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METHYLATION ANALYSES OF NRRL DEXTRANS BY CAPILLARY GAS–LIQUID CHROMATOGRAPHY

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ABSTRACT

Methylation analyses were conducted on 38 NRRL dextrans with the aid of capillary gas–liquid chromatography (g.l.c.)–mass spectrometry. The superior chromatographic resolution of capillary g.l.c. permitted better quantitation of linkage types and detection of trace components than had been obtained with packed columns. Only a few of the dextrans analyzed contain a single type of non-(1→6) linkage; seven contain at least traces of all three possible secondary links at points of branching. The results amend some earlier methylation analyses conducted by packed-column g.l.c., and extend the utility of the NRRL dextran collection by providing more-precise, quantitative data on the types of α-D-glucopyranosyl residues present.

INTRODUCTION

Numerous studies on carbohydrate-binding specificities of immunoglobulins and lectins have utilized dextrans from the NRRL collection[1]. For the most part, interactions were interpreted in terms of structural information based on periodate oxidations, optical rotations, and infrared-spectroscopic analyses[2], acetolysis (*e.g.*, ref. 3), and Smith degradations[4]. In recent years, more-precise structural information became available for many of the dextrans from methylation analyses by Seymour *et al.*[5,6], which were conducted by packed-column gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.). These results were used to assist interpretation of ^{13}C-n.m.r.[7–9] and Fourier-transform infrared[10] spectra of the dextrans, and to arrive at more-refined interpretations of earlier periodate-oxidation data[11].

The methylation analyses described herein were undertaken in order to obtain precise information on the nature and content of non-(1→6)-linked D-glucopyranosyl residues in many NRRL dextrans. Capillary g.l.c. with either flame-ionization detection or coupling to m.s. provides superior resolution and the

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capability for detecting particular linked residues that amount to <0.5% of those present. In contrast to packed-column g.l.c., quantitation is further aided by separation of the chromatographic peaks of interest from peaks that arise from non-carbohydrate impurities present in the original preparations or from decomposition products generated during the methylation–fragmentation procedure.

Criteria for selection of dextrans included: availability of published immunological or immunochemical data, but inadequate information on structure; apparent lack of correlation between biological activity and known structural features; the need to explore dextrans from related bacterial strains and fractions of heterogeneous dextrans; and confirmation or extension of structural information obtained by chemical, enzymic or spectroscopic methods.

MATERIALS AND METHODS

Permethylation of dextrans (2–10 mg) and subsequent hydrolytic fragmentations were performed as described by Jackson *et al.*[12]. Triphenylmethane was used as an indicator of methylsulfinyl carbanion excess[13]. Methylated dextrans were extracted from reaction mixtures by partition between chloroform (10 mL) and water (20 mL). The chloroform layer was subsequently washed twice with 20-mL portions of water, in order to remove traces of dimethyl sulfoxide, and then dried with anhydrous Na_2SO_4 prior to evaporation of solvent and hydrolysis of the permethylated dextran. Methyl ethers of D-glucose present in hydrolyzates were analyzed[14] as per-*O*-acetylated aldononitrile (PAAN) derivatives.

All of the PAAN derivatives of methylated D-glucose encountered in this work were fully separated by g.l.c. on either polar or apolar, open-tubular, fused silica, capillary columns. Table I lists the relative retention-times exhibited by these compounds, column characteristics, and chromatographic conditions. Sample-

TABLE I

RELATIVE RETENTION-TIMES OF PER-*O*-ACETYL-D-GLUCONONITRILES IN FUSED SILICA, CAPILLARY COLUMNS

Methyl ether	*Coated liquid phase*		
	SP 2100	*SE 54*	*Carbowax 20M*
2,3,4,6-	1.00[a]	1.00[b]	1.00[c]
2,4,6-	1.36	1.15	1.71
2,3,6-	1.53	1.19	2.38
3,4,6-	1.59		1.96
2,3,4-	1.64	1.23	2.26
2,4-	2.27	1.36	2.83
2,3-	2.49	1.38	3.31
3,4-	2.77	1.42	3.19

[a] 4.90 min; 25 m × 0.2 mm i.d.; 150° isothermal; He 276 kPa. [b] 16.26 min; 30 m × 0.25 mm i.d.; 120°, 5 min, 4°/min to 250°; He 69 kPa. [c] 6.42 min; 25 m × 0.2 mm i.d.; 135°, 5 min, 4°/min to 165°; He 276 kPa.

splitting injectors were adjusted to provide split ratios of ~100:1. Methyl silicone (SP 2100) and Carbowax 20M coatings were employed into order to obtain quantitative data. Retention times and integration of flame-ionization detector responses were obtained with the aid of an on-line, digital computer. Methyl phenyl silicone (SE 54) and Carbowax columns were used in conjunction with quadrupole mass spectrometers to confirm the identities of all PAAN derivatives, including those which occurred as trace components.

