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METHYLATION STRUCTURAL ANALYSIS OF UNUSUAL DEXTRANS BY COMBINED GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY*

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ABSTRACT

Eight bacterial dextrans from NRRL strains *Leuconostoc mesenteroides* B-742, B-1299, B-1355, B-1399, and B-1402, and from *Streptobacterium dextranicum* B-1254 were examined by methylation structural analysis. Methyl ethers of D-glucose that were present in hydrolyzates of permethylated dextrans were analyzed by combined g.l.c.–m.s. as the peracetylated aldononitriles. The various dextrans differed significantly in frequency and type of chain branching.

INTRODUCTION

We have previously applied combined g l.c.–m.s. analyses of the peracetylated aldononitrile (PAAN) derivatives of methyl ethers of D-glucose to the determination of glycosidic linkages in a series of dextrans[2]. We now report the application of this procedure to the methylation–fragmentation analysis of eight more dextrans. The dextrans were produced by the following bacteria, designated by the strain number in the ARS Culture Collection at the Northern Regional Research Center: *Leuconostoc mesenteroides* NRRL B-742, B-1299, B-1355, B-1399, and B-1402, and *Streptobacterium dextranicum* B-1254. A number of the dextrans are essentially homogeneous fractions separated from the corresponding, polydisperse, high-molecular-weight, native dextran by fractionation with ethanol[3]; these are B-742 dextran fraction S, B-742 dextran fraction L, B-1299 dextran fraction L, B-1355 dextran fraction L, B-1399 dextran fraction S, and B-1254 dextran fraction** L[$]. The physical properties and periodate oxidation data of these dextran fractions have been reported[4].

*Unusual Dextrans, Part III. References 1 and 2 may be considered to be Parts I and II of this series.
**The L[S] nomenclature refers to a reversal of dextran-fraction designation For a detailed rationale for designation of this B-1254 dextran fraction as L[S], see ref. 5.

The dextran fractions and nomenclature are identical to those previously reported[3,4], with one exception, that of B-1254 dextran fraction L[$]. Uncertainty that contributed to initial classification of this fraction in the S series[3,4] has now been resolved, and the amended designation L[$] places the fraction properly in the L series. In like manner, the other member of the fraction pair, originally designated in the L series, is now correctly designated[5] fraction S[L]. The data reported here, combined with previously obtained data[2], now allow comparison of the soluble (S) and less-soluble (L) fractions of dextrans obtained from native dextrans B-742, B-1254, B-1299, B-1355, and B-1399. The terms "soluble" and "less soluble" are relative, and indicate the relative order of precipitation from an aqueous solution of the native dextran by graded addition of ethanol[3,5].

RESULTS AND DISCUSSION

In contrast to the ease of methylation displayed by extra-cellular D-mannans from yeast[6], and analogous to previous dextran methylation[2], these dextran fractions failed to undergo complete permethylation by a single Hakomori[7] methylation. The permethylated dextrans also displayed considerable resistance to hydrolysis. Table I lists the results of methylation–fragmentation analysis of the eight dextrans. These data are the result of a uniform, analytical procedure consisting of three Hakomori methylations, followed by hydrolysis conditions (formolysis, and then sulfuric acid–acetic acid) that had previously proved most effective for permethylated dextrans that were difficult to hydrolyze. This procedure differs from that previously reported for dextrans[2] by employing extensive sonication during the Hakomori methylation steps and by the slow addition of water during the final hydrolysis step. Table II lists the quantitation of the overall permethylation procedure for each dextran after the third Hakomori methylation.

TABLE I

MOLE PERCENTAGE OF METHYLATED D-GLUCOSE IN HYDROLYZATES OF METHYLATED DEXTRANS

Organism	*NRRL strain*	*Dextran fraction*	*Methyl ethers of* D-*glucose*					
			2,3,4,6	*2,3,4*	*2,4,6*	*2,3*	*2,4*	*3,4*
Leuconostoc mesenteroides	B-1299	L	34.0	32.0	5 9		1.3	26.8
	B-1399	S	30 8	34 3	1.0		2 7	31.2
	B-1399 (P-37)		24.8	48.1	0 5		3.1	23.5
	B-1402		25 3	50.1			2 6	22.0
	B-742	S	45.4	4.4			50 2	
	B-742	L	14.4	72.5	0.7	12.4		
	B-1355	L	3 3	91 5	1.5		3.7	
Streptobacterium dextranicum	B-1254	L[$]	3.8	90 0	1.8		4 4	

