoses. Their identity was confirmed after larger scale fractionations on cellulose columns. The 2,3,4,6-tetra-, 2,4,6-tri-, and 3,4-di-*O*-methyl-D-glucoses were each obtained as the crystalline α-anomer of the pyranose form. The 2,3,4-tri-*O*-methyl-D-glucose (a syrup) was characterised by conversion into 2,3,4-tri-*O*-methyl-*N*-phenyl-D-glucosylamine. The results of two sets of quantitative determinations of the *O*-methyl-D-glucoses obtained from both dextrans are shown in Table I.

Periodate oxidation. — Dextran *S* reduced 1.49 mol. of periodate with the concomitant formation of 0.50 mol. of formic acid per "anhydroglucose" unit. The corresponding figures for dextran *L* were 1.52 and 0.55. These results are not significantly different from those obtained earlier[2].

Fragmentation of periodate-oxidised dextrans. — The controlled degradation of both dextrans by periodate oxidation, reduction, and hydrolysis gave four products which had paper-chromatographic and electrophoretic migration-rates identical with those of glycolaldehyde, glycerol, glyceraldehyde, and glucose. The determinations of glycolaldehyde, glyceraldehyde, and glucose by colorimetric methods were possible without their separation. The results, together with the origin of these compounds, are shown in Table I.

TABLE I

PERCENTAGE OF VARIOUSLY LINKED α-D-GLUCOPYRANOSE RESIDUES[a]

Dextran	*Position of linkage*	*Method*[b] (%)				*Average* (%)	*Approximate no. of "anhydro-glucose" units in average repeating-unit*
		(*A*)	(*B*)	(*C*)	(*D*)		
S	1 (terminal, non-reducing)	50	56[c]	32[f]	34[f]	33	5
	1,6			35[g]	26[h]	26	4
	1,3	1	7[d]		7[i]	7	1
	1,2,6	49	36[e]	33[j]	32[j]	34	5
L	1 (terminal, non-reducing)	55	49[c]	29[f]	28[f]	29	5
	1,6			38[g]	24[h]	24	4
	1,3	3	19[d]		15[i]	18	3
	1,2,6	42	32[e]	32[j]	33[j]	32	5

[a]The results are also expressed as molecular proportions of the compounds obtained by methods *B*–*D*. [b](*A*) Periodate oxidation; (*B*) fragmentation of periodate-oxidised dextran; (*C*) methylation–paper chromatography–hypoiodite method; (*D*) methylation–fractionation on cellulose column–optical rotation. [c]Glycolaldehyde. [d]D-Glucose. [e]Glyceraldehyde. [f]2,3,4,6-Tetra-*O*-methyl-D-glucose; [g]2,3,4- and 2,4,6-Tri-*O*-methyl-D-glucose. [h]2,3,4-Tri-*O*-methyl-D-glucose. [i]2,4,6-Tri-*O*-methyl-D-glucose. [j]3,4-Di-*O*-methyl-D-glucose.

N.m.r. spectroscopy. — N.m.r. spectroscopy can be a rapid means for determining the ratio of primary to secondary linkages in some dextrans[1,7]. However, our attempts to apply this method to dextrans *S* and *L* proved only partially successful due, in part, to the low solubilities of these dextrans in deuterium oxide, and the fact that the signals due to protons on the anomeric carbon atoms involved in primary and secondary linkages were insufficiently resolved. However, the spectra suggested that, in both dextrans, primary and secondary linkages might be present in equal proportions.

Structures of dextrans S *and* L. — The isolation of kojibiose, nigerose, and isomaltose and its homologues from both dextrans shows, together with the results of periodate oxidations, that these polysaccharides contain α-(1→2), α-(1→3), and α-(1→6) linkages. The presence of isomaltohexaose in partial hydrolysates of both dextrans also shows that they contain sequences of at least six D-glucopyranose residues linked by α-(1→6) linkages.

