Carbohydrate Research, 71 (1979) 231–250

STRUCTURAL ANALYSIS OF DEXTRANS CONTAINING 2-*O*-α-D-GLUCOSYLATED α-D-GLUCOPYRANOSYL RESIDUES AT THE BRANCH POINTS, BY USE OF ^{13}C-NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY*

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(Received July 10th, 1978, accepted for publication, July 24th, 1978)

ABSTRACT

Dextran fractions from NRRL strains *Leuconostoc mesenteroides* B-1299 and B-1399, and the native, structurally homogeneous dextrans from *L. mesenteroides* B-640, B-1396, B-1422, and B-1424, were examined by ^{13}C-n.m.r. spectroscopy at 34 and at 90°, and by g.l.c.–m.s. The ^{13}C-n.m.r. data indicate that the dextrans of this series branch exclusively through α-D-(1→2)-linkages, and differ from one another only in degree of linearity. Diagnostic, ^{13}C-n.m.r. resonances, correlating with 2,6-di-*O*-substituted α-D-glucosyl residues at branch points, have chemical shifts that are independent of the degree of linearity of the dextran. The intensities of these diagnostic resonances from branching residues, compared to the resonances associated with linear dextran (low degree of branching), are generally proportional to the degree of branching established by methylation–fragmentation analysis. The validity of assignment of the diagnostic, ^{13}C-n.m.r. resonances is substantiated by a critical review of methods previously used to provide structural information on dextrans having α-D-(1→2)-linkages, and by evaluation of the corresponding results on the basis of the ultimate standard—methylation structural analysis.

*Unusual Dextrans, Part V. For Part IV, see ref. 1.

INTRODUCTION

We have previously reported the ^{13}C-n m r spectra of a series of unusual dextrans[1,2] These spectra were interpreted, with the assistance of methylation structural analysis, by g l c –m s of the per-*O*-acetylated aldononitriles of the same dextrans[3,4]. The dextrans were produced by bacteria designated here by the strain number in the ARS Culture Collection at the Northern Regional Research Center[5] A number of the dextrans are essentially homogeneous fractions separated from the corresponding. polydisperse, high-molecular-weight, native dextrans; these dextran fractions are designated S (Soluble) and L (Less-soluble)[6]

On the basis of a limited number of examples[2], we concluded that certain ^{13}C-n.m.r. resonances are diagnostic for 2,6-, 3,6-, and 4,6-di-*O*-substituted α-D-glucosyl residues at branch points We concluded, also, that the spectra of the unusual dextrans are composites consisting of the series of six resonances associated with α-D-glucopyranosyl residues situated in linear chains of α-D-(1→6)-linked residues, and of resonances having chemical shifts and relative intensities that are dependent on the nature of the branching involved.

Diagnosis of ^{13}C-n m r resonances has now been successfully extended to identifying 2,6-di-*O*-substituted α-D-glucosyl residues* as the branch points in three additional dextrans, namely, native dextrans, previously prepared and characterized[5], from NRRL strains *Leuconostoc mesenteroides* B-1396, B-1422, and B-1424 Previous and present observations have also been made on ^{13}C-n m r spectra of six other native dextrans (or dextran fractions) in which the presence of branching at C-2 has been established through methylation structural analysis, these dextrans, each from a different strain of *L. mesenteroides*[5], are designated B-1299 fractions S (refs 1 and 3) and L (refs 1 and 4), B-1399 fractions S (ref 4) and L (ref 3), B-1399 (P-37) (ref 4), and B-1402 (refs 1 and 4) A dextran having low degree of branching ($< \sim 5\%$), from *L. mesenteroides* B-640, has been used to provide linear-type dextran control spectra

We have observed that ^{13}C-n m r spectra of carbohydrates are dependent on the temperature at which the spectrum is recorded[1,2] Previously, we reported the spectra at ambient temperature ($\sim 30°$), and also the dependence of chemical shifts on changes in recording temperature ($\Delta\delta/\Delta T$) for each spectral resonance obtained at low temperature This system permits direct comparison of our carbohydrate spectra with those reported by others, which usually have been recorded at ambient temperatures However, ^{13}C-n m r spectra of polysaccharides acquired at elevated temperature contain many additional resonances, and it is inconvenient to discuss these resonances in terms of low-temperature reference-spectra Therefore, beginning with this report, we have chosen for a reference the highest temperature (90°) con-

*Hereafter in this article, this terminology is often simplified, for convenience, to 2,6-substituted α-D-glucosyl residues Corresponding terminology is also used for α-D-glucosyl residues in dextrans that branch through C-3 or C-4

veniently compatible with an aqueous system For continuity, the 34° resonances of these dextrans are also listed, but, unless otherwise stated, all discussion of ^{13}C-n m r data refers to the 90° conditions Spectra recorded at 90° require only about a quarter of the number of acquisitions of 34° spectra for comparable signal-to-noise ratios[1]

Two major regions in these dextran spectra are of interest for diagnostic, resonance analysis (*a*) the 95–105-p p m region, which is the anomeric region, and (*b*) the 75–85-p p m region of the spectra of dextrans branched at C-2, C-3, or C-4 ^{13}C-N m r resonances associated with free positions C-2, C-3, and C-4 are clustered in the 70–75-p p m region, and, upon glycosidation at a specific position, the resonance is displaced down-field to the diagnostic 75–85-p p m region It has been proposed that, at 90°, these specific, diagnostic resonances for D-glucosyl residues in an α-D-glucan are approximately 77 8 p p m for 2,6-di-substituted residues, 80 2 p p m for 4,6-di-substituted residues, and 82 9 p p m for 3,6-di-substituted residues[1,2] The resonance for free C-6 occurs at 62 5 p p m , a value that is essentially independent of the type of branching present

Analysis of the ^{13}C-n m r resonances of a dextran in the anomeric region is somewhat more complicated, as essentially all of the anomeric positions are non-reducing When recorded at 34°, the anomeric resonances of D-glucans are situated in the 95–105-p p m region and, on raising the recording temperature to 90°, the resonances are increased by ~1 5 p p m It had been shown that, at ambient temperatures, resonances lying in the 97–101-p p m region are those of α anomers[7], and, when the $\Delta\delta/\Delta T$ corrections have been made, this relationship is also true for the 90° spectra Introduction of branching into a linear sequence of residues adds a minimum of two distinctly different types of D-glucosyl residue the branching residue and a side-chain, terminal group The anomeric carbon atoms of these residues must lie in environments different from those of the anomeric carbon atoms of (1→6)-linked D-glucosyl residues Therefore, these two new D-glucosyl groups could be expected to give different ^{13}C-n m r anomeric signals Fortunately, the chemical shifts of the anomeric resonances of the D-glucosyl groups are quite sensitive to the environment Previously, we have identified[2] two diagnostic resonances, at 97 37 and 98 22 p p m , that are associated with 2,6-di-*O*-D-glucosylated α-D-glucosyl residues at branch positions These anomeric resonances are distinct from other anomeric resonances associated with 3,6- or 4,6-di-*O*-D-glucosylated α-D-glucosyl residues at branch positions