TABLE II

RECOVERY OF PERMETHYLATED DEXTRAN AFTER THREE PERMETHYLATIONS

Organism	*NRRL strain*	*Dextran fraction*	*Weight of dextran before first methylation (mg)*	*Weight of dextran after third methylation (mg)*
Leuconostoc mesenteroides	B-1299	L	49	31
	B-1399	S	54	27
	B-1399 (P-37)		52	29
	B-1402		51	35
	B-742	S	51	29
	B-742	L	50	34
	B-1355	L	49	39
Streptobacterium dextranicum	B-1254	L[S]	51	45

Further Hakomori, and also Kuhn[8], methylations were performed on various samples of material described in Tables I and II; however, resultant changes in the mole percentages were minor. In addition, cumulative losses of material raised the question as to whether a representative sample of the original polymer was still being analyzed. Additional determinations were also performed after fewer than three successive Hakomori methylations. For all dextrans (with the exception of dextran B-1399 fraction S), a determination employing a single Hakomori methylation resulted in severe under-methylation—in many cases, the contribution of the dimethyl ether derivative represented >60% of the total chromatogram integral. However, this under-methylation was not random; a comparison of a chromatogram from a single Hakomori methylation with that of the same dextran after three Hakomori methylations revealed that, in all cases, the selective under-methylation had occurred essentially at the 3-hydroxyl groups. Sonication during the Hakomori methylation procedure emphasized this effect, apparently by promoting methylation at non-3-hydroxyl groups.

Samples of cellulose and pullulan were studied in determinations parallel with those for the dextrans. For pullulan, a single Hakomori methylation, especially with sonication, was sufficient to provide a chromatogram of the hydrolyzate in which the trimethyl ether derivatives provided >98% of the peak integrals. Identical conditions with cellulose yielded a chromatogram of the hydrolyzate that contained some 2,3,6-tri-*O*-methylated product, with the major peak representing the 2,6-dimethyl ether product. Other methylated derivatives, including mono-*O*-methylated and non-methylated D-glucose derivatives, contributed very minor peaks. Analysis of cellulose after three Hakomori methylations yielded a chromatogram of the hydrolyzate in which the 2,3,6-trimethyl ether provided >97% of the total integral. From this, it may be inferred that, under Hakomori conditions, both cellulose and the dextrans resist methylation at the 3-hydroxyl groups, whereas pullulan [a polymer composed of α-D-(1→4)- and α-D-(1→6)-linked D-glucopyranosyl residues] does not.

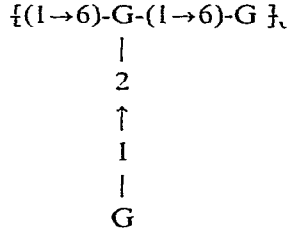
A convenient explanation for this phenomenon is to consider that Hakomori methylations are actually two-step reactions, with the first step involving a hydrogen exchange with the sodium hydride reagent. Were this exchange to be inhibited, the introduction of the methyl group from methyl iodide in the second step would not occur. Favored hydrogen-bonding of the 3-hydroxyl groups of the D-glucosyl residues in the original polysaccharide could cause this effect. Previous experimental results for dextrans, from electron-diffraction data[9,10], and, for cellulose-like oligomers, from ^{1}H-n.m.r. spectroscopy[11], indicated that such favored hydrogen-bonding at the 3-hydroxyl groups does indeed occur. Small amounts of 2,4,6-tri- or 2,4-di-*O*-methyl derivatives were present in several of the hydrolyzates of methylated dextrans shown in Table I; this could indicate (*a*) the actual presence of (1→3)-linkages in the dextran samples, or (*b*) selective under-methylation of D-glucosyl residues of the polymer that should actually be accounted for, respectively, as the 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl derivatives We have previously noted an excess of 2,4-di-*O*-methyl derivatives in the permethylation hydrolyzates[2] of dextrans B-1351 fraction S and B-1355 fraction S. The proportion of this 2,4-di-*O*-methyl component present in hydrolyzates of those dextrans, as well as of the dextrans reported here, could be lessened, but not eliminated, by successive Hakomori and Kuhn methylations. In general, for single or double Hakomori permethylations, the linear dextrans gave hydrolyzates containing much greater proportions of 2,4-di- and 2,4,6-tri-*O*-methyl derivatives than the more highly branched dextrans. It has been suggested[12,13] that most, if not all, dextrans contain (1→3)-linkages. It is possible that extensive hydrogen-bonding of the 3-hydroxyl groups in dextrans results in inaccurate estimates by other investigators of the contents of (1→3)-linkages.

