Carbohydrate Research, 81 (1980) 67–103

STRUCTURAL ANALYSIS OF α-D-GLUCANS BY ^{13}C-NUCLEAR MAGNETIC RESONANCE, SPIN–LATTICE RELAXATION STUDIES*†

FRED R. SEYMOUR,

Fleming Department of Rehabilitation, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030 (U.S.A.)

AND ROGER D. KNAPP

Division of Atherosclerosis and Lipoprotein Research, Baylor College of Medicine and Methodist Hospital, Texas Medical Center, Houston, Texas 77030 (U.S.A.)

(Received September 17th, 1979; accepted for publication, October 12th, 1979)

ABSTRACT

Relaxation measurements have been made for the resonances of the ^{13}C-n.m.r. spectra of the S fractions of dextrans from NRRL strains *Leuconostoc mesenteroides* B-742, B-1299, B-1355, and B-1498, the S[L] fraction of the dextran from *Streptobacterium dextranicum* B-1254, pullulan, and a synthetically branched, comb-like amylose. Resonance assignments have been made to carbon-atom positions on the basis that carbon atoms associated with large degrees of segmental motion will have resonances with larger than average dipole–dipole spin–lattice relaxation (T_1^{DD}) values. For a given spectrum, the relative magnitude of T_1^{DD} values are about the same as the observed spin–lattice relaxation (T_1^{obs}) values for each resonance. Comparisons of the relative magnitudes of relaxation data are most easily made with the resonances of anomeric positions. In general, the relative magnitude of the relaxation values for the spectrum of a given compound did not change between 34° and 90° recording conditions. Relaxation measurements were employed to establish the relative position of *O*-substituted residues in the average repeating unit of the S fractions of dextrans B-1355 and B-1498. The general parameters controlling the magnitudes of the chemical shifts of the α-D-glucopyranosyl residue were then considered in relation to the nature of the *O*-substitution for this residue.

INTRODUCTION

The two initial reports[4,5] in this series dealt with the identification of dextrans that contain variously *O*-substituted α-D-glucopyranosyl residues, in addition to the 6-mono-*O*-substituted α-D-glucopyranosyl residues of linear dextran. Data from ^{13}C-n.m.r. spectra were compared to data from methylation–fragmentation analysis in an attempt both to correlate ^{13}C-n.m.r. resonances to different branch-type residues

*Unusual Dextrans, Part XII. For Part XI, see ref. 1. For a preliminary note, see ref. 2.

†Presented, in part, at the National Meeting of The Chemical Institute of Canada, Winnipeg, Manitoba, June 1978 (see ref. 3).

and to extend our knowledge of the structure of the different dextrans. Methylation–fragmentation analysis (employing gas–liquid chromatography–mass spectrometry), the primary structural technique, provided a precise knowledge of the position of *O*-substitution and of the mole ratio (degree of linearity of the polymer) for the variously *O*-substituted residues present in the polysaccharide, with minimal dependence on standards or reference compounds. However, methylation–fragmentation analysis (*a*) only indicated the mole percentages of differently *O*-substituted residues in the original polymer, not the relative position of these residues, and (*b*) was dependent on the total permethylation and subsequent total hydrolysis of the polymer. Nondestructive, ^{13}C-n.m.r. spectrometry provided a convenient, complementary technique to methylation–fragmentation analysis.

The resonances of anomeric carbon atoms are quite dependent on the anomeric configuration of the associated residue[6,7], a structural feature regarding which methylation–fragmentation analysis provided no information. In addition, the literature indicated that relatively minor changes in the structure of a polysaccharide could cause measurable changes in the spectra of the polymer[8,9]. However, the ^{13}C-n.m.r. structural investigation of most polysaccharides had dealt with essentially linear polymers, rather than branched structures, such as these unusual dextrans. Our approach to the analysis of ^{13}C-n.m.r. spectra has been essentially empirical; that is, we have recorded the spectra of a variety of polysaccharides and have then compared the known structural features (known from other methods of structural analysis) of these polymers to the ^{13}C-n.m.r. spectra. ^{13}C-N.m.r. spectra provided a variety of data, in different spectral regions, about the positions of different carbon atoms of the individual residues. These spectra confirmed previous structural findings and provided new independent structural information.

As the fundamental, methylation–fragmentation, structural data had been expressed in mole percentages of specifically *O*-substituted residues, it was conceptually most convenient to compare these data to the ^{13}C-n.m.r. spectra in terms of such residues. Therefore, a set of hypotheses (which were either logical, or convenient) was employed for the analysis of the relationship of the ^{13}C-n.m.r. data, at least to a first approximation, to the methylation–fragmentation, structural-analysis data[10]. The technique of relating structural data, obtained from polysaccharides by different structural-determination methods, on the basis of specifically *O*-substituted residues has proved more useful than relating structural data based on a linkage-type approach. We have employed this residue-type approach in comparing polysaccharide structural data from Fourier-transform (F.t.), infrared difference-spectrometry[11], ^{1}H-n.m.r. spectrometry[12], and immunochemical techniques[13,14]. The relatively rapidly obtained ^{13}C-n.m.r. spectra, in conjunction with previously obtained periodate-oxidation data[16], have been used to survey an existing dextran collection, in order to identify candidates for the more laborious, methylation–fragmentation, structural determinations. A combination of these ^{13}C-n.m.r. and g.l.c.–m.s. techniques has allowed (*a*) the identification of two groups of dextrans; first, a group branching through differently substituted (2,6-, 3,6-, or 4,6-)di-*O*-α-D-glucopyranosyl residues[14], and

(*b*) the differentiation of these dextran groups from non-dextran biopolymers[17,18] produced by the same, or similar micro-organisms. These dextrans have also been shown to have considerable variation in degree of linearity.

This survey, which has provided the basis for the selection of dextrans employed in comparative studies with Fourier-transform, infrared difference-spectrometry[11] and ^{1}H-n.m.r. spectrometry[12], has now been employed for the choice of the polysaccharides which were to be studied by ^{13}C-n.m.r. relaxation techniques. The approximately thirty different dextrans that have been examined have been described in terms of the various groups of dextrans which are based on branch-type residues[1,14,15]. The present objectives are to (*a*) compare data from these four groups of dextrans in terms of a representative polymer from each group, (*b*) discuss ^{13}C-n.m.r.-resonance relaxation data that have been obtained for a representative polysaccharide from each group, and (*c*) relate the chemical-shift data for these representative dextrans to chemical shifts observed in the ^{13}C-n.m.r. spectra of other D-glucans.

The correlation of ^{13}C-n.m.r., chemical-shift data to polysaccharide structure assumes the following. (*a*) Each polysaccharide spectrum is considered to be a composite spectrum composed of individual spectra from the residues composing that polymer. (*b*) Each different type of *O*-substituted residue has a different ^{13}C-n.m.r. spectrum. (*c*) The spectrum of a specific residue is similar to that of the corresponding *O*-alkylated monosaccharide. (*d*) Upon glycosylation (as found for alkylation) of an oxygen atom on a specific carbon atom, the associated, ^{13}C-n.m.r. chemical-shift will be displaced $\sim$10 p.p.m. downfield. (*e*) To a first approximation, the chemical shifts of each residue are independent of the structure of adjacent residues. (*f*) The contribution of a specific type of residue to the polysaccharide spectrum is proportional to the mole percent of that residue present in the polymer.

The ^{13}C-n.m.r. spectra were also analyzed in conjunction with the following, general conventions for polysaccharide structure. Firstly, each polysaccharide molecule was considered to contain only one terminal, reducing residue. Secondly, as all branching occurred through nonreducing, hydroxyl groups, (*a*) each branch-point residue had a corresponding nonreducing, terminal group, (*b*) for large polysaccharides, the single, terminal, reducing residue became spectroscopically insignificant, and (*c*) therefore, for large polymers, the number of branch-point residues was essentially equal to the number of terminal residues. It has also been observed that (*a*) the chemical shifts of the anomeric carbon atoms are well separated ($\sim$95–110-p.p.m. region) from the chemical shifts of the other ring-carbon atoms, (*b*) the chemical shifts of these anomeric carbon atoms are very sensitive to small changes in polysaccharide structure, (*c*) the chemical shifts of the *O*-substituted ring-carbon atoms are also well separated (75–85-p.p.m. region), (*d*) the chemical shifts of the C-6 atoms (60–70-p.p.m. region) are only slightly influenced by structural changes, and (*e*) the remaining chemical shifts of dextrans are packed into the 70–75-p.p.m. region[3,4].

It was necessary to use care with the foregoing method of analysis of ^{13}C-n.m.r. spectra, based on the contributions of resonances from specifically *O*-substituted

residue types, as a number of the assumptions made were obviously approximations, or, possibly, simply not correct. Firstly, dextrans mainly containing 3-mono-*O*-substituted (*e.g.*, dextran B-1355 fraction S) or 3,6-di-*O*-substituted (*e.g.*, dextran B-1351 fraction S) α-D-glucopyranosyl residues yielded similar spectra[4], in terms of additional resonances not associated with linear dextran, indicating that, even if differently *O*-substituted residues had different, chemical-shift patterns, it was possible that such effects could not be easily observed at field strengths of 25 MHz. Secondly, the low (~30°) and high (90°) temperature spectra of dextran B-1355 fraction S had a distinctly different spectral "profile", especially in the 70–75-p.p.m. region, calling into question the assumption that the spectral contribution of a specific residue is proportional to the mole percent of that residue in the polymer. Thirdly, the independence of the chemical shifts of each residue from the adjacent residues was obviously an approximation, as (*a*) the anomeric chemical-shift of the terminal residues was different for dextrans of different branch-type, and (*b*) it had previously been shown that neighboring-residue effects occurred in pullulan, a polysaccharide that contains 4-mono-*O*-substituted α-D-glucopyranosyl residues in two environments[8,19]. In addition, we had previously used methylation–fragmentation analysis to examine the structures of several classes of highly branched D-mannans[20], and it proved difficult to relate the structures of these D-mannans, in terms of individual residues, to ^{13}C-n.m.r. spectra that had previously been recorded by Gorin[21,22]. The correlation of such D-mannan data will be dealt with in a subsequent report.

Despite the foregoing limitations, the *O*-substituted-residue approach to the analysis of ^{13}C-n.m.r. spectra has provided good correlation of structural features to the spectra of dextrans. The recording of the ^{13}C-n.m.r. spectra of a large number of dextrans under various conditions has helped to resolve several important questions that could not be dealt with on the basis of a few spectra from a few polymers. For example, (*a*) which minor resonances represented background noise (or impurities specific to a given polymer) and which resonances were diagnostic of a given branch-type residue, and (*b*) what was the accuracy and precision for the chemical shifts of spectra recorded for polysaccharides of similar structure? It was established that the change in the general, spectral pattern observed when increasing the recording temperature for dextran B-1355 fractions S was an unusual feature for dextrans[14], and that, for most dextrans, an increase in recording temperature does not greatly change the general, spectral profile. Employing high-temperature recording-conditions narrowed the width of the resonances, allowing improved spectral resolution and lessened recording times. A further improvement in resonance narrowing, and concomitant increase in the ease of visual, spectral examination, was achieved by employing the convolution-difference resolution-enhancement[23] (c.d.r.e.) technique on high signal-to-noise spectra. The c.d.r.e. technique did not create new resonances from a given data set, but did aid visual discrimination in establishing whether deflections that have been identified by digital slope-detection are actual, spectral features, or simply random noise. A second technique for improving chemical-shift accuracy for comparison of resonances in different spectra consisted of recording

blocks of spectra during a relatively short time (a few days) and then making corrections for small, consistent changes observed in the chemical shifts of the spectra[1].

In order to consider the structure of dextrans, we started with the following assumptions. (*a*) In dextran, we have considered the (1→6)-linkages to be located in the backbone of the biopolymer [although we now conclude that certain dextrans (*e.g.*, dextran B-1355 fraction S) contain (1→6)-linkages in the side chains of the biopolymer]. (*b*) The dextrans are based on a linear backbone (comb-like) structure, in contrast to the branching (dendritic) structure that has been established for glycogen. (*c*) The side chains are only one residue long. (*d*) For any specific polysaccharide, the number of residues interspaced between branch-point residues is a constant. When combined, these assumptions allow the construction of an average repeating unit (see **1**, where Glc*p* represents the D-glucopyranosyl

$$-\{-[-\alpha\text{-Glc}p^{t}\text{-}(1\rightarrow6)\text{-}]_{n}\text{-}\alpha\text{-Glc}p^{b}\text{-}(1\rightarrow6)\text{-}\}_{x}-$$

$$\begin{array}{c} m \\ \uparrow \\ 1 \\ \alpha\text{-Glc}p^{t} \end{array}$$

1

residue or group; *n*, the number of D-glucopyranosyl residues between branch-point residues; *m*, the carbon atom number of the position of branching (*O*-substitution); *t* identifies a terminal group, *b*, a branch-point residue, and *l*, a linear, chain-extending residue). The structure, as represented by **1**, provides a useful paradigm for comparison of data obtained by several techniques for a given polymer, and also for the structural comparison of different polymers. However, the foregoing assumptions may not be correct for all dextrans. Independent data suggested that some dextrans have side chains longer than one residue[5,24], and the uniformity of the number of residues between branch-points is difficult to evaluate by use of current, spectroscopic methods.

Several of the assumptions, or simplifications, employed in the original correlations of the ^{13}C-n.m.r. spectra to methylation–fragmentation analysis data have been fruitful. For example, it was assumed that neighboring-residue effects would not occur, and that the *l*-residues of the chain immediately before and after a *b*-residue would not exhibit different chemical shifts. However, pullulan (see **2**) is composed of a two to one ratio of 4-mono-*O*-substituted and

$$-[-\alpha\text{-Glc}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-Glc}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-Glc}p\text{-}(1\rightarrow4)\text{-}]_{x}-$$

2

6-mono-*O*-substituted α-D-glucopyranosyl residues, and the ^{13}C-n.m.r. spectrum of this biopolymer has been convincingly interpreted as representing a strictly ordered structure having different spectral contributions from 4-mono-*O*-substituted α-D-glucopyranosyl residues located immediately *before* and *after* a 6-mono-*O*-substituted residue[19]. On increasing the spectral resolution and the precision of the chemical-

shift data, we observed chemical shifts that are diagnostic of specific types of dextran branching and that have a small (~0.05 p.p.m.) distribution around a common, chemical-shift value observed for a given group of dextrans (see Table I of ref. 1, Table II of ref. 13, and Table I of ref. 14). However, we have been unable to correlate any of these small changes in chemical shift to the degree of linearity of the polysaccharides, and attribute these variations to limitations imposed by the general accuracy of the spectrometer, and, possibly, to small differences in individual sample, or solution, preparations. Therefore, we have not observed this neighboring-residue effect in these 25-MHz spectra, and continue to analyze these spectra in terms of only three residue types (*b*, *l*, and *t*). A possible reason for the absence of neighboring-residue effects for these dextrans will be discussed in a following section.