RESULTS AND DISCUSSION

Nine structurally homogeneous dextrans and dextran fractions have been identified as D-glucans composed of linear, 6-*O*-D-glucosylated α-D-glucosyl residues, and branched primarily, if not exclusively, through 2,6-di-*O*-D-glucosylated α-D-glucosyl residues Previously published spectra for dextrans containing α-D-(1→2)-linkages include dextrans B-1299 fraction S (ref 2), dextran B-1299 fraction L (ref 1),

TABLE I

CHEMICAL SHIFTS FOR ^{13}C-N M R SPECTRA, AT 34°, OF DEXTRANS CONTAINING 2,6-DI-*O*-SUBSTITUTED α-D-GLUCOPYRANOSYL RESIDUES[a]

	NRRL strain number of the organism producing the dextran						
	B-1299	*B-1399*	*B-1399*[b]	*B-1424*	*B-1422*	*B-1396*	*B-640*
	Dextran fraction (or other designation)						
	L	*S*	*P-37*				
A[c]	98 71	98 70	98 68	98 73	98 66	98 64	98 67
	97 24	97 18	97 21	97 24	97 18	97 14	
	96 39	96 40	96 43	96 40	96 36	96 32	
	76 47	76 49	76 46	76 55	76 46	76 43	
B	74 30	74 32	74 36	74 39	74 34	74 32	74 36
	73 86	73 83	73 86	73 89	73 83	73 80	
	72 77	72 80	72 86	72 89	72 75	72 74	
C	72 34	72 32	72 34	72 39	72 38	72 33	72 37
D	71 17	71 15	71 14	71 19	71 14	71 11	71 14
E	70 41	70 36	70 47	70 53	70 47	70 46	70 52
	66.76	66 64	66 90				
F	66 61	66 55	66 64	66 61	66 55	66 54	66 56
	61 38	61 38	61 38	61 41	61 37	61 36	

[a]The chemical shift is given in p p m relative to tetramethylsilane [b]This spectrum is essentially identical to that of dextran B-1402 [c]The letters A through F designate the major resonances of linear dextran (see ref 2)

dextran B-1399 fraction L (ref 2), and dextran B-1402 (ref 1) We now present ^{13}C-n m r. spectra acquired at 34° for five additional, similarly constituted dextrans, namely dextrans B-1396, B-1399 fraction S, B-1399 (P-37), B-1422, and B-1424 (see Table I). A control spectrum is shown, also, for the linear-type dextran B-640, which has a degree of branching below the current limits of detection by our ^{13}C-n m r spectroscopy These spectra allow direct comparison with ^{13}C-n m r. spectra previously recorded at ambient temperature.

^{13}C-N m r spectroscopy

The ^{13}C-n.m r. spectra were also recorded at elevated temperature (90°), and are shown in Fig. 1. Comparison of the spectra acquired at 90° with those acquired at 34° showed that the relative narrowing of each resonance is approximately the same Therefore, the general "profiles" of the high-temperature and low-temperature spectra are similar, as may be seen in Fig 1 by comparing the high- and low-temperature spectra of dextran B-1399 fraction S In addition, when the intensity ratios of the anomeric resonances were calculated for the low-temperature spectra, the results were similar to those calculated for the 90° spectra (as will be discussed later in relation to Table V)

The six spectra shown in Fig. 1 are representative of the nine dextrans that have been identified as branching primarily through C-2, and the corresponding chemical

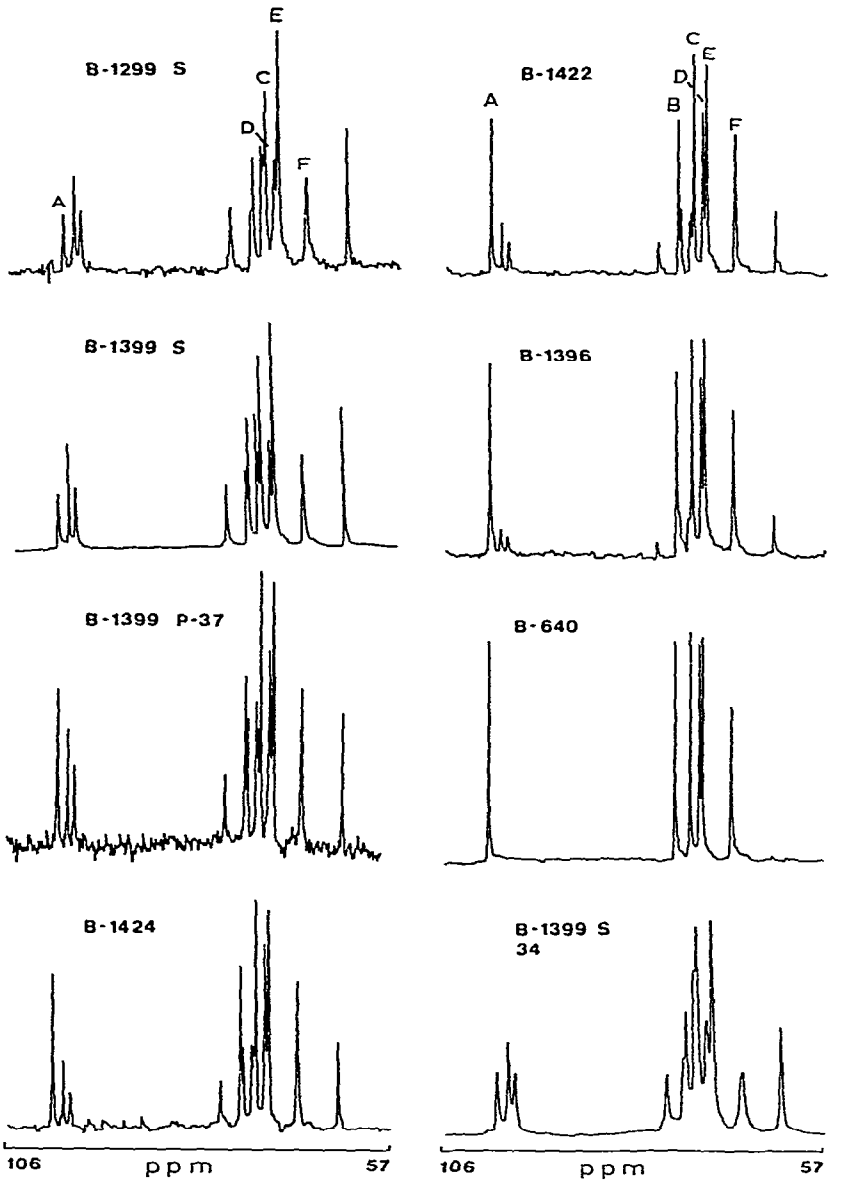


Fig 1 ^{13}C-N m r spectra at 90° (unless otherwise noted) for dextran B-1299 fraction S, dextran B-1399 fraction S, dextran B-1399 (P-37), dextran B-1424, dextran B-1422, dextran B-1396, and dextran B-640, and also at 34° for dextran B-1399 fraction S The letters A through F refer to the major resonances of linear-type dextran as represented here by dextran B-640

shifts are tabulated in Table II. The spectrum of dextran B-1399 fraction S is essentially identical to those previously published[1,2] for dextran B-1299 fraction S and fraction L, and the spectrum of dextran B-1399 (P-37) is identical to that of the previously published[1] spectrum of dextran B-1402 These type spectra constitute a