The identities of all compounds resulting from permethylated dextran hydrolyzates listed in Table I were confirmed through the peracetylated aldononitrile derivatives, separated by column conditions A, B, and C previously described[2]. Column C conditions confirmed the absence of mono- or non-methylated derivatives in the hydrolyzate. In addition to the retention times in g.l.c , the identities of the g.l.c. peaks were confirmed by recording their electron-impact, mass spectra. In all cases, these mass spectra were identical to those previously reported[1,2]. The integrals of the g.l.c. peaks reported in Table I were taken from chromatograms obtained with Column B conditions and a hydrogen-flame detector. Data from other column conditions, and from the peaks of the integrated, cumulative, total *m/e* values in the mass spectra, were in accord. The peak-area percentages were expressed directly as component percentages for various *O*-methylated D-glucose derivatives in the hydrolyzates of the permethylated dextran. The close correspondence of the amounts of tetramethyl *vs.* total dimethyl ether derivatives would appear to justify use of this approach. In addition, when previously employed[1], this assumption provided excellent agreement between the results of permethylation analysis of dextrans and those from ^{13}C-n.m.r. analysis of the identical compounds.

The following discussion of dextran structure employs the assumption that all (1→6)-linkages are located in the polymer backbone, and all non-(1→6)-linkages

are located at branch points. Permethylation analysis data, alone, provide no proof of this arrangement, but when such an assumption is made, the polymer structure can be expressed in terms of an average, repeating unit. Permethylation data also provide no evidence that permits discrimination between α- and β-linkages in the dextrans. However, specific optical rotation data[4] indicate that most of these linkages are α. ^{13}C-N.m.r. spectra recorded for the dextrans reported here showed all linkages, both chain-extending and branch-point, to be α-linkages[1].

Dextran B-1299 fraction L. — This dextran fraction is one of the two major fractions arising from fractional precipitation of the native dextran (from water, with ethanol). We previously reported the permethylation analysis of dextran B-1299 fraction S, the more soluble component. These dextrans are unusual, not only in the high proportion of branching, but also in the close similarity of the two fractions in type and degree of chain branching. The hydrolyzates of permethylated dextran B-1299 fraction L and of the dextran B-1299 fraction S, previously reported[2], yield very similar PAAN derivative chromatograms. The data from both fractions closely approximate those expected for a polymer composed of the following, repeat-unit structure (where G is a D-glucopyranosyl group or residue, or a D-glucopyranose residue).

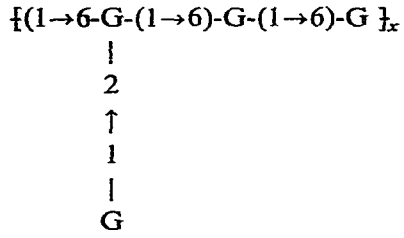


The S fraction appears to be somewhat more branched than the L fraction, as it contains a lower percentage of (1→6)-linked D-glucosyl residues. In addition, the data indicate a small proportion of (1→3)-linkages present in fraction L, but not in fraction S. These (1→3)-linkages could give rise to the difference in solubility of the S and L fractions Conversely, in terms of the foregoing discussion on the difficulty of methylation of 3-hydroxyl groups, the 2,4-di- and 2,4,6-tri-*O*-methyl derivatives could be merely a result of the generally lower solubility of this fraction, which apparently arises from currently unestablished structural factors (*e g.*, the molecular weight). For whatever reason, the L fraction is certainly more resistant to permethylation than the S fraction. In general, our data confirm previous observations[14,15] of the close structural similarity of the S and L fractions of dextran B-1299.

