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HIGH-TEMPERATURE ENHANCEMENT OF ^{13}C-N.M.R. CHEMICAL-SHIFTS OF UNUSUAL DEXTRANS, AND CORRELATION WITH METHYLATION STRUCTURAL ANALYSIS*

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ABSTRACT

Dextran fractions from NRRL strains *Leuconostoc mesenteroides* B-742, B-1299, B-1355, and *Streptobacterium dextranicum* B-1254 were examined by ^{13}C-n.m.r. spectroscopy at 34 and 90°, and by methylation structural analysis. The native, structurally homogeneous dextran from *L. mesenteroides* NRRL B-1402 was also examined. The data allow correlations to be made between the structure and physical properties of the S (soluble) and L (less-soluble) fraction pairs of dextrans B-742, B-1254, B-1299, and B-1355. For the dextrans under consideration here, increasing solubility of the dextran (both in water and in aqueous ethanol) was found to correlate with decreasing percentages of α-D-(1→6)-linked D-glucopyranosyl residues. Both the diagnostic nature of the 70–75-p.p.m. spectral region with regard to type of dextran branching, and the increase in resolution of the polysaccharide spectra at higher temperatures, have been further confirmed.

INTRODUCTION

We have previously reported the recording and analysis of the ^{13}C-n.m.r. spectra of several unusual examples of the dextran class of D-glucans[2]. Correlation of those spectra with data from methylation analysis indicated that the 75–85-p.p.m.

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

spectral region is diagnostic for establishing the presence of α-D-(1→2)-, α-D-(1→3)-, and α-D-(1→4)-linkages. Also, each carbohydrate chemical-shift was found to be temperature-dependent ($\Delta\delta/\Delta T$) and of positive sign when referenced to either the deuterium lock or an external tetramethylsilane standard. The data reported in this paper are in accord with our previous observations. Structural data, based on methylation analysis by combined g.l.c.–m.s. of the peracetylated aldononitriles[1,3], of nine dextrans are summarized in Table I.

The dextrans were produced by the following bacteria, designated by the strain number in the ARS Culture Collection at the Northern Regional Research Center: *Leuconostoc mesenteroides* NRRL B-742, B-1299, B-1355, and B-1402, and *Streptobacterium dextranicum* B-1254. The native dextrans from strains B-742, B-1254, B-1299, and B-1355 are polydisperse[4,5], and have been fractionated with ethanol[5], to obtain the major, essentially homogeneous fractions designated L (less-soluble) and S (soluble). The designation of the fractions indicates the relative order of precipitation from an aqueous solution of the native dextran by graded addition of ethanol[5], that is, L fractions are precipitated at concentrations of ethanol lower than those for S fractions.

Presented here are the properties of the L-type fractions from strains B-1254, B-1299, and B-1355; data on the corresponding S-type fractions have been reported[2,3]. The results of methylation structural analysis of the S-type fractions, and of the S and L fractions of dextran B-742, are included in Table I for comparison[1,3]. These data, combined in Table I, and the ^{13}C-n.m.r. analyses reported here and previously[2], now complete the structural information on these four unusual pairs of dextran fractions.

Previous experience had indicated that, in the ^{13}C-n.m.r. spectra of carbohydrates, $\Delta\delta/\Delta T$ values are linear within the error of the spectrometer[2]. Therefore, the ^{13}C-n.m.r. spectra of the currently reported dextrans were all acquired at 34 and at 90°. Due to lack of specific correlation of ^{13}C-n.m.r.-spectral peak-height with the amount of a given carbon species present, no great emphasis is placed on moderate differences in the intensities of the resonances. For carbohydrates, however, it has been found that peak height is, *in general*, proportional to the abundance of a particular carbon species present[6]. Consequently, we have designated the chemical shifts as major (M), or minor (m), as an aid to structural correlation and for convenience in referencing the data between the Tables and the Figures.

RESULTS AND DISCUSSION

The data reported here, when combined with those previously published[1–3], complete the structural analysis of the S- and L-types of dextran fractions from four bacterial strains. The combined data from methylation structure analysis of these four pairs of fractions are shown in Table I, along with data on a dextran (strain NRRL B-1402) that is structurally homogeneous in the native form. This dextran, and three others previously reported (B-1299 fractions S and L, and B-1399 fraction

TABLE I

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

Organism	*Data from ref no*	*NRRL strain*	*Dextran fraction*	*Methyl ethers of* D-*glucose*						
				2,3,4,6	2,3,4	2,3,6	2,4,6	2,3	2,4	3,4
Streptobacterium dextranicum	3	B-1254	S[L]	22 1	55 0	3 4		19 5		
	1	B-1254	L[S]	3 8	90 0		1 8		4 4	
Leuconostoc mesenteroides	3	B-1299	S	39.1	26 0					34.9
	1	B-1299	L	34 0	32 0		5.9		1.3	26 8
	3	B-1355	S	6 9	46 9		35.0		11 2	
	1	B-1355	L	3 3	91 5		1.5		3.7	
	1	B-742	S	45.4	4 4				50 2	
	1	B-742	L	14.4	72 5		0 7	12 4		
	1	B-1402		25.3	50.1				2 6	22 0

L)[2,3], constitute an unusual series in which branching is through the rare α-D-(1→2)-linkage.

The data show that, for three of the pairs of dextran fractions, the D-glucans differ radically in terms of the type of chain branching and the degree of linearity. For the members of the fourth pair, from NRRL B-1299, differences in structure appear to be minor, but, in their solubility, they are typical of the other pairs. In addition, the biopolymer fractions are remarkably free from cross contamination. For the B-742 and B-1355 pairs, the L-type fraction has the higher content of (1→6)-linked units, and a lower degree of branching, whereas the S-type fraction has the higher content of non-(1→6)-linkages and a higher degree of branching. However, the B-1254 dextran fractions (as originally designated) do not show this correlation, but they would conform were their designations reversed; the original classification gave more consideration to the concentration of ethanol effective in the preparative, fractionation procedure (although this differed only slightly for the two fractions) than to the structural differences of the fractions. As the striking, structural differences have been confirmed in the present study, the designation of the fractions has been reversed in order to show their correlation with the other fractions studied here. Henceforth, in this paper and subsequent articles, the fractions of B-1254 are named on the basis of the data presented here, followed by the old designation crossed-out with a solidus and bracketed. Thus, dextran B-1254 fraction L[S] has 90% of (1→6)-linkages[1] and dextran B-1254 fraction S(L) has 55% of these linkages[3].

