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Dextran Structural Details from High-Field Proton NMR Spectroscopy

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ABSTRACT

Proton nuclear magnetic resonance at 500 MHz resolves anomeric proton resonances of dextrans well enough to furnish useful structural information. Minor resonances which would take inordinately long accumulation times to detect by carbon-13 spectroscopy are readily accessible. Enzymic hydrolysis of the dextrans to homologous series of oligosaccharides was useful in assigning anomeric resonances to specific structural features in the polysaccharides. Neighbouring group effects are also discussed.

INTRODUCTION

During the course of a previous study (Taylor *et al.*, 1985) on branching in dextrans, we accumulated NMR data which indicated that high-field proton NMR spectroscopy would be useful in structural determination of these polysaccharides. During that work, and during structural studies on glucans produced from sucrose by strains of *Streptococcus sobrinus* (Walker *et al.*, 1990), we made use of a number of dextrans of known structure to assist with assignment of structural features. Enzymic hydrolysis to oligosaccharides whose structures were then determined

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yielded further information, which was used to assign some of the anomeric proton resonances in the parent polysaccharides.

In structural analysis of dextrans by NMR, the carbon-13 spectra have received a great deal of attention (Seymour *et al.*, 1976, 1979*b*, *c*; Seymour & Knapp, 1980*a*, *b*; Davis *et al.*, 1986).

The advent of high-field instruments with their enhanced sensitivity has made accumulation of carbon-13 spectra much more rapid. However, the heavy demand on such instruments often precludes their use for the long times required for good carbon-13 spectra, especially for those where some signals from minor (e.g. $< 5\%$ branching in some dextrans) structural features are of interest. Proton NMR instruments up to ~ 200 MHz have insufficient resolving power for detailed analysis of the anomeric resonances (Meyer *et al.*, 1978; Seymour *et al.*, 1979*c*).

NMR spectroscopy is not an absolute structural technique, i.e. it is not at present possible to interpret directly a spectrum without recourse to comparison spectra of known structures. In addition, correlation with other structural methods, such as methylation analysis and enzymic hydrolysis, is necessary to allow assignment of resonances to specific structural features.

Work on a number of samples is described to illustrate the use of high-field instruments in structural studies of dextrans.

EXPERIMENTAL

Materials

Carbohydrates

Dextran T_{10} and Dextran 2000 were obtained from Pharmacia. Dextran NRRL B-512(F) from *Leuconostoc mesenteroides* and dextran NRRL B-1351 from *Streptococcus viridans* were provided by Dr Allene Jeanes. A synthetic, branched dextran V39, was a gift to G.J.W. from Professor C. Schuerch. Dextrans SD9 and SD5 were synthesised by glucosyltransferases isolated from *Streptococcus sobrinus* strain 6715-13-201, and *Streptococcus sobrinus* strain K1-R, respectively, as previously described (Walker *et al.*, 1990).

Enzymes

Endodextranases from *Penicillium funiculosum* (EC 3.2.1.11) (Walker, 1972) and *Bacillus coagulans* QM474 (Walker, 1973) were used.

An endo α-(1→3)-D-glucanase was isolated from *Cladosporium resinae*. Enzymic hydrolyses were carried out for periods up to 6 days

(endodextranases) (Taylor *et al.*, 1985) or 13 days (endo α-(1→3)-glucanase) (Cheetham *et al.*, 1989).

HPLC separation of oligosaccharides

The end products of the hydrolysis of dextrans by the endodextranases were separated on a system consisting of a Varian model 5000 liquid chromatograph fitted with a manual loop injector, used in conjunction with a Dextropak cartridge (Millipore-Waters) held in a radial-compression separation system (Z-module, Waters Associates). The solvent was water, filtered through a Norganic purification system (Millipore). Flow rate was 2 ml/min unless otherwise stated. Gel-permeation chromatography was carried out in water at 0·8 ml/min flow rate on a TSK-gel G5000 P.W. column (7·5 × 600 mm, Toyo-Soda Co., Tokyo). Columns were monitored with a differential refractometer detector (R401, Waters Associates) and peak areas were measured with a chromatography data system (CDS111, Varian).