Although increased spectral resolution and improved precision for chemical-shift values have not indicated a neighboring-residue effect for dextrans, it has proved possible to distinguish clearly between various types of *O*-substituted residues. For example, the chemical shifts of the resonances associated with a (1→3)-α-D-linkage are similar, but not the same, for the mainly 3-mono-*O*-substituted (*e.g.*, the spectrum of dextran B-1355 fraction S) and the 3,6-di-*O*-substituted (*e.g.*, the spectrum of dextran B-742 fraction S) α-D-glucopyranosyl residues. Similar comparisons have been made to compounds other than dextrans[1,25]; the resonances associated with the (1→4)-α-D-linkage are different for 4-mono-*O*-substituted (*e.g.*, the spectrum of glycogen) and the 4,6-di-*O*-substituted (*e.g.*, the spectrum of dextran B-742 fraction L) α-D-glucopyranosyl residues.

Although it is known that different carbon-atom positions can give ^{13}C-n.m.r. resonances of different intensity, it was initially assumed that the relative intensity of a resonance from a specific type of *O*-substituted residue would be directly proportional to the mole percentage of that residue present in the polysaccharide. The correlation of relative intensities of ^{13}C-n.m.r. resonances to methylation–fragmentation analysis data for a series of dextrans branching through the 2,6-di-*O*-substituted α-D-glucopyranosyl residue[13] has demonstrated the general validity of such an additive approach. The narrowness of these resonances allows reasonable correlations to be made in terms of peak height, as well as peak area. Such an additivity relationship of resonances of specific residues would appear to have greater validity for the ^{13}C-n.m.r. spectra of these dextrans than for the corresponding ^{1}H-n.m.r. spectra[12]. These positive correlations between ^{13}C-n.m.r. resonance intensity and methylation–fragmentation analysis data have been welcome, as the ^{13}C-n.m.r. data provide independent confirmation of the general correctness of the methylation–fragmentation approach. However, for dextrans branching through the 4,6-di-*O*-substituted α-D-glucopyranosyl residue[1], the correlation of ^{13}C-n.m.r. intensity data to methylation–fragmentation analysis data has not been in exact parallel, and we are, at present, not certain whether this effect results from (*a*) unrecognized problems in recording ^{13}C-n.m.r. spectra, (*b*) incorrect methylation–fragmentation analysis data, or (*c*) a failure of the additive principle to apply in this specific case. We have observed that, for the best correlation of polysaccharide linearity to relative, ^{13}C-n.m.r. heights, it is

necessary to employ similar recording conditions[4,26] (*e.g.*, temperature) and data-processing techniques[1].

An important component of the total, structural-determination technique is the use of high ^{13}C-n.m.r.-recording temperatures. These elevated, ^{13}C-n.m.r.-recording conditions cause chemical-shift–temperature dependence ($\Delta\delta/\Delta$T) effects that must be considered when comparing spectra recorded at different temperatures, but, for most dextrans, the high- and low-temperature, spectral "profiles" are the same. Unless otherwise mentioned, all ^{13}C-n.m.r. data presented here will refer to aqueous solutions recorded at 90° and nominally referenced to tetramethylsilane. The ambient-temperature conditions for this series of reports on unusual dextrans require comment. The spectrometer is either allowed to equilibrate under ambient conditions, or adjusted to a desired temperature with the Varian probe, temperature-controller; in both cases, the final solution-temperature is confirmed with a calibrated thermometer. Measurements discussed in our initial, ^{13}C-n.m.r. report were recorded at an ambient temperature of 27°. After this initial series of dextran measurements had been made, the spectrometer was submerged inadvertently in brackish water, rebuilt, and moved to a new location. Ambient recording-conditions at this new location are 34°, however, but average values for ambient-temperature chemical-shifts of dextran spectra taken before and after this change are essentially the same. We are not certain why a $\Delta\delta/\Delta$T change was not observed for this apparent 7° change in ambient conditions; however, we are not planning to pursue this line of investigation.

^{13}C-N.m.r. chemical-shifts are very useful for establishing the anomeric configuration of the residues present in a polysaccharide. In general, it was known that the C-1 chemical-shifts of α-anomerically linked D-glucopyranosyl residues are upfield (~100 p.p.m.) from the corresponding C-1 chemical-shifts of β-anomerically linked residues (~104 p.p.m.). In invoking the argument that all upfield, anomeric resonances of these residues indicate α-D-linkages, it is difficult to avoid circular reasoning, as this series of studies of unusual dextrans has established that the anomeric resonances of these dextrans lie in a rather broad (97.3–101.6-p.p.m.) spectral region. However, the actual identification of the presence of α- or β-anomerically linked residues is dependent on observation of specific optical rotation. All of the dextrans we have studied have been found to have high specific optical rotations, and dextrans of high degree of branching are known for each of the four groups of dextrans. If any of the anomeric linkages of dextrans are associated with a β-anomerically linked residue, dextrans containing large proportions of these residues would exhibit abnormally low, specific optical rotations, and such an effect has not been observed for any dextran. Therefore, in accordance with other evidence, it was concluded that all resonances in the ^{13}C-n.m.r. spectra of these dextrans indicate α-linked residues.

Conversely, as no resonances downfield of 102 p.p.m. have been observed for dextrans, it was concluded that these biopolymers are, within the limits of spectroscopic observation, exclusively composed of α-linked D-glucopyranosyl residues. Due to the wide variety of α-D-glucose residues present in these dextrans, and in the

D-glucan-reference polymers, it was also concluded that few, if any, α-D-glucopyranosyl residues of D-glucans will exhibit anomeric, ^{13}C-n.m.r. resonances outside the 97–102- p.p.m., spectral region. Although fewer examples of specifically *O*-substituted, β-D-glucopyranosyl residues have been studied, none have been observed to exhibit an anomeric resonance upfield of 102 p.p.m., and it is therefore possible that the anomeric resonances of D-glucopyranosyl residues are cleanly separated into a downfield, β-linked region and an upfield, α-linked region.

The assignment of specific resonances to specific carbon atoms is of interest for accurate comparison of recorded data to proposed polymer structure, and is necessary for the prediction of the ^{13}C-n.m.r. spectrum of a proposed, polymer structure. Certain classes of chemical shifts are clearly differentiatable in a D-glucan, ^{13}C-n.m.r. spectrum (*e.g.*, the δ of C-1 and C-6). For highly branched D-glucans, the resonances from *t*- and *b*-residues dominate the ^{13}C-n.m.r. spectrum; the contributions from both *t*- and *b*-residues are approximately equal in intensity, and produce 12 new branching resonances. A more intimate knowledge of the effect of *O*-substitution on change in chemical shifts would be possible if the resonances associated with the *b*-residues could be separated from the resonances associated with the *t*-residues: this differentiation is possible by use of resonance-relaxation studies. The following discussion of resonance-relaxation data to structural relationships has been taken from general discussions for organic compounds[27,28], and applied to the structure of polysaccharides.

Spin–lattice relaxation (T_1) values are parameters associated with n.m.r. measurements. Each resolved, n.m.r. resonance is associated with a specific, carbon-atom position of the molecule, and has an associated, measurable T_1^{obs} (observed relaxation) value that indicates the average interaction of that carbon nucleus with the environment. Such effects can be divided into those arising from dipole–dipole (of the carbon nuclei with the nuclei of chemically bonded hydrogen atoms) interactions (T_1^{DD}), and a group of interactions dependent on spin rotation (T_1^{SR}), chemical-shift anisotropy (T_1^{CSA}), and scalar coupling (T_1^{SC}). These relationships can be expressed as:

$$1/T_1^{obs} = 1/T_1^{DD} + 1/T_1^{SR} + 1/T_1^{CSA} + 1/T_1^{SC}.$$

The nuclear Overhauser effect (n.O.e.) is a second measurable parameter associated with n.m.r. resonances. In actual practice, the quantity η (n.O.e. = η + 1) is directly measurable, and as $\eta \propto 1/T_1^{DD}$, a knowledge of η and T_1^{obs} allows T_1^{DD} to be factored out of the foregoing equation. In a solution free from paramagnetic species (*e.g.*, Fe^{3+}, dissolved oxygen), the T_1^{DD} of a resonance for a specific carbon atom may be considered to be a measure of the segmental motion of the part of the molecule containing that carbon atom.

When a relatively rigid molecule tumbles through solution with no favored mode of rotation (isotropic motion), segmental motion and external magnetic fields for all parts of the molecule will be similar. Therefore, the magnitude of T_1^{obs} and T_1^{DD} will be similar for each carbon nucleus. (However, those carbon atoms attached to two hydrogen atoms will have more rapid relaxation, and T_1^{DD} smaller by a factor

of half, than those carbon atoms attached to one hydrogen atom.) Parts of a molecule having motion greater than the average, segmental motion for the total molecule will contain carbon atoms exhibiting larger than average T_1^{DD} values. Of the four ^{13}C spin–lattice mechanisms, only T_1^{DD} and T_1^{SR} are generally observed in diamagnetic solutions, and, for large molecules, T_1^{DD} is known normally to dominate the spin–lattice relaxation process.

Therefore, for dextran ^{13}C-n.m.r. spectra, the following approach to spin–lattice relaxation data can be employed. (*a*) Consider dextrans in terms of **1**. (*b*) Assume that the dextran backbone has an average, segmental motion. (*c*) Assume that the side-chain residues have greater freedom of motion than the backbone residues. The additional, side-chain-residue motion is then superimposed on the average, backbone, segmental motion. Therefore, resonances associated with side-chain residues can be expected to have larger than average T_1^{DD} values, and these values will be of a similar order to the T_1^{obs} values.

The experimental procedure for obtaining T_1^{obs} values essentially consists of sequentially recording a series of ^{13}C-n.m.r. spectra for the same compound. Each spectrum in the series differs only by a change in the τ value (the delay time between the 180°-inversion pulse and the 90°-monitor pulse). A long τ value results in a fully relaxed spectrum (see Figs. 1–4), and a short τ value results in an inverted spectrum that is reflected across the ordinate (p.p.m.) axis. For each resonance, there exists a unique τ value that results in nulling of the magnitude of that resonance, and from which the T_1^{obs} value can be calculated. Alternatively, and more effectively, a single resonance may be examined in each of the series (with changing τ values) of spectra. These resonance-intensity values are then plotted *vs.* τ, a best-fit curve obtained, and a T_1^{obs} value calculated from this curve. This procedure would then be repeated for each resonance of interest in the spectrum. Therefore, a series of spectra (in practice, ~15) are needed in order to obtain the T_1^{obs} values for a single compound. The accuracy of these T_1^{obs} measurements is dependent on (*a*) the signal-to-noise ratio of each spectrum, (*b*) the number of spectra recorded at different τ values, and (*c*) the judicious choice of τ values for each spectrum in the series.

The difference between the T_1^{obs} and the T_1^{DD} values can be established with the n.O.e. values. The n.O.e. values are determined in a similar manner to the T_1^{obs} determinations, differing only in the details of proton decoupling during data acquisitions. Therefore, a number of spectra similar to the T_1^{obs} series of spectra are needed; and finally, to obtain η values, curve-fitting calculations must be performed for each specific resonance. A combination of T_1^{obs} and η values ultimately allows quantitation of the T_1^{DD} value for each resonance, which is a measure of the segmental motion of the associated carbon atom. Each F.t. n.m.r. experiment requires, in general, 0.5 s for completion, followed by a delay time of ~1 s to allow the system to re-equilibrate. Acquisitions can therefore be made at the rate of ~2,000/h, to obtain series of spectra for both T_1^{obs} and η values.

Accurate relaxation-measurements are difficult to obtain, and the presence of either dissolved oxygen or trace metals in solution can affect these values. Poly-

saccharide spectra have presented problems in the obtaining of acceptable, signal-to-noise ratios, and, often, spectra of ~50,000 acquisitions have been necessary in order that the signals could be clearly distinguished from the background. Finally, lack of resolution allowed the identification of only a few polysaccharide chemical-shifts that did not appear to be composite resonances. We have eliminated, or lessened, the aforementioned problems in the following way. Firstly, dissolved oxygen and trace metals have no detectable effect on polysaccharide relaxation-times under the experimental conditions employed here; both freeze–vacuum–thaw techniques and multiple, fractional distillation of solvent were employed without effect on T_1^{obs}. This lack of effect on T_1^{obs} for polysaccharides contrasts markedly with the results of a limited number of relaxation experiments that we performed on monosaccharides, which presented considerable diversity in relaxation values between successive preparations.

High-temperature spectrum-acquisition resulted in resonances having narrower linewidth; this has improved spectral resolution, and lessened the problems associated with low signal-to-noise ratios. As will be seen in the following section, for polysaccharides, $T_1^{obs} \simeq T_1^{DD}$, and it is therefore possible that the time-consuming n.O.e. measurements are not necessary. The nature of the polysaccharides employed is of importance, as all samples were soluble, and available in amounts greater than 100 mg. The preferred solution for study consisted of ~200 mg of polymer dissolved in 4 mL of deuterium oxide. Such a large, concentrated sample allowed acceptable spectra to be obtained in ~3,000 acquisitions, which, in turn, allowed an acceptable series of spectra of different τ values (*e.g.*, for T_1^{obs}) to be obtained in less than 24 h.

Our treatment of the spin–lattice relaxation approach is not rigorous. Two simplifications involve (*a*) the use of T_1^{obs} instead of T_1^{DD}, and (*b*) the neglect of solution viscosity. Theory suggests, and measurements on dextran B-742 fraction S indicate, that the order of the relative magnitudes for T_1^{obs} and T_1^{DD} is similar. The introduction of viscosity introduces complexities, especially when considering whether bulk or micro viscosities are the more important. We have attempted to avoid much of this viscosity problem by limiting the T_1 arguments to the *relative* magnitude of values obtained from resonances in the spectrum of the *same compound.* However, although the bulk viscosities of these solutions differed significantly at 90°, the absolute magnitudes of T_1^{obs} of similar carbon-atom positions (*e.g.*, the free C-6 atoms) are quite consistent for different polymers. For a given polysaccharide solution, the T_1^{obs} values can (for a given observation period) be obtained with greater accuracy than the η values. Therefore, the T_1^{obs} values are inherently more accurate than the T_1^{DD} values; this also encourages the use of T_1^{obs} values. Finally, we have based our arguments on differences in T_1^{obs} that are larger than 10%, which should avoid the possibility of these arguments being influenced by minor effects. It has been implicit in the foregoing discussion that the T_1 values are independent of the field strength. Under certain experimental conditions, T_1 values can be dependent on field strength, and the evaluation of such effects must be made at different magnetic-field strength. However, the magnitude of the n.O.e. values obtained for these dextrans indicates that the T_1^{obs}

values, especially at 90°, behave as though these polymers are in a region of isotropic motion, and therefore, the T_1^{obs} values are independent of the field strength[29].

