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STRUCTURAL STUDIES OF EXTRACELLULAR GLUCANS OF *Streptococcus mutans* BY PROTON MAGNETIC RESONANCE*†

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ABSTRACT

Proton magnetic resonance spectra at 100 MHz were obtained for water-soluble and water-insoluble glucans from 11 strains of *Streptococcus mutans*. The percentages of α-D-(1→6) and non-α-D-(1→6)-, namely, α-D-(1→3)-, linkages were calculated from the anomeric-proton resonances in the 4.7–4.8 and 5.0–5.1 p.p.m. ranges, respectively. The average content of α-D-(1→6) linkages in the polymer fractions precipitating from solution during synthesis of the glucans was generally much lower than that of fractions remaining in solution. The frequent appearance of the α-D-(1→3) resonances as doublets in the spectra suggested neighboring-group effects among the possible α-D-(1→3) and α-D-(1→6) linkage-configurations. These effects were confirmed from 100-MHz spectra of products of a dextranase-degraded, water-insoluble glucan, and a 270-MHz spectrum of an undegraded glucan. It was thus possible to assign the doublet resonances to α-D-(1→6) and α-D-(1→3), homogeneous, heterogeneous, and branch configurations, although complete differentiation among proportions of each configuration in the glucan chains could not be achieved.

INTRODUCTION

Proton magnetic resonance (^{1}H-n.m.r.) has been widely applied in the determination of the anomeric compositions of polysaccharides. Various publications have outlined results obtained with starches[2,3] and dextrans[4,5], for which ^{1}H-n.m.r. spectra typically demonstrate separate resonances of anomeric protons for each of the α-D linkages present.

During the past decade, there has been a growing interest in the extracellular glucans that strains of the oral organism, *Streptococcus mutans*, can synthesize from sucrose, as these glucans appear to be significant factors in the etiology of dental

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.
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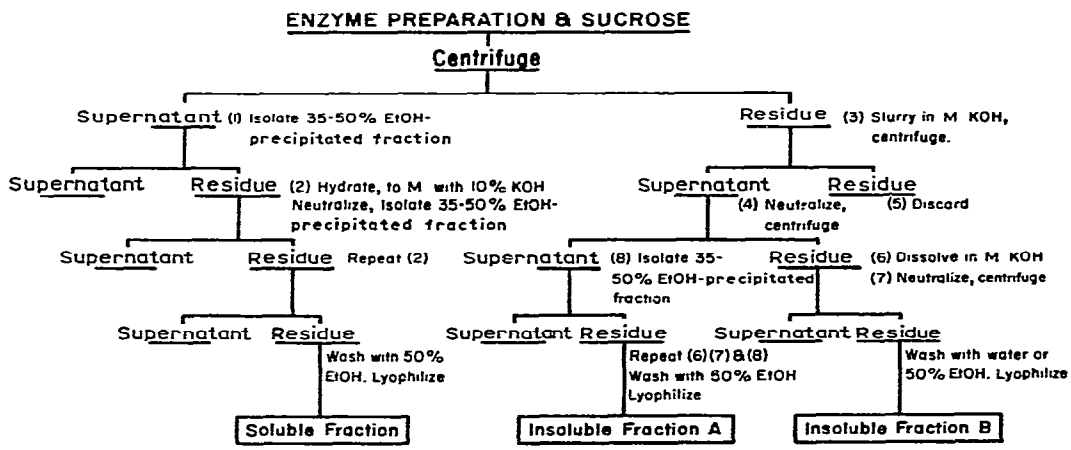
caries[6–8]. Structural analyses of the glucans from selected strains of *Streptococcus mutans*, performed by chemical methods[9–12] or by ^{13}C-n.m.r.[13,14] spectroscopy, have typically shown only α-D-(1→6) and α-D-(1→3) linkages. The water solubility of these glucans depends on their content of α-D-(1→3) linkages, the glucans of relatively high α-D-(1→3) linkage-content being quite insoluble. The amounts and proportions of the water-soluble and water-insoluble glucans produced by different strains grown under similar conditions may vary broadly[15]. The glucans appear to be highly branched[11,12,16], but there is also evidence that water-insoluble fractions, in particular, contain linear sequences of α-D-(1→3)-linked glucosyl residues[17,18].

