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DETERMINATION, BY CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY, OF THE COMPOSITION OF GLUCANS SYNTHESIZED BY ENZYMES OF THE CARIOGENIC ORGANISM *Streptococcus mutans**

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ABSTRACT

The ^{13}C-n m r spectra of water-soluble and -insoluble glucans synthesized by enzymes isolated from six strains of *Streptococcus mutans* are interpreted The glucans are shown to be composed primarily of α-(1→3)- and α-(1→6)-linked glucosyl residues, and the relative abundance of each linkage is estimated from peak areas Treatment of water-insoluble glucans with dextranase is found to result in water-soluble and -insoluble products, the former enriched in α-(1→6)-linkages and the latter in α-(1→3)-linkages The structural conclusions arrived at by ^{13}C-n m r spectroscopy are consistent with data from methylation analysis and ^{1}H-n m r spectroscopy

INTRODUCTION

Extracellular, water-insoluble glucans produced by cariogenic streptococci have been implicated in the formation of dental plaque[1–3] and have therefore been the subject of structural investigations These insoluble polysaccharides have been shown by chemical and enzymic studies[4–11] to contain high proportions of α-(1→3)-linkages In view of the tedious nature of these studies, we considered that ^{13}C-n m r spectroscopy would provide a useful and more rapid alternative thereto Usui *et al.*[4] have reported the only ^{13}C-n m r -spectroscopic study of a *S mutans* glucan namely that of *S mutans* JC-2

We describe here the determination, by ^{13}C-n m r spectroscopy, of the structures of glucans synthesized by enzyme isolates from six strains of *S mutans* E-49, Ingbritt, GS-5, SL-1, K-1R, and OMZ-176 In addition, glucans produced by OMZ-176 and K-1R enzymes were treated with a dextranase, and some of the resulting products were examined by ^{13}C-n m r spectroscopy To increase the solubility of

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TABLE I

^{13}C CHEMICAL SHIFTS[a] AND ASSIGNMENTS FOR GLUCANS PRODUCED BY *S mutans*, AND FOR RELATED GLUCANS

Carbon number	*(1→3)-α-Glucan*[b]	*(1→6)-α-Glucan*[b]	S mutans *glucans*
C-1	101 3		102 5–102 2 (1→3)
		99 4	99 7–99 4 (1→6)
C-2	72 2		72 4 (1→3)
		73 1	73 1–72 9 (1→6)
C-3	83 2		85 3–84 9 (1→3)
		75 4	75 8–75 5 (1→6)
C-4	71 7		71 9–71 7 (1→3)
		71 8	(1→6)
C-5	73 7		74 2–73 9 (1→3)
		71 1	71 5–71 3 (1→6)
C-6	62 2		62 4–62 2 (1→3)
		66 8	66 9–66 5 (1→6)

[a]In p p m relative to external tetramethylsilane, in D_2O, pD 14, 30° [b]Data taken from ref 12