With regard to saccharides, ^{13}C-n.m.r.-relaxation data have been used in studying the tumbling motion of monosaccharides[30,31], and disaccharides[32], and for the determination of glycosylation sites in nucleosides[23]. Relaxation values of ^{13}C-n.m.r. resonances can also provide information on the general motion of a polymer, and Benesi and Gerig[34] presented such data for linear, commercial dextrans. An approach similar to the correlation of the magnitudes of T_1 values of given resonances to various atom-positions that we describe herein has been applied to ^{1}H-n.m.r. spectra by Hall and Preston[35], who demonstrated that, for the anomeric resonances of oligosaccharides, a direct relationship exists between proton spin–lattice relaxation-times and the molecular weight of linear oligosaccharides. However, such proton spin–lattice relaxation-time relationships could not be demonstrated for various segments of the repeating unit of polysaccharides.

The dextrans employed in the present investigation were essentially homogeneous fractions separated from the corresponding, polydisperse, high-molecular-weight, native dextran[36] by fractionation with ethanol[37]. The dextran designations refer to the NRRL number for the bacterial strain from which the specific dextran was obtained. The bacterial strains employed were *Leuconostoc mesenteroides* B-742, B-1299, B-1355, and B-1498, and *Streptobacterium dextranicum* B-1254. The S (soluble) and L (less soluble) designations refer to fractions obtained from the ethanolic fractionation. Nigeran[38] and glycogen[39] were studied, as they are, respectively, well characterized examples of linear and of dendritically branched polysaccharides. The synthetically branched amylose[40] HAG-12 provides an excellent example of a polysaccharide having a known, comb-like structure of the general type denoted in **1**. These dextrans and reference polysaccharides make available a wide variety of differently *O*-substituted, α-D-glucopyranosyl residues and groups which, in turn, provide many examples of the effect of glycosidic *O*-substitution on change in ^{13}C-n.m.r. chemical-shifts and related parameters.

RESULTS AND DISCUSSION

The fundamental data under consideration in this study consist of ^{13}C-n.m.r. chemical-shifts, measured in aqueous solution at 90°, and the corresponding T_1^{obs} values obtained by the inversion-recovery method. The chemical shift for each resonance is shown in Table I, which also contains, in parentheses (after each chemical shift), the corresponding, inversion-recovery, T_1^{obs} value. Unless otherwise described, all ^{13}C-n.m.r. measurements refer to 90° acquisition-conditions. The relative intensity of the resonances is indicated by employing a series of numbers to reference the chemical shifts in Table I to the peak heights of the spectra in Figs. 1 and 2. Owing to the close spacing of resonances in the 69–76-p.p.m. region, expanded spectra of this region are also referenced to the chemical shifts of Table I (see Figs. 3 and 4). These referencing numbers have been chosen for convenience of referencing Table I

TABLE I

CHEMICAL SHIFTS AND T_1 VALUES FOR ^{13}C-N.M.R. SPECTRA, AT 90°, OF D-GLUCANS[a]

	Glycogen[b]	*Pullulan*	*Nigeran*	*Branched amylose HAG-12*	*Dextrans*					
					B-1254 L[S][c]	*B-1299 S*	*B-742 S*	*B-1254 S[L]*	*B-1355 S*	*B-1498 S*
	Major linkages present									
	(1→4)	*(1→4) & (1→6)*	*(1→3) (1→4)*	*(1→4) & (1→6)*	*(1→6)*	*(1→2) & (1→6)*	*(1→3) & (1→6)*	*(1→4) & (1→6)*	*(1→3) & (1→6)*	*(1→3) & (1→6)*
1[d]				104.37 (179)						
2		101.83 (188)[e]		101.65 (92)				101.57 (203)		
3	101.43	101.36 (160)	101.55	101.34 (108)						
4									101.07 (149)	101.04 (186)
5			100.44				100.81 (250)			
6								100.07 (140)	99.94 (111)	99.84 (187)
7(A)[f]		99.63 (160)			99.59	99.57 (203)	99.56 (150)	99.42 (197)	99.75 (222)	99.43 (289)
8						98.22 (241)				
9						97.37 (140)				
10									83.31 (120)	83.25 (108)
11							82.89 (110)			
12			81.83							

13		79.94 (152)		80.32 (115)				80.40 (113)		
14	79.37	79.63 (170)	79.34	79.30 (114)						
15				77.59 (203)		77.83 (130)				
16(B)	75.02	74.94 (168)	74.90		75.02	75.20 (215)	75.09 (140)	75.07 (141)	75.02 (116)	75.08 (147)
17				74.96 (146)			74.79 (180)			
18		74.79 (144)				74.84 (150)		74.87 (140)		
19			74.19					74.37 (138)		
20						73.76 (169)				
21	73.41						73.57 (180)			
22							73.50	73.50 (155)	73.46 (131)	73.43 (282)
23		73.30 (137)		73.29 (121)					73.30 (158)	73.31 (141)
24(C)	73.18	73.16 (128)	73.21	73.11 (117)	73.18	73.18 (190)	73.14 (140)	73.12 (152)		73.01 (231)
25		72.89 (162)								
26			72.24	72.36 (98)					72.37 (120)	72.32 (140)
27(D)		72.13 (181)	72.16		72.04	72.04 (126)		71.95 (126)	71.88 (104)	71.86 (118)
28							71.74 (110)			
29(E)		71.40 (155)	71.45	71.39 (184)	71.51	71.51 (156)	71.41 (170)	71.43 (147)	71.45 (143)	71.47 (140)
30				70.58 (83)				71.22 (130)		
31		68.49 (102)						68.47 (81)		
32(F)					67.83	67.83 (83)	67.67 (63)	67.68 (85)	67.67 (78)	67.65 (127)
33	62.51	62.55 (126)		62.48 (104)		62.46 (223)	62.49 (206)	62.43 (221)		
34		62.31 (124)	62.24						62.33 (169)	62.31 (151)

[a]The chemical shift in p.p.m., relative to Me_4Si. [b]Glycogen prepared from rabbit liver. [c]The letters S and L refer to dextran fractions. [d]These numbers are referenced to the spectra of the corresponding compounds in Figs. 1 through 4. [e]The bracketed value represents the T_1^{obs} value (in ms) for the corresponding chemical shift. [f]The letters A through F identify the resonances of linear dextran.

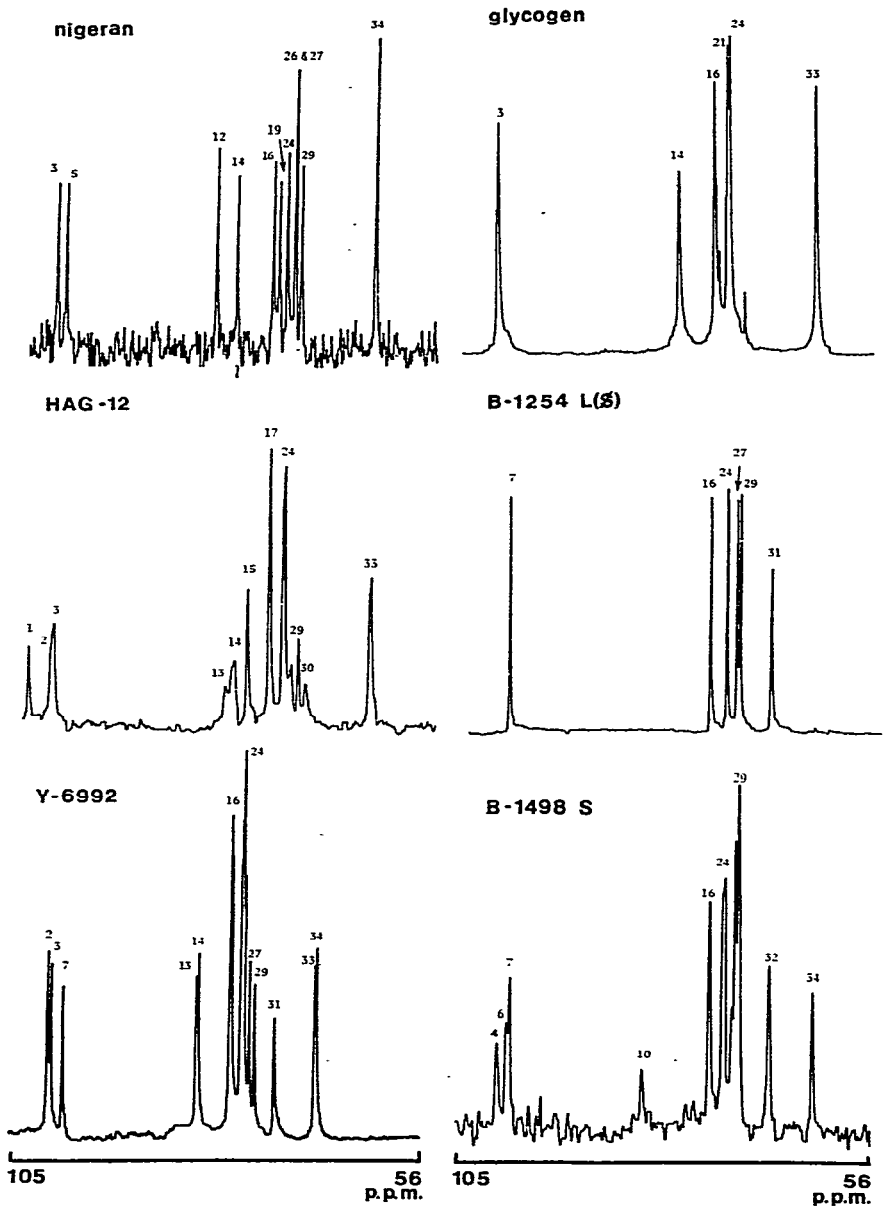


Fig. 1. Fully relaxed, ^{13}C-n.m.r. spectra at 90° for: nigeran, branched amylose (HAG-12), pullulan (Y-6992), rabbit-liver glycogen, dextran B-1254 fraction L[$], and dextran B-1498 fraction S. The numbered resonances correspond to the values in Tables I and IV.

to Figs. 1–4, and similar referencing numbers in Table I, for resonances from different polysaccharides (*e.g.*, comparison of glycogen to dextran B-1498 fraction S), are not intended to imply that these resonances arise from identical, carbon-atom positions, but are a result of the brevity used in Table I. However, especially for the dextrans,

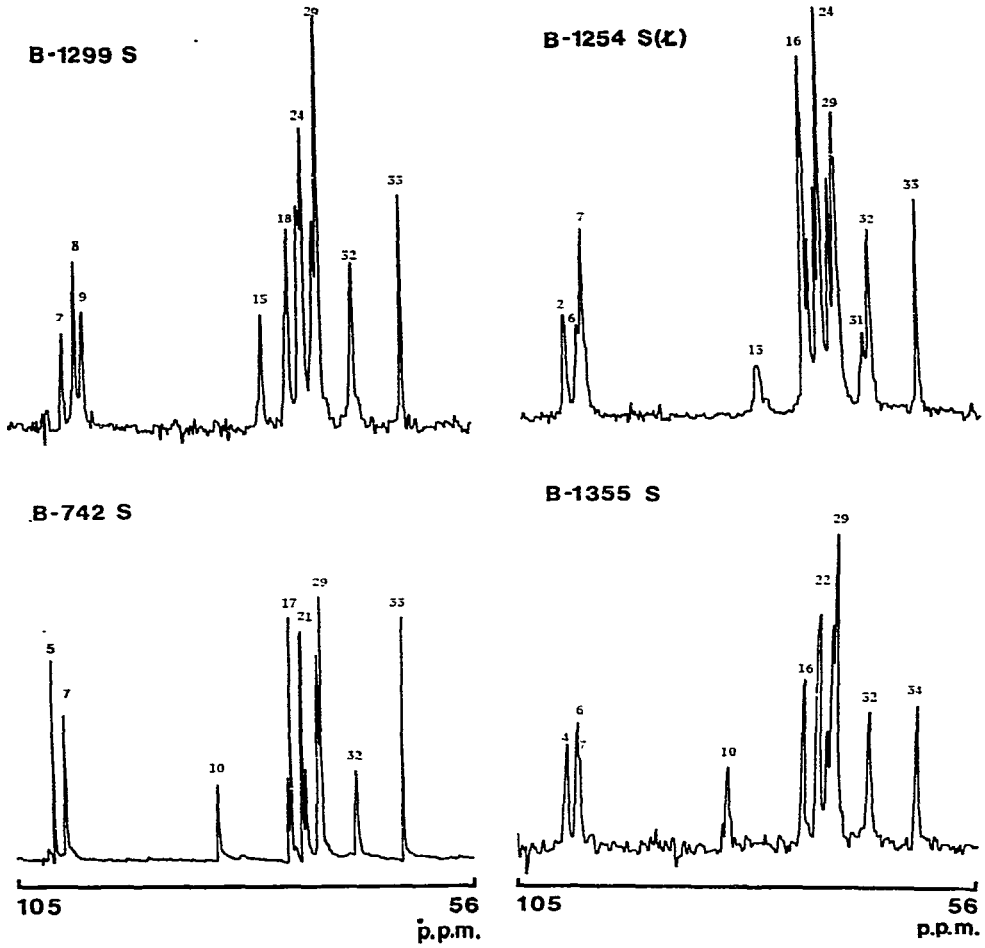


Fig. 2. Fully relaxed, ^{13}C-n.m.r. spectra at 90° for the S fractions of dextrans B-1299, B-742, B-1254, and B-1355. The numbered resonances correspond to the values in Tables I and IV.

many of these similarly numbered resonances in different polysaccharides apparently do refer to similar, carbon-atom positions.