Sidebotham *et al.*[19] have applied ^{1}H-n.m.r. spectroscopy to determine proportions of the α-D-(1→6) and non-α-D-(1→6) linkages in water-soluble glucans from *Streptococcus mutans* strains Ingbritt and OMZ 51, using deuterium oxide as solvent. To our knowledge, there have been no ^{1}H-n.m.r. studies of the water-insoluble glucans. In the present study, we have investigated the applicability of ^{1}H-n.m.r. spectroscopy to elucidate the structural characteristics of various glucan fractions from *Streptococcus mutans* strains AHT, HS-6, FA-1, BHT, NCTC 10449, Ingbritt, GS-5, SL-1, K-1R, OMZ 176, and LM-7. These strains constitute five serotypes of *Streptococcus mutans*, according to the classification of Bratthall[20,21]. The ^{1}H-n.m.r. studies were conducted at 80° in a 9:1 Me_2SO-d_6–D_2O mixture[22] to acquire information on the water-insoluble as well as the water-soluble glucans.

RESULTS AND DISCUSSION

Designation of glucan fractions. — The extracellular glucans were synthesized enzymically and fractionated according to the scheme shown in Scheme 1. By this procedure, the glucan material that was insoluble initially was separable, in many



Scheme 1. Scheme for isolation of polysaccharide fractions produced by extracellular enzymes of *Streptococcus mutans*.

cases, into two fractions. One fraction, termed Fraction "A", could be resolubilized and was recovered by ethanol precipitation within 35–50% limits. The other fraction, Fraction "B", which was not observed as frequently as Fraction "A", precipitated when the alkaline solution of the insoluble glucan material was neutralized. Analyses of hydrolyzed samples of the glucan fractions by a D-glucose oxidase procedure showed mean D-glucose percentages of 90.9, 83.2, and 80.8, respectively, for the soluble, insoluble "A", and insoluble "B" fractions. These values are in reasonably good agreement with other reports on the content of glucose found in *Streptococcus mutans* glucans by this technique[15,22,23].

Distribution of linkages in the glucans. — ^{1}H-N.m.r. spectra were obtained for NRRL reference dextrans B-512, B-742-L, B-742-S, and B-1355-S, for which structural data based on chemical analyses were available[24], and for the streptococcal glucans. Linkage percentages were determined by the method of Pasika and Cragg[3], by measuring the intensities of the doublet resonances found approximately between 4.7–4.8 and 5.0–5.1 p.p.m., representing the α-D-(1→6) and the non-α-D-(1→6) linkages, respectively. Intensities were measured both by electronic integration and by planimetry. Data reported represent the average of determinations by both methods; these averages did not differ by more than 3% from the values obtained by either method.

The α-D-(1→6) and non-α-D-(1→6) ratios calculated for the NRRL dextrans by ^{1}H-n.m.r. spectroscopy generally agreed with ratios determined by chemical analysis (shown in parentheses). The results were as follows: B-512, 95/5 (95/5); B-742-L, 82/18 (81/19); B-742-S, 64/36 (57/43); and B-1355-S, 58/42 (57/43).

The results for the streptococcal glucans are presented in Table I.

The soluble fractions ranged in content of α-D-(1→6) linkages from 81 to 64%. These values agree well with literature reports on the structure of soluble glucans from strains of *Streptococcus mutans*[11,19]. I.r. spectra of the fractions showed a strong absorption at 793 cm^{-1}, similar to that commonly observed with water-soluble dextrans[24,25].