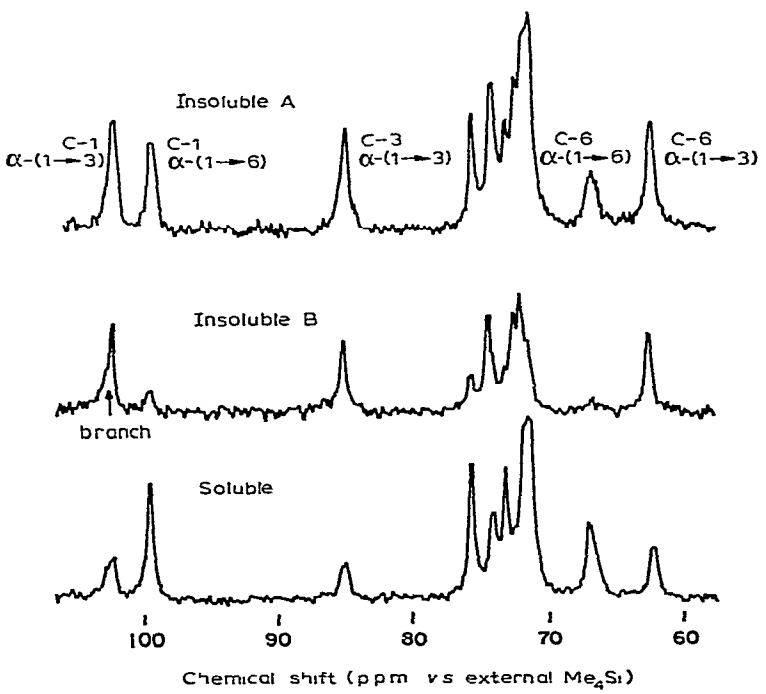

Fig 1. ^{13}C-N m r spectra, at 25 MHz, of aqueous solutions of glucans synthesized by the enzyme system from *S. mutans* OMZ-176 (80 mg/ml, pD 14, 32°). [~ 30,000 Accumulations of free-induction decays, pulse angle 75°, digital resolution 2 Hz, line broadening 2 Hz]

TABLE II

CHARACTERISTIC CHEMICAL SHIFTS[a] FOR LINKAGES IN SOME GLUCANS AND RELATED DERIVATIVES

Linkage type	*Carbon atom*					*References*
	C-1	*C-2*	*C-3*	*C-4*	*C-6*	
α-(1→2)	97 2	76 5				13[b]
	(96 4)					
β-(1→2)	105 0[c]	81 7				14[b]
α-(1→3)	101 3		83 2			12[d]
	100 3		81 6			13[b]
β-(1→3)	104 7		88 0			12[d]
	103 8		85 5			12[b]
α-(1→4)	102 9			80 6		12[d]
	100 9			78 4		12[b]
β-(1→4)	103 9[c]			80 3		14[b]
α-(1→6)	99 4				66 8	12[d]
	99 0				66 7	12[b]
β-(1→6)	104 0				70 0	14[b]

[a]In p p m relative to external tetramethylsilane [b]Sample solution at pD 7 [c]Data are for disaccharides [d]Sample solution at pD 14

those glucans that are insoluble in water at neutral pH, samples were studied at pD 14, where reasonable concentrations could be obtained

RESULTS AND DISCUSSION

Linkage and composition — The principal resonances of the various glucans are shown in Table I, representative spectra of the various fractions produced by the OMZ-176 system are presented in Fig 1 In order to assign the types of linkage present, the spectra of these polysaccharides were examined for resonances characteristic of a given linkage, and these are presented in Table II Thus, the absence of resonances in the regions around 97 and 77 p p m indicated that none of the polysaccharides contained a significant number of α-(1→2)-linked glucosyl residues Similarly, no appreciable proportion of α-(1→4)-linked glucosyl residues was present, as evidenced by the absence of signals at the region around 81 p p m The corresponding β-(1→2)- and β-(1→4)-linkages may be readily excluded by comparison of the C-1 chemical shifts observed for the *S mutans* polysaccharides with those reported for methyl α-sophoroside[14] and laminaran[12], respectively The presence of β-(1→6)- and β-(1→3)-linkages was excluded by similar arguments using data that had been reported for methyl β-gentiobioside[14] and a (1→3)-β-glucan[12]. In agreement with chemical studies, the main intersaccharide linkages were found to be α-(1→6) and α-(1→3) Resonances were assigned on the basis of the intersaccharide linkage present, and of extensive, spectral analyses of carbohydrates of known structure[12],

TABLE III

COMPOSITION OF GLUCANS PRODUCED BY *S. mutans*

Polysaccharide fraction		*Per cent of linkages*				
		^{13}C-n m r		*Chemical*[a]		*References*
		α-(1→6)	α-(1→3)	α-(1→6)	α-(1→3)	
E-49	soluble	62	38	78	22	5
	A	54	46			
	B	39	61			
Ingbritt	soluble	75	25	81	19	9
	A	51	49	50	50	[b]
	B	44	56	38	62	[b]
GS-5	soluble	87	13			
	B	33	67			
SL-1	soluble	60	40			
	B	33	67			
K-1R	soluble	72	28	56	44	[b]
	B	16	84			
OMZ-176	soluble	65	35			
	A	42	58	42	58	[b]
	B	15	85	16	84	15