The results of methylation–structural analysis are given in Table II. These data were obtained by employing the peracetylated aldononitrile derivatives[41], which were quantitated by hydrogen-flame g.l.c. and confirmed by electron-impact m.s.–g.l.c.[5,15]. For each polysaccharide, the same samples were employed in order to obtain the methylation–structural analysis data, the spin-relaxation ^{13}C-n.m.r. data, and the fully relaxed ^{13}C-n.m.r. spectra presented in Figs. 1–4. In general, each spectrum in Figs. 1–4 represents ~3,000 acquisitions, and therefore provides a reference for the signal-to-noise ratio of each of the spectra comprising the τ-value-change series. For the nigeran spectrum (see Fig. 1), a solution containing ~100 mg was employed. For the glycogen, dextran B-742 fraction S, and dextran B-1254 fraction L[$] spectra, ~40,000 acquisitions were employed for the fully relaxed

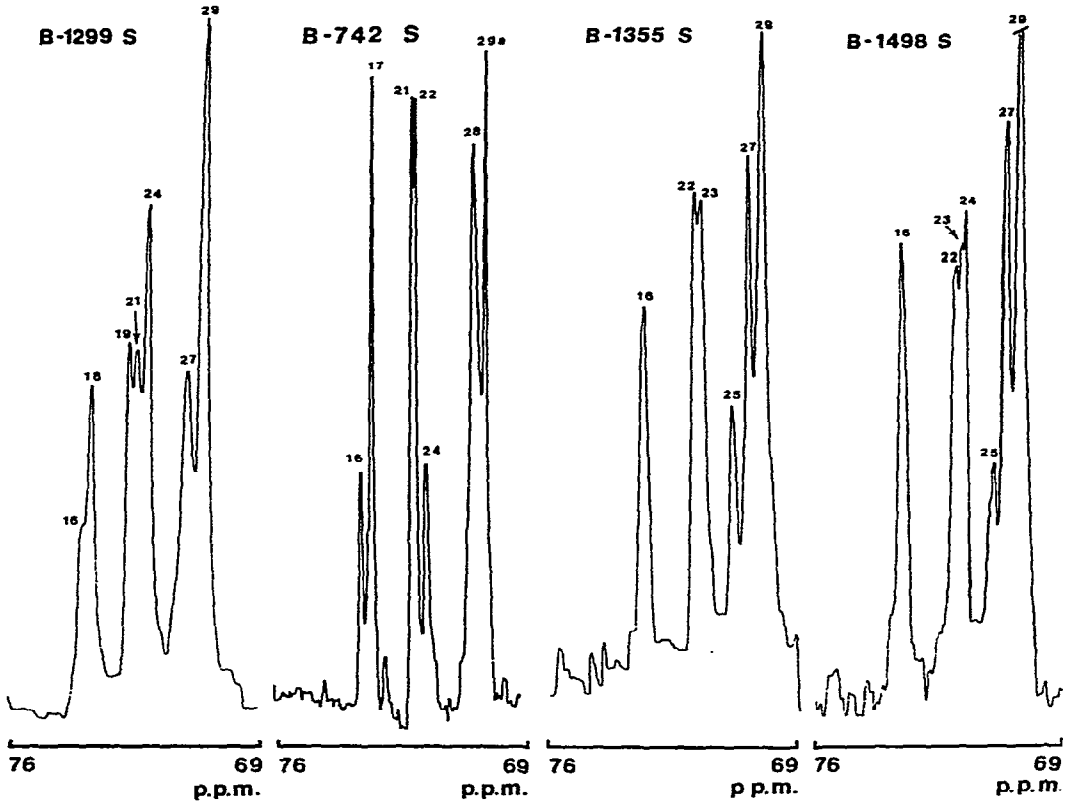


Fig. 3. The expanded, 60–75-p.p.m. region of the fully relaxed, ^{13}C-n.m.r. spectra at 90° of the S fractions of dextrans B-1299, B-742, B-1355, and B-1498. The numbered resonances correspond to the values in Tables I and IV and Figs. 1 and 2.

spectra. The fully relaxed, spectral data from glycogen and dextran B-742 fraction S were also processed by c.d.r.e., in order to obtain the high-resolution spectra shown in Figs. 1–3.

Glycogen. — The ^{13}C-n.m.r. spectrum of glycogen contains broad resonances that, for visual display, can be narrowed very effectively by the use of c.d.r.e. Also, when compared to dextrans, glycogen is very easily permethylated, and this permethylated product easily hydrolyzed. These properties of glycogen can be attributed to the dendritic nature of this biologically important and extensively studied compound[39]. Glycogen is a branching, tree-like molecule, primarily composed of 4-mono-*O*-substituted α-D-glucopyranosyl residues that are interspaced, at approximately 14 residue intervals (see Table II), with 4,6-di-*O*-substituted α-D-glucopyranosyl branching-residues. Owing to their small mole percentages, these glycogen branch-point residues do not make a significant contribution to the ^{13}C-n.m.r. spectrum, and therefore, the glycogen spectrum approximates 3, which is actually that of amylose. Were it more readily soluble, the linear amylose would no doubt give a

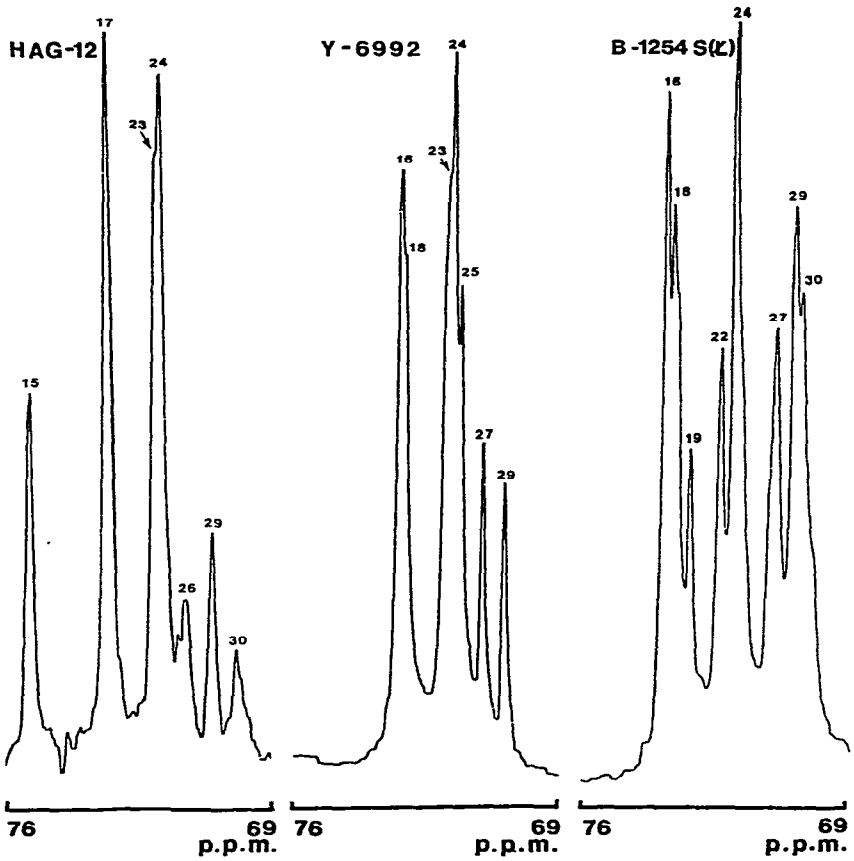


Fig. 4. The expanded, 69–75-p.p.m. region of the fully relaxed, ^{13}C-n.m.r. spectra at 90° of branched amylose (HAG-12), pullulan (Y-6992), and dextran B-1254 fraction S[L]. The numbered resonances correspond to the values in Tables I and IV and Fig. 1.

TABLE II

MOLE PERCENTAGES OF METHYLATED D-GLUCOSE COMPONENTS IN HYDROLYZATES OF PERMETHYLATED D-GLUCANS[a]

D-*Glucan*	*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*
Glycogen[b]		6.4		88.3		5.3		
Pullulan Y-6992		0.4	33.5	65.9			0.2	
Dextran								
B-1254[c]	L[S]	3.8	90.0		1.8		4.4	
B-1299[d]	S	39.1	26.0					34.9
B-742[c]	S	45.4	4.4				50.2	
B-1254[d]	S[L]	22.1	55.0	3.3		19.5		
B-1355[d]	S	6.9	46.9		35.0		11.2	
B-1498[e]	S	9.4	51.5		29.0		10.0	

[a]The mole ratio is expressed in terms of the uncorrected peak-integrals of the hydrogen-flame-detector chromatograms of the PAAN derivatives. The identities of the g.l.c. peaks were confirmed by electron-impact mass-spectrometry. [b]Data taken from ref. 12. [c]Data taken from ref. 26. [d]Data taken from ref. 5. [e]Data taken from ref. 14.

$$-[-\alpha\text{-Glc}p\text{-}(1\rightarrow4)\text{-}]_x-$$
$$\mathbf{3}$$

similar spectrum. The spectrum shown in Table I and Fig. 1 is that of a rabbit-liver glycogen. However, rabbit-liver glycogen and oyster glycogen, and their corresponding limit-dextrins, gave essentially the same ^{13}C-n.m.r. spectrum. The low intensities of the resonances of glycogen branching-residues preclude spin–lattice-relaxation studies, but the glycogen spectrum provides an example of the spectrum of 4-mono-*O*-substituted α-D-glucopyranosyl residues that are consecutively linked to similar residues.

Pullulan (Y-6992). — The linear polysaccharide pullulan contains α-D-glucopyranosyl residues similar to those preponderating in glycogen (4-mono-*O*-substituted) and in dextran (6-mono-*O*-substituted) in a 2:1 mole ratio (see **2**). Methylation–structural-analysis data (see Table II) confirm this ratio for the specific sample studied here. Pullulan is similar to glycogen in its ease of polymer permethylation and subsequent hydrolysis.

In examining the ^{13}C-n.m.r. spectrum of pullulan, Jennings and Smith[19] pointed out that the resonances of C-1, C-4, and C-6 of the 4-mono-*O*-substituted residue were each cleanly split into two sharp, well-defined resonances (respectively, resonances 2 and 3, resonances 13 and 14, and resonances 33 and 34 of Table I and Fig. 1). As a polysaccharide chain has a direction sense, with a reducing residue on one end and a nonreducing group on the other, it was concluded that (*a*) ^{13}C-n.m.r. data support a polymer of structure **2**, with a strictly ordered, residue sequence, as shown, and (*b*) the C-1, C-4, and C-6 resonance-splitting results from slightly different, spectral contributions from 4-mono-*O*-substituted residues *fore* and *aft* of the 6-mono-*O*-substituted residue. Essentially, such an interpretation postulates that the nature of *neighboring residues* can have an effect on the chemical shifts of a given residue, and that the environments of the two 4-mono-*O*-substituted-residue types in **2** are sufficiently different to produce detectable, chemical-shift changes at 25 MHz.

The T_1^{obs} values for each of the resonances of pullulan are given in Table I, and provide a general point of reference for comparing the magnitude of T_1^{obs} values of resonances arising from linear molecules. Most T_1^{obs} values of resonances of pullulan lie in the 140–170-ms region. The 60–70-p.p.m.-region resonances, representing carbon atoms attached to two hydrogen atoms, are an exception (in general, these T_1^{obs} values should be multiplied by 2, the number of attached hydrogen atoms, for direct comparison to the other T_1^{obs} values). As the pullulan backbone comprises (1→4)- and (1→6)-α-D-linkages, it is possible that the segmental motion of the backbone is not uniform, with regions of increased, segmental motion centered around the (1→6)-α-D-linkages. On this basis, the C-1 and C-4 resonances of the atoms of the parts of the 4-mono-*O*-substituted residues that are directly attached to the mobile, 6-mono-*O*-substituted residue will have the larger T_1^{obs} values. An assignment could then be made of resonances 2 and 13 to the C-1 and C-4 of the *aft* residue, and resonances 3 and 14 to the *fore* residue.

Nigeran. — Nigeran, as shown in **4**, is a linear polymer composed of

$$-[-\alpha\text{-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-Glc}p\text{-}(1\rightarrow4)\text{-}]_x-$$

4

alternating 3- and 4-mono-*O*-substituted α-D-glucopyranosyl residues[38]. Nigeran contains no (1→6)-α-D-linkages, and could be a better model than pullulan for testing the differences in magnitude of T_1^{obs} of a linear polysaccharide. However, the low solubility of this polymer presents technical problems for obtaining accurate T_1^{obs} values. The ^{13}C-n.m.r. spectrum of nigeran (see Table I and Fig. 1) shows ten, well resolved resonances, a partially resolved resonance-pair (26 and 27), and the unresolved C-6 resonances (34). By analogy to the glucan and pullulan chemical-shifts, resonances 3, 14, 16, 24 (either 26 or 27, and half of the contribution of 34) are assigned to carbon atoms of the 4-mono-*O*-substituted α-D-glucopyranosyl residue. The remaining resonances are then assigned to the 3-mono-*O*-substituted residue.

Synthetically branched amylose (HAG-12). — This compound is of interest as it is an authentic example of a comb-like, branching polysaccharide, as shown in **5**. Amylose HAG-12 was produced by attaching monomeric derivatives of D-glucose to linear

$$-\{-[-\alpha\text{-Glc}p^t\text{-}(1\rightarrow4)\text{-}]_n\text{-}\alpha\text{-Glc}p^b\text{-}(1\rightarrow4)\text{-}\}_x-$$

$$\begin{array}{c} 6 \\ \uparrow \\ 1 \\ \beta\text{-Glc}p^t \end{array}$$

5

amylose[40], and the resulting compound is therefore known to have (*a*) a linear, backbone structure, (*b*) side chains one residue long, and (*c*) the side-chain residues randomly attached along the linear backbone (therefore, *n* in **5** represents an average, rather than a specific, number of residues interspaced between branching residues). The spectrum of HAG-12 (see Table I and Figs. 1 and 4) is similar to previously obtained spectra of similar condensation-products of amylose[25]. The side-chain groups are known to be, within the limits of spectroscopic detection, exclusively β-D-glucopyranosyl groups, in contrast to the known, 4-mono-*O*-substituted α-D-glucopyranosyl residues of the original amylose. The β-D-glucopyranosyl residues have shifts for many carbon atoms that are clearly displaced from the chemical shifts of the α-D-glucopyranosyl residues. Therefore, many resonances of carbon atoms in the side-chain groups of HAG-12 are well identified, and definitely displaced from the resonances of the carbon atoms of the linear, backbone chain.

