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Structural Studies on Dextran from *Leuconostoc mesenteroides* NRRL B-512

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Structural studies on dextran, produced by *Leuconostoc mesenteroides* NRRL B-512 and on two fractions, prepared by graded acid hydrolysis of this dextran, are reported. Methylation studies revealed that all main chain residues in these dextrans are (1→6)-α-linked and that all branches start from 3-positions in the glucose residues. The predominant aldobiouronic acid formed on partial hydrolysis of carboxydextrans, prepared from the original dextrans by catalytic oxidation, was 6-*O*-(α-D-glucopyranosyluronic acid)-D-glucose, showing that in the dextran produced by this strain, most of the side chains are longer than one glucose residue.

Dextrans, produced by different strains of *Leuconostoc mesenteroides* and by other bacteria have been extensively studied.[1] Dextrans are used as plasma substitutes and this makes a detailed knowledge of their structure and the relation between structure and immunological properties a matter of some importance. Dextrans are essentially (1→6)-linked α-glucans but differ in some structural details. They show a varying degree of branching, at the 3- and/or 4-positions in the glucose residues. Some dextrans also have α-(1→3) and/or α(1→4) inter-chain residues.[2] Physical [3] and immunological [4] studies indicate that some dextrans contain branches (1→2)-linked to the main chain. Bourne and coworkers have studied the length of the side chains in some dextrans, by enzymatic methods [5] and by a method devised by Aspinall and coworkers,[6] involving catalytic oxidation of the hydroxymethyl groups in the polysaccharide to carboxyl groups, partial acid hydrolysis, and isolation and characterisation of the aldobiouronic acids formed. In the dextrans studied by Bourne and coworkers [7] (*L. mesenteroides* NRRL B-1375, B-1415, and B-1416), most, if not all of the side chains consisted of single glucose residues. A detailed methylation study of native dextran (B-512) by Van Cleve and coworkers [8] showed that the ratio of (1→3):(1→6) linkage was 1:21. In studies,[9,10] at present only summarily reported, on the partial hydrolysis of dextran, it is suggested that the non-reducing terminal linkages are more susceptible to hydrolysis. It is also well known that the α(1→3) linkage is

more easily hydrolysed than the α(1→6) linkage. It is thus anticipated that partial hydrolysis of dextran would lead to a decrease in branching. However, a methylation study [11] of a clinical fraction of dextran (B-512) rather unexpectedly revealed a ratio between α(1→6) units and α(1→3) units of 8:1.

These studies have now been repeated and extended using more refined techniques and in the present paper, studies on the dextran produced by *L. mesenteroides* NRRL B-512, and of two dextran fractions, Dextran 10 ($\bar{M}_w$ 12 000) and Dextran 80 ($\bar{M}_w$ 78 000),* produced by Pharmacia AB from the native dextran by graded hydrolysis, are reported.

Methylation analysis of the dextrans gave mixtures of 2,3,4,6-tetra-*O*-methyl-, 2,3,4-tri-*O*-methyl-, and 2,4-di-*O*-methyl-D-glucose, which were analysed by GLC as their methyl glycosides (Table 1). The methylated sugars were identified by GLC of their methyl glycosides [12] on two columns and of their alditol acetates.[13] The latter were also characterised by mass spectrometry.[14] The 2,4-di-*O*-methyl-D-glucose was further characterised as its crystalline *N*-*p*-nitrophenylglycosylamine. The presence of other tri- and di-*O*-methyl-D-glucoses which should have been detected by the methods used, could not be established. These results therefore demonstrate that all main chain residues are (1→6)-linked and that all branches start from 3-positions. A comparison of the relative amounts of methyl glucoses reveals that the degree of branching is reduced on graded acid hydrolysis, as expected.

In order to study the length of the branches, a method was developed, with low molecular weight substances,[15] by which the selective removal of the terminal, non-reducing glucose residues could be achieved. Selective introduction of 6-deoxy-6-*p*-tolylsulphonyl substituents, *via* 6-*O*-tosylates, into the primary hydroxyl positions in the polymer and subsequent alkali treatment should lead to the elimination of the non-reducing end groups. Methylation analysis before and after this operation would show whether

Table 1. Hydrolysis products from methylated dextrans.