After methylation and hydrolysis, both dextrans yielded only one di- and two tri-*O*-methyl-D-glucoses (3,4-di-, 2,3,4- and 2,4,6-tri-*O*-methyl-D-glucose). This fact makes it unlikely that the tri-*O*-methyl-D-glucoses are formed as a result of replacement of D-glucosyl units, attached to branching D-glucopyranose residues, by methyl groups during the methylation process. We are thus led to the following conclusions: (*a*) dextrans *S* and *L* have branched structures and most, if not all, branching linkages are of the α-(1→2) type; (*b*) most, if not all, α-(1→3) linkages present in both dextrans

represent terminal linkages on non-reducing chain ends (*i.e.* chains are terminated by nigerosyl units) and/or occur in unbranched segments of the dextran molecules where, for instance, a continuous chain of α-(1→6)-linked D-glucose residues is intersected by an α-(1→3) linkage.

It is now possible to calculate the percentages of the variously linked D-glucose residues in the two dextrans from (*a*) the results of periodate oxidations, (*b*) the molecular proportions of fragments obtained from the periodate-oxidised dextrans, and (*c*) the molecular proportions of the *O*-methyl-D-glucoses obtained by hydrolysis of the methylated dextrans (two methods). In calculating the average values, we have not taken into account the results obtained from the periodate oxidations, since small experimental errors in this method become magnified in these values. The results (Table I) show that, although the two dextrans contain the same types of glucosidic linkages, dextran *L* differs structurally from dextran *S* by its greater percentage of α-(1→3) linkages. From the average repeating-units of the dextrans, it might be concluded that dextran *L* possesses the same structural elements as does dextran *S* but its average repeating-unit contains two additional α-(1→3)-linked D-glucose residues. Such a structural difference makes it likely that the dextrans *S* and *L* represent successive stages in the synthesis, the latter stage being the synthesis of α-(1→3) linkages to give dextran *L*.

EXPERIMENTAL

Paper chromatography. — The solvents used were (*a*), (*b*), (*c*), and (*d*) of Part IX[1]; (*e*) butan-1-ol–benzene–pyridine–water (5:1:3:2); (*f*) butan-1-ol–ethanol–water–ammonia (40:10:49:1). Migration rates are expressed as in Part VI[8].

Paper electrophoresis. — The electrolytes used were those of Part VI[8].

Spray reagents. — The spray reagents used were (*a*), (*b*), and (*c*) of Part IX[1]; (*d*) *p*-anisidine hydrochloride[9].

Gas–liquid chromatography. — This was carried out as described by Aspinall[10], using columns containing polyphenyl ether [*m*-bis-(*m*-phenoxyphenoxy)benzene]. Retention times (*T*) are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Preparation and purification of dextrans. — The lyophilysed *Leuconostoc mesenteroides* NRRL B-1299 (NCDO 1875) micro-organism was twice subcultured (36 h) through media (10 ml), initially of pH 6.7, and containing D-glucose (1%), peptone (1%), yeast extract (1%), microcosmic salt (0.5%), sodium acetate (0.2%), potassium dihydrogen phosphate (0.1%), magnesium sulphate (0.02%), and manganese sulphate (0.005%). A suspension (1 ml) of growing cells was then used to inoculate media (1 litre) in which D-glucose was replaced by sucrose (10%). After incubation at 28° for 4 days (when the pH was *ca.* 3–4), the culture medium was centrifuged at 2500 *g* for 15 min. Crude dextran *S* was precipitated from the supernatant liquid by adjustment to 45% with ethanol. The centrifugate was warmed (1 h at *ca.* 60°) in M sodium hydroxide (*ca.* 150 ml) and re-centrifuged (2500 *g* for 15 min). From the supernatant

liquid thus obtained, crude dextran *L* was precipitated by adjustment to pH 4 with glacial acetic acid and to 45% with ethanol.

The crude dextrans were redissolved (dextran *S* in warm distilled water, dextran *L* in warm M sodium hydroxide) and the solutions centrifuged (2500 *g* for 15 min). The dextrans were precipitated by addition of an equal volume of ethanol (dextran *L* after adjustment to pH 4 with acetic acid). Each dextran was redissolved and deproteinised by the method of Sevag *et al.*[11]. The dextrans were again precipitated with ethanol, redissolved or suspended in distilled water, freeze-dried to white powders, and finally dried *in vacuo* over phosphoric oxide at 60°. The average yields (based on sucrose used) of dextrans *S* and *L* were 18 and 1%, respectively. The dextrans *S* and *L* had, respectively, ash 6.5 and 3.3, N<0.1%, $[\alpha]_D^{20}$ +211° and +220° (*c* 1.0, M sodium hydroxide).