[a]Compositions were determined by methylation analyses, and the results are expressed as a percentage of the total α-(1→3)- and α-(1→6)-linkages [b]Present study, polysaccharides were methylated according to Hakomori[19], and the methylated-sugar components were analyzed by g l c as their corresponding alditol acetates[20]

and especially by comparison with the spectra of (1→6)- and (1→3)-α-glucans[12 14]

In the ^{13}C-n m r spectrum of the soluble K-1R glucan in solution at pD 7 and at pD 14, C-1 (1→3) had chemical shifts of δ 100 5 and δ 102 9–102 5, respectively, reflecting the influence of pH on ^{13}C chemical shifts[12] A comparison of data obtained for the soluble K-1R glucan at pD 7 and pD 14 with data in Table II led to the same conclusion about linkage and composition

In a study of JC-2 glucans[4], the types of linkage were determined by ^{13}C-n m r spectroscopy, but the ratios of (1→6)- and (1→3)-linkages were determined by using ^{1}H-n m r spectroscopy. In this study, the relative proportions of (1→6)- and (1→3)-linkages were determined from the relative areas of the C-1 and C-6 resonances The results, given in Table III, represent the average values obtained by using C-1 and C-6 signals, and indicate that the water-soluble glucans have the highest content of α-(1→6)-linkages, whereas the "B" fraction, which is insoluble at neutral pH, showed the highest content of α-(1→3)-linkages Linkage distributions in the "A" fractions were intermediate between those of the soluble and insoluble "B" fractions The observation that the more-soluble glucans have a high content of α-(1→6)-linkages, whereas the least-soluble glucans have a high content of α-(1→3)-linkages, is in agreement with the suggestion of Guggenheim *et al* [8] that the ratio of α-(1→3)- to α-(1→6)-linkages in these streptococcal glucans may vary over a continu-

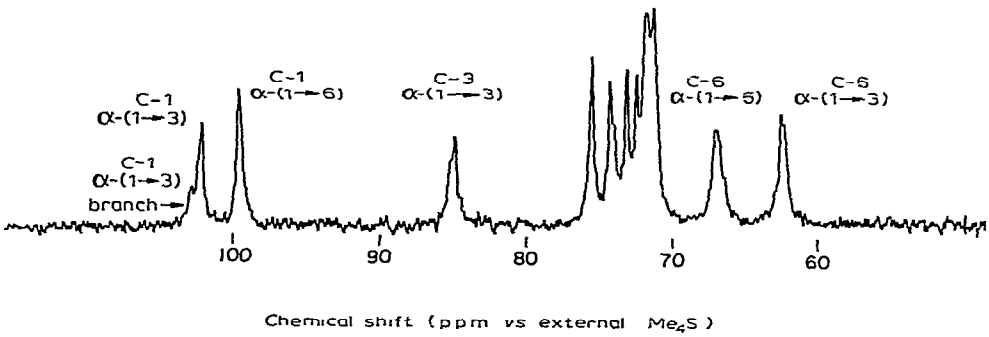

Fig 2 ^{13}C-N m r spectrum, at 68 MHz, of the soluble glucan synthesized by the enzyme system from *S mutans* OMZ-176 (40 mg/ml, pD 14, 40°) [26,000 Accumulations of free-induction decays, pulse angle 75°, digital resolution 2 Hz, line broadening 3 Hz]

ous range, with the glucans becoming less soluble as the proportion of α-(1→3)-linkages increases Linkage distributions determined by ^{13}C-n m r analysis are in reasonably good agreement with all but a few of those determined by chemical analysis In the course of these studies, we have found that the relative percentages of the two types of linkage vary somewhat from one preparation to another, therefore, our methylation and ^{13}C-n m r data are reported for the same sample This variability may be the source of some of the quantitative disagreements in Table III