The insoluble fractions generally showed much lower levels of α-D-(1→6) linkages than the soluble fractions, although the data encompass a broad range. It has been previously shown that the insoluble glucans are not homogeneous in structure or molecular weight[26]. The broad range we have found in linkage percentages among the insoluble glucans may result, in part, from small variations in experimental conditions affecting the point at which insoluble glucan appeared during the course of glucan synthesis. On the other hand, this range probably reflects real differences among the serological groups of *Streptococcus mutans*. The average content of α-D-(1→6) linkages for the "B" fractions, which were insoluble under neutral pH conditions, was somewhat lower than the average value of the "A" fractions. This was largely due to the serological group "d" glucans, which, in fact, were consistently low in α-D-(1→6) linkage content for the three types of glucan fractions as compared with the other serological groups. The lowest values were found for the three K-1R fractions. It has been reported[27] that linkage ratios for *Streptococcus mutans* glucans

TABLE I

PERCENTAGES OF α-D-(1→6) AND NON α-D-(1→6) LINKAGES IN *Streptococcus mutans* GLUCAN FRACTIONS AS ESTIMATED BY ^{1}H-N.M.R. SPECTROMETRY

Strain	*Serotype*	*Linkage type*	*Soluble*	*Insoluble "A"*	*Insoluble "B"*
AHT	a	1→6	78	63	
		Non 1→6	22	37	
HS-6	a	1→6	76	74	62
		Non 1→6	24	26	38
FA-1	b	1→6	72	74	
		Non 1→6	28	26	
BHT	b	1→6	78		
		Non 1→6	22		
NCTC 10449	c	1→6	69	68	51
		Non 1→6	31	32	49
Ingbritt	c	1→6		66	
		Non 1→6		34	
GS-5	c	1→6	81	72	
		Non 1→6	19	28	
SL-1	d	1→6	64		44
		Non 1→6)	36		56
K-1R	d	1→6	64	46	30
		Non 1→6	36	54	70
OMZ 176	d	1→6	65		37
		Non 1→6	35		63
LM-7	e	1→6	80	84	
		Non 1→6	20	16	
Averages:		1→6	73	68	45
		Non 1→6	27	32	55

were found to differ according to serological group, although no specific details were given. However, structural analyses of water-insoluble glucans of group "d" strains have characteristically shown a high content of α-D-(1→3) linkages[9,10,28].

I.r. spectra of the insoluble glucan fractions provided ancillary information that was in accord with the ^{1}H-n.m.r. findings. The "B" fractions invariably absorbed at about 822 cm^{-1}, with much less absorption at 793 cm^{-1} than was found with the soluble fractions. The absorption at 822 cm^{-1} has been previously reported[29] for insoluble glucans from *Streptococcus mutans* strains, and appears to be identical to the absorption at 12.2 μm observed in dextrans by Jeanes *et al.*[24], particularly for certain water-insoluble dextrans. Spectra of the "A" fractions, which had linkage ratios generally intermediate between those of the soluble and the "B" fractions, indicated these fractions to be either similar to the soluble fractions or to be mixtures of soluble and "B" fractions.

Neighboring-group effects. — Close examination of the 100-MHz, ^{1}H-n.m.r.

spectra revealed that the chemical-shift range for the non-α-D-(1→6) resonances actually contained two doublets. The two resonances were visible in the spectra of nearly all the glucans, but were particularly prominent in the cases of Ingbritt, K-1R, and OMZ 176 (Fig. 1). In addition to low-field doublets at 5.00 and 5.09 p.p.m., a spectrum recorded at 270 MHz further revealed that two high-field doublets were present in the chemical-shift range for α-D-(1→6) resonances. These included a major resonance at 4.75 p.p.m. and a minor one at 4.80 p.p.m., the latter appearing only as a shoulder in Fig. 1. This unusual multiplicity of resonances led to additional studies to determine its origin.