TABLE II

CHEMICAL SHIFTS FOR ^{13}C-N M R SPECTRA, AT 90°, OF DEXTRANS CONTAINING 2,6-DI-*O*-SUBSTITUTED α-D-GLUCOPYRANOSYL RESIDUES[a]

	NRRL strain number of the organism producing the dextran							
	B-1299[b]	*B-1399*[b]	*B-1399*[c]	*B-1424*	*B-1422*	*B-1396*	*B-1399*	*B-640*
	Dextran fraction (or other designation)							
	L	*S*	*P-37*				*L*	
A[d]	99 59	99 59	99 55	99 53	99 51	99 51	99 53	99 54
	98 22	98 19	98.21	98 19	98 19	98 19	98 20	
	97 37	97 35	97 33	97 32	97 29	97 29	97 30	
	77 83	77 79	77 80	77 79	77 74	77 76	77 78	
B	75 20 s[e]	75 17 s	75 21	75 17	75 17	75 17	75 20	75 19
	74 84	74 99	74 85 s	74 82 s	74 81 s	74 81 s	71 82 s	
	73 76 s	73 74 s	73 75 s	73 74 s	73 73 s	73 73	73 75 s	
	73 55 s	73 52 s	73 54 s	73 48 s	73 50 s	73 50	73 50	
C	73 18	73 15	73 19	73 18	73 18	73 18	73 19	73 20
D	72 04	72 00	72 02	72 01	72 00	72 00	72 01	72 01
E	71 51	71 45	71 59	71 58	71 58	71 59	71 60	71 62
F	67 83	67 80	67 82	67 80	67 80	67 80	67 80	67 80
	62 49	62 47	62 49	62 46	62 45	62 46	62 46	

[a]The chemical shift is given in p p m relative to tetramethylsilane [b]The spectra for dextrans B-1299 fraction L and B-1299 fraction S are essentially identical [c]This spectrum and that of dextran B-1402 (not shown) are essentially identical [d]The letters A through F designate the major resonances of linear dextran (see ref 2) [e]The symbol s designates a shoulder on the major peak

TABLE III

SPECIFIC ASSIGNMENT OF ^{13}C-N M R RESONANCES[a] [b] IN SPECTRA, AT 90°, OF DEXTRANS CONTAINING 2,6-DI-*O*-α-D-GLUCOSYLATED α-D-GLUCOPYRANOSYL RESIDUES

Range in chemical shift for dextrans	*Typical resonance shift (p p m)*	*Specific assignment of resonance*		
		Carbon atom		*Type of residue*
		Number	*Anomeric form*	
99 59–99 51	99 59	1	α	chain-extending
98 22–98 19	98 22	1	α	branch-terminating
97 37–97 30	97 37	1	α	branch point
77 83–77 74 75 20–75 17 74 99–74 81 73 76–73 73 73 55–74 48 73 19–73 15 72 04–72 00	77 83	2		branch point
71 60–71 45	71 6	4		(1→6)-linked pyranosidic, of linear-type chain
67 83–67 80	67 8	6, linked		both chain-extending and branch-point types
62 49–62 45	62 5	6, free		branch-terminating

[a]The chemical shift is given in p p m relative to tetramethylsilane [b]Taken from Table II

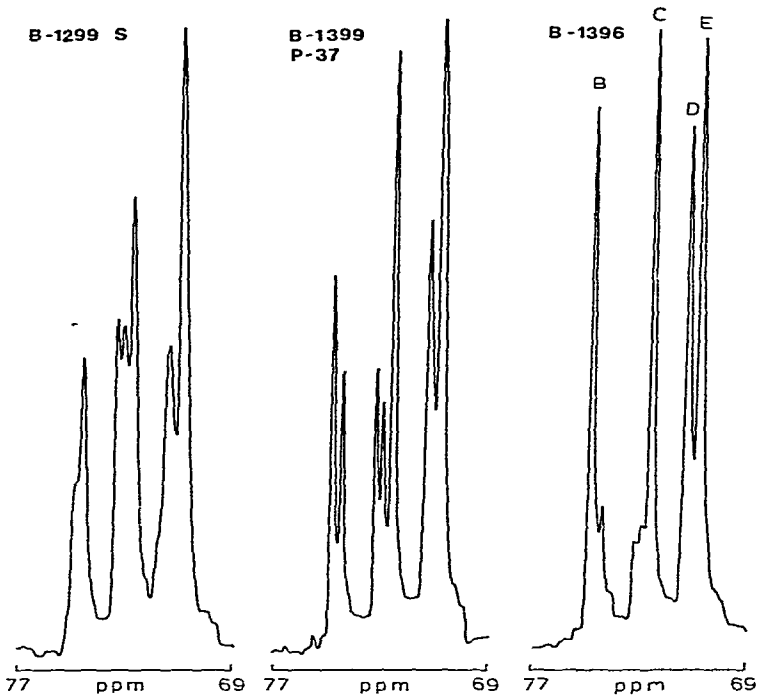


Fig 2 ^{13}C-N m r spectra of the expanded 69–77-p p m region of the data presented in Fig 1, for dextran B-1299 fraction S, dextran B-1399 (P-37), and dextran B-1396 The letters *B* through *D* refer to major resonances of linear-type dextran (as shown in Fig 1 for the linear-type, control dextran B-640)

series, the members of which are alike in having the same (six) spectral lines of the linear-type dextran control B-640, plus seven additional lines These type spectra differ distinctively, however, in the relative intensities (peak heights) of the lines The order of arrangement of these spectra is that of increasing height of peak A, which, in dextran B-1396, reaches a value near that of the linear-type dextran control B-640

The range of chemical shifts in the resonances of the six dextrans, from Table II, are summarized in Table III, and correlated with the specific assignment of the resonance