Proton NMR spectroscopy was performed at 500 MHz on a Brüker AM500 spectrometer operating in the Fourier-transform mode, at 90°C in D_2O. The reference was internal acetone (2·2 ppm).

RESULTS AND DISCUSSION

Dextran T_{10} Dextran B-512(F)

The anomeric proton resonances for the 500 MHz spectrum of dextran T_{10} are shown in Fig. 1(a). The three peaks centred at ≈ 5·28 ppm are due to the anomeric protons of α-1→3-linked-D-glucosyl residues attached to the α-1→6-D-glucosyl residues of the main chain, whose anomeric proton resonances are centred at ≈ 4·94 ppm. Resonances from the reducing end group anomeric protons are also detectable: α-centred at 5·22 ppm (J3·5 Hz) and β-centred at 4·63 ppm (J8·0 Hz). Integration, and comparison of the areas of the α + β-free anomeric resonances with the total anomeric resonances yields a value of 2·9% for the free anomerics, or a number average molecular weight (M_n) of ≈ 5400. This compares reasonably with an M_n of 6200, supplied by Pharmacia.

The degree of branching was determined by comparing the integrals (α-1→3 anomerics)/total anomerics = 4·9%. This compares favourably with 4–5% for the native B-512(F) dextran (Jeanes & Wilham, 1950; Van Cleve *et al.*, 1956; Lindberg & Svensson, 1968) from which T_{10} is derived by hydrolysis. Borohydride reduction of T_{10} removed the

reducing anomeric proton signals completely. In the native B-512(F) spectrum the reducing anomeric proton signals are not detectable (Fig. 1(b)). Structurally, the three signals at ≈ 5·28 ppm are significant. We have shown (Taylor *et al.*, 1985), that dextran B-512(F) possesses approximately equal amounts of α-1 → 3-linked-D-glucosyl groups and α-1 → 3-linked isomaltosyl groups attached to the main chain. Enzymic hydrolysis of dextran B-512(F) with *P. funiculosum* endodextranase yielded two homologous series of branched oligosaccharides (in addition to the linear isomalto- series). The structural features of dextran B-512(F) which lead to these two branched oligosaccharide series are shown in Fig. 2. The boxes outline the pentasaccharide structures from each series. The higher homologues of each series are those with one, two, etc., extra α-1 → 6-D-glucosyl units attached to the non-reducing end of the corresponding pentasaccharide main chain. The combination of one- and two-unit side chains as in Fig. 2 gives rise to the three signals near 5·28 ppm which appear in Figs 1(a) and 1(b). The apparent triplet is actually two overlapping doublets — one from the α-1 → 3-linked glucosyl of the B_5-2 series (single-unit side chain) and one from the corresponding α-1 → 3-linked (two-unit side chain) glucose of the B_5-1 series.

This was confirmed by examination of the spectrum of dextran B-1351 (Fig. 3) and V39 (not shown). These polysaccharides give rise to only one branched oligosaccharide series (yielding B_5-2 etc.) on digestion by *P. funiculosum* endodextranase (Taylor *et al.*, 1985). V39 is a chemically synthesized dextran having single-unit α-1 → 3-D-glucosyl side chains, and B-1351 dextran has also been shown to have *single*-unit side chains only. Figure 3 shows a simple doublet at ≈ 5·28 ppm for this single-unit side chain. It corresponds to the downfield pair of the three peaks near 5·28 ppm in Fig. 1. Thus the higher-field component in this

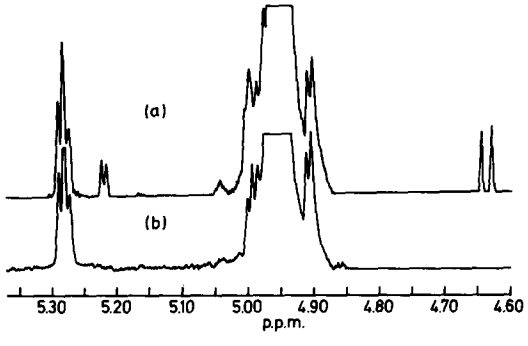

Fig. 1. The 500 MHz proton spectrum of (a) Dextran T_{10} and (b) Dextran B-512(F).