Thirteen peaks are resolved in the HAG-12 spectrum shown in Figs. 1 and 4, although a polysaccharide having a repeating unit of the kind shown in **5** could have 18 carbon resonances. By comparison with methyl β-D-glucopyranoside, it is known that peak 15 is a composite of two resonances from the β-D-glucopyranosyl group, and that resonance 33 is a composite of the free, C-6 atoms of the *t*- and *l*-residues[25];

thus, 15 of the 18 expected resonances of **5** are identified. Resonance 1 and the composite resonance 15 are known[25] to correspond to carbon atoms of the *t*-group, and both resonances have T_1^{obs} values (~190 ms) distinctly larger (see Table I) than the average T_1^{obs} values found for the resonances of this polysaccharide. Furthermore, resonances 2 and 13 have been shown[25] to correspond, respectively, to C-1 and C-4 of the *b*-residue, with resonances 3 and 14 corresponding, respectively, to C-1 and C-4 of the *l*-residue. The *l*- and *b*-residues form the backbone chain, and the associated resonances 2, 3, 13, and 14 have small T_1^{obs} values (~100 ms). These measurements support the concept that side-chain groups will have segmental motion greater than the average, and that the expected, relatively larger, T_1^{DD} values of resonances associated with carbon atoms in these groups will be paralleled by corresponding T_1^{obs} values that are larger than the average for T_1^{obs} values associated with resonances of a given spectrum. In addition, the T_1^{obs} value of resonance 29 is large (184 ms), indicating that this is also a resonance of the *t*-group, whereas the T_1^{obs} of resonance 30 is abnormally small, indicating that it represents the linked C-6 (primary alcohol) positions of the *b*-residue. The remaining three, unidentified resonances are assumed to be associated with the relatively intense peaks 17 and 24, and, as the T_1^{obs} of 17 is large (146 ms), it is possible that this peak contains the remaining, unidentified, *t*-group resonance.

Compared to previous spectra of synthetic amylose[25], and also, compared to a value of $n = 2.6$, which has been established for this compound by measuring the nitrogen content of a precursor* (we have not performed on this polymer methylation–structural analysis, similar to those in Table II), the branching resonances of HAG-12 are surprisingly intense. However, it is possible that this divergence of apparent *n* value, as established by the two methods, indicates a phenomenon that must be considered for other polysaccharides. Some polysaccharide solutions are opalescent; that is, they appear clear under indirect light, but cloudy when illuminated with direct light. The HAG-12 solution employed for ^{13}C-n.m.r. study was highly opalescent. Such light-scattering in solution normally results from suspended, particulate matter.

The following argument is a possible explanation for both the solution opalescence and the difference in the measure of polymer linearity, the *n* values, as established by nitrogen content and by ^{13}C-n.m.r. relative peak-height. The original, linear, amylose polymer is relatively insoluble in water, and the addition of side-chain groups is a random, chemical process. It is possible that certain regions of the amylose chain could be highly substituted, promoting solubility, whereas portions of the original amylose chain remain unaffected and essentially insoluble. Such a polysaccharide could then be solubilized in water, but still have microregions of insolubility. The insoluble regions (which, in this case, are assumed to be the linear regions) could scatter light to give the opalescent effect, and also the carbon atoms of this insoluble region would have the exceptionally large relaxation-times of solid material, and would contribute little to a ^{13}C-n.m.r. spectrum. The net effect of such

*Personal communication, Dr. B. Pfannemüller.

a situation would be to produce a ^{13}C-n.m.r. spectrum that indicates an average degree of branching greater than that obtained by chemical means. Such arguments have interest for the branched dextrans, as we are desirous of establishing average degrees of branching for these polysaccharides.

In some cases (*e.g.*, the dextrans branching through 4,6-di-*O*-substituted residues), divergence has been noted between *n* values established by methylation–structural analysis and by ^{13}C-n.m.r.-spectral, relative peak-heights[1]. We have examined problems arising from methylation–structural analysis[15], and it is possible that effects associated with methylation are totally responsible for such divergence between the methods; however, it must be considered that microregions of insolubility could alter the relative intensity of the diagnostic branching-resonances of a polysaccharide. Such differences of *n* values, as established by independent, chemical and n.m.r.-spectral techniques, would then be a measure of the randomness of branching residues in the backbone. The examination of relative *n* values of dextrans, as indicated by ^{1}H-n.m.r. spectroscopy[12] has yielded results that parallel the ^{13}C-n.m.r. results. However, as both the ^{1}H- and ^{13}C-n.m.r. data were dependent on the total solubility of the material studied, such solubility effects must be considered when comparing these two methods to that of methylation–structural analysis. Fortunately, the foregoing, total-solubility arguments would appear to have little effect on relative, segmental-motion differences of the small portions of the polysaccharide involved in the correlation of the magnitude of T_1^{obs} to the carbon-atom positions of the *t*-groups or *b*-residues.

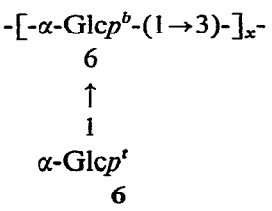
Dextran B-1254 fraction L[$]. — This polysaccharide, mainly composed of 6-mono-*O*-substituted α-D-glucopyranosyl residues (see Table II), is an essentially linear polymer. When employing methylation–fragmentation analysis, it is difficult accurately to establish dextran *n* values below $n \simeq 10$, and, therefore, difficult to calibrate minor, branching features observed in the ^{13}C-n.m.r., ^{1}H-n.m.r., and difference, F.t. i.r. spectra. Dextran B-1254 fraction L[$] may be represented by **1**, where $n \sim \infty$, and the spectroscopic contribution of the *b*- and *t*- residues become negligible. The spectrum of dextran B-1254 fraction L[$] (see Table I and Fig. 1) is given as a reference spectrum; it contains the six, well defined, narrow resonances (A through F) that dominate, or persist in, most dextran spectra (dextran B-742 fraction S is the exception).

The dextrans that have been studied in this series of reports (summarized in Table I of ref. 18), with the exception of the group containing dextran B-1355 fraction S, have all physical and spectroscopic properties in common. The 90°, ^{13}C-n.m.r. spectra of these dextrans show sharp, well defined resonances, and the "profile" for the spectrum of a dextran remains constant at high and low recording-temperatures. Such ^{13}C-n.m.r. properties are quite similar to those of synthetically branched amylose, which is known to have a comb-like structure, with single-residue side-groups. These dextrans are all difficult to permethylate (like cellulose), and the permethylated polymer is difficult to hydrolyze. In addition, the solubilities of these dextrans are similar. Although S and L designations (Soluble and Less soluble,

under ethanolic-fractionation conditions) are employed to define dextran fractions, the dextrans are similar, in that (*a*) their rate of dissolution in water is low, and (*b*) even the L fractions are soluble to the extent of ~50 mg/mL of water[26]. These dextran properties contrast with those of glycogen, of extracellular mannans known to have long side-chains[20], and of the polysaccharide class containing dextran B-1355 fraction S. The dendritic glycogen is very soluble in water, and very easily permethylated (and the product hydrolyzed), and gives a ^{13}C-n.m.r. spectrum containing broad resonances. The dextran B-1355 fraction S series (which will be discussed later) also gives broad, n.m.r. resonances. In addition, a group of extracellular mannans believed to have more-complex and more highly branched structures than the dextrans exhibit more-complex, ^{13}C-n.m.r.-spectral patterns, and are much more easily permethylated than the dextrans.

These observations combine to support indirectly the concepts that (*a*) essentially all dextrans we have studied are comb-like variants of linear dextran, and (*b*) **1** is not only a conveniently simple paradigm for data comparison, but also a good approximation of the major repeating-units contributing to the spectral features of dextrans. The following three classes of dextrans discussed (represented by the S fractions of dextrans B-742, B-1299, and B-1254) are examples of the general dextran described in **1**. The last two dextrans discussed (the S fractions of dextrans B-1355 and B-1498) constitute a separate polysaccharide group that is based on a general structure differing radically from the general form of **1**.

Dextran B-742 fraction S. — The most highly branched dextran we have found, namely, dextran B-742 fraction S, is an example of **1** with $m = 3$, and $n \sim 0$. In the following discussion, it will be assumed that $n = 0$, although methylation–fragmentation analysis[26] indicated that $n \sim 0.1$, and high-resolution (c.d.r.e.), ^{13}C-n.m.r. spectroscopy suggests a somewhat larger value ($n \sim 0.2$). Therefore, only two saccharide components will be considered, the 3,6-di-*O*-substituted α-D-glucopyranosyl residue, and a terminal α-D-glucopyranosyl group. The only alternative to **1** ($m = 3$, $n \sim 0$) is **6**, which



places (1→3)-α-D-linkages in the backbone chain. However, **6** does not seem to be a likely structure for dextran B-742 fraction S. Pseudonigeran[42] is an essentially linear polymer composed of 3-mono-*O*-substituted α-D-glucopyranosyl residues, and essentially insoluble under the conditions we have employed; we could not obtain a reference spectrum under acquisition times where ~10 mg in 4 mL would have given observable, ^{13}C-n.m.r. signals. We discuss elsewhere[43] the solubility-to-structure relationship for dextrans containing contiguously linked non-(1→6)-α-D-linked

TABLE III

CHEMICAL SHIFTS AND SPIN–LATTICE RELAXATION-TIMES (T_1^{obs}) FOR ^{13}C-N.M.R. SPECTRA, AT 34°, OF DEXTRANS[a]

	Dextran	
	B-1299 fraction S	*B-742 fraction S*
	Linkage types (*1→2*) & (*1→6*)	(*1→3*) & (*1→6*)
5[b]		100.33 (93)
7(A)[c]	98.74 (117)	98.84 (82)
8	97.18 (74)	
9	96.40 (68)	
10		81.66 (46)
15	76.42 (60)	
16		74.39 (77)
17		73.79 (95)
18(B)	73.84 (83)	
21		72.80 (92)
24(C)	72.39 (42)	~72.50 (68)
27(D)	71.17 (77)	71.08 (59)
29(E)	70.37 (79)	70.50 (74)
32(F)	66.52 (29)	66.54 (36)
33	61.35 (75)	61.50 (66)

[a]The chemical shift in p.p.m., relative to tetramethylsilane. [b]These numbers are referenced to the spectra in Fig. 5, and correspond to the 90° resonances in Table I. [c]The letters A through F identify the resonances similar to those of linear dextran.

TABLE IV

COMPARISON OF OBSERVED SPIN–LATTICE RELAXATION-TIMES (T_1^{obs}), NUCLEAR OVERHAUSER EFFECTS (N.O.E.), AND DIPOLE–DIPOLE RELAXATION-TIMES (T_1^{DD}) FOR THE ^{13}C-N.M.R. SPECTRUM, AT 34° AND 90°, OF DEXTRAN B-742 FRACTION S

	Chemical shift[a]		T_1^{obs}		*n.O.e.*		T_1^{DD}		T_1^{obs}	T_1^{DD}/T_1^{obs}
	34°	*90°*	*34°*	*90°*	*34°*	*90°*	*34°*	*90°*	*90°/34°*	*90°*
5[b]	100.33	100.81	93[c]	224	0.64	1.75	286[c]	259	2.44[d]	1.16[e]
7	98.84	99.56	82	153	0.63	1.62	260	189	1.87	1.23
10	81.66	82.89	46	108	0.69	1.98	135	109	2.34	1.01
16(w)	74.39	75.09	77	108	0.58	1.25	268	173	1.39	1.60
17	73.79	74.79	95	168	0.72	1.48	261	227	1.78	1.35
21[f]	72.80	73.57	92	186	0.74	1.48	249	251	2.01	1.34
24(w)	~72.50	73.14	68	161	—	1.37	—	235	2.36	1.46
27	71.08	71.74	59	115	0.70	1.46	170	157	1.94	1.36
29	70.50	71.41	74	168	0.60	1.36	247	247	2.26	1.47
32	66.54	67.67	36	60	0.73	1.36	120	112	1.74	1.87
33	61.50	62.49	66	204	1.18	1.48	223	551	3.09	2.70

[a]The chemical shift in p.p.m., relative to tetramethylsilane. [b]These numbers are referenced to the spectra in Figs. 2, 3, and 5. [c]T_1 values in ms. [d]The 90°, T_1^{obs} value divided by the 34°, T_1^{obs} value. [e]The 90°, T_1^{DD} value divided by the 90°, T_1^{obs} value. [f]The unresolved, combined values for resonances 21 and 22.

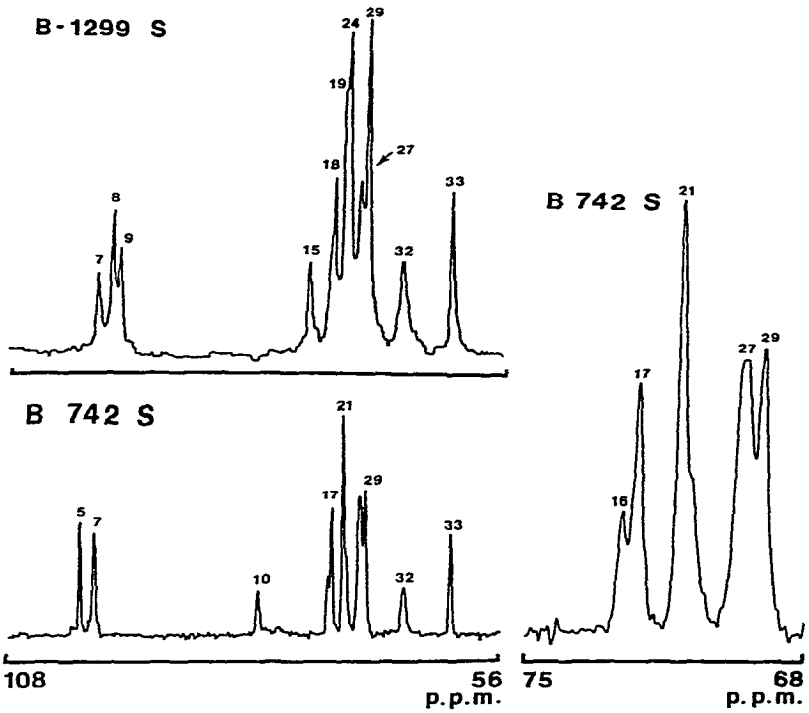


Fig. 5. Fully relaxed, ^{13}C-n.m.r. spectra at 34° of the S fractions of dextrans B-1299 and B-742, and the expanded, 68–75-p.p.m. region for dextran B-742 fraction S. The numbered resonances correspond to the values in Tables III and IV.