The narrow range of the values of chemical shift for each resonance in this series of spectra is evident throughout the entire group (see first column, Table III) Detailed inspection of the closely packed resonances in the 69–77-p p m region is afforded by the expanded spectra shown in Fig 2 for three representative dextrans in this series

The anomeric resonances and their assignments — Three resonances are shown in the anomeric region of each spectrum in Fig 1, and are the first listed in Table III The relative intensity of peak A (99 59 p p m) increases steadily for the following series of dextrans B-1399 fraction S, B-1399 (P-37), B-1424, B-1422, and B-1396 This series is in the order of increasing linearity, as is shown in Tables IV and V,

TABLE IV

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

NRRL strain[a]	*Dextran fraction*	*Methyl ethers of* D-*glucose*				
		2,3,4,6	*2,3,4*	*2,4,6*	*2,4*	*3,4*
B-1299[b]	S	39 1	26 0			34 9
B-1299[c]	L	34 0	32 0	5 9	1 3	26 8
B-1399[c]	S	30 8	34 3	1 0	2 7	31 2
B-1399[b]	L	12 8	74 5		5 9	6 8
B-1399[c]	P-37	24 8	48 1	0 5	3 1	23 5
B-1402[c]		25 3[c]	50 1		2 6	22 0
B-1424		24 5	51 9		3 0	20 6
B-1422		20 1	60 0		1 0	18 9
B-1396		9 8	77 8		3 3	9 1

[a]All dextran-producing organisms were *Leuconostoc mesenteroides* [b]Methylation analyses are from ref 3 [c]Methylation analyses are from ref 4

TABLE V

DEXTRANS BRANCHED THROUGH 2,6-DI-*O*-SUBSTITUTED α-D-GLUCOPYRANOSYL RESIDUES DEGREE OF LINEARITY CALCULATED FROM ^{13}C-NMR SPECTRA, AT 90°, AND FROM GLC–MS PERMETHYLATION ANALYSIS DATA

NRRL strain[a]	*Dextran fraction*	^{13}*C-nmr, anomeric-resonance ratio*[b]	n *(calculated from* ^{13}*C-nmr data*[c]*)*	n *(calculated from glc–ms data*[d]*)*
B-1299	S	0 58	0 87	0 67
B-1299	L	0 64	0 96	0 94
B-1399	S	0 55	0 83	0 91
B-1399	P-37	1 33	2 0	1 94
B-1402		1 34	2 0	1 98
B-1424		2 38	3 6	—
B-1422		3 05	4 6	3 0
B-1399	L	5 27	7 9	5 8
B-1396		7 10	10 7	7 9
B-640		—	—[e]	~23

[a]All dextran-producing organisms were *Leuconostoc mesenteroides*. [b]The peak height of the resonance at 99 6 p p m divided by the peak height of the resonance at 98 2 p p m [c]This column contains the values of column 3 upon normalization to $n = 2\,0$ for dextrans B-1399 (P-37) and B-1402 [d]The amount of 2,3,4-tri-*O*-methyl derivative divided by the amount of 2 3,4,6-tetra-*O*-methyl derivative, data taken from Table IV [e]Methylation structural analysis shows ~4% of branching residues in this dextran, a proportion that is below the limit of detection in these nmr spectra (see Tables II and III)

and, as will be discussed later, the peak A resonance must be that of C-1 of linearly (1→6)-linked residues This assignment accords with the presence of this resonance in the spectrum of the linear-type dextran B-640, which is shown in Fig 1 and Tables I and II for comparison Methylation structural analysis shows that 96% of the α-D-glucosyl residues of dextran B-640 are linked solely by (1→6)-linkages (refer to

Table V) It is probable that the 98 2-p p m resonance represents C-1 of the terminal residue, and the 97.3-p p m resonance represents C-1 of the branch-point residue These assignments are based on the following logic firstly, there must be an exact one-to-one correspondence of branch-point to terminal residues, secondly, carbon atoms with greater mobility have narrower and more intense resonances, thirdly, terminal side-chain residues can be expected to have greater mobility than branch-point residues, which are incorporated into the dextran back-bone Therefore, the more intense, 98 2-p p m resonance represents C-1 of the terminal residue, and the less intense, 97 3-p p m resonance represents C-1 of the branch-point residue The 95–110-p p.m , ^{13}C-n m r -spectral region clearly shows only the presence of α-anomeric carbon atoms (whose resonances are found in the 95–102-p p m region) and no β-linkages (whose resonances are found down-field from 102 p p m)

The ratio of the intensities (peak heights) of the anomeric resonances at 98 22 p p m and 97 37 p p m in the spectra of the various dextrans is essentially constant (or approximately unity) throughout this series of dextrans, as would be expected for the ratio of the branch-terminating residue to the branch-point residue The ratio of the intensities of the resonances at 99 59 p p m 98 22 p p m (or 99 59 p p m 97.37 p p m) is specific for each dextran, and, as it is a measure of the number of (1→6)-linked, interchain residues per branch point, it is a measure of linearity The increasing order of these ratios, shown in Table V, column 3, correlates with the order of the corresponding, spectral types in Fig 1 When calculated from methylation structural analysis, the ratio of chain-extending residues per branch-point residue is designated n (as is discussed in relation to structure A in the next section) For comparison, the n values [arbitrarily normalized to $n = 2$ for dextran B-1399 (P-37)] have been calculated (see Table V, column 4) from the ratios of intensities of the anomeric resonances The general agreement of these n values calculated from ^{13}C-n m r spectra with those calculated from the methylation–fragmentation analysis data, verify that the ^{13}C-n m r resonances provide a reliable estimate of the degree of linearity of a dextran or, inversely, the degree of branching

Other assigned and unassigned resonances — The 77 8-p p m resonance represents C-2 of the branch-point residue, and the 62 5-p p m resonance represents C-6 of the branch-terminating residue The chemical shift of the C-6 atom is relatively insensitive to structural changes, as, otherwise, the 67 8-p p m resonance would be split to represent signals of both chain-extender and branch-point types of D-glucosyl residues, an effect not observed In addition, peak E (71 6-p.p m , previously identified as the C-4 resonance of linear dextran[1]) is unaffected by branching at 2,6-di-*O*-substituted α-D-glucosyl residues These resonances, 71 6 p.p m and 67 8 p p m , appear in the spectra of the linear-type, control dextran B-640

Not all possible resonances are resolved For the structure A (see the next section), when $n = 1$ (*e g* , dextran B-1399 fraction S), there could be 6 × 3, or 18 resonances, but only 13 resonances are resolved Were only the spectra of dextran B-1299 fraction S and dextran B-1396 examined, it could be concluded that the spectrometer error was sufficiently great as to allow the 74 84-p p m (dextran B-1299

fraction S) and the 75 19-p p m (dextran B-1396) resonances to represent the same type of carbon atom However, this series of spectra, including intermediate ones, such as that of dextran B-1399 (P-37), clearly shows that resonances at 74 84 and 75 19 p p m. represent two different types of substituted carbon atoms