Detection of branching — Some of the streptococcal glucans, particularly the soluble glucans, have been shown to be highly branched[5-7] A close examination of the spectra of the soluble and insoluble "B" glucans from OMZ-176 revealed an additional resonance in the region of the α-(1→3), C-1 signal, as indicated by the vertical arrow in Fig 1 It was speculated that this additional resonance might arise from the occurrence of α-(1→3)-linkages both as branch points in the glucan chains and as linear-backbone sequences in the chains Spectra at 68 MHz permitted the observation of two resonances, at δ 102 8 and 102 3 (see Fig 2), which were assigned to C-1 of α-(1→3)-linked units, with the former being attributed to linkages at branch points Estimates of the ratio of the two types of linkage were made from deconvoluted, spectral-peak areas The soluble glucan from OMZ-176 was determined to have a 2 8 1 ratio of linear to branched α-(1→3)-linkages, in good agreement (considering the extensive overlap of the two ^{13}C resonances) with the value of 3 5 1 determined by methylation analysis The ratio was found to be even higher, 4 1, for the insoluble "B" glucan, compared to the value of 3 6 1 determined by methylation studies However, in several cases, such as the soluble glucan from E-49, in which some α-(1→3)-linkages were found at branch points[5], the present method fails to distinguish the linear from the branched linkages, only a single, broad resonance was observed in the glucan spectrum at 25 MHz Thus, the ^{13}C chemical shifts are sensitive to the presence of branch points, but this information can be masked by the relatively large linewidths often observed for this type of glucan, this difficulty may sometimes be circumvented by obtaining the ^{13}C-n m r spectra at higher magnetic fields, as

already mentioned However, we have had only limited success with this method, often, the resonances are merely broadened by a factor equal to the ratios of the magnetic fields at which the spectra were obtained, this implies that the widths of the resonances are not simply ^{13}C–^{1}H dipolar in origin, but also comprise contributions due to heterogeneity of chemical shift This heterogeneity is undoubtedly due to the possibility of a variety of neighboring residues for a particular, observed carbon atom Thus, a glucosyl residue whose anomeric carbon atom is linked α-(1→3) has the possibility of a main chain or branch-point linkage at C-3 or C-6, as well as several of these for the neighboring residue to which it is attached by a glycosidic bond Such sensitivity to sequence effects has been demonstrated in the ^{13}C-n m r spectra of pullulan and of the major glucan elaborated by *Tremella mesenterica*[12] It is conceivable that these sequence-related, chemical shifts could be observed in spectra having broad resonances if the dipolar contributions could be removed by high-power, decoupling techniques, such as ^{13}C (^{1}H) cross-polarization[17] In some instances, higher temperatures lead to better resolution of resonances[13]

Comparison with ^{1}H-n m r data — Meyer *et al*[18] studied the ^{1}H-n m r spectra of similar preparations In particular, parallel ^{13}C- and ^{1}H-n m r studies were performed on the soluble glucan produced by the OMZ-176 enzyme system The ^{1}H spectra yielded a composition of 35% of α-(1→3)- and 65% of α-(1→6)-linkages, to be compared with the present, ^{13}C-derived values of 35 and 65%, respectively Thus, the results of the two methods are in perfect agreement. ^{1}H-N m r spectroscopy has the advantage of greater sensitivity, but the overall disadvantage of a smaller scale of chemical shifts The two methods make an excellent, complementary pair for the study of complex carbohydrates

The influence of treatment with dextranase — Interest in lessening formation of dental caries has resulted in the treatment of glucans produced by *S mutans* with dextranase[8,10 15] Ebisu *et al*[10] prepared, in the presence of a dextranase, a water-insoluble glucan from OMZ-176, and showed by methylation analysis that the glucan contained 96 2% and 3 8% of α-(1→3)- and α-(1→6)-linkages, respectively In the present study, dextranase treatment of the insoluble "B" glucan from OMZ-176, which contained 83% of α-(1→3)-linkages and 17% of α-(1→6)-linkages, yielded a water-soluble and a water-insoluble product The water-soluble glucan was estimated, by ^{13}C-n.m r. spectroscopy, to contain 41 4% and 58 6% of α-(1→3)- and α-(1→6)-linkages, respectively, whereas the insoluble product contained a higher proportion, namely, 88 5%, of α-(1→3)-linkages Dextranase treatment of insoluble "B" glucan from K-1R, which contains 73% of α-(1→3)-linkages, resulted in the formation of an insoluble glucan that had 91 6% of α-(1→3)- and 8 4% of α-(1→6)-linkages A water-soluble product also obtained was shown by ^{13}C-n m r spectroscopy to contain 45 7% of α-(1→3)- and 54 3% of α-(1→6)-linkages