The data also provide new examples of D-glucans having α-D-(1→2), α-D-(1→3)-, and α-D-(1→4)-linkages. This report and Part II (ref. 3) together provide data for a total of four α-D-(1→2)-, six α-D-(1→3)-, and two α-D-(1→4)-linked D-glucans, and further confirm the respective, diagnostic, 75–85-p.p.m.-spectral region

TABLE II

CHEMICAL SHIFTS, RELATIVE INTENSITIES, AND $\Delta\delta/\Delta T$ FOR ^{13}C-N.M.R. SPECTRA OF D-GLUCANS

	NRRL number of the organism producing the D-*glucan*						
	B-1254 Dextran fraction	*B-1299*	*B-1355*	*B-742*	*B-742*	*B-1402*	*Methyl α-*D-*glucopyranoside*
	L[*8*]	*L*	*L*	*S*	*L*		
	Linkage						
	α-(1→6)	*α-(1→2),α-(1→6)*	*α-(1→6)*	*α-(1→3),α-(1→6)*	*α-(1→4),α-(1→6)*	*α-(1→2),α-(1→6)*	
				100.33 (M, 1.11)[a,b]	100.97 (m, 1.20)[a]		100.13 (1.56)
					99.25 (m, 1.67)		
A[c]	98.65 (1.57)[d]	98.71 (1.57)	98 76 (1.43)	98.84 (M, 1.51)	98.66 (M, 1.67)	98.76 (1.75)	
		97.24 (1.82)				97.21 (2.00)	
		96.39 (1.75)				96.39 (1.90)	
				81.66 (m, 2.34)			
					79.46 (m, 1.81)		
		76.47 (2.43)				76.47 (2.70)	
B	74.35 (1.54)	74.30 (1.61)	74.45 (1.37)	74.39 (m, 1.48)	74.34 (M, 1.64)	74.36 (M, 1.67)	
		73.86 (1.89)		73.98 (M, 1.62)		73.85 (m, 2.00)	74.01 (1.62)
		72.77		72 80 (M, 1.49)		72.81 (m, 1.89)	
					72 77 (s, 1.57)[a]	72.68 (m, 1.74)	
C	72.34 (1.37)	72.34 (1.54)	72.46 (1.37)		72.39 (M, 1.61)	72.35 (M, 1.70)	72.46 (1.48)
				71.99 (m, 2.21)			72.13 (1.63)
D	71.14 (1.57)	71.17 (1.57)	71.25 (1.41)		71.18 (M, 1.64)	71.14 (M, 1.77)	
				71 08 (M, 1.41)			
				70.97 (s, 1.39)			
E	70.50 (2.05)	70.41 (1.96)	70 65 (1.82)	70.50 (M, 1.82)	70.51 (M, 2.16)	70.44 (M, 2.33)	70.50 (1.82)
F	66.72 (2.31)	66.61 (2.17)	66.72 (2.07)	66.54 (m, 2.18)	66.63 (M, 2.36)	66.84 (2.10)	
		61.38 (1.98)		61.50 (M, 1.90)	61.52 (m, 2.16)	61.41 (2.18)	61.52 (1.82)
							55.94 (1.32)

[a]The letter stands for a major (M) or a minor (m) peak, or a shoulder (s). [b]The number in parentheses stands for the Δ(p.p.m.)/ΔT ($\times$ 100) of the corresponding chemical-shift. [c]Letters A–F refer to major resonances observed for linear dextran (see ref. 2). [d]The first number is the chemical shift in p.p.m. relative to tetramethylsilane, recorded at 34°.