The 70–85-p p m , ^{13}C-n m r.-spectral region provides strong support for the exclusive presence of the D-glucopyranoid ring-form, as all major ring-carbon resonances (except the anomeric and the C-6 resonances) are in the 70–75-p.p m. region, which is diagnostic of the D-glucopyranoside ring The additional resonances in the 70–85-p p m. region are all attributable to linked carbon atoms of pyranoside rings, rather than to corresponding carbon atoms of D-furanosides, which can also be found in this region

The diagnostic, ^{13}C-n m r resonances — Of the resonances shown in Table III, those specifically unique to dextrans having 2,6-di-*O*-substituted α-D-glucopyranosyl residues are present Significantly absent from the resonances listed in Tables II and III are the 82 9–p p m and the 80 2-p p m resonances, which have been proposed as specifically diagnostic for 3,6-di-*O*-substituted and 4,6-di-*O*-substituted α-D-glucopyranosyl residues in D-glucans This hypothesis is verified in subsequent Parts of this series of articles[8]

The effect that the presence of unbranched, 2-*O*-substituted α-D-glucopyranosyl residues would contribute to a dextran spectrum is not certain At present, we have no evidence for the presence of this mono-*O*-substituted residue in any of the dextrans studied However, dextran-like biopolymers that contain both mono-3-*O*-substituted and 3,6-di-*O*-substituted D-glucosyl residues have similar, but distinctly different, diagnostic resonances[8]

Methylation structural analysis

Methylation–fragmentation analysis data currently available for dextrans branched at C-2 are assembled in Table IV Almost all of the hydrolyzates of methylated dextrans contained 2,4-di- and 2,4,6-tri-*O*-methyl derivatives of D-glucose[4] Because of the resistance of the 3-hydroxyl group of dextrans to methylation[4], it is difficult to establish whether the presence of ~3% or less of these partially methylated D-glucose derivatives indicates undermethylation, or structural features involving (1→3)-linkages With the exception of dextran B-1299 fraction L, which exhibited a weak resonance at 83 p p m (90° spectrum), no ^{13}C-n m r resonances associated with (1→3)-linkages were observed for these dextrans However, at the signal-to-noise level of these spectra, resonances from small proportions of (1→3)-linked D-glucosyl residues would not be observable. The data in Table IV indicate that all of the α-D-(1→2)-linkages are associated with 2,6-di-*O*-substituted D-glucosyl residues at branch points.

The dextrans of this series may be considered in three general groups, which differ in the degree of branching as indicated by the mole percentages of 2 3,4,6-tetramethyl ethers and the corresponding 3,4-dimethyl ethers (see Table IV) The three dextrans of the first group, B-1299 fractions S and L and B-1399 fraction S,

are outstanding for their high degree of branching, and all give similar ^{13}C-n m r spectra The data, however, show each to have individual structural details that resulted in their being chosen as spectrally typical (see Fig 1, and Tables I and II) The second group, having intermediate degrees of branching, consists of the very closely similar dextrans B-1399 (P-37) and B-1402, and the apparently somewhat different dextran B-1424 The dextrans of the third group, B-1422, B-1399 fraction L, and B-1396, differ distinctly in their decreasing degree of branching The structural details for the last two groups, like those for the first group, accord with the spectral observations

The ratio of the molar percentages of the 2,3,4-tri- to the corresponding 2,3,4,6-tetra-methyl ether indicates the average number of α-D-(1→6)-linked D-glucopyranosyl residues per branch in the dextran This constant, which is designated *n*, and is tabulated for the dextrans of this series (see Table V, column 5), reveals rather well the group similarities of, and individual differences between, the dextrans

For convenience of comparison of the dextrans (or other α-D-glucans) of this, as well as other, series of our dextrans[1-3], a generalized repeating unit (A) is used,

$$-[(1\rightarrow6)\text{-}\alpha\text{-G-}\{(1\rightarrow6)\text{-}\alpha\text{-G-}\}_n]_x\text{-}$$
$$\begin{matrix} & & 2 \\ & & \uparrow \\ \alpha\text{-G-}(1) & & \end{matrix} \qquad \text{A}$$

where G represents the D-glucopyranose or D-glucopyranosyl residue, and *n* represents the average number of α-D-(1→6)-linked D-glucopyranosyl residues in such a unit The $-\{(1\rightarrow6)\text{-}\alpha\text{-G-}\}_n$ sub-unit in structure A represents that part of the repeat unit corresponding to linear dextran As *n* becomes larger, the spectral and other properties of the dextran approach those of linear dextran Only for simplicity of comparison is the length of the side chain shown as one α-D-glucopyranosyl group The actual distribution of (1→6)-linked residues between the backbone chain and linear positions in side chains is very difficult to establish, and reliable information is available for only two dextrans that branch through C-2 In dextran B-1299 fraction S, branches appear to consist mainly, but possibly not exclusively, of single α-D-glucopyranosyl groups[10] and, in dextran B-1397, branch lengths of one residue, and more than two residues, occur[11] Among the few other dextrans studied[9], branches one residue long constitute the majority in one, but less than half in another

Observations from ^{13}C-n m r spectroscopy add tenuous support to the hypothesis that the (1→6)-linked α-D-glucopyranosyl residues of dextran may form a backbone that is comb-like rather than dendritic (*a*) values of ^{13}C-n m r resonance relaxation suggest that all of the (1→6)-linked residues are in a backbone chain[12], and (*b*) the narrow peak-widths of the ^{13}C-n m r resonances of dextrans are similar to those of comb-like synthetic amylose, and differ from the broader resonances of dendritic glycogen[13] Evidence for three possible structural forms of dextrans has been reviewed[9]

There is no information on the uniformity of branch distribution in the dextrans

reported by us, either here or previously[1 3] Of the few studies that have been made of such branch distribution[9], several have employed the linear-type dextran from NRRL B-512(F) and dextranases that have specificity for α-D-(1→6)-linkages[14,15]. The availability of other (1→6)-α-D-dextranases having different action-patterns[16] now permits extension of such studies to other structural types of dextrans

Judging by the criteria available, the dextrans we have employed in this and preceding investigations[1 3] appear to consist of structurally individual species Ten of these dextrans constitute the fraction pairs (S and L) from five NRRL strains In the pairs from strains B-742, B-1254, and B-1355, the signal for the type of non-(1→6)-linkage that is prominent in one member of the pair does not appear in the ^{13}C-n m r spectrum of the other member, or in significantly interpretable proportion among the methylation products of the other member This essential absence of carry-over between the B-742 fraction pairs is also shown in enzymic hydrolyzates[16], which constitute a more sensitive test than the methylation products The seven unfractionated dextrans used in our investigations gave no indication of structural heterogeneity when tested by our analytical fractionation procedure[6] Fractionation might, however, be accomplished by more-refined procedures should they be effective on closely related macromolecules