Acidic hydrolysis of dextrans. — (*i*) The dextrans (*ca.* 500 mg) were separately hydrolysed with 0.1M sulphuric acid (10 ml) at 70° for 1 h[12]. Paper chromatography of each hydrolysate using solvent (*a*) and spray (*b*) revealed traces of a component having properties identical with those of glucose, but did not reveal fructose.

(*ii*) The dextrans (1.5 g) were separately hydrolysed with M sulphuric acid (20 ml) at 100° for 8 h[13]. Paper chromatography [solvent (*a*)] of each de-ionised hydrolysate revealed a single component having properties identical with those of glucose. Evaporation of the hydrolysates and crystallisation of the residues gave α-D-glucopyranose (0.76 g from dextran *S*; 0.78 g from dextran *L*) having m.p. and mixed m.p. 140–144°, $[\alpha]_D^{20}$ +52° (equilibrium; *c* 0.2, water).

The amount of D-glucose produced by hydrolysis of another sample (100 mg) of each dextran was determined with the D-glucose oxidase–peroxidase reagent (Boehringer Biochemicals). The results, corrected for ash and D-glucose lost under the above conditions, were: dextran *S*, 100.0%, dextran *L*, 97.5%.

(*iii*) Each dextran (*ca.* 30 mg) was hydrolysed with 0.5M sulphuric acid (3 ml) at 100° for 1.5 h. Paper chromatography [solvents (*a*) and (*c*)] of each de-ionised hydrolysate revealed the presence of glucose and the oligosaccharides of the isomaltose series.

Chromatography of the de-ionised hydrolysate of a larger sample of dextran *S* (22 g) on a charcoal–Celite column[14] (7.5×70 cm) gave samples of glucose, oligosaccharides of the isomaltose series, and a small amount of kojibiose (all identified by determination of molecular size[15], paper chromatography, electrophoresis[16,17], and optical rotations[18]). The components identified as isomalto-tetraose, -pentaose, -hexaose, and -heptaose were treated with the dextranase of *Penicillium lilacinum*[19] (NRRL 896; I.M.I. 79197) to give products consistent with the assigned structures. The components identified as isomaltose and isomalto-triose, -tetraose, -pentaose, and -hexaose reduced, respectively, 6.1, 8.0, 10.0, 12.1, and 14.0 mol. of periodate. The corresponding figures for formic acid liberated by treatment with periodate[20] were 5.0, 6.0, 6.9, 7.9, and 8.9 mol. The component identified as isomaltose was converted into octa-*O*-acetyl-β-isomaltose[18], m.p. and mixed m.p. 139–141°.

Acetolysis of dextrans. — (*i*) Each dextran (*ca.* 150 mg) was heated with a

mixture (1.2 ml) of acetic anhydride and conc. sulphuric acid (100:9, v/v) at 35°. The reaction mixture was worked up as described before[1,21]. Each mixture of saccharides thus obtained was fractionated by paper chromatography [solvent (*c*)] to give materials which had migration and staining [spray (*c*)] properties identical with those of glucose, nigerose, kojibiose, isomaltose, isomaltotriose, and isomaltotetraose. (The mixtures also contained tri- and tetra-saccharides possessing primary and secondary linkages; their separation and identification will be reported elsewhere.) The separated disaccharides maintained their identity during electrophoresis in sodium borate solution. The products obtained by reduction with sodium borohydride had electrophoretic mobilities in molybdate buffer identical with those of the corresponding *O*-D-glucosylhexitols, *i.e.* nigeritol, kojibiitol, and isomaltitol.

