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Proton and carbon-13 NMR studies on xanthan derivatives

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High-resolution NMR spectra of a number of xanthan derivatives have been obtained. Enzymic hydrolysis with cellulases was used to produce xanthan fragments as small as the pentasaccharide repeat unit. Spectral resolution improved as the molecular weight of the samples was decreased. Changes in the chemical shift of some anomeric proton and carbon resonances indicated that substantial conformational transitions had taken place on depolymerisation.

Partial and complete removal of pyruvyl and acetyl substituents, plus proton-carbon-13 correlation NMR spectroscopy and homonuclear Hartmann-Hahn spectroscopy, were used to assist in the assignment of a number of resonances. Comparison of spectra from samples run at ambient (27°C) and high temperature (90°C) revealed chemical shift changes, particularly of anomeric resonances, which were probably a consequence of conformational transitions induced about the glycosidic linkages by heating or cooling.

INTRODUCTION

Xanthan, the extracellular polysaccharide produced by the bacterium *Xanthomonas campestris*, has a -(1 → 4)-β-linked-D-glucopyranosyl main-chain (as in cellulose), and a trisaccharide side-chain attached at C_3 to each alternate glucosyl residue. The side-chain, β-D-mannopyranosyl-(1 → 4)-β-D-glucopyranuronosyl-(1 → 2)-α-D-mannopyranosyl-, is often fully substituted on the inner mannose at C_6 by 6-O-Acetyl groups. The outer mannose contains 4,6-O-pyruvyl ketal groups to varying extents depending on the polymer source. The side-chains assist in keeping the main-chain of the polymer in an ordered, helical conformation over a large range of conditions. Ordered xanthan behaves like a rigid rod (Morris *et al.*, 1977; Milas & Rinaudo, 1979) and the maintenance of this conformation is responsible for many of its unusual rheological properties.

NMR spectra of xanthan have been studied by several groups. Rinaudo *et al.* (1983) used samples partly depolymerised by cellulase in proton and carbon-13 NMR investigations. They used proton NMR spectra at 90°C to determine the ratio of pyruvyl/acetyl groups, and partially assigned the resonances of the carbon-13 spectrum. Horton *et al.* (1985) used carbon-13 NMR spectroscopy in structural and biosynthetic studies of xanthan. They assigned many of the resonances, using heteronuclear gated decoupling experiments to confirm assignments in the anomeric region of the spectrum. Hall and Yalpani (1981) used solid-state carbon-13 spectroscopy as an early demonstration of the utility of the magic angle spinning/cross polarization technique in polysaccharide chemistry. Gorin *et al* (1967) and Garegg *et al.* (1980) used proton and carbon-13 spectroscopy to assign the S-configuration to the ketal carbon atom of pyruvic acid in xanthan.

One feature of xanthan is its ability to form firm, stable gels with certain galactomannans at low total carbohydrate concentrations. The xanthan molecule undergoes a thermally-induced order-disorder conformational transition, characterised by the transition temperature, T_C. The disordered conformation is favoured by low concentrations of external salt, when electrostatic repulsions between carboxyl groups are maximised. There is continuing debate about the precise nature of the conformational transition, about whether xanthan in solution is single (Milas & Rinaudo, 1986) or double-stranded (Lecourtier *et al.*, 1986) and about the conformation adopted by xanthan in the junction zones of mixed xanthan-galactomannan gels (Cairns *et al.*, 1986, 1987). A model for junction zones proposing interaction between xanthan side-chains (rather than main-chains) and the galactomannan backbone has been developed (Tako &

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Nakamura, 1984; Tako *et al.*, 1984; Tako & Nakamura, 1986.)

As part of studies on the conformational aspects of xanthan-galactomannan gelation (Cheetham & Mashimba, 1988; 1991*a* the authors have isolated a number of xanthan samples resulting from cellulase hydrolysis of the native and modified material (Cheetham & Mashimba, 1991*b*) and now report details of their NMR spectra.

EXPERIMENTAL

The xanthan used was derived from crude Keltrol or from Keltrol XT (Kelco Division of E. Merck & Co. Inc., USA) as described previously (Cheetham & Mashimba, 1991), where enzymic hydrolysis and isolation of the products was also reported.

NMR spectra were obtained using a Brüker AM500 spectrometer operating in the Fourier-transform mode, at 500 MHz (protons) and 125 MHz (carbon-13). Samples were lyophilised twice in D_2O, dissolved in the same solvent, and placed in 5 mm tubes at a concentration of ~10 mg/0·6 ml. References were external acetone (2·2 ppm) for protons and external dioxane (67·8 ppm) for carbon. Chemical shifts in quoted references have been corrected to allow for different shift reference compounds. One-dimensional spectra were collected from samples run at 27°C or 90°C (probe temperature).