The concurrent biosynthesis of at least two, distinctly different, dextran products by sucrose-grown cultures of NRRL strains B-1299 has been elucidated by Brooker's application[17] of electron microscopy and related techniques to studying the ultra-structural, surface changes in the growing cells

The dextrans used in this research, from strains B-1299 (refs 1, 3, and 9), B-1399 (P-37) (refs 3 and 4), and B-1424 (refs 9 and 18), have been the subjects of much previous research This research is cited in the references indicated, and in the following sections of this article The dextrans from strains B-1396, B-1399, B-1402, and B-1422 have received less attention The close, structural similarity of dextran B-1402 to dextran B-1399 (P-37) is unusually significant for several reasons First, strain B-1402 serves to replace[4] B-1399 (P-37), which produced a homogeneous dextran, but was "lost" through genetic change to strain B-1399, which produces a heterogeneous dextran[3], and second, the existence of dextrans B-1399 (P-37) and B-1402, which have essentially the same molecular structures and yet are synthesized by strains of unrelated natural origin, indicates that each is a true structural entity, formed in accordance with certain biosynthetic principles and not a fortuitous mixture of different molecular forms

STRUCTURAL DATA PREVIOUSLY REPORTED ON DEXTRANS

Structural investigations extending over the past three decades on the dextrans from the NRRL collection[5], have gradually established proof that the rare, α-D-(1→2)-glucopyranosyl linkage occurs in a number of the dextrans The resultant information has usually been expressed in terms either of the type of linkages present, or the constituent α-D-glucopyranosyl residues Each of the structural analyses that

has been applied to the dextrans has contributed somewhat different information Periodate-oxidation analysis gives the percentage of linkages present, but no specific information related to branching Acetolysis provides information on branch-point linkages (semi-quantitative) and anomeric form Optical rotational shift data in cuprammonium solutions identified and quantitated the (1→2)-linkages in certain dextrans Methylation structural analysis and ^{13}C-n m r spectroscopy emphasize the component residues of the dextran as whole entities, *i e*, the interchain residues in the main backbone and those positioned at branch points and nonreducing ends Methylation structural analysis is distinctive in the quantitative precision of its results, ^{13}C-n m r spectroscopy has the advantage of also giving direct evidence on the anomeric configuration Viewing dextran structure on the basis of entire residues, rather than mere linkages, fosters correlation with spatial and conformational concepts

The relationship between ^{13}C-n m r spectroscopy and polysaccharide structure is as yet only partially developed, and necessitates a semi-empirical approach to the subject The large series of dextrans, which comprises several structural classes[5], is therefore of unusual value in the study of ^{13}C-n m r spectroscopy The varied and extensive structural evidence already available on these dextrans (see refs 9 and 18 for citation of original research publications) provides a substantial basis for interpreting the ^{13}C-n m r spectra of these polysaccharides and for establishing general rules governing this spectral–structural relationship It is pertinent, therefore, to evaluate the experimental methods by which these data have been obtained Each of the methods enumerated in the preceding paragraph is considered in turn, and, in addition, certain immunochemical procedures are also considered Furthermore, contributions that ^{13}C-n m r spectroscopy may make to the further development of, and progress with, these methods is pointed out

Configuration of anomeric linkages — Evidence that the linkages of *Leuconostoc* dextrans are exclusively of the α-anomeric form is abundant and consistent The ultimate proof[19] is that oligosaccharides resulting from enzymolysis[20], hydrolysis[21,22], acetolysis[23,24], and methanolysis[25] have exclusively α-D-linked residues From a dextran having a low degree of branching, isomaltose and homologous series of isomalto-oligosaccharides have been isolated from enzymic[15,16,20] and acid[21,22] hydrolyzates and methanolyzates[25] From branched dextrans, kojibiose, nigerose, and maltose have been isolated in good yields after acetolysis[23] Concordant evidence comes from the specificity of action of dextransucrases and dextranases[9,18] To these various sources of evidence, ^{13}C-n m r spectroscopy adds a new principle of anomeric determination

Periodate oxidation — Jeanes *et al*[26,27] adapted the newly developed technique of analytical, periodate oxidation of carbohydrates to dextrans, and used the method to quantitate three categories of linkage in a large number of dextrans in a relatively short time[5] The alternative method then available was methylation structural analysis which, until the recent introduction of extensive improvements[3,4,28], was impracticably slow and tedious

The structural basis for differential oxidation by periodate ion is the presence in dextrans of contiguous, unsubstituted hydroxyl groups in the D-glucopyranosyl residues, a condition that is determined by which of the hydroxyl groups, on C-2, C-3, or C-4, is glycosidically bound All OH-1 groups, exclusive of that at the reducing end, are involved in D-glucosidic bonds Thus, residues having three hydroxyl groups contiguous are linked only through C-1, or through both C-1 and C-6, one mol of such a residue reduces two mols of periodate and liberates one mol of formic acid Residues having two free hydroxyl groups contiguous reduce one mol of periodate per mol, and can be linked at either C-2 or C-4 Residues having no free hydroxyl groups contiguous do not react, it is not determinable by this method whether a single position, O-3, is bound, or two, namely, O-2 and O-4, are bound For the second and third of these major, structural categories, O-6 may or may not be substituted Thus, on the basis of periodate-oxidation analysis, the proportion of non-(1→6)-linkages cannot be equated with the degree of branching In view of adequate explanation of these principles[27], it was originally convenient to express the data in terms of the linkages found, that is, (1→6)-like, (1→4)-like, and (1→3)-like, as this terminology provided a common basis for correlating various data obtained with these dextrans However, for interpretation of specialized research findings, individual consideration might reasonably have been given to the role of factors other than linkages *per se*, such as steric conformation, and hydrodynamic and biocolloidal properties Through numerous research approaches other than periodate oxidation, more-specific information has gradually been acquired on the identity of the non-(1→6)-linkages and on some details of branching

For the sixteen dextrans for which we have now published the results of methylation structural analysis, comparison shows that the accuracy of the periodate-oxidation values for (1→6)-like linkages is generally within $\pm 10\%$, and for a number of dextrans, it is within $\sim \pm 5\%$ Agreement is usually best for dextrans having 90% or more of these linkages [*i e*, dextrans B-512(F) (ref 29), B-640, B-1308 (ref 3), and B-1254 fraction L[S] (refs 1 and 4)] For dextrans in which the (1→6)-like linkages are $< \sim 90\%$ and the major other linkage is (1→2) or (1→4), the periodate values for the (1→6)-like linkages are lower (usually, 5–10%) than the methylation values, and the percentage of (1→2)- or (1→4)-linkages, or both, is correspondingly higher[30] This deficiency in the periodate-oxidation, structural analysis is apparently caused by involvement of the dialdehyde formed by the first bond-cleavage of the 2,3,4-triol in a cyclic hemiacetal, this prevents the second cleavage that is necessary to liberation of a molar proportion of formic acid[31] This mechanism, which has previously been shown to be applicable to several, linear-type dextrans[31 32], now appears to operate for all of our dextrans in which branching through O-2 or O-4 has been established[30]