(*ii*) The mixture of saccharides (13.5 g) obtained by acetolysis, followed by deacetylation, of a larger sample of dextran *S* (21 g) was fractionated on a charcoal–Celite column (7 × 75 cm) to give α-D-glucopyranose (5.3 g), m.p. 143°, $[\alpha]_D^{20}$ +51° (equilibrium; *c* 0.2, water) (a sample was converted into penta-*O*-acetyl-β-D-glucopyranose, m.p. 130–131°); isomaltose (60 mg, syrup); kojibiose (4.2 g), m.p. 187–189°, $[\alpha]_D^{20}$ +137° (equilibrium; *c* 0.7, water) (a sample was converted into octa-*O*-acetyl-β-kojibiose, m.p. 117–121°); nigerose (60 mg, syrup), $[\alpha]_D^{20}$ +108° (equilibrium; *c* 0.6, water); and higher saccharides (*ca.* 1 g).

N.m.r. spectra. — The n.m.r. spectra were recorded as described before[1], using tetramethylsilane as internal standard. Overlapping peaks of approximately equal height at τ 4.90 and 4.70 were present in the spectrum of each dextran.

Methylation of dextrans. — Dextrans *S* (13 g) and *L* (8.2 g) were separately methylated by five and four treatments, respectively, with sodium and methyl iodide in liquid ammonia; the methoxyl contents had then become constant[22]. The methylated dextrans *S* (7.3 g) and *L* (5.5 g) had, respectively, OMe 41.7 and 41.5% (corrected for ash), and exhibited no significant absorption between 3200 and 3700 cm^{-1}.

Isolation, characterisation, and determination of O-*methyl*-D-*glucoses obtained from methylated dextrans.* — (*i*) Solutions of the methylated dextrans *S* (30 mg) and *L* (30 mg) in 7.2% methanolic hydrogen chloride (10 ml) were boiled under reflux[10] for 24 h. After neutralisation (silver carbonate), the methanolysates were extracted with chloroform (10 ml) and the extracts evaporated to syrups. G.l.c. of the syrups revealed components having the retention times shown in Table II and identical with those of the methyl glycosides of the named *O*-methyl-D-glucoses.

(*ii*) The methylated dextrans *S* (5.4 g) and *L* (4 g) were separately treated with 72% (v/v) sulphuric acid (40 ml) at room temperature for 1 h. Water (320 ml) was added to each mixture and the hydrolysis completed[23] at 100° for 4 h. Paper chromatography [solvent (*f*)] of each hydrolysate revealed four major components having the migration rates shown in Table II and identical with those of the named *O*-methyl-D-glucoses. (Each hydrolysate contained traces of a component with a migration rate similar to that of a mono-*O*-methyl-D-glucose.)

A portion of each of the above hydrolysates was used for the quantitative determination of the components by the hypoiodite method[24]. Since the two tri-*O*-

TABLE II

YIELDS AND PROPERTIES OF *O*-METHYL-D-GLUCOSES OBTAINED FROM METHYLATED DEXTRANS *S* AND *L*.

Dextran	*Sugar*									*Aniline derivative*	
	O-*Methyl*-D-*glucose*	*Isolated yield* (g)	T[a]	$R_{TMG}(f)$[b]	M_G (B)	*M.p.* (*degrees*)	*Mixed m.p.* (*degrees*)	$[\alpha]_D^{20}$ (*equil.*) (*degrees*)	*MeO* (%)	*M.p.*	*MeO* (%)
S	2,3,4,6-tetra-	1.5	1.00, 1.36	1.00	0.0	84–86	83–85	+83	51.4		
	2,3,4-tri-	1.3	1.34, 1.86	0.89	0.0					138–140	32.7
	2,4,6-tri-	0.1	1.63, 2.30	0.84	0.0	117–120	117–120	+74			
	3,4-di-	1.1	2.43	0.68	0.29	110–112		+74	29.3	168–169	21.1
L	2,3,4,6-tetra-	1.03	1.00, 1.35	1.00	0.0	84–86	82–85	+83	51.1		
	2,3,4-tri-	0.7	1.34, 1.88	0.89	0.0						
	2,4,6-tri-	0.2	1.71, 2.42	0.84	0.0	118–121	117–121	+73	39.1		
	3,4-di-	0.6	2.43	0.68	0.30	111–113		+74	29.4		

[a]As methyl glycosides. [b]TMG, 2,3,4,6-tetra-*O*-methyl-D-glucose.

methyl-D-glucoses were not completely resolved, they were combined and determined together. The results, expressed as molecular proportions, are shown in Table I.