Two-dimensional proton-carbon-13 correlation spectra were obtained as described by Bax (1983) and modified by Rutar (1984); 4Kcarbon acquisition; 1063·8 Hz carbon sweep-width; 1050 Hz proton sweep-width; 256 scans per t_1; 178 experiments zero-filled to 512 points, with 1 s relaxation delay; processed with an exponential window function in the $f2$ direction; line-broadening factor 5 Hz, and a shifted sine bell squared for $f1$, shifted by $\frac{\Pi}{4}$. A homonuclear Hartmann-Hahn (HOHAHA) spectrum was obtained as described by Bax and Davies (1985) using an MLEV-17 sequence to spin-lock the protons.

Methylation analysis of an acetate-free/pyruvate-free pentasaccharide repeat unit was carried out by the method of Harris *et al.* (1984).

Cellopentaose was a gift from Dr G. J. Walker, of the Institute of Dental Research, Sydney.

RESULTS AND DISCUSSION

Proton spectra

Figure 1 shows the 500 MHz, 90°C proton spectra of a series of xanthan samples. Figure 1(a) shows the native-sized, acetate-free sample, while (b)–(d) show other, low-acetate samples that have been treated with cellulase for 6, 12 and 24 h, respectively. Removal of the acetate alone resulted in a much improved proton spectrum, even before enzymic hydrolysis was carried out. Peaks in the 4·0–5·5 ppm region were assigned as follows: Peak I, anomeric proton resonance of the inner, α-linked-D-mannopyranosyl; Peak II, anomeric resonances of the β-D-glucuronosyl and β-D-mannopyranosyl unit; Peak III, anomeric resonances of the two β-D-glucosyl units in the main-chain; Peak IV, resonance of the proton attached to carbon 2 of the α-D-mannopyranosyl unit. The assignments will be fully justified later. The series reveals some interesting features, in addition to merely narrower line widths.

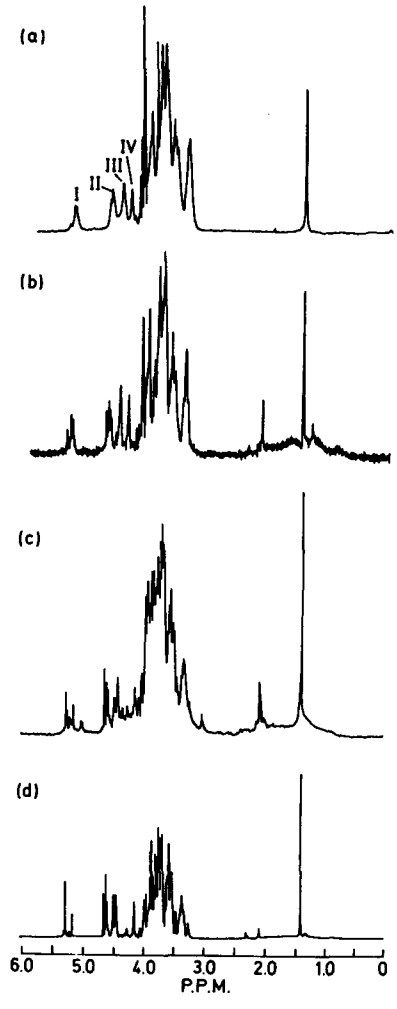

Fig. 1. The 500 MHz proton spectra, at 90°C in D_2O, of xanthan derivatives. (a) An acetate-free but otherwise native polymeric sample. (b)–(d) Low acetate samples similar to that in (a), which have been treated with cellulase for 6, 12 and 24 h respectively. For discussion of regions marked I–IV see text.

Selecting peak I (one proton, H_1, α-mannose) and progressing downward in molecular weight from Fig. 1(a) to Fig. 1(d), one can observe the increase in signal quality and the shift downfield of this resonance from