Dextran B-1399 fraction S. — The methylation structural analysis of the complementary L fraction of this dextran has been reported[2]. The data in Table I indicate that the structures of dextran B-1399 fraction S and dextran B-1299 fraction L are very similar. The lower proportion of the 2,4,6-tri-*O*-methyl derivative indicates either a smaller percentage of (1→3)-linkages, relative to dextran B-1299 fraction L, or a greater ease of methylation of the 3-hydroxyl groups for this fraction.

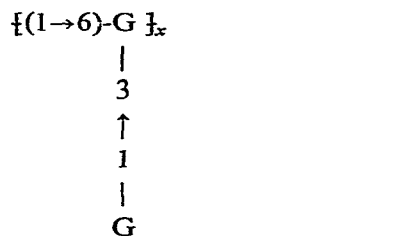
In terms of the evidence for low proportions of (1→3)-linked residues, dextran B-1399 fraction S closely resembles dextran B-1299 fraction S. Unlike the dextran pair obtained from native dextran B-1299, members of the pair from native dextran B-1399 differ distinctly in degree of branching.

Dextran B-1399 (P-37). — This dextran is not the same as the native dextran B-1399 employed for the foregoing fractionation. This earlier product, obtained from a culture of the newly isolated strain, appeared to be homogeneous, and to require no fractionation; it was characterized chemically[4,12,16] and immunochemically[12,17]. Table I indicates that dextran B-1399 (P-37) is a more linear analog of dextran B-1299 fractions S and L and of dextran B-1399 fraction S. Dextran B-1399 (P-37) yields permethylation data similar to those expected of a polymer composed of the following repeat-unit structure.



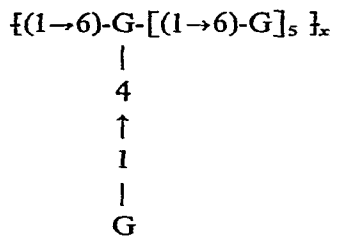
Dextran B-1402. — This homogeneous dextran yields permethylation data essentially identical to those obtained for dextran B-1399 (P-37). Therefore, on the basis of present information, this dextran may prove to be an excellent replacement for dextran B-1399 (P-37).

Dextran B-742 fraction S. — The S and L fractions of native dextran B-742 are of interest, as both are highly branched. The results in Table I indicate that fraction S contains essentially equal proportions of D-glucosyl groups and 1,3,6-tri-*O*-substituted residues, with a minor proportion of (1→6)-linked residues. These data are similar to those expected for a polymer composed of the following repeat-unit structure.



In addition, the dextran appears to contain ~4% of (1→6)-linked, chain-extending residues. Dextran B-742 fraction S is the most highly branched dextran that we have identified. Due to this high degree of branching, there are few alternative, repeat-unit structures possible from these data, the most obvious alternative being a highly branched, pseudonigeran backbone having (1→6)-linked D-glucosyl side-groups.

Dextran B-742 fraction L. — This (less-soluble) dextran fraction, complementary to the foregoing S fraction, is also highly branched. The permethylation data given in Table I show a remarkably low degree of cross-contamination between the S and L fractions, and are in accord with a structure containing one D-glucosyl residue, five (1→6)-linked D-glucosyl residues, and one (1→6)-linked D-glucosyl residue branching through C-4 per average repeating unit. This would constitute a dextran similar to, but slightly more linear than, the previously reported[2] dextran B-1254 fraction S[Ł]. An average repeating-unit structure may be represented as shown.



Dextran B-1254 fraction L [$]. — This dextran fraction, previously reported[3,4] simply as the S fraction, has now been reclassified to the "L" series[5]. Table I indicates that this fraction is composed of one D-glucosyl residue, 24 (1→6)-linked D-glucosyl residues, and one (1→6)-linked D-glucosyl residue branching through C-3 per average repeating-unit. A small proportion of (1→3)-linked D-glucosyl residues is also indicated, but at such a low level that determination of whether this represents a structural feature, or an artifact resulting from the resistance of the 3-hydroxyl groups to permethylation, is difficult.