Sugar [a]	Molar percentage			Relative retention time [b]		
	Native dextran	D-80-dextran	D-10-dextran	Methyl glycoside		Alditol acetate
				column a	column b	column c
2,3,4,6-Tetra-*O*-methyl-G	4.2	4.6	5.1	1.00 1.35	1.00 1.42	1.00
2,3,4-Tri-*O*-methyl-G	91.2	91.6	91.9	1.35 1.86	2.55 3.58	2.49
2,4-Di-*O*-methyl-G	4.6	3.8	3.0	2.36 3.34	—	5.10

[a] G=D-glucose.
[b] Relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside for the methyl glucosides and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol for the alditol acetates.

* "Macrodex", a clinical dextran, has $\bar{M}_w$ 70 000.

Table 2. Hydrolysis of carboxydextrans.

	Native dextran	D-80-dextran	D-10-dextran
Terminal D-glucose residues in original dextran, mol %	4.2	4.6	5.1
Uronic acid content in carboxydextran, mol %	2.5	2.6	3.1
Yield of aldobiouronic acid fraction, mol %	1.8	2.1	2.6
Relative proportions of (1→3)- to (1→6)-linked aldobiouronic acid [a]	1:4	1:9	1:19

[a] Calculated from the relative proportions of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,4-tri-*O*-methyl-D-glucose obtained from the hydrolysis of glucosidated-esterified, reduced and methylated aldobiouronic acid fractions.

the branches were single glucose units or longer. Repeated degradations would demonstrate the presence of short chains. Attempts to prepare tosylates of the dextrans, however, failed, probably because of the low solubility and swelling of the polysaccharide in solvents suitable for tosylation. Other attempts, involving the preparation of lipophilic dextran derivatives with 6-positions free, *e.g.* by trimethylsilylation and selective removal of the substituents in 6-position,[16] were no more successful, and finally this approach had to be abandoned.

The problem was instead studied by catalytic oxidation of the polysaccharides to carboxydextrans, followed by partial acid hydrolysis, isolation and characterisation of the aldobiouronic acids formed. The results (Table 2) show that a significant part of the terminal glucose residues were oxidised, that the yields of aldobiouronic acids were about 50 % and that the major part of the aldobiouronic acid fractions consisted of 6-*O*-(α-D-glucopyranosyluronic acid)-D-glucose. The presence of some α-(1→3)-linked aldobiouronic acid was also demonstrated, the amount being highest for the native dextran and least for the Dextran 10. The aldobiouronic acid from the oxidised Dextran 10 was converted into crystalline isomaltitol and its nona-acetate, indistinguishable from authentic samples.

From the oxidised Dextran 10, an aldotriouronic acid fraction was also obtained. On partial hydrolysis it yielded glucose and the above aldobiouronic acid. After treatment with methanolic hydrogen chloride, reduction, methylation and hydrolysis, it yielded comparable amounts of 2,3,4,6-tetra-, 2,3,4-tri-, and 2,4,6-tri-*O*-methyl-D-glucose, indicating that its major component is *O*-(α-D-glucopyranosyluronic acid)-(1→6)-*O*-α-D-glucopyranosyl-(1→3)-D-glucose.

The results thus demonstrate that most of the branches in the original polysaccharides are more than one glucose residue long and indicate that some,

at least in Dextran 10, are disaccharide residues. The NRRL B-512 dextran therefore differs from the dextrans studied by Bourne and coworkers, in which most of the side chains consist of single glucose residues only. The determination of chain length or the chain distribution of the branches in the NRRL B-512 dextran by purely chemical means, presents a number of difficulties. Extensive studies on the enzymatic synthesis and degradation of the polysaccharide, using highly purified enzymes, as has been done with starch and glycogen, may, however, solve this problem.

EXPERIMENTAL

General methods. Melting points are corrected. Evaporations were carried out under reduced pressure at temperatures not exceeding 40°. Paper chromatograms were run on Whatman No. 1 and 3 MM papers, using the following systems (v/v): (*a*) ethyl acetate-acetic acid-water (3:1:1); (*b*) ethyl acetate-pyridine-water (8:2:1); (*c*) butanone, saturated with water. Electrophoretic analyses were performed on Whatman 3 MM papers in 0.05 M germanate at 40°, pH 10.7.[17] Components were detected with alkaline silver nitrate or *p*-anisidine hydrochloride. GLC was performed on a Perkin-Elmer model 881 instrument, using the following columns: (*a*) 15 % butan-1,4-diol succinate (BDS) on Chromosorb G at 175°; (*b*) 15 % polyphenyl ether (OS138) on Chromosorb G at 165°; (*c*) 3 % nitrile silicone-polyester copolymer (ECNSS-M) on Chromosorb G at 180°.