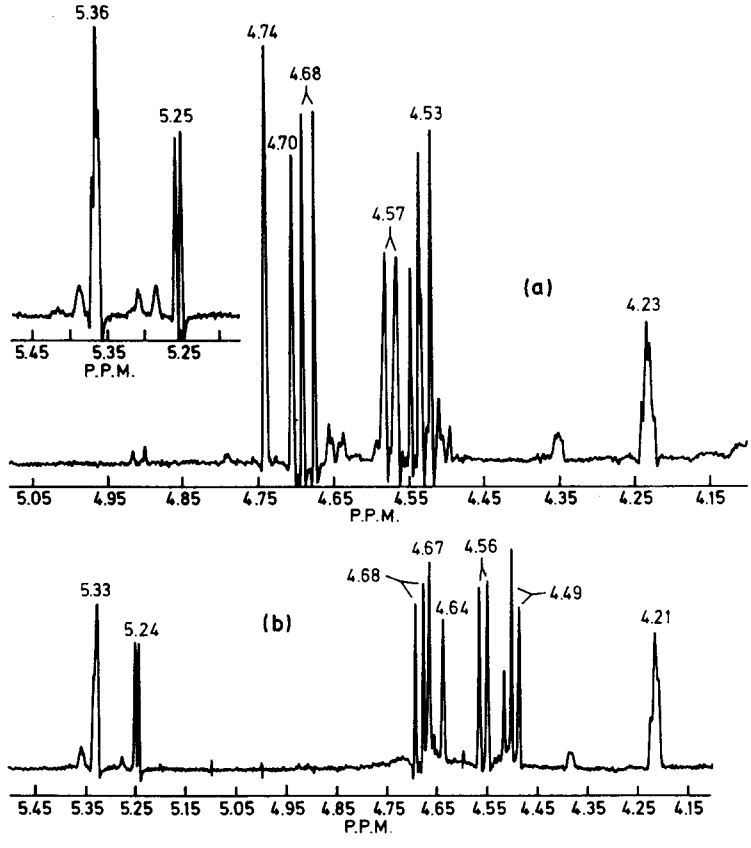

Fig. 2. The low-field resonances of the 500 MHz proton NMR spectra of a pentasaccharide derived from acetate-free xanthan by enzymic hydrolysis (a) at 90°C in D_2O; (b) at 27°C in D_2O.

~5·1 to 5·36 ppm. In Fig. 1(c) there is also present a signal at 5·25 ppm which is due to the α-D-glucopyranosyl anomeric proton on the reducing end of the partially depolymerised sample. This develops to a clean doublet at 5·25 ppm (J = 3 Hz) in the spectrum of the oligomeric material (Fig. 2(a)). The apparent triplet at 5·35 ppm, and the presence of several other peaks in Fig. 2(a) and 2(b) occur as the pentasaccharide is not a single species. There are several combinations of acetate and pyruvate present.

Peak II also moves slightly downfield, and becomes sharper going from Fig. 1(a) to 1(d). It finally splits into three distinct signals:

(i) A doublet centred at 4·68 ppm (J = 7·9 Hz) is due to the β-D-glucopyranosyl anomeric proton on the reducing end of the oligomeric material. Both this signal and the corresponding α-signal have identical chemical shifts and coupling constants to a sample of cellopentaose, which was run as a standard.

(ii) A singlet at 4·74 ppm. This is clearer in Fig. 2(a) and is attributed to the β-D-mannopyranosyl unit of the side-chain, with pyruvate present.

(iii) A singlet at 4·70 ppm, also attributed to the β-D-mannosyl unit of the side-chain, but without attached pyruvate. These assignments will be justified fully in the section on oligomeric fragments.

Peak III (2 protons) has divided in Fig. 1(c) and 1(d) into two doublets, centred at 4·57 ppm (J = 7·9 Hz) and at 4·53 ppm (J = 7·8 Hz) (better seen also in Fig. 2(a)). The lower field doublet corresponds closely in chemical shift and coupling constant to those of the anomeric proton on the non-reducing end of cellobiose and cellopentaose. The higher-field resonance is therefore that of the β-D-glucuronosyl residue in the xanthan side-chain. This assignment was confirmed by the proton–carbon-13 correlation spectrum (see later).

In the light of the above assignment, the original assignment of the β-D-glucuronosyl anomeric resonance to Peak II in the polymeric material is open to question. The authors believe, however, that in the high-polymer, the glucuronic acid anomeric resonance is indeed in Peak II, but that on depolymerisation (Fig. 1(b)–(d)) there is a shift, which could be due to a conformational change, resulting in the glucuronic acid anomeric resonance moving from the Peak II to the Peak III region. Other evidence for this proposal comes from carboxyl-reduced xanthan. In high molecular weight, reduced xanthan, the size of Peak II is greatly decreased, and that of Peak III increased (Cheetham, N. W. H. & Nik Norma, N. M., unpublished). Evidence

for considerable differences between anomeric carbon chemical shifts of the polymeric versus the oligomeric material is presented in the discussion on the carbon-13 spectra.

Peak IV (1 proton) is the signal for H_2 of the α-D-mannosyl residue of the side-chain. In the polymeric material this is centred at ~4·35 ppm (Fig. 1(a)) but shifts upfield to 4·23 ppm in samples of lower molecular weight (Fig. 1(d) and 2(a)).