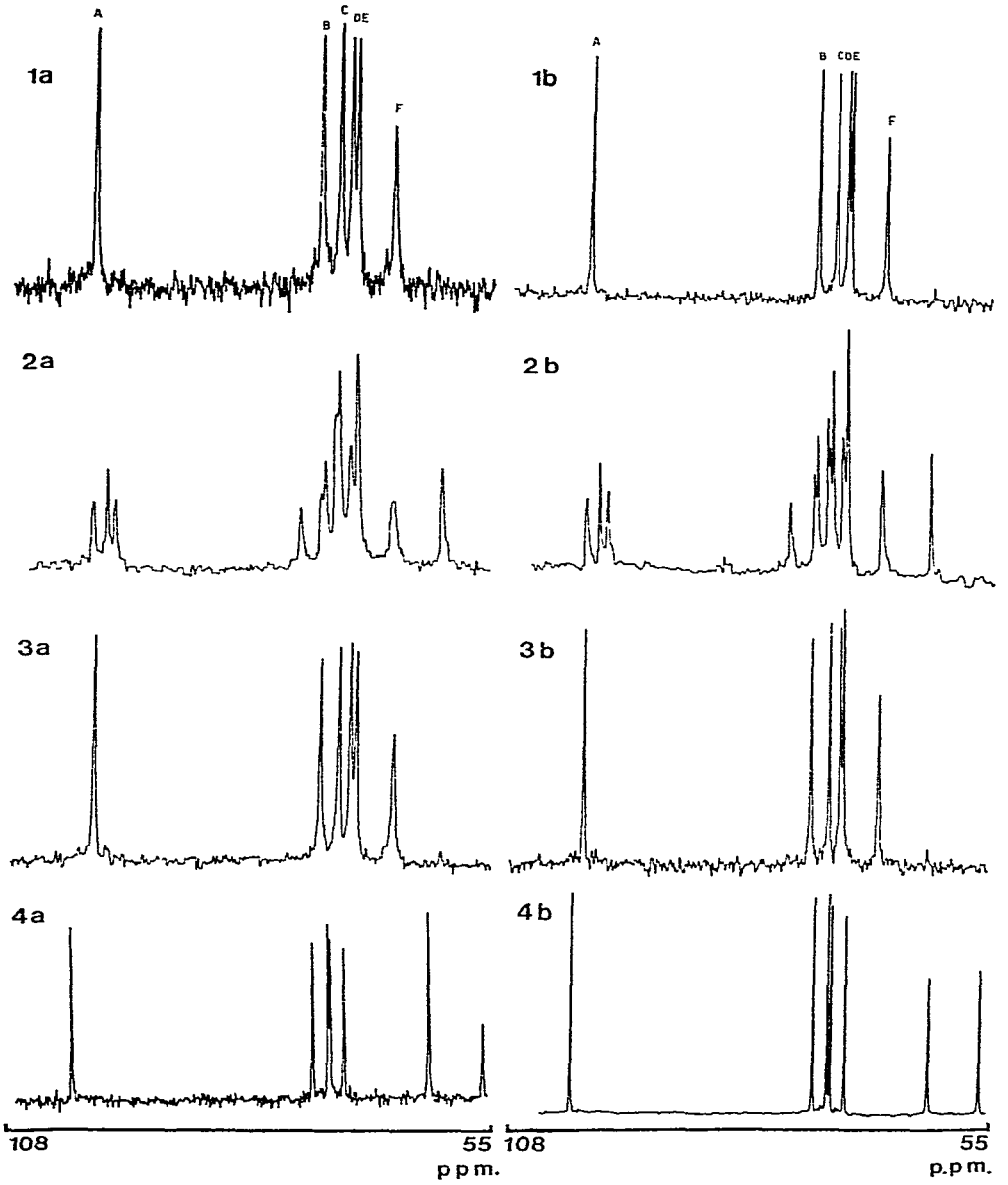


Fig. 1. ^{13}C-N m r. spectra at 34° (designated a) and at 90° (designated b) for 1, dextran B-1254 fraction L[S]; 2, dextran B-1299 fraction L; 3, dextran B-1355 fraction L; and 4, methyl α-D-glucopyranoside.

chemical-shifts for these linkage types[2] as shown in Table II. The advantages of high-temperature, ^{13}C-n.m.r. spectra are again apparent. The enhancement of signal-to-noise ratio is seen (see Figs. 1 and 2) on comparison of spectra 1a and 1b, which represent a roughly equivalent number of transients (see Table III) performed on

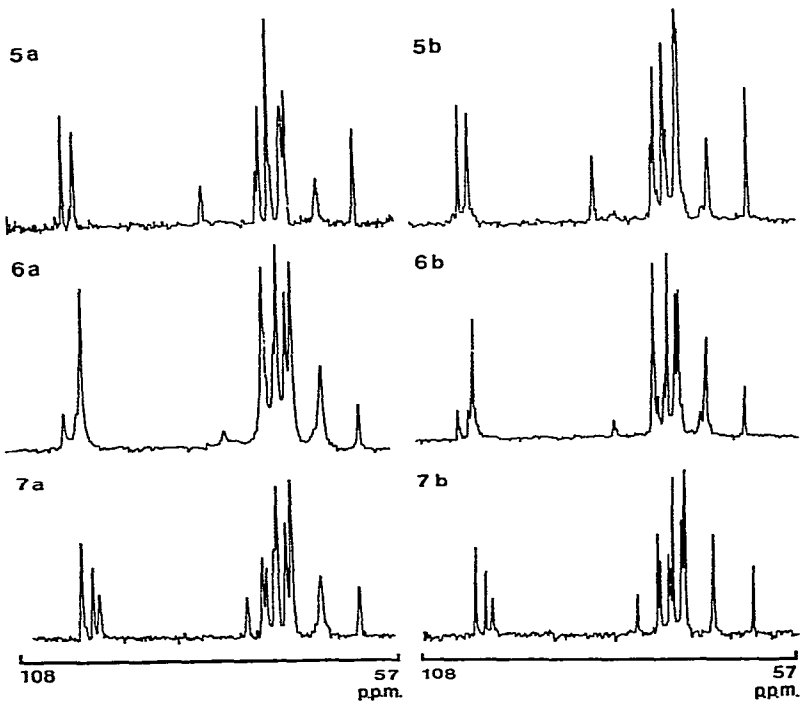


Fig. 2. ^{13}C-N.m r. spectra at 34° (designated a) and at 90° (designated b) for 5, dextran B-742 fraction S; 6, dextran B-742 fraction L; and 7, dextran B-1402.

TABLE III

NUMBER OF ^{13}C-N M R.-SPECTRAL ACQUISITIONS FOR EACH D-GLUCAN

NRRL number of the D-glucan	*Fraction*	*Acquisitions in thousands*	
		Spectra at 34°	*Spectra at 90°*
B-1254	L[S]	6	8
B-1299	L	23	8
B-1355	L	25	8
B-742	S	32	8
B-742	L	25	7
B-1402		32	8

the same sample at different temperatures. Spectra 2a and 2b through 7a and 7b show that comparable signal-to-noise ratios can be obtained by employing approximately one-third the number of transients at high temperature. The high-temperature, line-width diminution allows a considerable increase in chemical-shift resolution, best demonstrated in the expanded, 75–85-p.p.m. regions of the spectra of dextran B-742 fraction S and dextran B-1402 (see Fig. 3).