Methylation analysis of dextrans. The polysaccharide was methylated by the method of Srivastava *et al.*,[18] one treatment being sufficient to obtain a chloroform soluble product, and then permethylated using methyl iodide and silver oxide, some chloroform being added to obtain dissolution. Two treatments were required to give a fully methylated product (methoxyl analysis, IR). Fully methylated dextrans were obtained, in one step, by treatment of the polysaccharides in dimethyl sulphoxide first with dimethylsulphinyl anion and then with methyl iodide, following the procedure of Sandford and Conrad.[19]

The methylated dextran (5 mg) was dissolved in a mixture of chloroform (0.5 ml) and 6 % methanolic hydrogen chloride (0.5 ml) and heated, in a sealed tube, at 100° for 12 h. The resulting mixture of methyl glycosides was analysed by GLC on columns *a* and *b* (Table 1). The response factors of the components were determined using mixtures of authentic samples.

Methylated dextran was treated for 2 h with 90 % formic acid at 100°, and then hydrolysed with 0.25 M sulphuric acid at 100° for 18 h. The hydrolysate was converted into alditol acetates and analysed by GLC on column *c* [13] (Table 1). The components were identified by GLC-mass spectrometry.[14]

The hydrolysate from methylated Dextran 10 (1 g) was fractionated on a cellulose column (2 × 50 cm), using solvent system *c*. The di-*O*-methyl-D-glucose fraction was converted into the *N*-*p*-nitrophenylglycosylamine, m.p. 249—251°, in good agreement with the recorded m.p. for the corresponding derivative of 2,4-di-*O*-methylglucose.[20]

Preparation and partial hydrolysis of carboxydextrans. A stream of oxygen was passed through a stirred solution of dextran (10 g) in water (500 ml), kept at 70° and containing platinum catalyst (from 2 g platinum oxide), for 1 month. The pH of the solution was kept at around 8 by adding sodium hydrogen carbonate. The catalyst was then removed by centrifugation and the polysaccharide precipitated in ethanol, dissolved in water (500 ml), treated with Dowex 50 (H^+), dialysed and lyophilised to yield the carboxydextran (8 g). The carboxydextran prepared from Dextran 80 had $\bar{M}_w$ 40 000, compared to $\bar{M}_w$ 78 000 for the starting material, showing that depolymerisation is relatively insignificant during the oxidation. (The $\bar{M}_w$ values were determined by light scattering, at the research laboratory of Pharmacia AB). The glucuronic acid content was determined by the carbazole method,[21] and corrected for the colour reaction given by glucose by similar analysis of unoxidised dextran.

Carboxydextran (1.0 g) was hydrolysed with 0.5 M sulphuric acid (15 ml) at 100° for 7 h, cooled, neutralised with barium carbonate and filtered. The barium salts were washed with hot water and the combined aqueous solutions concentrated to a syrup.

Acidic and neutral components were separated by paper chromatography using solvent system *b*, in which acidic components hardly move. As expected, glucose was the major neutral component but significant amounts of di- and trisaccharides were also obtained. The acidic components were further separated by paper chromatography using solvent *a*. The aldobiouronic acid fraction was collected and weighed (Table 2).

A hydrolysate from carboxy-Dextran 10 (10 g), prepared as above, was added to the top of a Dowex 2 (OAc^-) column. Neutral components were eluted with water and acidic with 0.5 M sulphuric acid. The latter fraction was neutralised with barium carbonate and worked up as above. Fractionation of the product by paper chromatography (solvent system *a*) yielded an aldobiouronic acid (480 mg), R_{Gluc} 0.52, and an aldotriouronic acid (80 mg), R_{Gluc} 0.35. The presence of a small amount of glucuronic acid was also observed on the chromatograms.

Structural analysis of the acidic oligosaccharides. The aldobiouronic acid fraction (5 mg), in 2 % methanolic hydrogen chloride (5 ml), was heated at 100° for 2 h, cooled, neutralised with silver carbonate, filtered and concentrated. The product was dissolved in water (5 ml) containing sodium borohydride (50 mg). After 12 h at room temperature further sodium borohydride (25 mg) was added. 10 h after this addition, the solution was neutralised with Dowex 50 (H^+), filtered and concentrated. Boric acid was removed by codistillation with methanol. The product was then methylated by treatment, in dimethylsulphoxide, first with dimethylsulphinyl anion and then with methyl iodide.[19] The methylated product was hydrolysed with 0.5 M sulphuric acid for 18 h at 100° and the methylated sugars in the hydrolysate converted into alditol acetates.[13] A quantitative analysis of the mixture was performed by GLC (column *c*) and the components were identified by GLC-mass spectrometry.[14] The results are given in Table 2.