Further comparison of Fig. 2(a) and (b) reveals significant differences in the chemical shifts of the β-mannosyl (4·74 to 4·67, pyruvate present and 4·70 to 4·63, pyruvate-free) anomeric resonances, when the sample is cooled from 90 °C to room temperature. Such changes would be expected, as the mannose and glucuronic acid residues are on the outer portion of side-chain which is almost certainly involved in conformational changes during heating and cooling. The α-mannosyl anomeric resonance changes from 5·36 to 5·33 ppm on cooling. This seems a small shift, as this inner mannose links the side-chain to the main-chain. Similarly on cooling, the low-field doublet of the non-reducing β-D-glucosyl anomeric proton changes from 4·57 to 4·56 ppm, and that of the β-D-glucuronosyl unit from 4·53 to 4·49 ppm. The major shift for the β-glucuronosyl resonance occurred on depolymerisation, as discussed above. The signal for H_2 of the α-D-mannosyl residue moves from 4·23 to 4·21 on cooling.

Figures 1 and 2 show the spectra of samples with most of the acetyl groups removed. Figure 3(a) shows at 90 °C, the anomeric region of oligomeric material, obtained by the enzymic hydrolysis of xanthan with acetate and pyruvate still present. Four extra doublets between 4·35 and 4·45 ppm are present. They arise from H_{-6} and H_{-6}^1 of the inner, α-linked mannose which is O-acetylated at C_6. Their coupling constants ($J_{5,6}$ = 2·4 Hz; $J_{5,6}^1$ = 5·6 Hz; $J_{6,6}^1$ = 12·2 Hz) are consistent with previous assignments for cellotriose (Ikura & Hikichi, 1987) and cellobiose (Hall *et al.*, 1980) for C_6 acetylation. At room temperature (Fig. 3(b)) additional peaks occur in the 4·15–4·25 ppm region only in samples containing acetate (cf. Fig. 2(b)). These have not been assigned positively, but as the 4·21 ppm resonance is that of H_2 of the α-mannosyl unit *without* acetate (Fig. 2) these additional resonances most probably belong to H_2 mannose in the acetylated samples.

In Figs 2 and 3, the relative intensities of the 'reducing' anomeric resonances to any of the other four anomeric resonances is close to unity. This supports the proposal that the samples giving rise to the spectra in Figs 2 and 3 are pentasaccharides. The monosaccharide ratios of close to 2 : 2 : 1 for glucose : mannose : glucuronic acid respectively, show that the samples have the normal values for a xanthan repeating unit.

Carbon-13 spectra

Figure 4 shows two of the repeat-unit sections of the xanthan chain together with their carbon-13 assign-

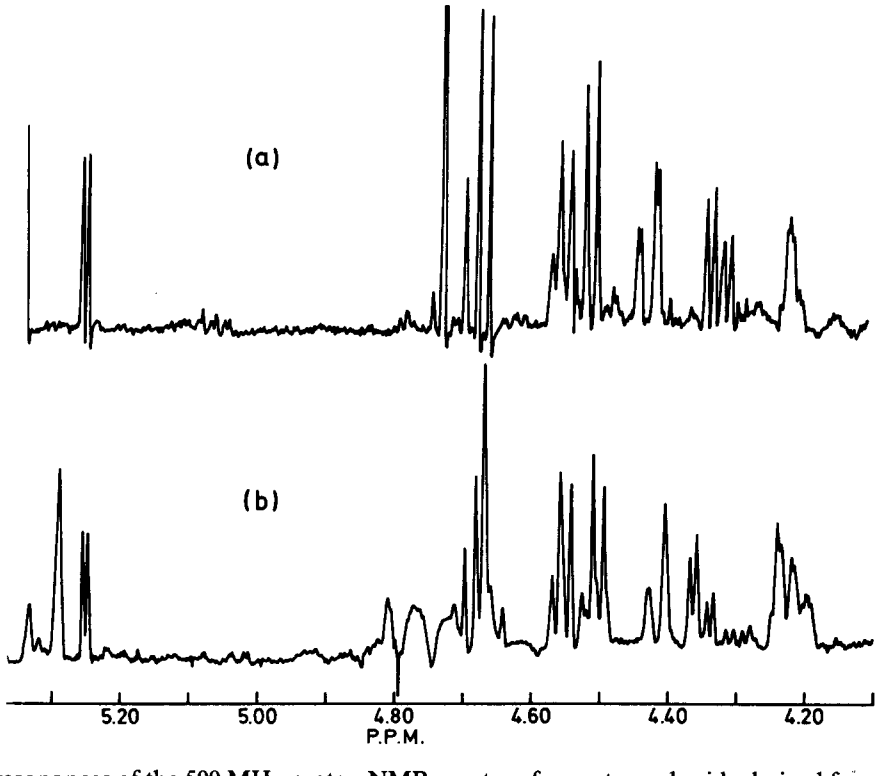

Fig. 3. The low-field resonances of the 500 MHz proton NMR spectra of a pentasaccharide derived from xanthan (containing both pyruvate and acetate) by enzymic hydrolysis (a) at 90 °C; (b) at 27 °C.