The remainder of each hydrolysate (4.4 and 3.8 g from methylated dextrans *S* and *L*, respectively) was fractionated on a cellulose column (5 × 80 cm) to give, from each, four chromatographically pure materials. The tetra- and tri-*O*-methyl-D-glucoses were eluted with a mixture (3:7) of butan-1-ol–light petroleum (b.p. 100–120°), whereas the di-*O*-methyl-D-glucose was eluted with a 4:6 mixture of butan-1-ol–light petroleum (b.p. 100–120°). Their yields, paper-chromatographic and electrophoretic migration-rates, melting points (where crystalline), mixed melting points with authentic substances, optical rotations, and methoxyl contents are shown in Table II. The melting points and methoxyl contents of the "anilides" of two of these materials are also shown in Table II. The tabulated properties are identical with those of the named *O*-methyl-D-glucoses.

The fractionations were followed polarimetrically. The optical rotations of the fractions were used to estimate each of the four components of the two hydrolysates. The results, expressed as molecular proportions, are shown in Table I.

Periodate oxidation of dextrans S *and* L. — The method was as described before[1]. However, the values for the amount of periodate reduced and formic acid liberated did not become constant even after oxidation for 200 h. The values reported here were obtained by extrapolation to zero time. The number of moles of periodate reduced per unit of "anhydro-glucose" of dextran *S* and *L* were then 1.49 and 1.52, respectively. The corresponding figures for formic acid liberated from dextrans *S* and *L* were 0.50 and 0.55, respectively.

Fragmentation of periodate-oxidised dextrans. — Each dextran (*ca.* 8 g) was oxidised with sodium metaperiodate (23.5 g in 2 litres of water), as described earlier[1]. The reactions were terminated by the addition of ethylene glycol when dextrans *S* (7 days) and *L* (16 days) had, respectively, reduced 1.54 and 1.56 moles of periodate per unit of "anhydro-glucose", with the concomitant formation of 0.53 and 0.54 mole of formic acid. The solutions were then dialysed against running water for 3 days. Sodium borohydride (4 g) was added to each dialysed solution, followed by boric acid (9.6 g in 100 ml of water). After 24 h, more sodium borohydride (2 g) was added. After a further 12 h, the solutions were dialysed against running water for 5 days, concentrated *in vacuo*, and finally freeze-dried (dextran *S* polyalcohol 3.5 g, ash 5.3%; dextran *L* polyalcohol 7.7 g, ash 0.0%).

Each dextran polyalcohol (*ca.* 100 mg) was hydrolysed with 0.5M sulphuric acid (5 ml) at 100° for 7 h in a sealed tube. Paper chromatography [solvents (*d*) and (*e*)] and electrophoresis in sodium borate solution of each de-ionised hydrolysate revealed components having properties identical with those of glycolaldehyde, glyceraldehyde, glycerol, and glucose. After neutralisation, aliquots were removed from each hydrolysate for the determinations described below. The results, expressed as molecular proportions and corrected for degradation under the above conditions, are shown in Table I. (Solutions containing D-glucose, glycolaldehyde, or glyceraldehyde in 0.5M sulphuric acid were separately heated at 100° for 7 h in a sealed tube; the

degradations occurring under these conditions were determined by the methods described below.)

(*a*) D-Glucose was determined with the D-glucose oxidase–peroxidase reagent (Boehringer Biochemicals).

(*b*) Glycolaldehyde was determined[25], after treatment with diphenylamine, trichloroacetic acid, and acetic acid, by measurement of the absorbance at 660 nm. Measurement of the absorbance of standard solutions of glycolaldehyde also containing equal quantities of D-glucose, glycerol, and glyceraldehyde showed that the added three compounds caused a combined error of not more than 3% in the colorimetric determination of glycolaldehyde.

(*c*) Glyceraldehyde can be determined[25], after treatment with diphenylamine, trichloroacetic acid, and acetic acid, by measurement of the absorbance at 510 nm. Measurements using standard solutions (see above) showed that only glycolaldehyde interfered with the determination of glyceraldehyde. The measured absorbance was thus corrected by the value at 510 nm due to the previously determined amount of glycolaldehyde.

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