For dextrans B-742 fraction S and B-1355 fraction S, which have high contents of (1→3)-like linkages[1 5], comparison shows that the periodate-oxidation values are high for both the (1→4)-like and (1→6)-like linkages, and low for the (1→3)-like linkages[30]. The presence of the (1→3)-like linkages appears to cause excessive re-

duction of periodate The mechanism is unknown, but it differs from that generally designated[30] "over-oxidation"

Optical rotation of cuprammonium complexes — Scott *et al*[33] applied to the structural analysis of dextrans a previously established principle that great differences in optical rotation were determined by whether the 2,3- or the 3,4-glycol grouping of the D-glucopyranosyl ring in D-glucosides and D-glucans is free to complex with cuprammonium It was supposed that, through computation, the (1→4)-like linkages found by periodate oxidation analysis could be resolved into the presumed, component (1→2)- and (1→4)-linkages, but the objective was achieved only for measurement of the (1→2)-linkages For dextrans B-1299 fraction S and L and B-1399 (P-37), for which periodate oxidation showed a high content of (1→4)-like linkages, the (1→2)-linkages were found to be 38, 34, and 29%, respectively These values are all in good agreement with the corresponding results from methylation structural analysis (see Table IV) Dextrans from seven other strains examined, including the two fractions from strain B-742, did not show any (1→2)-linkages, and other structural procedures have confirmed this absence

The values calculated for (1→4)-linkages are invalid, because of the dependence of the calculations on (*a*) periodate-oxidation values for (1→4)-like linkages that are now known to be erroneous[30], and (*b*) coefficients derived mostly from monosaccharide standards, rather than from dextrans of known structure[33] Structural information now available on these and other dextrans could well permit the calculation of more-dependable coefficients.

The proof (for the first time), from rotational-shift data in cuprammonium, of the presence of (1→2)-linkages in dextrans was a singular accomplishment, and it made possible the correlation of important immunological observations (see the following section on structural indications from immunology) The value of the optical-rotatory analysis of cuprammonium complexes for structural analysis of polysaccharides is further attested to by other, unique evidence obtained[33] Thus, in dextrans B-1355 fraction S and B-1149 (for which periodate-oxidation analysis showed 35 and 40% of (1→3)-linkages, respectively), ~50% and 70% of these respective linkages were indicated to be in linear portions of the dextran chain, rather than at branch points, this prediction has been confirmed[3] for dextran B-1355 fraction S and accords with the "anomalous" i r absorption spectra[5] of the refractory dextran B-1149 Other α-D-glucans are now known that, like "dextran" B-1149, have contiguous D-glucosyl residues linked through (1→3)-linkages and are characteristically difficult to dissolve One such polysaccharide is a fraction of[24] dextran B-1299, and another, from *Streptococcus mutans*, was the first recognized member of the proposed, mutan class of α-D-glucans[34].

Acetolysis fragmentation analysis — Acetolysis is the simplest, most sensitive, and most generally applicable method for identification and semi-quantitation of the non-(1→6)-linkages in dextrans These linkages are relatively stable to the reagent, and are found in the acetolyzate in good yield as the corresponding acetylated disaccharides The (1→6)-linkage is, however, more labile, and the low yields of

isomaltose (6-*O*-α-D-glucopyranosyl-D-glucopyranose) are not related to the actual proportion of this linkage in the dextran The method has most frequently been applied to dextrans having (1→2)-linkages, namely dextran B-1299 and its fractions[10,24 35 36], dextran B-1424 (ref 37), dextran B-1397 (ref 11), and dextran B-1298 (ref 38) Suzuki and Hehre[23] established a semimicro technique, defined its limits of detection of disaccharide as 0 1% of the dextran, obtained an average recovery of disaccharide of 56 4% of the dextran, and reported their findings on 16 dextrans (12 from the NRRL collection) Considerable proportions of kojibiose (2 5–19.6%) or maltose (1 0–5 7%) were obtained from some dextrans, but a few showed only small proportions of these disaccharides (0 1–0 3% and 0 5%, respectively), and others showed none Nigerose was found for all of the dextrans studied, in proportions in the range of 0 1–6 4%, and it was shown not to be an artifact No β-D-glucobioses were found Through this study[23], the structural basis was established for differentiation of two serotypes of dextran, these serotypes were initially defined by a highly sensitive and specific, serological analysis that focused attention on several unique dextrans that are among those now known to have (1→2)-linkages (see ref 3, Table IV, and the following section)

Structural indications from immunology — One of the initial investigations on the NRRL dextrans divided them into two serotypes on the basis of whether they did or did not cross-react with type XII pneumococcal antiserum (serotypes A and B, respectively)[39] (see also, ref 23 for the origins of this differentiation, and the dextrans initially observed) Dextrans from ~100 strains were tested 15 of the dextrans gave a strongly positive reaction, and seven reacted weakly The finding of (1→2)-linkages in one of the most reactive of these serotype-A dextrans (dextran B-1299 fraction S)[33] supported the hypothesis that this type of linkage is the distinctive, structural feature of all serotype-A dextrans, and provided a criterion for selecting dextrans for other studies The correctness of this assumption was first confirmed by acetolysis studies[23], and, more recently, by methylation structural analysis (see ref 3 and citations therein, and also ref 9) All of the dextrans shown here (see Tables IV and V), and previously by us[3 4], to contain 2,6-di-*O*-substituted α-D-glucopyranosyl residues, are strongly reacting, serotype-A dextrans Serotype dextrans that precipitate strongly with type XII pneumococcal antiserum also show specific, vigorous precipitation with the jack-bean globulin[39] (concanavalin A)

A further, early indication of the involvement of (1→2)-linkages in serotype-A activity was shown for dextrans B-1299 fractions S and L, and B-1399 (P-37) These dextrans were found to be unusually effective quantitatively in precipitation of antibody N from type XII pneumococcal antisera, and kojibiose was the best inhibitor[40] of this cross-reaction of dextran B-1299 fraction S Another serotype-A dextran, B-1424 (see Table IV), acted equivalently to the dextrans just named in precipitin-type and quantitative, inhibition-type studies[41] Furthermore, the serotype-A dextrans gave strong, precipitin reactions with antisera to dextran[42] B-1299 fraction S and to dextran[41] B-1424 These reactions with antisera having specificity for α-D-(1→2)-glucopyranosidic linkages were also inhibited most effectively by kojibiose[40,41].