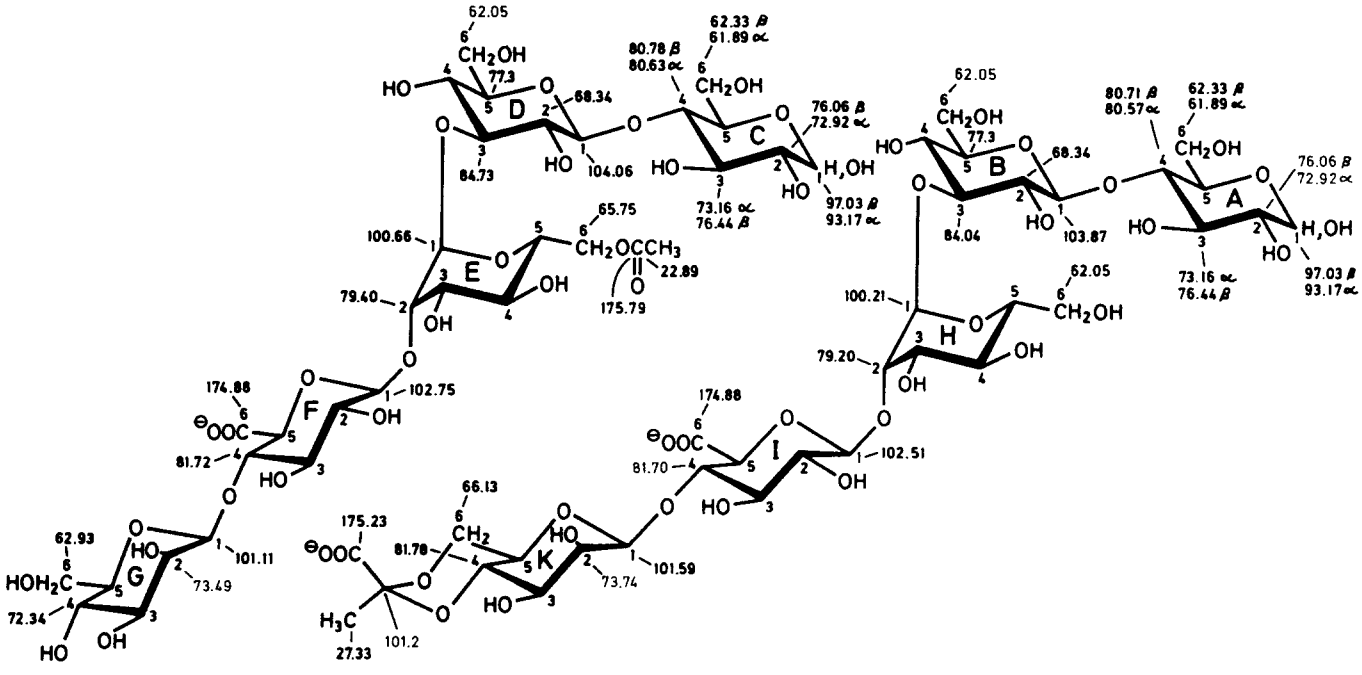

Fig. 4. Two of the pentasaccharide repeat-units of xanthan, with carbon-13 NMR assignments for the principal carbon resonances. The structure on the left is O-acetylated at C_6 of the inner, α-D-mannopyranosyl residue of the side-chain. That on the right has a 4,6-O-pyruvyl ketal residue on the -β-linked, outer -D-mannosyl residue. See text for a full discussion.

ments. One of the two side-chains in Fig. 4 has the α-mannosyl residue acetylated at C_6. This was done to demonstrate the manner in which the carbon-13 assignments vary depending on the presence or absence of the acetyl group. The second unit is treated similarly to illustrate the effect of pyruvate. The assignments were made by

(a) Inspection of the one-dimensional, proton decoupled spectra. By use of a variety of samples, e.g. fully acetylated and pyruvylated, pyruvate-free, acetate-free, and acetate-pyruvate-free, most of the signals could be assigned.
(b) The proton-carbon-13 correlation spectrum.
(c) Homonuclear Hartmann-Hahn (HOHAHA) spectroscopy.