EXPERIMENTAL

General — The ^{13}C-n m r spectra were recorded with Varian XL-100 (25 MHz)

and Bruker HX-270 (68 MHz) spectrometers, employing sample tubes of outside diameter 12 mm and 10 mm, respectively, with complete proton-decoupling The solvent was deuterium oxide, the deuterium resonance was used as a field-frequency lock, and chemical shifts are expressed relative to tetramethylsilane contained in a coaxial sample-tube of outside diameter 5 mm For studies at 25 MHz, the concentration of the samples of glucan was ~80 mg/ml in deuterium oxide, at pD 14 (pD = pH-meter reading + 0 4) and 32 ±1°, containing ~0 5 mg of sodium borohydride per ml to inhibit base-catalyzed hydrolysis Due to the greater detection sensitivity of the 68-MHz instrument, lower concentrations of glucan could be used with it heating of the samples due to high-power irradiation resulted in an equilibrium temperature of 42° in this spectrometer The less-soluble glucans were dissolved by gradual addition, during several hours, of the lyophilized material to a continuously stirred solution at room temperature

Enzymes — Extracellular enzymes were obtained from each *S mutans* strain by successively growing the organisms, under 19 1 N_2–CO_2, in Todd–Hewitt broth (Difco Laboratories, Detroit, Michigan) and in a chemically defined medium (CDM), as reported previously[18] The SL-1 glucans were synthesized by enzymes precipitated from the CDM culture-fluid by ammonium sulfate[18] For synthesis of glucans for each of the other strains, 250 ml of the CDM culture was centrifuged at 16,300 *g* for 20 min at 3–5° to remove bacterial cells, and the supernatant fluid was combined with phosphate–buffered sucrose to a volume of 1 liter having a final concentration of 10% of sucrose in 0 02M phosphate, pH 6 8 Toluene (0 5 ml) was added as a preservative, and the mixture was incubated for 5 days at 37°, producing a mixture of soluble and insoluble glucans The soluble-glucan fraction was separated from the supernatant liquid by ethanol precipitation within 35–50% limits The insoluble material from the incubation mixture was dissolved in M potassium hydroxide for further purification, this led in most instances to the separation of two fractions, termed "A" and "B" The "B ' fraction, which was not found so frequently as the "A" fraction, was precipitated upon neutralization of the alkali in the solution The "A" fraction, soluble at neutral pH, was recovered from the supernatant liquor by ethanol precipitation within 35–50% limits All of the glucan fractions were lyophilized Other details of the isolation have already been described[18]

The dextranase degradation was conducted on 2 0-g samples of "B" fractions from strains K-1R and OMZ-176 Each sample was incubated for 5 days at 37° with 95 units of *Penicillium sp* dextranase (Worthington) in 5 ml of 0 05M phosphate buffer, pH 6 0 The mixtures were swirled several times daily during this period The undissolved residues were washed three times, using water volumes each approximately equal to the volume of the residue, and were then lyophilized (1 10 g from K-1R, 1 35 g from OMZ-176) The supernatant liquors and respective residue washes were combined, heated for 5 min at 90–100° to denature the dextranase, and clarified by centrifugation Dialysis of these preparations against water for 24 h, and lyophilization, yielded, in each experiment, a fluffy, water-soluble product (0 29 g from K-1R, 0 25 g from OMZ-176).

CONCLUSION

Although chemical methods are, ultimately, the most reliable for structural determinations, in the case of the *S mutans* glucans, ^{13}C-n m r. spectroscopy readily provides information on the gross structure of these polysaccharides, and, in some instances, also indicates the degree of branching In addition to establishing the anomeric configurations and positions of intersaccharide linkages, the relative abundance of each type of linkage may be readily determined The method is non-destructive, and requires only simple manipulation of the samples for analysis

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