triplet in dextran T_{10}/B-512(F) is due to the α-1 → 3-glucosyl anomeric proton of the *two*-unit side chain. Integration shows B-1351 dextran to contain 14% of these single-unit side chains. Figure 3 also illustrates neighbouring-group effects in the small doublet on the low field side of the α-1 → 6-linked glucose resonances at ≈ 4·95 ppm. This doublet has an area similar to that of the α-1 → 3-linked glucosyl unit. It should be caused by the effect of that unit on the chemical shift of the main chain glucosyl to which it is attached. The corresponding region in dextran T_{10}/B-512(F) (Fig. 1) is more complex, reflecting the presence of both one- and two-unit side chains.

Highly branched dextrans

The neighbouring group effect is prominent in the highly branched dextran SD9 (Fig. 4(a)). This is a so-called S1-type dextran synthesised

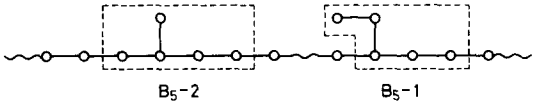

Fig. 2. Structural features of Dextran T_{10}/Dextran B-512(F). The dotted boxes show the origins for branched pentasaccharides released by the action of endodextranase. ○—○, α-1 → 6-linked-D-glucosyl units; ○ with ○ below, represents α-1 → 3-linked glucosyl units.

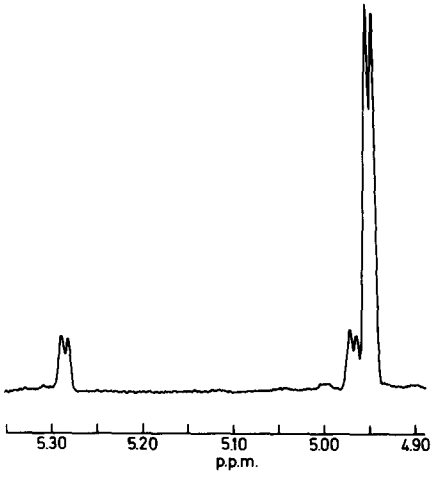

Fig. 3. The anomeric proton region of the 500 MHz proton spectrum of Dextran B-1351.

from sucrose by a glucosyltransferase (termed GTFS1) obtained from the culture of filtrate of *S. sobrinus* strain 6715-13-27 (Walker *et al.*, 1990). Dextran SD9 is highly branched (25% methylation, 30% NMR) and contains mainly single-unit (α-1→3-linked-D-glucosyl) side chains. Hydrolysis by endodextranase is limited, and little of the linear isomaltose series of oligosaccharides is produced. There are essentially three types of glucosyl residue present, in approximately equal amounts (Fig. 4(b)). This is reflected in the anomeric regions of the proton spectrum, with the simple doublet for the single side chain stubs at 5·29 ppm and a more complex region, estimated to be approximately twice that total area, for the α-1→6-linked backbone region. Comparison of the α-1→3-linked region of SD9 (doublet, 5·29 ppm) with the corresponding α-1→3-linked region of dextran B-1351 (doublet, 5·28 ppm) (Fig. 3) reveals a slight difference in chemical shift presumably due to the more highly substituted structure of SD9. For the α-1→6-linked region of SD9 one might expect, by analogy to the corresponding region in B-1351, two reasonably well resolved doublets. That the more complex spectrum is observed (Fig. 4(a)) again demonstrates the effect of the highly branched SD9 structure, which presumably imposes conformational restraints not found in dextrans such as B-1351 which have a lower degree of branching.