The structures shown in Fig. 4, i.e. with the side-chain attached to position three of the non-reducing D-glucosyl residue, was deduced from the carbon-13 spectra, and from methylation analysis. Methylation analysis showed the presence (approximately equimolar) of 2,3,6-tri-O-methyl glucose and 2,4,6-tri-O-methyl glucose. Had the side-chain been attached to the reducing glucosyl residue, one would have obtained 2,6-di-O-methyl glucose and 2,3,4,6-tetra-O-methyl glucose. Carbon-13 NMR spectroscopy showed resonances corresponding to the α- and β-forms of a -D-glucose residue unsubstituted at positions 2 and 3, and substituted at position 4.

Anomeric carbon resonances

Figure 5(a) shows the anomeric region of the spectrum of a xanthan-derived pentasaccharide, acetate and pyruvate-free. The assignments are identified by reference to Fig. 4, where the ten residue types are labelled A-K. For instance, the resonance at 103·87 pm is C_1 B, i.e. the anomeric carbon on residue B, which is one of the -1 $\longrightarrow$ 4-β-D-glucosyl residues in the main-chain, without acetate on the side-chain. Figure 5(b) represents part of the spectrum of a sample that is high in pyruvate, and lacking acetate. The resonance at 101·59 ppm belongs to the anomeric carbon in the β-mannosyl unit, with pyruvate present (C_1K). The assignment of this peak was substantiated by the proton-carbon correlation spectrum which clearly linked this to the proton resonance at 4·70 ppm, which is due to the anomeric proton of the β-linked mannosyl unit of the side-chain, with pyruvate present (C_1K). The peak at 101·10 ppm is that of the same β-D-mannosyl unit *without* pyruvate (C_1G).

The quaternary carbon (C_2) resonance of the pyruvate ketal was located at 101·2 ppm, i.e. under the C_1-β-mannosyl resonance. It can be seen as a lower-field hump (perhaps as it was not fully relaxed) in Fig. 5(b). The assignment was made by the following means:

(a) By reducing the power of the decoupler. This made the partly-coupled anomeric resonances appear as doublets of reduced size. The resonance

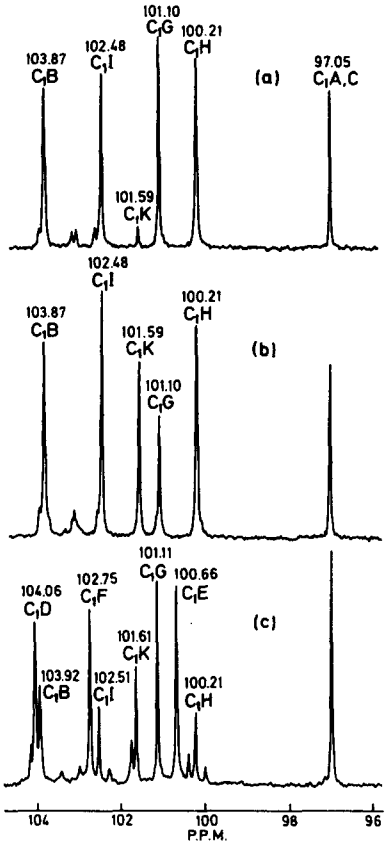

Fig. 5. The anomeric carbon resonances of the 125 MHz NMR spectra of three xanthan-derived pentasaccharides, at 27 °C (a) a pyruvyl-free/acetyl-free sample; (b) an acetyl-free sample; (c) an 80% acetylated, partially pyruvylated sample. The origin of each C1 resonance in Fig. 4 is indicated above each peak.

of the pyruvate C2 remained in the same position, virtually unchanged in size (the authors thank Professor K. Bock for this suggestion).

(b) Examination of the 90 °C spectrum. The C_1-β-D-mannosyl resonance shifted downfield sufficiently to reveal the smaller pyruvate resonance (not shown).