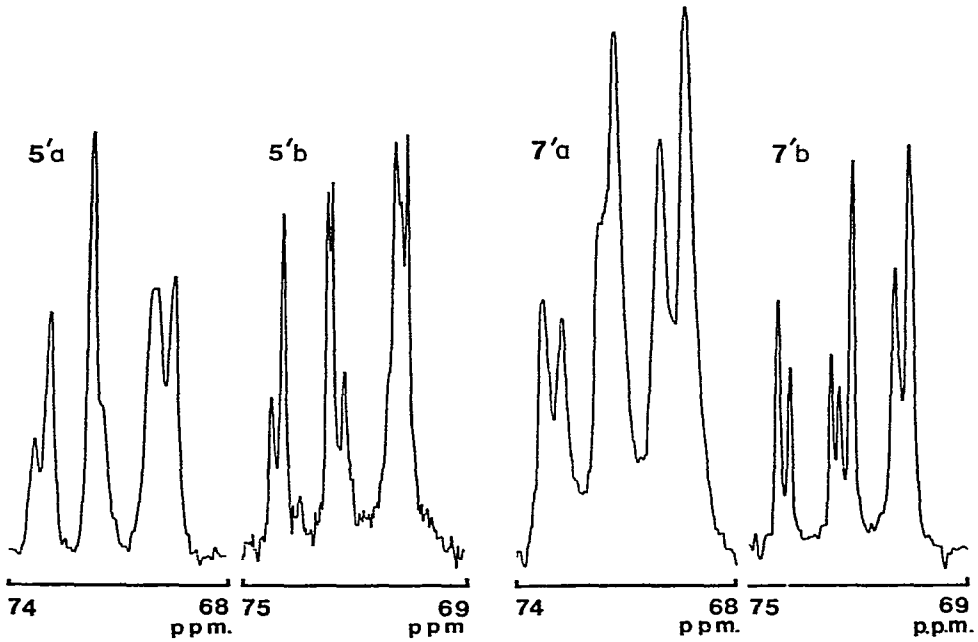


Fig 3 ^{13}C-N.m r. spectra at 34° (designated a) and at 90° (designated b) of the expanded, 70–75-p.p m region of the spectra presented in Fig. 1. [The resonance of each chemical shift may be established from Table II (*5'*, dextran B-742 fraction S; 7', dextran B-1402).]

Dextran B-1254 fraction L[$]. — This (less-soluble) fraction of the dextran produced by *Streptobacterium dextranicum* B-1254 differs greatly in structure from the corresponding (more-soluble) fraction S(L). Previously[3], dextran B-1254 fraction S(L) was indicated to be an α-D-(1→6)-linked D-glucan having every fourth D-glucopyranosyl residue 4-*O*-substituted by a D-glucopyranosyl end-group. The corresponding dextran B-1254 fraction L[$] is shown here to be a simple, linear α-D-(1→6)-linked D-glucan, as evidenced by methylation data and a ^{13}C-n.m.r. spectrum displaying only the six chemical-shifts (previously designated A through F) associated with linear dextrans. The anomeric resonance, at 98.65 p.p.m., clearly indicates α-linkages[7]. The ^{13}C-n.m.r. data give no indication of branching, although methylation structure analysis shows one branch point for about every 20 backbone α-D-(1→6)-linked residues[1]. Our previous observations[2] on other dextrans would indicate that the limit of detection by ^{13}C-n.m.r. spectroscopy is about one branch point per 10 backbone units.

Dextran B-1299 fraction L. — This (less-soluble) fraction from *L. mesenteroides* NRRL B-1299 appears to have only minor structural differences from the corresponding[2,3] fractions S, but it is probably more complex. The mole percentages of methylated D-glucose components (see Table I) differ. As rigorous proof of the homogeneity of fraction L has not been established, a structure cannot be depicted with certainty.

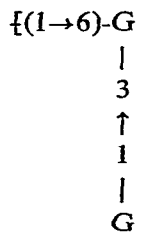
The ^{13}C-n.m.r. spectrum of dextran B-1299 fraction L is very similar to that of the corresponding fraction S. In fact, this spectrum is quite similar to those of a series, which will be reported later, of dextrans containing only α-D-(1→2)-branching. At 90°, peak B is cleanly separated into two resonances, a weaker resonance at 75.20 p.p.m. and a stronger one at 74 86 p.p.m. Similarly, at 90°, peak C separates into three well-defined resonances, at 73.76, 73.55, and 73.18 p.p.m. Two minor features of these spectra should be noted: firstly, at both 34 and 90°, digital-slope detection indicated a minor resonance-shoulder 0.15 p.p.m. downfield from peak F: secondly, at 90°, a minor resonance is indicated at 83.0 p.p.m. Both features are indicative of a small proportion of α-D-(1→3)-linkages. These data are in accord with the permethylation data given in Table I, which indicate the presence of traces of (1→3)-linkages. Therefore, the small proportions of 2,4-di- and 2,4,6-tri-*O*-methyl derivatives found in the chromatogram[1] of the hydrolyzate of permethylated dextran B-1299 fraction L may represent structural features, and not under-methylation.

Thus, our ^{13}C-n.m.r.-spectral and methylation structural analyses disclose no new distinction between fractions L and S of dextran B-1299. There has been consistent agreement[4,8] that fraction L has the higher content of (1→3)-linkages. Through application of various chemical techniques, Bourne *et al.*[8] established the ratio of (1→3)-linked residues to the total number of residues per average repeat-unit at 1:15 for fraction S, and 3:17 for fraction L. Kobayashi *et al.*[9] resolved native dextran B-1299 into five fractions by successive application of two solutions (sodium tetraborate and Cetavlon); characterization[10] showed polymolecularity and polydispersity among these fractions. For four of the fractions, a higher content of (1→3)-linkages correlated with lower solubility during the first treatments with the fractionating reagents. For two of these four fractions, there was also correlation with a high molecular weight (2×10^6). The other two of these fractions had lower molecular weights (150,000–200,000), and were consistent in having greater solubility in the subsequent fractionation steps and an intermediate content of (1→3)-linkages. The fifth fraction, although the most soluble of all in the fractionating reagents, and relatively low in its content of (1→3)-linkages, had a molecular weight of 2×10^6. The greatest difference (other than solubility) originally found[4] between fractions S and L was in the intrinsic viscosity (see Table IV), which may relate to molecular weight and degree of branching.

Dextran B-1355 fraction L. — This (less-soluble) fraction of the dextran produced by *L. mesenteroides* NRRL B-1355 differs greatly from the corresponding, soluble fraction S, although the difference in degree of branching is minor. Fraction S contains approximately equal proportions of α-D-(1→6)- and α-D-(1→3)-linkages; however, only ~7% of the α-D-(1→3)-linkages are at branch points, whereas 35% are in linear-chain positions[3]. Fraction L, as shown by methylation structure analysis (see Table I), has ~4% of its linkages at (1→3)-branch points. ^{13}C-N.m.r. spectroscopy showed that fraction L is essentially an α-D-(1→6)-linked, linear polysaccharide. Only the six ^{13}C-n.m.r. signals associated with the α-D-(1→6)-linkages are observed.