RESULTS AND DISCUSSION

Table II lists the results of methylation–fragmentation analyses of selected NRRL dextrans. Methyl ethers present in the hydrolyzates of the permethylated dextrans are given in the order of emergence as PAAN derivatives from apolar, capillary, g.l.c. columns. The dextrans are grouped according to predominant non-(1→6) linkage type and in the approximate order of increasing degree of branching. A majority of the dextrans selected have more than one type of branching and some have all three possible types.

Components that amount to <0.5% of a dextran are designated "trace". Because capillary chromatographic peaks are sharp, on the order of 0.1% of a chromatogram can be quantitated by f.i.d. response, and identified by high-resolution m.s. Peak sharpness and consequent improved chromatographic separations resolved impurities, inherent in certain samples, from either the 2,3,4,6-tetra- or di-*O*-methyl PAAN derivatives of D-glucose. For the same reason, minor components that chromatograph close to major peaks are fully resolved (see Table I). Dextrans analyzed previously in this laboratory[5] by packed-column g.l.c. were re-examined (see Table II). It became clear after capillary g.l.c. that the excesses of tetra- and di-methyl ethers earlier reported were due either to unidentified sample-impurities or to hydrolytic-degradation products, but not, as had been ascribed, to incomplete hydrolysis or undermethylation.

Methylation linkage analyses had been performed earlier[5,6,8,9,15] on additional NRRL dextran preparations listed in Table II and on dextrans prepared by others[16–18] from cultures of certain of the bacterial strains listed. As indicated in Table II, the present results agree with many of the earlier analyses. There are, however, exceptions that merit further discussion.

Dextran B-1299 fraction S. — Approximately one third of the dextrans selected for this study were among those designated by Hehre[19] as serotype A. All such dextrans listed in Table II contain significant proportions of 2,6-di-*O*-substituted D-glucosyl residues. In addition to having the highest content of such residues, the serologically most active type A dextran, B-1299 fr S, uniquely contains 5% of 2-*O*-mono-substituted D-glucopyranosyl residues. These residues, detected earlier by Kobayashi *et al.*[17], are the likely sources of kojitriose and tetrasaccharides containing kojitriose that were later obtained by acetolysis[20]. The analysis given in Table II most closely resembles that given for fraction CS by Kobayashi *et al.*[21].

TABLE II

MOLE PERCENTAGES OF METHYL ETHERS OF D-GLUCOSE FROM SELECTED NRRL DEXTRANS

NRRL dextran B-[a]	*Fraction or prepn.*	*2,3,4,6-*	*2,4,6-*	*2,3,6-*	*3,4,6-*	*2,3,4-*	*2,4-*	*2,3-*	*3,4-*
1254	12	4				92			4
1399	L	12				75	3		9
1382		15				71	2	4	9
1396		13				73	3	2	9
1397[b]		18	tr[c]			66	2	tr	14
1299	L	20	5			53	5		16
1422[d]		19				62	2	tr	16
1424[d]		22				57	4		17
1298		28	3			44	3		22
1299	S	31	1		5	32	1		30
1399[d,e]	P-37	29				45	2		24
1399	S	32	1			35	1		31
1308		4				91	4		1
523	E	4	3			90	3		
1351	S	8				84	6		1
1351	L	13	2			73	12		
1255	E-1	9				82	7	2	
1374		15				70	15		
1398		20	1			58	15	6	
1192		17	1			64	17	1	
1191[f]		18				62	18	2	
1191[f]		25				50	20	5	tr
1141		16	1			64	18		1
1142		25	1			47	26		1
1118		11	13			66	10		
523	A	6	27			60	4		2
523	L	8	27			58	4		3
1149	A	9	38			45	3		5
1498[e]	S	10	29			51	9		1
742	S	38				25	28	8	
742	6-R	40				21	33	6	
1254	L[S]	6				88	2	4	
1420		20	1			62	1	17	
1526[d]	I	18				65	3	13	1
1526		18				66	2	14	
742	L	17	tr			65	2	15	
1254	S[L]	21	1	4		52	tr	19	2

[a]Bacterial strain designations are given in Jeanes *et al.*[2]. [b]Result agrees with methylation analyses by Miyaji *et al.*[18]. [c]tr = trace (<0.5%). [d]Result agrees with methylation analysis by Seymour *et al.*[6,8]. [e]Fragmented according to Seymour *et al.*[5]. [f]Different preparations.