(c) Figure 5(c) shows the spectrum of an oligosaccharide with pyruvate present, but 20% deacetylated. Acetate stabilises the ordered conformation in the polymer (Holzwarth & Ogletree, 1979), and is likely to be associated with particular φ and ϕ conformational angles between the backbone β-D-glucosyl residues. Removal of acetate could change the values of the φ and ϕ. This seems to have caused the shift from 104·06 (C_1D) to 103·92 ppm (C_1B), as seen in Fig. 5(c). The discrepancy in area between the two resonances reflects the fact that about 20% of the acetate has been removed from the sample in Fig. 5(c). A sample with most (~80%) of the acetate removed had the areas of the 104·06 and 103·92 ppm resonances reversed. The chemical shifts of these resonances differ from those of the corresponding C_1 resonances in cellopentaose, which were found at 103·57 ppm in a reference compound. This indicates that the conformation of the -1 ⟶ 4-β-D-glucosyl linkage in this xanthan derivative is not identical to that in cellulose oligomers, presumably due to the presence of the bulky side-chain. The resonances at 102·75 and 102·51 ppm in Fig. 5(c) are attributed to C_1 of the 1 ⟶ 4-linked-β-D-glucuronosyl residue in the presence and absence of acetyl groups respectively. Confirmation of the assignment involved comparison with Fig. 5(a) and (b). The difference between the chemical shifts probably indicates the involvement of this part of the side-chain in overall conformational transitions. The small but significant peak at 101·74 ppm in Fig. 5(c) is as yet unassigned, together with some of the minor ones in Fig. 5(c). This sample obviously contains combinations of acetyl and pyruvyl groups not represented in Fig. 4. The resonance in Fig. 5(c) at 100·66 ppm is due to the C_1 of the inner, -α-linked-D-mannosyl residue linked to C-3 of the backbone in the presence of acetyl (C_1E). A marked shift up-field to 100·21 ppm (C_1H) (Fig. 5(a) and (b)) resulted upon removal of acetyl groups.

Effect of temperature

It is appropriate to mention here further effects of raising the temperature to 90 °C, i.e. well above T_C, the temperature for the melting of the ordered conformation in the polymer. Though there is no doubt much less scope for the ordered conformation to be present in the oligomers, there is still a significant temperature effect on most of the anomeric carbon resonances:

(i) An up-field shift of the β-D-glucosyl resonances from 104·06(C_1D)/103·92(C_1B) to 103·67/103·61 ppm. These latter shifts are very close to that for the C_1 carbons in cellopentaose (103·57 ppm). Thus heat energy overcomes the resistance imposed by the presence of the side-chains, and the backbone conformation becomes more 'cellulose-like'.

(ii) An up-field shift from 102·75(C_1F)/102·51(C_1I) to 102·61/102·36 in the C_1 resonance of glucuronic acid. This acid residue is also likely to be involved in the overall conformational changes.

(iii) A small but significant downfield shift (of ~0·2 ppm) in the resonance of the C_1 of β-D-mannosyl residues at the end of the side-chain as the temperature is raised to reveal the C_2 pyruvyl resonance.

(iv) Substantial up-field shifts of the α-D-mannosyl C_1 resonances from 100·6 (C_1E) to 100·21 ppm, and 100·21 (C_1H) to 99·98 ppm. This is also to be expected in such a key linkage.

The above assignments of the anomeric carbon resonances differ from those of Rinaudo *et al.* (1983) which were given for the polymeric xanthan at 90 °C. The downfield C_1 resonances were assigned by Rinaudo *et al.* to the three side-chain moities, and the higher field ones to the β-D-glucosyl backbone. This is the reverse of the present assignments, which agree with respect to the order of side-chain versus main-chain anomeric resonances, with the assignments by Horton *et al.* (1985). The latter authors assign to C_2 of the pyruvyl residue the resonance at 101·7 ppm (102·1 corrected) in the polymer at 90 °C. This compares with the present authors' value of 101·2 ppm for the C_2 pyruvyl in the pentasaccharide at 27 °C.

Carbons 2–6 resonances

The higher-field regions of the spectra are complex, but well-resolved. Using the respective upfield spectra of the samples in Fig. 5(a)–(c), most key assignments were made as shown in Fig. 4, using one- and two-dimensional spectra. Figure 6 shows part of the non-anomeric carbon region of a pyruvyl-free, acetyl-free pentasaccharide.

The lowest field resonances of non-anomeric carbons occur at 84·73 (Fig. 6, peak (a)) and 84·04 ppm. The latter belongs to the C_3 of the -β-D-glucosyl residue to which the side-chain is attached (C_3B in Fig. 4) but which has no acetate on the nearby C_6 of α-D-mannose. When acetate *is* present on the mannose, this resonance is shifted downfield to 84·73 ppm (C_3D). (The 84·73 ppm peak is lost on deacetylation.) The assignment of 84·04 ppm compares with 84·33 ppm (corrected for a different reference) reported for the corresponding C_3 of methyl-β-nigeroside (Jansson *et al.*, 1988). This assignment is somewhat up-field of Jansson's value, but nigerose is not a perfect model for the pentasaccharide.