Dextrans B-1299 fraction S, B-1399 (P-37), and B-1424 also gave precipitin reactions with antisera having specificity for α-D-(1→6)-linkages[43] A plot of the precipitin activity of these three dextrans, and a wide variety of others, with the antisera *versus* the proportion of 1-linked groups and (1→6)-linked residues (as indicated by the original, periodate-oxidation analyses) in the dextrans, showed that all points fell on a smooth curve, except those of dextrans B-1299 fraction S and B-742 fraction L (see Fig 2 in ref 43) If the proportions of structural units are taken from the corrected, periodate-oxidation values (see preceding section and ref 30) or from the results of methylation structural analysis (see Table IV), dextran B-1299 fraction S becomes coincident with the curve, dextran B-742 fraction L is displaced still farther, and dextrans B-1399 (P-37) and B-1424, which showed little activity, are not changed significantly The values (molar percentages) from methylation structural analysis used for this comparison are the sum of the (1→6)-linked residues and either the 1-linked (nonreducing) end-groups, or their numerical equivalent, namely, the branch-point residues, which, for dextran B-1299 fraction S are 2,6-di-*O*-substituted, and for dextran B-742 fraction L, are 4,6-di-*O*-substituted No correlation results if the proportion plotted is that of the 1- or the (1→6)-linked residues from Table IV Apparently, factors other than linkages *per se* are involved Obviously, there is a need to resolve the individual roles in the precipitin reaction of the nonreducing end-groups, the branch-point residues, and the linearly (1→6)-linked residues

The original observations were interpreted[9 42 43] in terms of the general concept that the major (if not total) activity occurs in the terminal sequences of branch ends, the lengths of which are assumed to be related to the combining dimensions of the antidextran (see subsequent publications by these authors as listed in ref 18, and also a review[9]) The combining site of these antidextrans is believed to be complementary to an open chain of at least three, and probably as many as six, 6-*O*-substituted α-D-glucopyranosyl residues[43]. No data are yet available, however, on the length of branches in the dextrans under discussion here, and only for dextran B-1424 is there an indication of possible sequences of as many as three 6-mono-*O*-substituted α-D-glucopyranosyl residues (see Tables IV and V) Nevertheless, there are tentative indications, from ^{13}C-n m r spectroscopy and various other data[9], that the majority of branches in many dextrans are only one unit long For the dextrans we have reported on, serotype-A as well as others[1 3], proof is given for the first time of the presence and frequency of branching Also, for all these dextrans, *n* values have been established (see Table V and refs 1 and 3) that indicate the average extent of α-D-(1→6)-linked interbranch residues in linear sequences, no matter whether these lie in main, backbone chains, or in a few, unusually long, side chains Examination of immunochemical precipitin- and inhibition-type data on the NRRL dextrans in terms of these experimentally established parameters may be expected to open up new perspectives

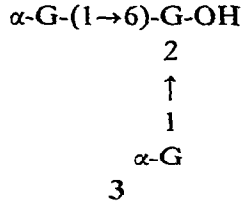
In addition to the influence of specific, structural parameters on the immunochemical behavior of dextrans, consideration must be given to spatial effects that

extend beyond the confines of a glycosidic bond to include at least the area of one or more substituted D-glucosyl residues ^{13}C-N m r.-resonance displacements show that introduction of a new linkage into an α-D-glucopyranosyl residue can alter the conformation of the residue. An example involves the inhibitory role of kojibiose on the precipitin-type reactions of the dextrans that are here shown to have, exclusively, 2,6-di-*O*-substituted α-D-glucopyranosyl residues at the branch-points Methylation structural analysis and ^{13}C-n m r spectroscopy give no evidence of the simple kojibiosyl linkage in unbranched, 2-*O*-substituted α-D-glucopyranosyl residues in any of these dextrans examined. Results of other structural investigations on serotype-A dextrans are concordant[1 3 9] with these findings Thus, the true recognition-site for antisera to these dextrans, and also to pneumococcus type XII, must involve this 2,6-di-*O*-substituted α-D-glucopyranosyl residue

Initially, kojibiose was the only saccharide containing the α-D-(1→2)-linkage that was available for inhibition-type studies Later, three trisaccharides containing this rare linkage were obtained[11] by acetolysis of serotype-A dextran B-1397, these are· *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**1**); *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**2**), and *O*-α-D-glucopyranosyl-(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose (**3**)·

α-G-(1→2)-α-G-(1→6)-G-OH **1**

α-G-(1→6)-α-G-(1→2)-G-OH **2**



where G represents the D-glucopyranosyl or D-glucose residue, and OH, the reducing end Of these, the linear structure **1** best satisfied the combining-site requirements of the type XII pneumococcal antisera and of human antisera to dextrans B-1299 fractions S and L and B-1424 (refs 40 and 41) Of the other trisaccharides, **3** qualified as intermediate and **2** as poor, for combining-site activity Thus, only **1**, with its 2-*O*-substituted α-D-glucopyranosyl residue shows a spatial resemblance to the apparent, critical recognition-site of a 2,6-di-*O*-substituted α-D-glucopyranosyl residue In contrast, **3** has a 2,6-di-*O*-substituted D-*glucose* residue, and structure **2** has a 2-mono-*O*-substituted D-*glucose* residue. Both of these D-glucose residues are capable of assuming various cyclic forms and conformations

Another example of an immune response that is directed more towards a specifically linked residue rather than to a linkage, involves dextran B-1355 fraction S (which contains both 3,6-di-*O*-substituted and 3-mono-*O*-substituted α-D-glucopyranosyl residues[3]) and dextran B-742 fraction S (which has 3,6-di-*O*-substituted α-D-glucopyranosyl residues exclusively[1]) These residues have been shown to have distinctly different, immunochemical responses[44]

EXPERIMENTAL

The preparation and characterization of the dextrans[5] and dextran fractions[6] and the ^{13}C-n m r conditions and methods of preparing dextran samples[1,2] have been described In general, a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 system was employed in the Fourier-transform mode The dextran samples, ~0 3 g per 4 mL of D_2O, were maintained at 34° or at 90° Chemical shifts are expressed in p p m relative to external tetramethylsilane, but were actually calculated by reference to the lock signal

The g l c –m s conditions employed for the separation of the PAAN derivatives of the methyl ethers of D-glucose have been described[3,4] In general, for mass-spectral confirmation of the g l c peaks, a Hewlett–Packard 5980 GC/MS 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

ACKNOWLEDGMENTS

This work was supported, in part, by a Robert A Welch Foundation Grant (Q294), a National Science Foundation Grant (BMS-74-10433), and National Institutes of Health Grants (HL-17269 and HL-17372)

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