D-glucopyranosyl residues. However, on the basis of solubility, it is probable that repeating units described by **6** make no, or little, contribution to the structure of dextran B-742 fraction S. Alternative support for **1** as being the predominant repeating-unit of dextran B-742 fraction S was provided by Ito and Schuerch[44], who synthesized a D-glucan (designated V32) having an anticipated structure of **1** ($m = 3$, $n = 0$), and this synthetic D-glucan and dextran B-742 fraction S have essentially identical, ^{13}C-n.m.r. spectra.

A spectrum having 12 possible resonances is indicated by **1** ($m = 3, n = 0$)—6 resonances from the less-mobile *b*-residue and 6 resonances from the *t*-group. The spectrum of dextran B-742 fraction S is relatively simple, with 11 peaks (see Table I and Figs. 2 and 3) consisting of 5 peaks of low intensity and 6 intense peaks. It is tempting to consider that the five less-intense peaks correspond to carbon atom positions of the *b*-residue, that the five intense peaks correspond to the carbon atom positions of the *t*-group, and that the intense peak remaining is a composite of a resonance from the *b*-residue and a resonance from the *t*-group. The spectrum of dextran B-742 fraction S at 34° (see Tables III and IV, and Fig. 5) has also been recorded. In addition, n.O.e. values for dextran B-742 fraction S have been obtained, at both 34 and 90°, which allow the calculation of the T_1^{DD} values (see Table IV) at these temperatures. The n.O.e. determinations presented difficulties, as the narrower

dynamic range for the available technique requires ~4 times the spectral acquisition time (compared to T_1^{obs}) in order to afford values of equivalent, statistical accuracy. An examination of the data in Table IV allowed the following observations. For a given temperature, different resonances of the spectrum of the polysaccharide have different T_1^{obs} values. The relative order of the T_1^{obs} values for different resonances persists (*a*) when the T_1^{obs} values are converted into T_1^{DD} values, and (*b*) for comparison of high- and low-temperature measurements. However, as shown by the n.O.e. values for a specific polysaccharide at a specific temperature, the contribution to T_1^{obs} from non-T_1^{DD} relaxation-processes can vary considerably for the different resonances. In general, the less-intense resonances of dextran B-742 fraction S (resonances 7, 10, 16, 24, and 32) have small T_1^{obs} values, again encouraging the correlation of these resonances to the *b*-residue. As will be shown, extreme caution must be exercised for the general correlation of resonances to carbon-atom positions on the basis of relative T_1^{obs} magnitude. However, the highly branched dextran B-742 fraction S has a 90° spectrum of unusual simplicity and resolution, and allows a unique inspection of resonances lying in the 70–75-p.p.m. region.

Table IV does show that much care must be taken in relating T_1^{obs} values for structural determination. The penultimate column of Table IV indicates the variations observed in the relative order of magnitude between T_1^{obs} values at 34 and 90°. The last column of Table IV indicates the relative variations observed by correlating T_1^{obs} to T_1^{DD} at 90°. Both columns indicate that changes in the relative orders of values can result upon comparing T_1 data for various resonances, upon comparing these data acquired at different temperatures, or after correcting with n.O.e. values. Despite the complexities involved for ^{13}C-n.m.r. relaxation-data from polysaccharides, we consider that certain resonance-assignments can be made, provided that some precautions are observed.

We shall first examine several cases where resonance assignments cannot easily be made on the basis of T_1^{obs} values. Firstly, the 70–75-p.p.m. region contains all of the non-*O*-substituted C-2, C-3, C-4, and C-5 resonances for α-D-glucopyranosyl residues. The close packing of these resonances makes resolution difficult, and, for most dextrans, there are no completely resolved resonances in this region. The lack of resolved resonances, coupled with the lack of certainty that a detached peak represents a single resonance, discourages the application of T_1 studies. For all three classes of dextran **1** (m = 2, 3, or 4), it is possible to record high signal-to-noise spectra and then employ c.d.r.e. processing to observe approximately 18, well defined resonances (assumed to correspond to the 18 carbon-atom positions of **1**). Therefore, at 90°, 25-MHz spectra have nearly enough, but not sufficient, resolution for relaxation studies in the 70–75-p.p.m. region. Secondly, the 60–70-p.p.m. region, containing C-6 resonances, is not amenable to relaxation studies. The chemical shifts of the C-6 resonances are relatively insensitive to structural changes, and therefore, most C-6 resonances of α-D-glucopyranosyl residues or groups are either at 67.8 p.p.m. (linked C-6) or 62.3 p.p.m. (free C-6). In addition, C-6 positions are free to exhibit motion independent of the pyranoside ring-system. Furthermore, the C-6 atoms that are not

directly a part of the backbone chain (*e.g.*, C-6 of the *t*-group of **1**) can move in several additional modes, including rotation around the C-5–C-6 bond axis. No doubt, the latter freedom contributes to the unusually large increase observed for the T_1^{obs} values of resonance 33 (C-6 of the *t*-group) on comparison of the 34° and the 90° spectra (see the penultimate column of Table IV).

However, the T_1^{obs} values may be useful for assigning linked C-6 resonances. For the variously substituted α-D-glucopyranosyl residues, there is little problem in assigning the linked C-6 resonances (~67.8 p.p.m.), as all resonances of non-C-6 atoms are upfield of 70 p.p.m. For other residue types (*e.g.*, β-D-glucopyranoside and α-D-mannopyranoside residues), separation between the resonances of the linked C-6 atoms and the resonances of the free C-2, C-3, C-4, and C-5 atoms is not so complete. As the linked C-6 atom is bonded to two hydrogen atoms, the T_1^{obs} value of C-6 is approximately half the average T_1^{obs} value of other resonances in the same spectrum, and this magnitude of change is readily observable, even in complex spectral-regions.

In contrast to the foregoing, relaxation-data problems, the resonance data for the anomeric carbon atoms are promising. Firstly, the anomeric resonances are not only well spaced from other resonances, but, as these resonances are quite dependent on structure changes, the anomeric resonances are normally well resolved from one another. Secondly, as each residue contains a single anomeric carbon atom, and as these anomeric positions are separated from one another by approximately the distance across a pyranoside ring, these positions are uniformly distributed throughout the various parts of a repeating unit of a polysaccharide. Thirdly, as all of the observable, anomeric carbon atoms are directly linked to another residue, these positions both indicate the *general degree* of segmental motion of the polymer for the associated residue, and are, to the same general extent, shielded from solvent effects. This similar, steric shielding from solvent effects should be reflected in all of the anomeric carbon atoms having similar n.O.e. values (and $T_1^{obs} = T_1^{DD}$).

Steric effects may also be expected to be important for resonances of the 75–85-p.p.m. region (the *O*-substituted C-2, C-3, and C-4 atoms). Such steric shielding for these linked carbon positions could result in large n.O.e. values (indicating little interaction with the solvent), and, in turn, this n.O.e. effect would lessen the intensity (on a mole basis) of the associated resonances. Therefore, resonances in the 75–85-p.p.m. region could have less-intense resonances and larger n.O.e. values than average, due to both (*a*) the lower mobility resulting from a backbone-chain *b*-residue, and (*b*) greater than average (the average of the *b*-residue positions) steric hindrance. The foregoing discussion explains why most of the following discussion of T_1 values will deal with differences in the relative magnitudes of relaxation values associated with anomeric resonances.

Several observations can now be made that relate specifically to the relaxation data for dextran B-742 fraction S. Comparison of the relative magnitudes of the set of T_1^{obs} values to those of the set of T_1^{DD} values (compare the 4th and 8th columns of Table IV) indicates both the strengths and weaknesses of employing T_1^{obs} values as

measures of segmental motion. At 34° and at 90°, both the T_1^{obs} and the T_1^{DD} values of the anomeric resonances maintain a constant relationship, with the more-intense resonance 5 consistently having larger T_1 values (at a given temperature) than the corresponding T_1 values of resonance 7. For this reason, resonance 7 is assigned to C-1 of the *t*-group.

In general, the relative magnitudes of the resonances in the 70–75-p.p.m. region (resonances 16 through 29 of Table IV, which represent non-*O*-substituted, carbon-atom positions of the pyranoside ring) remain the same, with large values in one column being paralleled by large values in another (compare columns 4, 5, 8, and 9 of Table IV); however, changes in some of the relative orders of these T_1 values do occur. Lastly, it may be observed that both changes in recording temperatures and corrections based on n.O.e. values can contribute significant changes in T_1 values of resonances in the 60–70-p.p.m. region (resonances 32 and 33, representing the C-6 positions).

Similar measurements, and calculations, of T_1^{obs} and T_1^{DD}, and n.O.e. values at 34° and 90°, were made for dextran B-1299 fraction S. These spin–lattice relaxation-values of dextran B-1299 fraction S parallel the general results obtained from dextran B-742 fraction S, and showed that (*a*) the relative order of the magnitudes of the T_1 values of any data set remained the same for the anomeric resonances, and (*b*) although the relative order of these T_1 values for the resonances of the 70–75-p.p.m. region are generally the same, there are some changes in order, depending on the recording temperature and the n.O.e. corrections. Therefore, based on comparisons of the T_1 changes shown in Table IV, it is concluded that (*a*) the relative magnitudes of T_1^{obs} values of the anomeric resonances are reasonable indices of the relative degrees of the segmental motion associated with the corresponding carbon-atom positions (especially for differences of magnitude greater than 10%), (*b*) the relative magnitudes of the different data-sets for the 70–75-p.p.m. region (either at different recording temperatures, or on comparing T_1^{obs} to T_1^{DD}) are similar, in that large values for one set tend to remain large for a corresponding set, but differences in these relative orders do occur, and (*c*) temperature and n.O.e. corrections must be carefully considered when comparing the magnitudes of the T_1^{obs} and T_1^{DD} values in the 60–70-p.p.m. spectral-region (the C-6 positions).

The 90° n.O.e. value of the linked C-3 (resonance 10) of the *b*-residue of dextran B-742 fraction S is large (essentially equal to the theoretical, maximum n.O.e. value of 1.99), indicating a resonance of a carbon-atom position that is dominated by the T_1^{DD} process, and implying that this position is extensively shielded from solvent effects. It should be noted that a high-resolution, c.d.r.e.-processed spectrum for dextran B-742 fraction S is shown in Figs. 2 and 4. These spectra actually suggest greater resolution than obtainable for our relaxation measurements. Resonance 24 is, actually, poorly resolved from the nearby, intense resonance, and therefore, the relaxation values for this peak have greater than average statistical deviation. Also, high resolution confirms that resonance 21 is actually split (into resonances 21 and 22), this being a feature that we do not yet understand.

Dextran B-1299 fraction S. — The highly branched dextran B-1299 fraction S can be represented by **1** ($m = 2, n = \sim 1$). We have previously examined the structure of this compound by methylation–structural analysis[5], ^{13}C-n.m.r. chemical-shift measurements[4], ^{1}H-n.m.r. chemical-shift measurements[12], and F.t., infrared difference-spectrometry measurements[11]. Although estimates of linearity vary for data from the various techniques, the average values obtained for n lie between 0.7 and 1.0.

The interpretation of the anomeric region of the spectrum of dextran B-1299 fraction S (see Table I and Figs. 2 and 3) is as follows. Resonance 7, with a chemical shift equivalent to the anomeric resonance of linear dextran (A, 99.6 p.p.m.), is identified as corresponding to C-1 of the *l*-residue. We have previously made such an assignment, for the intensity of resonance 7, compared to resonances 8 and 9, is proportional to n of **1** for a series of dextrans branching through the 2,6-di-*O*-substituted α-D-glucopyranosyl residue[13]. Resonance 8 ($T_1^{obs} = 241$ ms) is identified, on the basis of the magnitude of T_1^{obs}, as corresponding to C-1 of the *t*-group, and resonance 9 ($T_1^{obs} = 140$ ms) is identified as corresponding to the *b*-residue. A set of data for n.O.e. and T_1^{DD} values, at 34° and 90°, similar to the data in Table IV, also supports the assignments of resonances 8 and 9 (as, in each case, the T_1 value for resonance 8 is larger than the corresponding value for resonance 9).

The correspondence of resonance 15 ($T_1^{obs} = 120$ ms) to C-2 of the *b*-residue, which was previously made on the basis of chemical-shift values, is further confirmed by the small T_1^{obs} value. In addition, the 90° n.O.e. value of resonance 15 (n.O.e. = 1.70) is larger than the average n.O.e. values observed for this spectrum (n.O.e. ~ 1.5), and indicates that this position is again shielded from solvent effects, although not so effectively shielded as C-3 of the *b*-residue of dextran B-742 fraction S.

Dextran B-1254 fraction S[£]. — This polysaccharide is a representative of the final class ($m = 4$) represented by **1**, where $m = 4$ and $n = \sim 3$. Only the 90° relaxation-data (T_1^{obs}) were recorded for this polymer (see Table I and Figs. 2 and 4). On the basis of analogy to the linear-dextran resonance, resonance 7 is again assigned to C-1 of the *l*-residue. Resonance 2 ($T_1^{obs} = 203$ ms) is identified, on the basis of the magnitude of T_1^{obs}, as corresponding to C-1 of the *t*-group, and resonance 6 ($T_1^{obs} = 140$ ms) is identified as corresponding to C-1 of the *b*-residue. Although c.d.r.e. resolution-enhancement can achieve base-line resolution between resonances 6 and 7, the unprocessed spectrum in Fig. 2 gives a better indication of the resonance overlap of the data available for relaxation studies.

The correspondence of resonance 13 ($T_1^{obs} = 113$ ms) to C-4 of the *b*-residue, previously made on the basis of chemical-shift data, is further confirmed by the small T_1^{obs} value. In addition, the identity of resonance 31 ($T_1^{obs} = 81$ ms), which was previously assigned to C-6 of the *b*-residue on the basis of resonance intensity and chemical shift, is further confirmed, as this very small T_1^{obs} value is diagnostic for carbon atoms linked to two hydrogen atoms.