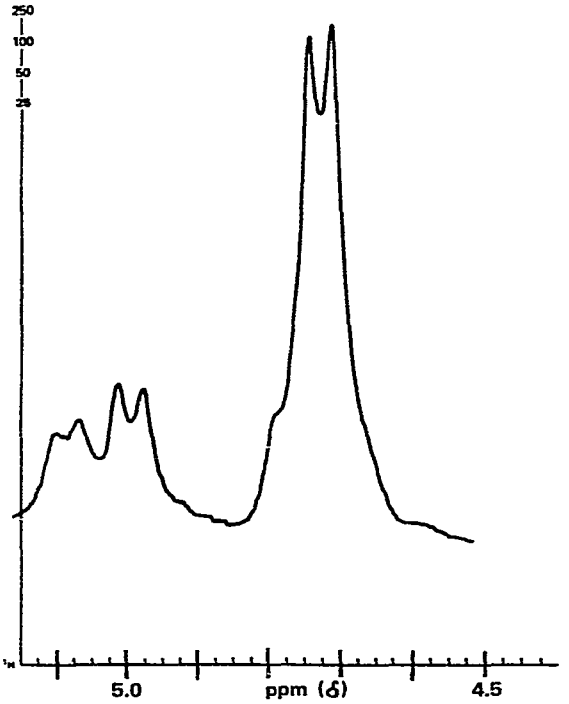


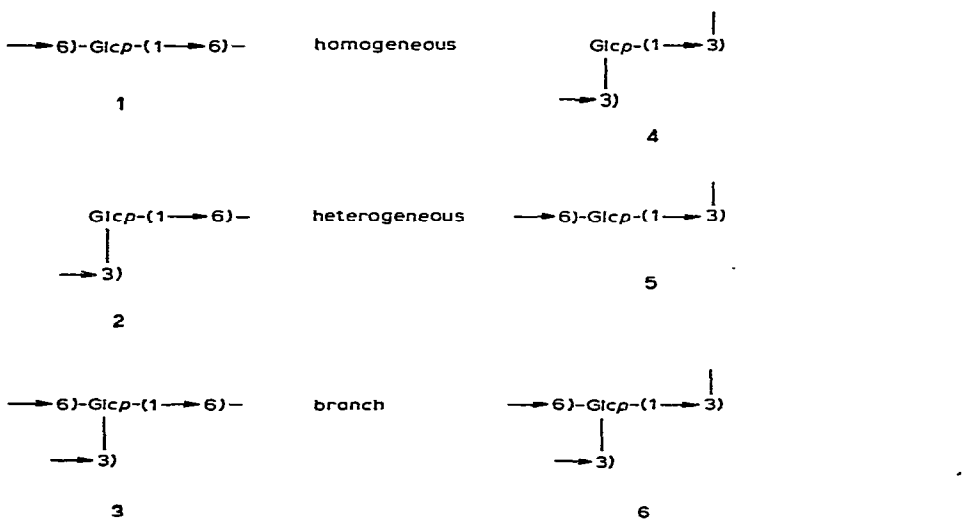
Fig. 1. A 100-MHz, ^{1}H-n.m.r. spectrum of the anomeric region of OMZ 176 soluble glucan.

Although glucans of the type examined in this study were known to contain predominantly α-D-(1→3) and α-D-(1→6) linkages, it was initially assumed, in view of at least one report[30], that α-D-(1→2) and α-D-(1→4) linkages might also be present as the source of the additional anomeric resonances. However, ^{13}C-n.m.r. studies conducted by Dr. Ian C. P. Smith[14,28], based on his previous studies[31,32] and those of others[33,34], clearly revealed that only α-D-(1→3) and α-D-(1→6) linkages were present. This led to the supposition that the doubling of peaks might arise from a neighboring-group effect—namely α-D-(1→3) linkages adjacent to α-D-(1→3) or α-D-(1→6) neighbors for the downfield pair, and α-D-(1→6) linkages adjacent to

α-D-(1→3) or α-D-(1→6) neighbors for the high-field pair, with the latter condition probably associated with the major peak.