In fact, the ^{13}C-n.m.r. spectra of dextran B-1355 fraction L and dextran B-1254 fraction L[S] are identical (see Fig. 1).

Dextran B742 fraction S. — Combined g.l.c.–m.s. analysis of the per-*O*-acetyl-aldononitriles from a hydrolyzate of permethylated dextran B-742 fraction S (see Table I) established that the molar ratio of its methylated D-glucose components was 2,3,4,6-tetra:2,3,4-tri:2,4-di = 10:1:11. Structurally, therefore, ~96% of this dextran fraction consists of a disaccharide residue as the repeating unit.



The remaining 4% consists of α-D-(1→6)-linked residues for which the only clue to distribution in the fraction is obtained from X-ray diffraction observations[11]. Under conditions that induced "crystallinity" in dextrans having 89% (or more) of their residues linked through C-1 only, or C-1 and C-6, fraction S of dextran B-742 developed an X-ray diffraction line-pattern, but the corresponding fraction L (see Table I) remained amorphous. The type of X-ray pattern developed, L-3, had previously been obtained for fractions of NRRL B-512(F) dextran[12] partially hydrolyzed with acid. These fractions had relatively low molecular weights, and were linked almost exclusively α-D-(1→6), owing to favored cleavage of α-D-(1→3)-links; they showed a strong tendency to retrograde from aqueous solutions, and to develop the X-ray line-pattern. Three possible molecular structures that might "crystallize" in dextran B-742 fraction S were postulated[11]: (*a*) α-D-(1→6)-linked residues disposed consecutively in segments sufficiently long and numerous to crystallize, (*b*) molecular heterogeneity, or (*c*) regular sequences of single-unit branches arranged along the main chains. For the first possibility, conformational rigidity of the molecule would enhance ordering in predisposed regions. The second possibility, molecular heterogeneity, cannot be excluded. If, however, traces of fraction L were present, they would not be expected to crystallize[11]. The third possibility is incompatible with the origin of the L-3 pattern[12] from a structure markedly different from that of dextran B-742 fraction S.

Dextran B-742 fraction S, as depicted, has no residues linked exclusively α-D-(1→6). Even if 4% of such units should occur in the molecular structure, this fraction S is "anomalous" as a dextran, because, by definition, these α-D-glucans contain a majority of units linked α-D-(1→6).

On the basis of the disaccharide repeat-unit structure of dextran B-742 fraction S, it would be expected that (*1*) the ^{13}C-n.m.r. spectra would be unusually simple, and consist merely of the twelve peaks arising from the respective carbon atoms, and (*2*) these same spectral peaks should be prominent in the spectra of dextrans having

a high content of (1→3)-D-glucosidic linkages, particularly dextran B-1355 fraction S.

Our ^{13}C-n.m.r. data are in accord with these structurally-based predictions. Our 34° spectrum of B-742 fraction S (see Table II) is similar to a spectrum reported[13] for dextran B-742 (fraction not identified), recorded at 32°, that displayed a complicated 70–75-p.p.m. region, and showed only α-D-anomeric linkages. The 90° spectrum, however, shows greatly increased resolution, especially in the 70–75-p.p.m. region (see Fig. 3); this spectrum has twelve peaks, as expected for a disaccharide unit, with a few features indicating other minor contributions.

For dextran B-742 fraction S, the anomeric peak at 100.3 p.p.m. is identical to that observed[2] with dextrans having a high content of α-D-(1→3)-linkages. These dextrans are B-1351 fraction S (in which these linkages occur at branch points exclusively) and B-1355 fraction S (in which a minor part of these linkages is at branch points and a major part is in linear-chain positions[3]). The second anomeric peak, at 98.8 p.p.m. (peak A), superficially the same as that for all dextrans previously reported[2] as well as for those shown in Table II, apparently arises from (1→6)-linked residues. However, for dextran B-742 fraction S and the other dextrans (B-1351 fraction S and B-1355 fraction S) in which its branched disaccharide unit occurs, a third anomeric peak, arising from α-D-(1→3)-linked residues involved in branching, might be expected, but has not been observed. Apparently, one of the anomeric chemical-shifts of the proposed, repeating disaccharide unit of dextran B-742 fraction S is essentially identical to the anomeric resonance of α-D-(1→6)-glucan itself.

For dextran B-742 fraction S, there are four minor peaks. at 81.66, 74.39, 71.99, and 66.54 p.p.m. (see Table II). Two of these peaks, at 81.66 and 66.54 p.p.m., are clearly identified with C-3 and C-6, respectively, of the 1,3,6-tri-*O*-substituted α-D-glucosyl residue The 81.66-p.p.m. chemical shift is essentially the same as that observed[2] for the linked C-3 of dextrans B-1351 fraction S and B-1355 fraction S. Although the α-D-(1→3)-linked residues of dextran B-1355 fraction S are mainly di-*O*-substituted, whereas those of both dextran B-742 fraction S and B-1351 fraction S are mainly 1,3,6-tri-*O*-substituted, the diagnostic chemical-shifts in the 75–101-p.p.m. region are the same for both residues. The 66.5-p.p.m. peak originates from a linked C-6 atom. It is possible that all four of these minor chemical-shifts for dextran B-742 fraction S arise from tri-*O*-substituted-D-glucosyl, backbone residues, and that their lower peak-heights result from the fact that all backbone residues in this dextran are probably tri-*O*-substituted and, consequently, are held more rigidly than similar residues adjacent to di-*O*-substituted D-glucosyl residues (as in dextran B-1351 fractions) would be.

For the 70–75-p.p.m. region, the 90° spectrum contains seven sharp peaks, in accord with the disaccharide repeating-unit model. In the 27° spectrum, a broad peak occurs at 71.1 p.p.m.; digital slope-detection indicated this to consist of peaks at 71.08 and 70.79 p.p.m. At 90°, this peak is resolved into a peak at 71.94 p.p.m. having a shoulder at 71.79 p.p.m. Only the minor peak, at 74.4 p.p.m. (B), and a major peak, at 70.5 p.p.m. (peak E), remain from the B through E chemical-shifts identified for the 1,6-di-*O*-substituted α-D-glucosyl residue of linear dextran—clear

evidence that this unit no longer contributes significantly to the spectra of dextran B-742 fraction S.