Summary of relaxation data from dextrans of the type of structure **1**. — The foregoing relaxation data for dextran examples having $m = 2$, 3, or 4 (in **1**) may be summarized as follows. (*a*) All 90°, T_1^{obs} values of resonances associated with non-C-6

positions are in the 110–250-ms region. (*b*) The 90°, T_1^{obs} values associated with linked C-6 positions are approximately half the average T_1^{obs} value. (*c*) For **1**, with both $m = 2$ and $m = 4$, the T_1^{obs} values of the C-1 resonances decrease in the order: C-1 for the *b*-residue, C-1 for the *l*-residue, and C-1 for the *t*-group. (*d*) The foregoing T_1^{obs} order cannot be checked for $m = 3$, as C-1 of the resonances of the *b*-residue and the *l*-residues are not resolvable under current recording conditions. However, owing to the fortuitous existence of the highly branched dextran B-742 fraction S ($m = 3$, $n = \sim 0$), the C-1 resonances of the *b*-residue and *t*-group for this class ($m = 3$) can be identified, and the corresponding, relatively large and small T_1^{obs} values of these two resonances indicate a similar order of magnitude for the T_1^{obs} values of dextrans with $m = 3$. (*e*) For each case of $m = 2$, 3, or 4, T_1^{obs} data indicate that the resonance of C-1 of the *b*-residue is *downfield* of the corresponding resonance of C-1 of the *t*-group (although both resonances may vary, in terms of chemical shifts, relative to the consistent 99.6-p.p.m. resonance of C-1 of the *l*-residue). (*f*) As *m* increases, the C-1 chemical-shift of the *b*-residue, and also of the *t*-group, moves to downfield (larger) values. (*g*) The T_1^{obs} values of resonances of linked C-2, C-3, and C-4 (the resonances of the 75–85-p.p.m. region) are smaller than the average T_1^{obs} values for the polysaccharide. (*h*) Although all T_1^{obs} value–structure considerations have been based on the relative magnitudes of T_1^{obs} values in the *same* spectrum, a comparison of the 90°, T_1^{obs} values in Table I indicates similar, absolute values for carbon atoms known to be in similar positions of the general polysaccharide structure.

The S fractions of dextran B-1355 and dextran B-1498. — In the course of our methylation–structural analysis and ^{13}C-n.m.r.-spectral survey, we have examined approximately 30 dextrans. Three of these dextrans have yielded data that indicate a structure that is incompatible with the general structure **1**, and all (the S fractions of dextrans B-1355, B-1498, and B-1501) contain significant mole percentages of 3-mono-*O*-substituted α-D-glucopyranosyl residues[14]. Although all dextrans containing (1→3)-α-D-linkages have approximately the same diagnostic, branching resonances, an examination of the ^{13}C-n.m.r. spectra of these three dextrans revealed that (*a*) all three S fractions of dextrans B-1355, B-1498, and B-1501 have the same chemical shifts, and (*b*) the chemical shifts of these diagnostic, branching resonances are slightly (although well beyond experimental error) displaced from the resonances of dextrans branching through 3,6-di-*O*-substituted α-D-glucopyranosyl residues (*e.g.*, dextran B-742 fraction S). In addition, the ^{13}C-n.m.r.-spectral properties of these three dextrans differ from those of the other dextrans in the following ways: (*a*) they yielded low-temperature spectra having broad resonances, (*b*) at increased recording temperature, there is a change in the general, spectral "profile", which corresponds to increasing relative intensity of the A through F resonances associated with linear dextran, and (*c*) in general, c.d.r.e. processing is much more effective in narrowing the resonances in the spectra of these compounds (when compared to other dextrans). On the basis of methylation–structural analysis (see Table II) and the chemical shifts of ^{13}C-n.m.r. resonances (see Table I), we have proposed a general structure for this group of compounds (see **7**, where all residues are α-D-linked, and,

for

$$\text{-}\{\text{-}[\text{-Glc}p\text{-}(1\rightarrow3)\text{-Glc}p\text{-}(1\rightarrow6)\text{-}]_n\text{-Glc}p\text{-}(1\rightarrow3)\text{-Glc}p\text{-}(1\rightarrow6)\text{-}\}_x\text{-}$$
$$\text{Glc}p\text{-}(1\rightarrow6)\text{-}[\text{-Glc}p\text{-}(1\rightarrow6)]_q\text{-Glc}p\text{-}(1\rightarrow6)$$
7

dextran B-1355 fraction S, $n = 5.1$, $q = 0.7$; for dextran B-1498 fraction S, $n = 3.1$, $q = 1.4$; and for dextran B-1501 fraction S, $n = 3.4$, $q = 3.9$), which is the same as structure C in Part VIII of this series[14]. [Table II (column *c*) of ref. 14 incorrectly cites the mole ratio of the 2,4,6-tri-*O*-methyl derivative of the hydrolyzate from dextran B-1355 fraction S as 1.6; the originally reported, correct value in Table II of ref. 5 is 35.0 mole% (or a mole ratio of 5.1). The accompanying (b-c)-1 value of Table III of ref. 14 then remains 0.7]. This proposed, general structure is based on the following observations for data from the S fractions of dextrans B-1355, V-1498, and B-1501. (*a*) The anomeric, ^{13}C-n.m.r.-spectral region is simple, and yet different from resonances observed for dextrans branching through 3,6-di-*O*-substituted residues. (*b*) There is no detectable, neighboring-residue effect (the anomeric resonances observed are narrow, with single maxima), a phenomenon that can be explained by the presence of a chain of alternating, 3-mono-*O*- and 6-mono-*O*-substituted α-D-glucopyranosyl residues. (*c*) The "excess" (as established by methylation–fragmentation analysis), 6-mono-*O*-substituted α-D-glucopyranosyl residues are then placed in consecutively (1→6)-α-D-linked regions of the polysaccharide, and can make a "linear dextran-like" contribution to the total spectrum. (*d*) The increased, "linear dextran-like" contribution to the ^{13}C-n.m.r. spectra at elevated temperatures is a result of these regions of consecutively linked, 6-mono-*O*-substituted residues being located in side chains (from the backbone) composed of alternating (1→3)-α-D- and (1→6)-α-D-linked residues. Elevated temperature then increases the segmental motion of the side chains, relative to the backbone residues, and the resonances of the residues of the "linear dextran-like" side-chains then narrow, and rise to dominate the high-temperature, ^{13}C-n.m.r. spectra.

The T_1^{obs} data at 90° for dextran B-1355 fraction S and dextran B-1498 fraction S (see Table I and Figs. 1, 2, and 3) are also consistent with structure **7**. The initial discussion of T_1^{obs} for this class of dextrans will deal with data for dextran B-1355 fraction S, but, in all cases, these results are paralleled in the data obtained from dextran B-1498 fraction S. Resonance 10 (T_1^{obs} = 120 ms) has a small T_1^{obs} value, similar to T_1^{obs} values obtained for the resonances of the linked C-2, C-3, and C-4 atoms of the *b*-residues in **1**, suggesting that the linked C-3 (and the corresponding 3-mono-*O*-substituted residue) is also in the backbone. The foregoing discussion, and observations on the n.O.e. contributions to T_1^{obs} values, indicate that it is possible that the small T_1^{obs} value of the C-3 resonance could largely result from steric hindrance, and, therefore, this T_1^{obs} value must be viewed with caution; however, each anomeric position may be assumed to have similar steric hindrance. The correspondence of resonance 7 (T_1^{obs} = 222 ms) to C-1 of a 6-mono-*O*-substituted residue located in a

linear, dextran-like side-chain, previously made on the basis of methylation–fragmentation analysis and ^{13}C-n.m.r. chemical-shift data, is, therefore, further confirmed on the basis of the relatively large, associated T_1^{obs} value. The remaining anomeric resonances, resonance 4 (T_1^{obs} = 149 ms) and resonance 6 (T_1^{obs} = 111 ms), have previously been attributed, respectively, to 3- and 6-mono-*O*-substituted α-D-glucopyranosyl residues located in the backbone chain, and the small T_1^{obs} values of these resonances further confirm such an assignment.

The foregoing discussion of resonance assignment and structural relationship of the different, *O*-substituted residues is highly dependent upon the T_1^{obs} values of the anomeric resonances. However, the anomeric, spectral region of dextran B-1355 fraction S is poorly resolved. Additional evidence for the relative magnitudes of the T_1^{obs} values of resonances 4, 6, and 7 were obtained for dextran B-1498 fraction S. The spectrum of dextran B-1498 fraction S contains a much greater contribution of "linear dextran-like" resonances (compared to dextran B-1355 fraction S), and it is therefore easier to measure the T_1^{obs} value of resonance 7. Again, the pattern of the magnitudes of the T_1^{obs} values for the anomeric region of the spectrum of dextran B-1498 fraction S supports a polymer structure of type 7.

General summary of chemical-shift and spin–lattice-relaxation data from the spectra of α-D-*glucans.* — We have previously indicated that there is reason to believe that the chemical shifts, T_1^{obs}, and n.O.e. values associated with the various resonances of the ^{13}C-n.m.r. spectrum of a polysaccharide are dependent on the nature of the *O*-substitution of the residue associated with the specific resonances under consideration. Two questions that have been consistently addressed in this series of dextran studies have been: (*a*) what structural features of the polymer have a major influence on these resonance parameters, and (*b*) in relation to resonance resolution and to the precision of the measurements, how far away (in terms of sequentially linked carbon atoms) will a given structural feature affect resonance parameters? The following discussion will attempt to summarize and systematize a number of our observations, and the relationships outlined are based on the assumption that our resonance assignments are correct. The discussion will deal primarily with resonance perturbations resulting from structural changes, *i.e.*, in the type of substitution on the α-D-glucopyranosyl residue or group.

All branching polysaccharides that we have studied by ^{13}C-n.m.r. spectroscopy contain di-*O*-substituted residues having one linkage through C-6. Therefore, the present empirical approach provides no information for resonance displacements associated with a residue that is di-*O*-substituted through two non-C-6 atoms. The chemical-shift displacements resulting from *O*-substitution are most readily considered as two groups: the resonances of the residues, and the resonances of the terminal groups. As the largest chemical-shift displacements occur for the carbon-atom positions directly participating in the linkage, the following discussion will primarily deal with resonances in the 70–85-p.p.m. and the 90–105-p.p.m. regions. Data are collated from Table II, in terms of *O*-substituted residue type, and in position of substitutions, both for the residue (or group) under consideration, and for the

TABLE V

VALUES OF CHEMICAL SHIFT OF THE RESONANCES OF LINKED CARBON ATOMS OF VARIOUSLY *O*-SUBSTITUTED, α-D-GLUCOPYRANOSYL RESIDUES AND GROUPS[a]

Compound[b]	*Fraction*[b]	*δ (p.p.m.)*		*O-substitution position of adjacent residue attached to C-1*
		anomeric	*nonanomeric*	
	Residues containing (1→4)-α-D-linkages			
4-Mono-O-				
Glycogen		101.43	79.37	4
Pullulan		101.83	79.94	6
Pullulan		101.36	79.63	4
Nigeran		101.55	79.34	3
HAG-12 amylose		101.34	79.30	4
4,6-Di-O-				
HAG-12 amylose		101.65	80.32	4
[c]Dextran B-1254	S[L]	100.07	80.40	6
	Residues containing (1→3)-α-D-linkages			
3-Mono-O-				
Nigeran		100.44	81.83	4
Dextran B-1355	S	101.07	83.31	6
3,6-Di-O-				
[c]Dextran B-742	S	99.56	82.89	6
	Other residue-linkage types			
2,6-Di-O-				
[c]Dextran B-1299	S	97.37	77.83	6
6-Mono-O-				
[c]Dextran B-1254	L[$]	99.63		6
	Terminal α-D-glucopyranosyl groups			
Dextran B-1299	S	98.22	—	2
Dextran B-742	S	100.81	—	3
Dextran B-1254	S[L]	101.57	—	4

[a]The resonance assignments are based on the discussion in the text. [b]The specific compound in which this residue type occurs, and for which the accompanying resonances were measured. [c]Comparison set of variously *O*-substituted dextrans of type exemplified by **1**.

adjacent residue attached to the anomeric position of the residue under consideration (see Table V).

Table V indicates that the anomeric resonance of an α-D-glucopyranosyl residue is predominantly determined by the position of *O*-substitution on that residue. Direct comparison of the major, anomeric-resonance effect may be observed in the dextran series (designated *c* in Table V) where, for 2,6-di-*O*-substitution, $\delta = 97.4$; for 3,6-di-*O*-substitution, $\delta = 100.8$; and, for 4,6-di-*O*-substitution, $\delta = 101.6$. In a similar way, the linked, nonanomeric resonance of an α-D-glucopyranosyl residue is dependent on the (carbon atom) position of attachment, and the foregoing dextran series indicates that for C-2, $\delta = 77.8$; for C-3, $\delta = 82.9$; and for C-4, $\delta = 80.4$.

Although based on limited data, an additional parameter apparently also affects the chemical shift of the linked, carbon-atom positions, namely, the position of *O*-substitution of the *adjacent residue* attached to the anomeric carbon atom of the residue under consideration.

For example, the resonances assigned to the 4-mono-*O*-substituted residue of pullulan (79.94 p.p.m.), and the 3-mono-*O*-substituted residue of dextran B-1355 fraction S (83.31 p.p.m.), are both of residues that are anomerically attached to C-6 of an adjacent residue, and both of these residues have anomeric, and linked, carbon resonances that are downfield (by ~0.5 p.p.m.) of the resonances of similar residues anomerically attached to C-3 or C-4 of the adjacent residue. Resonance displacements are also found on comparing a given, *O*-substituted, α-D-glucopyranosyl residue (*e.g.*, the 3-mono-*O*-substituted residue of dextran B-1355 fraction S) to the corresponding di-*O*-substituted residue containing a 6-*O*-substituted atom (*e.g.*, the 3-di-*O*-substituted residue of dextran B-742 fraction S); in this situation, the addition of a second, *O*-substituted position (*e.g.*, comparing the 3-mono-*O*- to the 3,6-di-*O*-substituted α-D-glucopyranosyl residue) displaces the chemical shift of the anomeric resonance upfield by ~1 p.p.m. However, it will be necessary to observe more examples from specifically situated residues in order to see whether such a pattern of resonance displacements is general.