This supposition was confirmed and specific resonance-assignments were possible when the insoluble "B" fraction from K-1R glucan was treated with *Penicillium sp.* dextranase [α-(1→6)-D-glucan 6-glucanohydrolase, EC 3.2.1.11] and degradation products were examined by ^{1}H-n.m.r. spectrometry. The water-insoluble residue remaining after the dextranase treatment and containing 90% or more α-D-(1→3) linkages, exhibited only the 5.09 p.p.m. resonance in its ^{1}H-n.m.r. spectrum, whereupon that resonance was assigned to α-D-(1→3) linkages adjacent to α-D-(1→3) neighbors. The ^{1}H-n.m.r. spectrum of a slowly dialyzable, water-soluble fraction recovered from the supernatant revealed major resonances at 5.00 and 4.80 p.p.m.; these were assigned respectively to α-D-(1→3) linkages adjacent to α-D-(1→6) neighbors and α-D-(1→6) linkages adjacent to α-D-(1→3) neighbors. The resonance at 4.75 p.p.m. associated with α-D-(1→6) linkages adjacent to other α-D-(1→6) neighbors was very much decreased compared to this resonance in the original "B" fraction of K-1R glucan that did not receive the dextranase treatment.

A polymer consisting of only α-D-Glc*p* (1→6) and α-D-Glc*p* (1→3) residues (when end groups are excluded) may contain three types of α-D-(1→6) linkages (**1**, **2**, and **3**) and three types of α-D-(1→3) linkages (**4**, **5**, and **6**), representing homogeneous, heterogeneous, and branch configurations, respectively (Scheme 2). As only two,



Scheme 2. Possible configurations of glucopyranosyl residues in a polymer containing only α-D-(1→6) and α-D-(1→3) linkages (excluding end-groups).

rather than three, α-D-(1→6) or α-D-(1→3) resonances were observed, the anomeric-proton chemical shifts are sensitive only to substitution at the C-3 hyrdoxyl group and are unaffected by changes at the more distant C-6 hydroxyl group. Thus ^{1}H-n.m.r.

spectrometry can differentiate between **1** and the combination of **2** and **3**, as well as **5** from the combination of **4** and **6**.

Two preparations of OMZ 176 soluble glucan fraction and one preparation of Ingbritt insoluble "A" fraction, having similar proportions of the various linkages, were studied in detail. The results, collected in Table II, illustrate the application of these neighboring-group effects in the ^{1}H-n.m.r. spectra.

TABLE II

PERCENTAGES OF NEAREST NEIGHBORS TO α-D-(1→3) AND α-D-(1→6) LINKAGES IN *Streptococcus mutans* GLUCAN FRACTIONS, AS ESTIMATED BY ^{1}H-N.M.R. SPECTROMETRY

Glucan fraction	α-D-(*1→3*) *Adjacent to*		α-D-(*1→6*) *Adjacent to*	
	α-D-(*1→3*) **4, 6**[a]	α-D-(*1→6*) **5**	α-D-(*1→6*) **1**	α-D-(*1→3*) **2, 3**
Ingbritt Insoluble "A"	19	15	17	49
OMZ 176 Soluble prep. no. 1	15	17	——68——	
OMZ 176 Soluble prep. no. 2	13	19	——68——	

[a]Refer to Scheme 2 or text for explanation of numerals.

A recent publication[35] has reported a similar effect in lichenan, a glucan containing β-D-(1→4) and β-D-(1→3) linkages. Additionally, ^{1}H-n.m.r. studies[33] on NRRL B-1299, a water-soluble dextran obtained from *Leuconostoc mesenteroides*, have revealed neighboring-group effects on α-D-(1→6) resonances caused by the presence or absence of adjacent α-D-(1→2) linkages. However, this is the first report of such a neighboring-group effect in streptococcal glucans, and we believe it offers an important new insight into the structures of these compounds.