Branched dextrans containing significant α-1→3-linked sequences

Streptococcus sobrinus strains also produce glucosyltransferases (designated GTFS-4), which from sucrose produce the corresponding S4

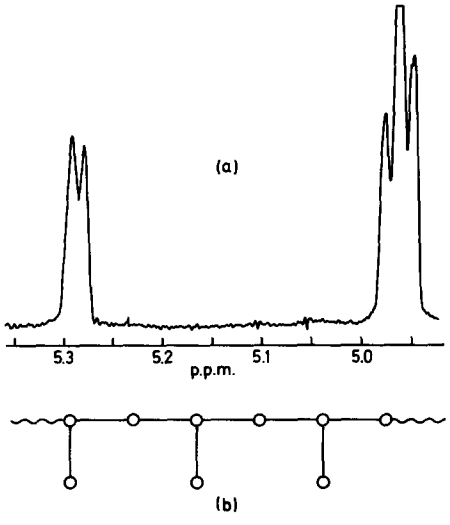

Fig. 4. (a) The anomeric proton region of the 500 MHz proton spectrum of a highly branched, S1-type dextran, SD9. (b) Idealised structural features of Dextran SD9. O—O, α-1→6-linked-D-glucosyl units; ⚲, represents α-1→3-linked glucosyl units.

dextrans. These polysaccharides have 10–14% branching, as shown by methylation analysis (Cheetham *et al.*, 1989). We have also shown the S4 polysaccharides to have, in addition to single α-1→3-linked branching stubs, sequences of α-1→3-linked-D-glucosyl units attached as side chains to the α-1→6-linked glucosyl backbone. The anomeric proton resonances from the α-1→3-linked region of a typical S4 dextran, SD5, are shown in Fig. 5(a). After digestion with an α-1→3-endoglucanase and dialysis, the molecular size of the remaining polysaccharide, as determined by gel-permeation chromatography, was virtually unchanged. Methylation analysis showed (by the decrease in the amount of 2,4,6-tri-*O*-methyl-D-glucose) a considerable reduction in the amount of α-1→3-linked glucosyl sequences. We thus conclude that the α-1→3-linked sequences are attached as side chains, rather than being incorporated in the main chain. The anomeric proton resonances of the α-1→3-linked region after enzyme digestion are shown in Fig. 5(b). The higher-field doublet (partly overlapped in Fig. 5(a)) is now better resolved and is attributed to single α-1→3-linked glucosyl side chains.

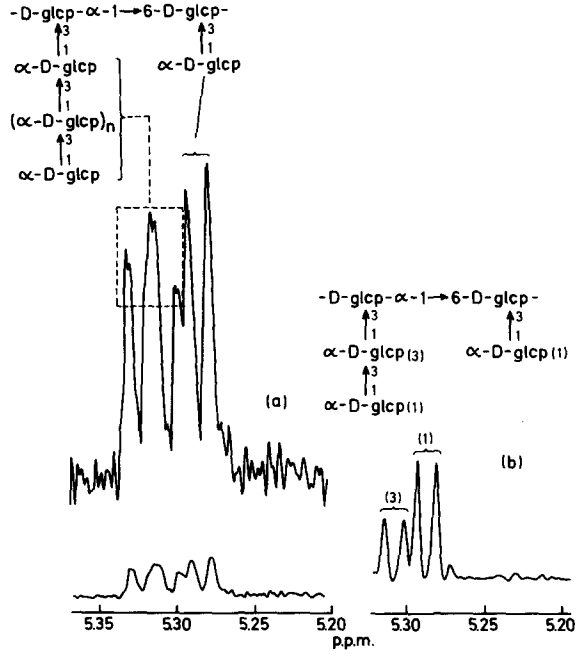

Fig. 5. The α-1→3-linked anomeric region of the 500 MHz proton spectrum of an S4-type dextran, SD5. (a), before digestion with an endo-α-1→3-D-glucanase; (b) after digestion with the endo-α-1→3-D-glucanase.