Dextran B-1355 fraction L. — The permethylation data given in Table I indicate that this less-soluble fraction also exhibits a low degree of branching. On the basis of these data, dextran B-1254 fraction L[$] and dextran B-1355 fraction L are essentially identical. We have, to date, found no other dextrans having a greater degree of linearity. Again, the data in Table I, compared to previous permethylation data[2] for fraction S, indicate a low degree of cross-contamination between the S and L fractions of dextran B-1355.

EXPERIMENTAL

Permethylation of dextran. — Dextran (50 mg) was dried for 3 h at 60°/1 torr over phosphorus pentaoxide, and then transferred to a test tube fitted with a serum cap and a magnetic stirring-bar, and containing dimethyl sulfoxide (12 ml) (dried over 3A molecular sieve). The vial was purged with nitrogen, and kept in a sonic bath for 1 h at room temperature. Except for the linear dextrans, clear solutions resulted during sonication. The linear dextrans were further magnetically stirred for 1 h and resonicated for 1 h, to yield clear solutions. The mixture was injected with Hakomori reagent (3 ml) and sonicated for 30 min, to yield a clear, amber solution. The Hakomori reagent was prepared by adding Me_2SO (10 ml) to 1.1 sodium

hydride–oil suspension (1.0 g), and sonicating at 80° until gas evolution ceased. Methyl iodide (3 ml) was cautiously injected (with venting) into the vial, cooled in ice–water, and this mixture was sonicated for 30 min to yield a clear solution, which was poured directly into a dialysis bag. After dialysis overnight against running tap-water, the resulting solution was lyophilized to dryness. This procedure was then twice repeated with the following modifications: Me_2SO (3 ml), Hakomori reagent (1 ml), and methyl iodide (1 ml).

Hydrolysis of permethylated dextran. — Permethylated dextran (6 mg) and 90% formic acid (3 ml) were magnetically stirred for 2 h on a boiling-water bath. The resulting solution was evaporated in a rotary evaporator, and water (5 ml) was added to, and evaporated to dryness from, the residue. To this residue was added 0.5M sulfuric acid–acetic acid[18] (0.5 ml), and the solution was magnetically stirred for ~14 h at 70°. Water (0.1 ml × 4) was added at 1-h intervals, with continued stirring, and heating at 70°. Five hours after the first addition of water, the hydrolysis solution was passed through a column (0.7 ml) of Dowex-2 X-8 (OAc^-) resin (200–400 mesh), followed successively by water (2 ml) and methanol (2 ml). This methanol–water eluate was evaporated to dryness, and water (5 ml) and absolute ethanol were successively added to, and evaporated from, the residue. Both the final formolysis solution and the final acetic acid hydrolysis solution were clear and colorless, and free from suspended material. Under the vacuum provided by a water aspirator, the bath temperature is not critical, insofar as selective losses of the underivatized saccharides are concerned.

Derivatization of the contents of the hydrolyzate. — The foregoing hydrolyzate was converted into the peracetylated aldononitrile (PAAN) derivatives as previously described[19]. A decrease in the g.l.c. solvent-front was obtained by a five-fold preconcentration of the final, 1-ml chloroform solutions by use of a nitrogen stream at room temperature.

G.l.c.–m.s. conditions. — The g.l.c. conditions employed for the separation of the PAAN derivatives of the methyl ethers of D-glucose have been described[2]; both column B (butanediol succinate) and column C (Apiezon L) conditions were employed for product identification and quantitation. Column A (neopentyl glycol succinate) conditions were employed in order to confirm the absence of any PAAN derivatives resulting from mono-*O*-methylated or non-methylated D-glucose. For routine-survey analyses, and for peak-area quantitation, a Barber–Coleman Series 5000 gas–liquid chromatograph equipped with hydrogen-flame detectors was employed. For mass-spectral confirmation of the g.l.c. peaks, a Hewlett–Packard 5980A GC/MS integrated g.l.c.–m.s.–computer system was employed in the electron-impact mode. The spectrum was scanned from 25 to 600 at 5-s intervals, and these data were stored for later regeneration of the chromatogram. Both chromatographs employed glass columns (1.23 m × 2 mm) with the flow rate lower by a factor of 0.41 (to correspond to the velocity in the 3.18-mm columns originally reported).

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