Within the limits of detection provided by the method, there is little, if any, evidence for the presence of either α-D-(1→2)- or α-D-(1→4)-linkages in the spectrum of this dextran fraction.

Dextran B-742 fraction L. — The (less-soluble) component of dextran B-742, complementary to fraction S already discussed, has a completely different structure. Methylation data indicate that a fundamental, average, repeating unit is as follows.

$$\{\text{G-(1}\rightarrow\text{6)-}\{\text{G-(1}\rightarrow\text{6)}\}_n\}_x$$
$$\begin{array}{l} | \\ 4 \\ \uparrow \\ 1 \\ | \\ \text{G} \end{array}$$

where $n = 5$.

The side chain is merely represented as being one unit long; the actual distribution of unbranched α-D-(1→6)-linked D-glucosyl residues between backbone and linear side-chain positions has not been established. The methylation data are analogous to those obtained for dextran B-1254 fraction S[L], for which n is[3] 3. Likewise, the ^{13}C-n m r. spectrum (see Fig. 2, spectra 6a and 6b) of dextran B-742 fraction L is very similar to that (ref. 2, Fig. 1c) of dextran B-1254 fraction S[L]. In the spectrum of dextran B-742 fraction L, as compared with that of dextran B-1254 fraction S[L], the greater linearity (less-frequent branching) results in greater intensity in each chemical shift (A through F) that arises from residues linked only through C-1 and C-6. The anomeric, ^{13}C-n.m.r. region indicates that all of the D-glucosyl residues in this D-glucan are α-linked.

Dextran B-1402. — Methylation data indicate that this native, unfractionated dextran has the following fundamental, repeating unit.

$$\{\text{-G-(1}\rightarrow\text{6)-}\{\text{G-(1}\rightarrow\text{6)}\}_n\}_x$$
$$\begin{array}{l} | \\ 2 \\ \uparrow \\ 1 \\ | \\ \text{G} \end{array}$$

where $n = 2$.

For this dextran, as for dextran B-742 fraction L, the length(s) of side chains is not established. As is discussed later, however, evidence from ^{13}C-n.m.r. chemical shifts for this dextran and two others that branch through C-2 supports our use of side-chains that are one unit long in the respective, repeat-unit structures.

This fundamental, repeating unit is similar to those in dextran B-1299 fraction S (where $n = 1$) and dextran B-1399 fraction L (where[2,3] $n = 8$). Even though there is a significant difference in the degree of branching ($n = 1$, 2, and 8) within this dextran series, the chemical shift of the linked C-2 atom is ~76.45 p.p.m. for each dextran. The anomeric region of the spectrum of dextran B-1402 displays three well-defined, chemical-shifts, at 98.76, 97.21, and 96.39 p.p.m. These three anomeric chemical-shifts are present in the spectrum of dextran B-1399 fraction L, with peaks at 97.21 and 96.39 p.p.m. that show a constant, relative peak-height ratio, and a peak at 98.76 p.p.m. (peak A) that increases as n increases in the aforementioned, repeating unit. This independent increase in the 98.76-p.p.m. peak confirms that it is contributed by the anomeric carbon atoms of the backbone-extending, α-D-(1→6)-linked D-glucosyl residues. The other two anomeric chemical-shifts arise from the 1,2,6-tri-*O*-substituted residues and the (nonreducing) terminal D-glucosyl groups. Of this pair, the more intense resonance (at 97.21 p.p.m.) represents the anomeric carbon atoms of the (nonreducing) terminal D-glucosyl groups; chain rigidity would be expected to decrease the intensity of the anomeric carbon shift (96.39 p.p.m.) of the tri-*O*-substituted group at branch positions. The enhanced resolution of the 90° spectra is again apparent in the 70–75-p.p.m. region of the spectrum of dextran B-1402 (see Fig. 3). At 90°, peak B resolves into two resonances, at 75.17 and 74.99 p.p.m., with peak C resolving into three resonances, at 73.74, 73.52, and 73.15 p.p.m.

Correlation of structure and physicochemical properties of fraction pairs. — Each type of dextran fraction has distinctive characteristics, both in structure and in physicochemical properties. The L-type fractions are the more linear in structure,

TABLE IV

PHYSICAL PROPERTIES AND STRUCTURE OF DEXTRANS

NRRL strain	*Dextran fraction*	*m*[a]	*n*[a]	*Ethanol at precipitation maximum (%)*[b]	[η][c] *(in water at 25°)*
B-1254[d]	S[L]	4	3	37 5	0 19
B-1254	L[S]	3	24	36.5	0 54
B-1299	S	2	1	40	0.47
B-1299	L	2	1	38	0.87
B-1355[d]	S	3	[e]	40	0.19
B-1355	L	3	24	36.5	1.12
B-742	S	3	0	43	0.33
B-742	L	4	5	37	0.15
B-1402		2	2		

[a]The symbols *m* and *n* refer to the terms designating the position of branching and degree of linearity as expressed in Fig. 4. [b]The percentage of ethanol at the maximum of the analytical, fractional-precipitation curve (ref. 5). [c]Intrinsic viscosity (from ref 4). [d]Structural information taken from refs. 1–3. [e]This structure is characterized by alternating α-D-(1→6)- and α-D-(1→3)-linked residues

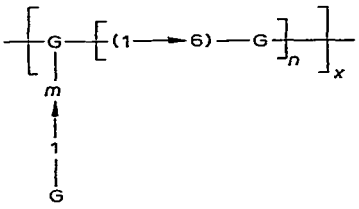


Fig. 4. The generalized structure of the average repeating-unit of branched dextrans [The symbol *m* designates the position of branch attachment (C-1, C-2, or C-3), and *n* is the number of unbranched, α-D-(1→6)-linked D-glucosyl residues per (nonreducing) α-D-glucosyl end-group (and, therefore, per branch-point unit). Only for simplicity of comparison is the side chain shown as one unit long The actual distribution of (1→6)-linked residues between the backbone and linear positions in side chains has not been established.]