EXPERIMENTAL

Enzymic synthesis of the glucans. — Each strain of *Streptococcus mutans* was cultured at 37° under 95% N_2–5% CO_2 successively in Todd–Hewitt broth (THB) and in a chemically defined medium (CDM)[36], both media being supplemented with 2% of D-glucose. The procedure was as follows: The organisms were grown in 3% THB for 24 h, and then transferred and grown in fresh THB for an additional 24 h before transfer to CDM. After 24 h, a 0.3-ml portion of the CDM culture was added to 30 ml of CDM, the mixture was incubated for 24 h, and this culture was inoculated into 1 liter of CDM for the enzyme synthesis. Following incubation for 18–20 h, the bacterial cells were separated from the mixture by centrifugation for 15 min (16,300*g*) at 3–5° and the extracellular enzymes were precipitated by ammonium sulfate at 80%

saturation. The precipitated material was dissolved in 150 ml of 25mM phosphate buffer, pH 6.8, dialyzed at 3–5° successively against the phosphate buffer and water, and lyophilized. Other details of this procedure have been previously reported[36].

For the glucan synthesis, 20–25 mg of the lyophilized enzyme-preparation was dissolved in 500 ml of 10% sucrose in 25mM phosphate (pH 6.8), 0.5 ml of toluene was added as preservative, and the mixture was incubated for 1–4 days at 37°. The procedure for isolation of the glucan fractions is shown in chart 1. As a final step prior to lyophilization (not included in chart 1), each fraction was dissolved in M potassium hydroxide and dialyzed against water until the pH of the dilayzed fraction had decreased to 7.0 or below. Other details of the procedure are similar to those previously reported[22] for the isolation of glucan fractions from cultures of *Streptococcus mutans*.

Partial degradation of water-insoluble glucan by dextranase. — A sample (500 mg) of lyophilized insoluble "B" fraction from strain K-1R was incubated in a 50-ml centrifuge tube with approximately 135 units of *Penicillium sp.* dextranase (Worthington) in 25 ml of 50mM phosphate buffer, pH 6.0, for 5 days at 37°. The mixture was swirled several times daily during this period. The undissolved residue was then washed with water three times, with water volumes each approximately equal to the residue volume, and was lyophilized (312 mg). The supernatant liquid was combined with the residue washes, heated for 5 min to 90–100° to denature dextranase, clarified by centrifugation, dialyzed against water for 24 h, and lyophilized, yielding 38 mg of a fluffy, water-soluble product.

Analytical methods. — ^{1}H-N.m.r. spectra at 100 MHz were obtained for the majority of the glucans on a Varian Associates HA-100 spectrometer. The spectrum shown in Fig. 1 was obtained with a Varian Associates XL-100-15 instrument, in the pulsed FT mode. A 9:1 mixture of Me_2SO-d_6 and D_2O was employed as the solvent and spectra were recorded at 80°. Each sample was prepared by hydrating approximately 100 mg of the glucan with 0.2 ml of D_2O for 24 h at ~5°. Me_2SO-d_6 was added in small aliquots with continual agitation until a total of 1.8 ml had been added. The gel formed was shaken for an additional 24–48 h and then transferred without filtration into a 5-mm tube for ^{1}H-n.m.r. spectrometry. The 270-MHz spectrum was obtained on a Bruker HR-270 spectrometer operating in the FT mode. The chemical shifts were measured with reference to tetramethylsilane as the internal standard.

Glucose analyses were performed on hydrolyzates of glucan samples by the D-glucose oxidase method (Fisher). Details of the procedure have been previously reported[22]. I.r. spectra were obtained with a Perkin–Elmer Model 467 double-beam spectrophotometer, by using discs pressed from mixtures consisting of 3 mg of lyophilized glucan and 300 mg of potassium bromide.

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