The chemical shift of the anomeric resonance of the 4,6-di-*O*-substituted α-D-glucopyranosyl residue is quite different for the synthetically branched amylose HAG-12 and for dextran B-1254 fraction S[*L*], reflecting the fact that a (1→6)-β-D-linkage is involved at C-6 of the di-*O*-substituted residue of HAG-12. The differences in the chemical shifts for the anomeric resonances of the *b*-residues of HAG-12 and of dextran B-1254 fraction S[*L*] show that the nature of an *adjacent residue* attached to the residue under consideration (*e.g.*, the *b*-residue) by a nonanomeric linkage can affect the chemical shifts of the latter residue, this pair of di-*O*-substituted residues being the only example of such an adjacent residue effect, and also the only situation in this study where a β-D-linked residue is involved. Owing to the similarity of the values of the 101.65-p.p.m. resonance in the spectrum of HAG-10 (a polysaccharide similar to HAG-12) and the 101.57-p.p.m. resonance in the spectrum of dextran B-1254 fraction S[*L*], it was suggested that both resonances represent the 4,6-di-*O*-substituted residue, and, therefore, that a given *b*-residue is very insensitive to the nature (α-D- or β-D-linked) of the nonanomerically attached residues[25]. However, the current resonance-relaxation values do not support this concept, as the 101.57-p.p.m. resonance of dextran B-1254 fraction S[*L*] is now assigned to the terminal group.

The chemical shifts of the anomeric resonances of α-D-glucopyranosyl (terminal) groups, shown at the bottom of Table V, are of interest when compared to the chemical shifts of the α-D-glucopyranosyl residues. The dominant factor for the anomeric resonances of these terminal groups is, apparently, the position of *O*-substitution on the *adjacent* residue to which the terminal group is attached; *e.g.*, for the C-2 linkage, δ 98.2; for the C-3 linkage, δ = 100.8; and for the C-4 linkage, δ = 101.6. These

chemical-shift differences for the anomeric resonances of the C-2, C-3, and C-4 linkages of α-D-glucopyranosyl *groups* parallel, in terms of the order of the resonances and the $\Delta\delta$ between the residue types, the differences found for the C-2, C-3, and C-4 linkages of the α-D-glucopyranosyl *residues*. However, for each type of linkage [*e.g.*, the (1→2)-α-D-linkage], the chemical shift of the anomeric resonance of the α-D-glucopyranosyl *group* is ~1.5 p.p.m. downfield from that of the corresponding α-D-glucopyranosyl *residue*.

A specific example for the displacement of the anomeric resonances of similarly linked residues and groups has previously been noted. Gorin[21] examined the ^{13}C-n.m.r. spectrum of a series of oligosaccharides composed of D-mannopyranosyl residues that were exclusively (1→2)-α-D-linked. It was concluded that the anomeric resonances (at 70°) of the interior, nonterminal, (1→2)-α-D-linked residues were at 102.3 p.p.m. and independent of the specific position in the chain, whereas the anomeric resonance of the corresponding (1→2)-α-D-linked terminal group was shifted downfield, by ~1.5 p.p.m., to 103.8 p.p.m., an effect paralleling the residue–group relationship observed for the anomeric resonances of the dextrans.

In general, it is concluded that the chemical shifts of resonances of a given residue are primarily dependent on the position of *O*-substitution of the given residue, and, to a lesser extent, on the nature of the residues immediately adjacent to the residue under consideration, especially the position of substitution of the residue attached to the anomeric position of the residue under consideration. As previously noted, Ito and Schuerch[44] have synthesized polysaccharides, similar to dextran B-742 fraction S, that unambiguously contain, in the backbone of the polymer, all (1→6)-α-D-linkages. The ^{13}C-n.m.r. spectrum of their highly-branched, synthetic dextran (V-32) is essentially identical to that of dextran B-742 fraction S. Based on the foregoing discussion of adjacent-residue effects, we conclude that all, spectroscopically observable (1→6)-α-D-linkages of dextran B-742 fraction S are situated in the backbone chain. In addition, owing to the persistence of a limited number of specific, chemical shifts in the spectra of all dextrans branching through the 3,6-di-*O*-substituted residue, it is concluded that, within the limits of spectroscopic detection, all (1→6)-α-D-linkages of polysaccharides of structure of **1**, when $m = 3$, are situated in the backbone chains. Furthermore, because of the persistence and invariance of the diagnostic chemical-shifts in the spectra of the series of dextrans branching through 2,6-di-*O*-substituted[13] and 4,6-di-*O*-substituted[1] α-D-glucopyranosyl residues, it is concluded that all, spectroscopically observable (1→6)-α-D-linkages are situated in the backbone chains of these polysaccharides. This uniformity of (1→6)-α-D-linkages in the backbone chain of dextrans (except for the S fractions of dextrans B-1355, B-1498, and B-1501), and the influence of the *position* of *O*-substitution of the adjacent residue to which the residue under consideration is anomerically linked, then explains why neighboring-residue effects are not seen in the spectra of the dextrans, although they are observed in the ^{13}C-n.m.r. spectrum of pullulan.

Finally, it should be emphasized that the structural analyses presented here

deal only with the more obvious structural features (*e.g.*, structural features associated with residues that are present at, or above, ~10 mole percent). It is possible that further examination of these polysaccharides will reveal minor (on a mole percent basis) structural features that can contribute to knowledge of the physicochemical and biochemical properties of these biopolymers.

EXPERIMENTAL

^{13}C-N.m.r. spectra were obtained at natural abundance, with samples of carbohydrate (~200 mg) dissolved in deuterium oxide (4 mL) being spun at ~20 r.p.s. in 12-mm (o.d.) tubes. Experiments were performed at 25.2 MHz with a Varian XL-100-15A spectrometer equipped with a Varian variable-temperature probe and a Nicolet TT-100 pulse, Fourier-transform, data system. The chemical shifts are expressed in p.p.m. relative to external tetramethylsilane, but were actually calculated by reference to the internal, deuterium solvent, lock signal[4]. Spectral widths of 2–4 kHz were used, the time for the 90° monitor-pulse was 16.8 ms, and an 8,096-point, complex, F.t. from quadrature phase-detection was employed. Full, proton-decoupling power (~9.5 W) was used, except during gated decoupling.

The relaxation experiments were conducted by two methods. Firstly, the general pulse-sequence $(180°–\tau–90°–T)_n$ was employed, where the delay time between experiments (T) was adjusted so that $T > 5\ {}_{max}T_1^{obs}$. Secondly, a method allowing the simultaneous determination of T_1^{obs} and ^{13}C-{^{1}H}n.O.e., the dynamic n.O.e. pulse-sequence[45], was employed. One- to four-thousand acquisitions were accumulated for each of 10–20 τ values. In general, τ values were chosen inside the 5-ms to 5-s range, with each successive τ value being twice that of the previous value. In actual practice, long and short τ values were interspersed, in order of accumulation, to avoid the introduction of errors resulting from sample change due to prolonged, elevated temperatures. Values of T_1 were calculated with the Nicolet Transform Technology algorithms in the 1004 version of the NTCFT program. The experiments were repeated for a specific sample until reproducibility of the data was better than 10%.

The preparation and characterization of the pullulan[4], nigeran[38], synthetically branched amylose[40], and dextrans[36] and dextran fractions[37] have been reported. Rabbit-liver glycogen was obtained from Sigma Chemical Co., St. Louis, MO. Deuterium oxide (at 99.7 atom%) from Merck Sharp and Dohme, Canada, Ltd., Montreal, was normally employed without further treatment.

ACKNOWLEDGMENTS

This work was supported, in part, by National Institutes of Health Grants (HL-17373 and HL-17269). We thank Drs. A. Jeanes and M. E. Slodki, Northern Regional Research Center, Peoria, Illinois, U.S.A.; Dr. B. Pfannemüller, Institut für Makromolekular Chemie der Universität Freiburg, West Germany; Dr. J. H.

Nordin, University of Massachusetts, Amherst, Mass., U.S.A.; and Dr. T. Sawai, Aichi Kyoiku University, Kariya, Japan, respectively, for the samples of dextran, pullulan, branched amylose, pseudonigeran, and nigeran.

REFERENCES

1 F. R. SEYMOUR, R. D. KNAPP, E. C. N. CHEN, A. JEANES, AND S. H. BISHOP, *Carbohydr. Res.*, 75 (1979) 275–294.
2 F. R. SEYMOUR, R. D. KNAPP, AND S. H. BISHOP, *Carbohydr. Res.*, 72 (1979) 229–234.
3 F. R. SEYMOUR, "Polysaccharide Branching and C-13 N.M.R.", in M. PASIKA (Ed.), *ACS Symp. Ser.*, 103 (1979) 27–51.
4 F. R. SEYMOUR, R. D. KNAPP, AND S. H. BISHOP, *Carbohydr. Res.*, 51 (1976) 179–196.
5 F. R. SEYMOUR, M. E. SLODKI, R. D. PLATTNER, AND A. JEANES, *Carbohydr. Res.*, 53 (1977) 153–166.
6 D. E. DORMAN AND J. D. ROBERTS, *J. Am. Chem. Soc.*, 92 (1970) 1355–1360.
7 A. S. PERLIN, B. CASU, AND H. J. KOCH, *Can. J. Chem.*, 48 (1970) 2596–2606.
8 P. COLSON, H. J. JENNINGS, AND I. C. P. SMITH, *J. Am. Chem. Soc.*, 96 (1974) 8081–8087.
9 A. S. PERLIN, N. M. K. NG YING KIN, S. S. BHATTACHARJEE, AND L. F. JOHNSON, *Can. J. Chem.*, 50 (1972) 2437–2441.
10 F. R. SEYMOUR, "Nuclear Magnetic Resonance and Mass Spectrometry of Polysaccharides", in P. A. SANDFORD (Ed.), *ACS Symp. Ser.*, 45 (1977) 114–127.
11 F. R. SEYMOUR AND R. L. JULIAN, *Carbohydr. Res.*, 74 (1979) 63–75.
12 F. R. SEYMOUR, R. D. KNAPP, AND S. H. BISHOP, *Carbohydr. Res.*, 74 (1979) 77–92.
13 F. R. SEYMOUR, R. D. KNAPP, E. C. M. CHEN, A. JEANES, AND S. H. BISHOP, *Carbohydr. Res.*, 71 (1979) 231–250.
14 F. R. SEYMOUR, R. D. KNAPP, E. C. M. CHEN, S. H. BISHOP, AND A. JEANES, *Carbohydr. Res.*, 74 (1979) 41–62.
15 F. R. SEYMOUR, E. C. M. CHEN, AND S. H. BISHOP, *Carbohydr Res.*, 68 (1979) 123–140.
16 A. JEANES AND F. R. SEYMOUR, *Carbohydr. Res.*, 74 (1979) 31–40.
17 F. R. SEYMOUR, R. D. KNAPP, J. E. ZWEIG, AND S. H. BISHOP, *Carbohydr. Res.*, 72 (1979) 57–69.
18 F. R. SEYMOUR, R. D. KNAPP, AND A. JEANES, *Carbohydr. Res.*, 72 (1979) 222–228.
19 H. J. JENNINGS AND I. C. P. SMITH, *J. Am. Chem. Soc.*, 95 (1973) 606–608.
20 F. R. SEYMOUR, M. E. SLODKI, R. D. PLATTNER, AND R. M. STODOLA, *Carbohydr. Res.*, 44 (1975) 181–198.
21 P. A. J. GORIN, *Can. J. Chem.*, 51 (1973) 2375–2383.
22 P. A. J. GORIN, *Carbohydr. Res.*, 39 (1975) 3–10.
23 I. D. CAMPBELL, C. M. DOBSON, R. J. P. WILLIAMS, AND A. V. XAVIER, *J. Magn. Reson.*, 11 (1973) 172–181.
24 H. MIYAJI, A. MISAKI, AND M. TORII, *Carbohydr. Res.*, 31 (1973) 277–287.
25 F. R. SEYMOUR, R. D. KNAPP, B. PFANNEMÜLLER, AND T. E. NELSON, *Carbohydr. Res.*, 70 (1979) 125–133.
26 F. R. SEYMOUR, R. D. KNAPP, S. H. BISHOP, AND A. JEANES, *Carbohydr. Res.*, 68 (1979) 123–140.
27 G. C. LEVY AND G. L. NELSON, *Carbon-13 Nuclear Magnetic Resonance for Organic Chemists*, Wiley–Interscience, New York, 1972.
28 J. R. LYERIA AND G. C. LEVY, in G. C. LEVY (Ed.), *Topics in Carbon-13 NMR Spectroscopy*, Vol. 1, Wiley–Interscience, New York, 1974, pp. 81–148.
29 F. A. L. ANET, in G. C. LEVY (Ed.), *Topics in Carbon-13 NMR Spectroscopy*, Vol. 1, Wiley–Interscience, New York, 1974, pp. 211–215.
30 K. BOCK AND L. D. HALL, *Carbohydr. Res.*, 40 (1975) c3–c5.
31 A. NESZMÉLYI, A. LIPTÁK, AND P. NÁNÁSI, *Carbohydr. Res.*, 58 (1977) c7–c9.
32 J. M. BERRY, L. D. HALL, AND K. F. WONG, *Carbohydr. Res.*, 56 (1977) c16–c20.
33 J. KOBE AND J. C. VALDES, *Carbohydr. Res.*, 65 (1978) 278–282.
34 A. L. BENESI AND J. T. GERIG, *Carbohydr. Res.*, 53 (1977) 278–283.
35 L. D. HALL AND C. M. PRESTON, *Carbohydr. Res.*, 49 (1976) 3–11.
36 A. JEANES, W. C. HAYNES, C. A. WILHAM, J. C. RANKIN, E. H. MELVIN, M. J. AUSTIN, J. E. CLUSKEY, B. E. FISHER, H. M. TSUCHIYA, AND C. E. RIST, *J. Am. Chem. Soc.*, 76 (1954) 5041–5046.

37 C. A. WILHAM, B. H. ALEXANDER, AND A. JEANES, *Arch. Biochem. Biophys.*, 59 (1955) 61–75.
38 S. A. BARKER, *Methods Carbohydr. Chem.*, 5 (1965) 165–167.
39 D. FRENCH, in W. J. WHELAN (Ed.), *Control of Glycogen Metabolism*, Churchill, London, 1964, pp. 7–29.
40 B. PFANNEMÜLLER, G. C. RICHTER, AND E. HUSEMANN, *Carbohydr. Res.*, 43 (1975) 151–161.
41 F. R. SEYMOUR, R. D. PLATTNER, AND M. E. SLODKI, *Carbohydr. Res.*, 44 (1975) 181–198.
42 M. HORISBERGER, B. A. LEWIS, AND F. SMITH, *Carbohydr. Res.*, 23 (1972) 183–188.
43 F. R. SEYMOUR AND R. D. KNAPP, *Carbohydr. Res.*, 81 (1980) 105–129.
44 H. ITO AND C. SCHUERCH, *J. Am. Chem. Soc.*, 101 (1979) 5797–5806.
45 R. FREEMAN, H. D. W. HILL, AND R. KAPTEIN, *J. Magn. Reson.*, 7 (1972) 327–329.