as may be seen from the summary in Table IV for the idealized, fundamental repeating-unit (see Fig. 4). The magnitude of the factor *n*, which is directly related to structural linearity, and inversely to frequency of branching, is >10 for the L-fractions of B-1254 and B-1355 dextrans. For B-742 fraction L, *n* is 5 (compared to 0 for the S-type fraction). For B-1355 fraction S, *n* is meaningless, as non-(1→6)-linkages alternate with (1→6)-linkages in the backbone of the molecule. The summary of values of *m* and *n* given in Table IV affords convenient structural comparisons, but, as already stated (see legend to Fig. 4), it is not meant to imply that the branches are really known to be one unit long. Information regarding branch lengths in dextrans has been reviewed[3,14], and it was concluded[14] that "most of the branches in the dextrans that have been examined to date consist of a single α-D-glucopyranosyl group". With a few possible exceptions[14,15], however, branches have not been shown to be exclusively one unit long. Dextran B-742 fraction S may be one of the exceptions.

Graded addition of ethanol to a dilute solution of the native dextran under analytical fractionation conditions[5] resulted in precipitation of the L fraction at a concentration of ethanol lower than that required for the corresponding S fraction. The precipitation of the maximum increment of the L member of each pair occurred at 36.5–38% of ethanol (see Table IV), compared with 37.5, 40, and 43% concentrations for the respective S fractions. The differential in these ethanol concentrations for the members of a pair determines the ease of preparative fractionation. Separation of the B-1254 pair was difficult: the differential in ethanol concentration was only 1%, and the ratio of concentration of fraction L to that of fraction S was 8:1. In fact, under the conditions for large-scale, preparative fractionation, fraction S[L], separated out[5] before fraction L[S]. Remarkably clean separation of the B-1254 pair, as well as of the others, was achieved by the initial "cutting" of fractions, and several refractionations.

The relationship of the structure of individual members of type L and S fractions to the ethanol concentration at which the maximum increment of precipitation occurred in analytical fractionation is depicted in Table V. If the dextran fractions are listed in the order of this ethanol concentration, the fractions are also ordered in terms of increasing percentages of non-(1→6)-linkages [or, more specifically, in

TABLE V

PRECIPITABILITY OF DEXTRANS BY AQUEOUS ETHANOL

Dextrans	*Order*[a]
B-1254 Fraction L[S] and B-1355 fraction L	↓ 1 ↑ 2
B-742 Fraction L and B-1254 fraction S[L]	
B-1299 Fraction L	
B-1299 Fraction S and B-1355 fraction S	
B-742 Fraction S	

[a]Arrow 1 indicates the order of increasing concentration of ethanol needed to precipitate the dextran from aqueous solution. Arrow 2 indicates the order of increasing percentage of α-D-(1→6)-linked, D-glucopyranosyl residues.

terms of decreasing percentage of α-D-(1→6)-linked D-glucopyranosyl residues]. The terms "S" and "L" are relative for a given pair of fractions, and are not absolute values, as is emphasized in Table V.

The L fractions of dextrans B-1254, B-1299, and B-1355 have higher intrinsic viscosities than the corresponding S fractions (see Table IV), in accord with the generalization that branching contributes to lower viscosity of solutions. Other factors may, however, be influential, as shown by the very low solution-viscosity of dextran B-1355 fraction S, which has only ~7% of nonreducing end-groups[3]. This dextran, for which M_w is 40 × 10^6 (light-scattering)*, must assume a very compact form. The alternation of (1→6)- and (1→3)-linkages, and the conformational effects expected of (1→3)-linkages, cause the dextran to have novel properties.

The viscosities of both of the B-742 fractions are low, that of fraction L being the lower. Generalization is inadvisable, as the molecular weights of the fractions are not known, the non-(1→6)-linkages are different, and the degree of branching of fraction S is exceptionally high.

Correlation of chemical shifts to specific carbon positions in carbohydrates. — The case of the simpler spectra, such as those of the linear dextrans (*e.g.*, B-1355 fraction L), encourages assignment of chemical shifts to specific carbon atoms. ^{13}C-N.m.r. spectra of linear dextrans, which display six discrete resonances, are analogous to the corresponding spectrum of methyl α-D-glucopyranoside (see Fig. 1 and Table II) which, with the exception of the 56-p.p.m. resonance, also displays six resonances.

Certain problems will now be identified and discussed: firstly, the actual assignment of the chemical shifts for the 70–75-p.p.m. region of the spectrum of methyl α-D-glucopyranoside; secondly, whether the order of assignment of these monomer chemical-shifts is the same as for the B through E peaks of linear dextran; and, thirdly, for dextrans, how glycosylation (that is, attachment of a side chain)

*R. Tobin, unpublished results.

TABLE VI

ASSIGNMENT OF ^{13}C-N M R. CHEMICAL SHIFTS

Value of δ for monomer[a]	*Assignment for monomer*[b]	*Value of δ for analogous dextran (unsubstituted oxygen atoms*[c]*)*	*Observed value of δ for dextran (glycosylated oxygen atoms)*	*Effect of glycosylation (Δδ)*[d]
74.0 (1.62)[e]	C-3	74.4 (1.54) B	81.6	7 2
72.5 (1.48)	C-5	72.5 (1.37) C		
72.1 (1.63)	C-2	71.3 (1 41) D	76 5	5 2
70.5 (1 82)	C-4	70 7 (1.82) E	79 5	8.8
61.5 (1.82)	C-6	61.7 (2.07) F[f]	66 7	5 2

[a]Data for methyl α-D-glucopyranoside, from Table II. [b]From ref 17. Data for these peaks, which were obtained at an intermediate temperature, differ somewhat from those in column 1. [c]Unsubstituted oxygen atoms, based on values for dextran B-1355 fraction L at 34°; values in parentheses represent $\Delta\delta/\Delta T$. [d]The value in column 4 less the value in column 3. [e]Chemical shifts at 34°; values in parentheses give $\Delta\delta/\Delta T$. [f]Based on data for dextran B-742 fraction S

affects the magnitude of the displacement of the corresponding chemical-shifts in the 70–75-p.p.m. region.