Several methylation analyses have been performed by us on the NRRL preparation, and we have consistently found a content of <2% of (1→3)-linked residues. Seymour and Julian[10] attributed an infrared absorbance by dextran B-1299 fr S at 1030 cm^{-1} to the presence of non-contiguous, 3-*O*-mono-substituted residues, which, in dextran B-1355 fr S, give a characteristic absorption band at

1033 cm^{-1}. It is possible that the infrared absorbance at 1030 cm^{-1}, is indicative of contiguous 2-*O*-mono-substituted D-glucopyranosyl residues, as found in the aforementioned oligosaccharides. As discussed by Jeanes[1], the unexpectedly strong α-(1→3) immunoactivity of the dextran fraction may reflect peripheral location of α-(1→3)-linked D-glucosyl residues in a ramified structure[21].

Dextrans B-1298 and B-1299 fr S appear related. The values for contents of nonreducing end-groups agree with the results of an earlier methylation analysis[22] of a fraction of B-1298 dextran and with degrees of hydrolysis of both dextrans by a specific α-(1→2) debranching enzyme[23]. A more-recent examination[24] of dextran B-1298 records a lower degree of hydrolysis by the debranching enzyme. This result corresponds to the sum of 3,4-di- and 4-mono-methyl ethers found by Lewis *et al.*[22] in the hydrolyzate of permethylated B-1298 dextran. (We found no mono-*O*-methyl ethers). From comparisons of ^{13}C-n.m.r. spectra of B-1298 and B-1299 dextrans and limit dextrans obtained by use of debranching enzyme alone or together with an endodextranase, Kobayashi *et al.*[24] concluded that B-1298 dextran more closely resembles B-1396 dextran. Possibly, these discrepant results reflect differences among various preparations of the dextran.

Dextran B-1299 fraction L. — The chromatograms of PAAN derivatives of methyl ethers obtained from this dextran are unusual, in that substantial amounts of unidentified impurities emerge from the Carbowax column. These peaks occur adjacent to the peaks that represent the 2,3,4,6-tetra- and 3,4-di-methyl ethers. When the areas of adjacent peaks are combined, the results approach those obtained by Seymour *et al.*[6] on the same dextran preparation by use of a packed column with polar liquid phase. These impurities are not seen in the chromatograms from the apolar methyl silicone and methyl phenyl silicone capillaries. It should be noted that the NRRL L and S fractions are the main products obtained from a complex fermentation mixture[1].

Dextran B-742 fraction S. — The present analyses of this dextran fraction, and of the similar preparation 6-R, differ from the analysis of Seymour *et al.*[6]. We find significant amounts of 1,4,6-tri-*O*-substituted D-glucose and much more 1,6-di-*O*-substituted D-glucose than reported previously. It is remarkable that our values for (1→3) and (1→4) linkages agree precisely with estimates of non-(1→6) linkages from Smith degradations[4] carried out on the same fraction S preparation. Although high-temperature, ^{13}C-n.m.r. spectra are in accord with the methylation analysis of Seymour *et al.*[9], earlier ^{13}C- and ^{1}H-n.m.r. spectra[25] of the preparation were interpreted as indicating (1→3) and (1→4) linkage contents similar to those reported here. Further evidence for the presence of (1→4)-linked residues, possibly located in the interior of the dextran, came from two sources: (*1*) biosynthetic studies[26] with B-742 dextransucrase, which indicated that the L-type fraction (see Table II) may be a precursor of the S-type, and (*2*) release of a trisaccharide, containing the (1→4) linkage, only after prolonged digestion by *exo*-acting isomaltodextranase (see Fig. 2 of ref. 27).