The remainder of the α-1→3-linked residues signal is reduced to the smaller, downfield doublet. We attribute this to the residual inner glucosyl units of the α-1→3-linked sequences. The signals which disappeared completely were due to the α-1→3-linked units well away from the branch point. The length of these residual sequences is not known, but is probably two. The α-1→3-endoglucanase cannot remove all the glucosyl residues close to a branch point. The difference between the short (two-unit) side chains of these modified S4 polysaccharides, and those of dextran T_{10}/B-512(F) can be observed by high-field NMR. Figure 6 compares the structures and the corresponding resonances from SD5 before (Fig. 6(a)) and after (Fig. 6(b)) α-1→3-glucanase digestion, with those of B-512(F) (Fig. 6(c)). Resonance (3) in Fig. 6(b) is distinguishable by chemical shift from resonance (2) in Fig. 6(c). Structurally they differ only in having respective single α-1→3- and α-1→6-D-glucosyl residues attached. This is quite a subtle difference. To confirm the assignments in Fig. 6, it was decided to use a template of dextran B-512(F) on which to attach α-1→3-linked glucosyl sequences as side chains. This was done by incubating B-512(F) dextran (as dextran 2000) with sucrose, plus a glucosyltransferase (GTF-I) capable of synthesising α-1→3-linked sequences. The GTF-I used was isolated from the same culture filtrate of the *S. sobrinus* strain 6715-13-201 used for the synthesis of SD9 dextran (Walker *et al.*, 1990).

The anomeric region of the proton NMR spectrum of the polysaccharide resulting from the above incubation is shown in Fig. 7. The

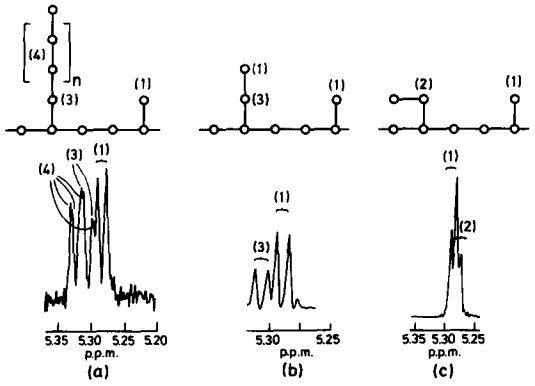

Fig. 6. Comparison of the structural features and corresponding 500 MHz proton spectra (α-1→3-linked anomeric regions) of (a) Dextran SD5 (S4-type dextran) before α-1→3-glucanase digestion. (b) Dextran SD5, after α-1→3-glucanase digestion. (c) Dextran NRRL B-512(F).

α-1→3-linked region displays all the features one would expect for a combination of B-512(F) (triplet at 5·28 ppm) plus α-1→3-linked sequences attached to it as side chains (multiplet below 5·30 ppm) though the proportion of α-1→3-linked side chains present in Fig. 7 is less than that in Fig. 5(a) for the SD5 dextran. The α-1→6-linked region of the Fig. 7 spectrum ($\approx$ 4·95 ppm) is also more complex than that from the B-512(F) starting material (Fig. 1) due to neighbouring group effects. Further work is envisaged to determine whether neighbouring group effects can be used to provide information about the sequence and distribution of side chains along the main chain.

CONCLUSION

Enzymic hydrolysis, HPLC separations and methylation analysis allowed NMR resonances to be assigned to specific structural features in several dextrans. Single- and double-unit α-1→3-linked glucosyl side chains could be distinguished from one another, and from longer side chains of α-1→3-linked-D-glucose sequences. Considerable NMR time savings for the analysis of minor resonances were achieved by the use of proton rather than carbon-13 spectra.

ACKNOWLEDGMENT

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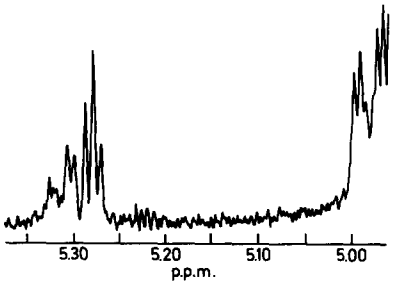

Fig. 7. The anomeric proton region of the 500 MHz proton spectrum of dextran NRRL B-512(F), after incubation with sucrose and the glucosyltransferase GTF-I. (For the spectrum of this polysaccharide before incubation with GTF, see Fig. 1(b).)

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