The chemical shifts associated with methyl α-D-glucopyranoside were originally assigned by both Perlin *et al.*[16] and Dorman and Roberts[7]. The values for C-1 and and C-6 are well established, but certain questions have persisted about those for C-2 through C-5, normally located in the 70–75-p.p.m. region. Walker *et al.*[17] employed methyl α-D-glucopyranoside having C-1 specifically enriched with carbon-13. The ^{13}C-n.m.r. spectrum of this compound definitely established the assignment of the C-2 resonance, and, by inference through neighboring effects, allowed assignment of other chemical shifts. These assignments are summarized in column 2 of Table VI.

Columns 1 and 3 of Table VI indicate a close parallelism between our observed chemical-shifts for methyl α-D-glucopyranoside and those for linear dextran, which suggests that these chemical shifts arise from carbon atoms in corresponding positions. This correlation is tentatively assumed to be correct. The 70-p.p.m. peak of the monomer (peak E for dextran) has a very large $\Delta\delta/\Delta T$ value for both the monomer and the polymer. The major divergence between chemical shifts in columns 1 and 3 occurs at 72.1 p.p.m. for the monomer, and at 71.3 p.p.m. for the dextran; for the monomer, the peak at 72.1 p.p.m. is known[17] to correspond to C-2. It is possible that the chemical shift for C-2 in dextran is slightly affected by the adjacent C-1 of the D-glucopyranosyl group. Assignment of the C-6 resonance is clearly established by its upfield position, and the identification of C-3 and C-4 resonances is unambiguous[17]. Identification of the C-5 resonance as being[16] at 72.5 p.p.m. is less certain; Walker *et al.*[17] argued that perturbation of the chemical shift at 72.5 p.p.m. by ^{13}C-enrichment of C-1 of methyl α-D-glucopyranoside identifies this as the C-5 resonance.

Chemical shifts observed in essentially unbranched dextrans (see Table VI, column 3) are closely analogous to those in column 1, and can, therefore, be assigned

to specific, unsubstituted carbon atoms of the monomeric unit. Chemical shifts of *O*-substituted C-2, C-3, and C-4 of α-D-glucopyranosyl residues shown in column 4, are from data presented here and in ref. 2 on dextrans in which a high degree of branching occurs at these respective atoms. By subtracting the values for unsubstituted carbon atoms (column 3) from those observed for the corresponding *O*-substituted atoms (column 4), $\Delta\delta$ values resulting from glycosylation of each carbon atom were obtained (column 5).

The $\Delta\delta$ effect of glycosylation on specific carbon atoms of α-D-glucopyranosides has not been reported. The data merely show that methylation of a specific hydroxyl group results in a change in chemical shift for the corresponding carbon atom of (approximately constant) 10 p.p.m. downfield for each position[18]. The average $\Delta\delta$ for α-D-glucosylation has been found to be smaller, ~6.6 p.p.m. downfield.

The identification of the chemical shift of each carbon atom of the unsubstituted D-glucopyranosyl residues of D-glucans is important to an understanding of the perturbation of these resonances resulting from glycan substitution. An excellent example is dextran B-742 fraction S, which contains only ~4% of α-D-(1→6)-linked D-glucopyranosyl residues; the rest consists of equal proportions of 1,3,6-tri-*O*-substituted α-D-glucopyranosyl residues and (terminal) nonreducing D-glucosyl groups. Here, it is evident (see Fig. 1, spectra 3a and 3b) that 3-*O*-glycosylation results in the perturbation of all of the chemical shifts in the 70–75-p.p.m. region, except that (peak E) at 70.7 p.p.m.

CONCLUSIONS

^{13}C-N.m.r. spectroscopy and methylation structural analysis by g.l.c.–m.s. accord in showing that pairs of fractions separated from four native dextrans (from NRRL strains B-742, B-1254, B-1299, and B-1355) by solubility differences have distinctly different structures. Another structural type, a homogeneous native dextran (from NRRL strain B-1402), extends a series of dextrans branched through C-2, and permits correlation between the peak height of a chemical shift and the number of backbone residues lying between branch points. The sensitivity of detection by ^{13}C-n.m.r. spectroscopy is within ~10% of that of methylation structural analysis, and thus it may contribute to the unusually "clean" aspect of some of the spectra. Recording ^{13}C-n.m.r. spectra at higher temperatures enhances the resolution significantly, and greatly lessens the number of acquisitions needed. The spectra show that all observable linkages are α and, assuming (for convenience of comparison) that all α-D-(1→6)-linkages are in the backbone chain, the average repeating unit of each dextran can be represented by a simple formula. However, the actual distribution of α-D-(1→6)-linkages between backbone and side chains remains unknown. The data presented are in agreement with the concept that α-D-glucans can, to a first approximation, be treated as an aggregate of variously substituted D-glucopyranoside monomers. Upon substitution by branching at a single position of a D-glucopyranoside

ring of a dextran, most of the resonances in the 70–75-p.p.m. region are increased by ~5–9 p.p.m.

EXPERIMENTAL

Spectral conditions. — Proton-decoupled, ^{13}C-n.m.r. spectra were obtained at natural abundance, with a total carbohydrate concentration of 50 mg/ml of deuterium oxide. A Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 system was employed in the Fourier-transform, data-processing mode. The spectral width was 6 kHz; the acquisition time, 1.4 s; and the pulse-width, 14 μs The number of acquisitions was, in general, a function of the desired signal-to-noise ratio, and is described in Table III for each spectrum. The samples were measured in 12-mm diameter tubes spun at ~20 r.p.s. Temperatures were controlled to within ±1°. Sample temperatures were measured with an immersion thermometer, to within ±0.5°, before and after each experiment, and were held constant with a Varian temperature-controller. Chemical shifts are expressed in p.p.m. relative to external tetramethylsilane, but were actually calculated by reference to the lock signal.

Materials. — The preparation and characterization of the dextrans[4] and dextran fractions[5] have been reported. Before acquisition of the spectra, the solutions were passed through a glass frit to remove any traces of particulate matter. Methods previously described[1,3] were used for methylation of the dextrans and for structural analysis by g.l.c.–m s. of the peracetylated aldononitriles.

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