The aldotriouronic acid (20 mg) from the oxidised Dextran 10 was hydrolysed with 0.5 M sulphuric acid for 7 h at 100° and the hydrolysate fractionated by paper chromatography (solvent system *a*). Glucose and an aldobiouronic acid with the same mobility as 6-*O*-(α-D-glucopyranosyluronic acid)-D-glucose were the main components. Part of the aldotriouronic acid was treated with methanolic hydrochloric acid, reduced, methylated and hydrolysed as described above for the aldobiouronic acid. Analysis of the methylated sugars, as their alditol acetates, showed comparable amounts of 2,3,4,6-tetra-, 2,3,4-tri-, and 2,4,6-tri-*O*-methyl-D-glucose.

Reduction of the aldobiouronic acid to isomaltitol. Part of the aldobiouronic acid obtained from the large scale hydrolysis of the oxidised Dextran 10 (50 mg) was dissolved in methanol and esterified with diazomethane in ethyl ether. The methyl ester was reduced with borohydride and the mixture worked up as described above for the corresponding reduction of the methyl glycoside-methyl ester mixture. The product (40 mg) which had the same electrophoretic mobility as isomaltitol in germanate buffer, crystallized from methanol on seeding, m.p. 163—165°, $[\alpha]_{578}^{22}$ +89° (*c* 0.2, water). It was indistinguishable from an authentic sample of isomaltitol (mixed m.p., IR). Part of the product was acetylated with acetic anhydride in pyridine to give the nona-acetate, m.p. 114—115°, also indistinguishable from an authentic sample of isomaltitol nona-acetate.

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REFERENCES

1. Neely, W. B. *Advan. Carbohydrate Chem.* **15** (1960) 341.
2. Abbot, D. and Weigel, H. *J. Chem. Soc.* **C 1966** 816.
3. Scott, T. A., Hellman, N. N. and Senti, F. R. *J. Am. Chem. Soc.* **79** (1957) 1178.
4. Allen, P. Z. and Kabat, E. A. *J. Am. Chem. Soc.* **81** (1959) 4382.
5. Bourne, E. J., Hutson, D. H. and Weigel, H. *Biochem. J.* **86** (1963) 555; **88** (1963) 588.
6. Aspinall, G. O., Cairncross, I. M. and Nicholson, A. *Proc Chem. Soc. (London)* **1959** 270.
7. Abbot, D., Bourne, E. J. and Weigel, H. *J. Chem. Soc.* **C 1966** 827.
8. Van Cleve, J. W., Schaefer, W. C. and Rist, C. E. *J. Am. Chem. Soc.* **78** (1956) 4435.

9. Jones, R. W., Dimler, R. J., Jeanes, A., Wilham, C. A. and Rist, C. E. *Abstracts Papers Am. Chem. Soc.* **126** (1954) 33 D.
10. Jeanes, A. *Polysaccharides in Biology*, Josiah Macy, Jr. Foundation, New York 1958, p. 140.
11. Jones, J. K. N. and Wilkie, K. C. B. *Can. J. Biochem. Physiol.* **37** (1959) 377.
12. Aspinall, G. O. *J. Chem. Soc.* **1963** 1676.
13. Björndal, H., Lindberg, B. and Svensson, S. *Acta Chem. Scand.* **21** (1967) 1801.
14. Björndal, H., Lindberg, B. and Svensson, S. *Carbohyd. Res.* **5** (1967) 433.
15. Lindberg, B. and Lundström, H. *Acta Chem. Scand.* **20** (1966) 2423.
16. Hurst, D. T. and McInnes, A. G. *Can. J. Chem.* **43** (1965) 1998, 2004.
17. Lindberg, B. and Swan, B. *Acta Chem. Scand.* **14** (1960) 1043.
18. Srivastava, H. C., Sing, P. P., Harse, S. N. and Virk, K. *Tetrahedron Letters* **1964** 493.
19. Sandford, P. A. and Conrad, H. E. *Biochemistry* **5** (1966) 1508.
20. Schaefer, W. C. and Van Cleve, J. W. *J. Am. Chem. Soc.* **77** (1955) 5341.
21. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.

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