Dextrans B-523 fractions A and L, and B-1149 fraction A. — These dextrans are relatively insoluble in water. Their insolubility is ascribed to high content of contiguous α-(1→3)-linked residues, as reflected in the values obtained for 2,4,6-tri-*O*-methyl-D-glucose. Although heretofore not analyzed by methylation–fragmentation, the dextrans were examined by ^{13}C-n.m.r.[28] and Fourier-transform infrared difference (Seymour *et al.*[29]; only for B-1149 fr A) spectroscopies. The methylation analyses (Table II) agree with estimates of (1→3) linkage content made by oxidation with periodate[2] and by optical rotation of cuprammonium complexes[30]. Dextran B-1149 fr A, however, gave anomalously weak ^{13}C-n.m.r. resonances diagnostic for non-(1→6) linkages. Although fractions A and L of dextrans B-523 were originally separated on the basis of solubility, they appear to be structurally similar.

Dextran B-1254 complex. — We have re-examined dextran B-1254 fr S[𝕃] (see ref. 5) and have found small proportions of at least two additional types of linked D-glucosyl residues (3-*O*- and 2,6-di-*O*-). The complexity of this fraction suggests the presence of a small proportion of a second molecular species. Fraction 12, a hitherto unpublished component of the B-1254 dextran complex, is of interest, in that it appears to be a simple dextran which has (1→2) secondary linkages, but lacks (1→3)-linked residues.

Byproducts of PAAN formation. — In batch preparations at 70°, from which byproducts were isolated and characterized, Furneaux[31] reported formation of PAAN derivatives from unmethylated aldoses in yields as low as 63%. The major byproducts were *N*-hydroxy-D-glycosylamine hexaacetates; furanose isomers far exceeded the pyranose. We have confirmed these results by capillary g.l.c. analyses (methyl silicone; 160 to 220°, 5°/min) of crude PAAN derivatives formed at 60° from unmethylated aldoses. The PAAN yields were ~10% higher than reported by Furneaux. From thin-layer chromatographic (t.l.c.) separations made according to Furneaux[31], a significant portion of byproduct was present in the fastest-moving t.l.c. band, which, when eluted, gave two liquid-chromatography peaks. In g.l.c., the t.l.c. band gave the PAAN derivative. Apparently, that t.l.c. band consisted of *syn*- and *anti*-oxime acetates that were converted into the PAAN in the g.l.c. injector (275°). The 72–82% range of PAAN yields observed in g.l.c. probably accounts for the agreement between polysaccharide-component analyses, involving a variety of sugar types, carried out with both PAAN and trimethylsilyl ether derivatives[12,32].

With regard to the use of PAAN derivatives in methylation analyses, formation of glycofuranosylamine should be blocked in methylated sugars obtained from polysaccharides linked other than (1→4). For this reason, only 3–5% of byproduct was detected in g.l.c. of a PAAN reaction-mixture from 2,4-di-*O*-methyl-D-glucose. In those dextrans in which they are present, the (1→4)-linked residues are in the minority. Except in dextran B-1254 fr S[𝕃], such residues are exclusively involved in chain branching, and are also (1→6)-linked. The results of partial acetolysis[3] and Smith degradation[4] agree with the relative amounts of secondary linkages we now find in these same preparations.

TABLE III

MOLE PERCENTAGES OF METHYL ETHERS OF D-GLUCOSE IN HYDROLYZATES OF METHYLATED DEXTRANS[a] ANALYZED AS PAAN AND ALDITOL ACETATE (AA) DERIVATIVES

NRRL dextran B-	*Derivative*	*2,3,4,6-*	*2,4,6-*	*2,3,4-*	*2,4-*	*2,3-*
742[b]	PAAN	22		59	6	13
	AA	22		58	6	14
1355[c]	PAAN	10	35	45	10	
	AA	10	34	45	11	

[a] Dextran fractions repeatedly methylated (Na–liq.NH_3–CH_3I) in 1954 by J. E. Hodge and W. C. Schaefer. [b] A less-soluble fraction similar to the B-742 fr L preparation of Table II. [c] The more-soluble (S) fraction. Infrared analysis indicated complete methylation. Result agrees with methylation analysis by Hare *et al.*[15]

Further support is given in Table III for the suitability of PAAN derivatives. Dextrans methylated in 1954 were fragmented under the conditions employed in this work. The hydrolyzates were divided, and the sugars derivatized as PAANs and alditol acetates. The correspondences observed may reflect suppressed formation of amine acetate byproducts from partially *O*-methylated aldoses.

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