A resonance at 81·78 ppm (Fig. 6, peak (b)) is present in samples containing some pyruvate (C_4K) together with a smaller one at 72·34 ppm (C_4G) due to the pyruvate-free resonance. The former resonance is lost completely, and the latter increases in size (Fig. 6, peak (d)) when pyruvate is removed. The resonance of C_4 for the non-reducing glucose of methyl-β-cellobioside is ~71·9 ppm (corrected) (Usui *et al.*, 1973). The 72·34 ppm resonance is the nearest to this which increases enough in size to be considered. In all samples there is a resonance at 81·72–81·70 corresponding to C_4 of the β-D-glucuronic acid of the side-chain (C_4I, C_4F). This compares to 80·5 ppm for C_4 in methyl-β-cellobioside (Usui *et al.*, 1973). There are only minor chemical shift changes on removal of pyruvate and acetate.

Resonances at 80·71 and 80·57 ppm appear in approximately the free anomeric α/β ratio of 35/65, and are attributed to C_4A (no acetate present). When some acetate is present, e.g. in residue E, two extra peaks appear at 80·78 and 80·63 ppm, in the same size ratio. The authors assign these to C_4C, as the presence of acetate on the nearby C_6E mannose is likely to have some interactive effect.

A resonance at 79·20 ppm was definitely identified by the proton–carbon-13 correlation spectrum as belonging to C_2H, i.e. to carbon-2 of the inner mannosyl residue,

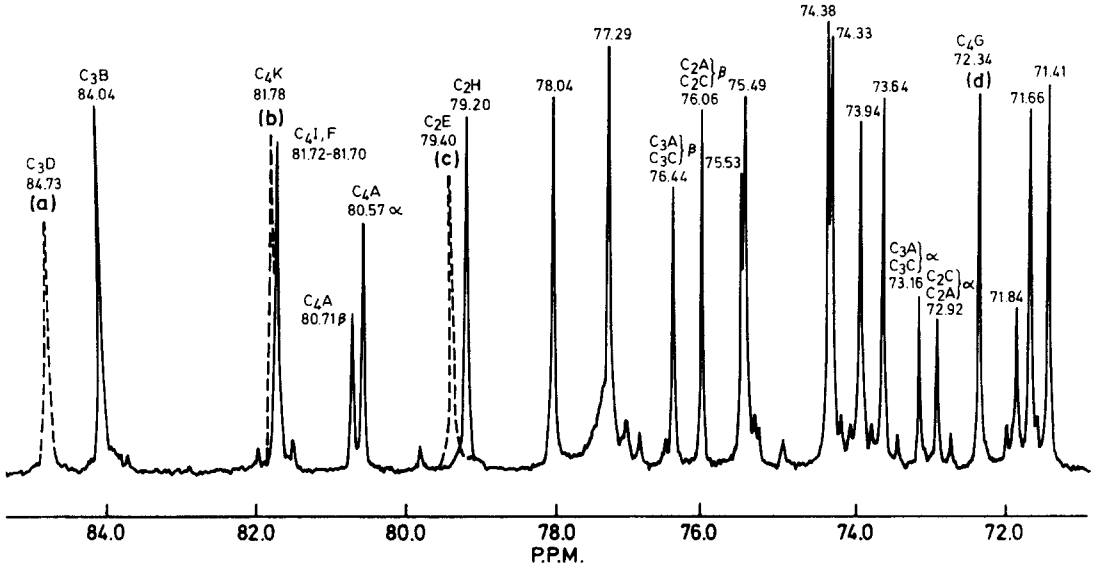

Fig. 6. Carbon-13 spectrum at 27 °C, of part of the non-anomeric region of a pyruvyl-free/acetyl-free xanthan-derived pentasaccharide. Peak (a): the C_3 resonance of the -b-D-glucosyl residue (84·73 ppm; C_3D in Fig. 4) when acetyl is *present* (cf. 84·04 ppm, C_3B in Fig. 4, acetyl-free). Peak (b): the C_4 resonance (81·78 ppm; C_4K in Fig. 4) of the -β-D- mannosyl residue when pyruvyl is *present* (cf. 72·34 ppm; C_4G in Fig. 4, pyruvyl-free). Peak (c): the C_2 resonance (79·40 ppm; C_2E, Fig. 4) of the inner, α-linked -D-mannosyl residue, when acetyl is *present* (cf. 79·20 ppm; C_2H in Fig. 4, acetyl-free). Peak (d): the C_4 resonance (72·34 ppm, C_4G) of the β-mannosyl residue, pyruvate-free.

without acetate. (The proton resonance at ~4·21 ppm is in a clear part of the spectrum. Fig. 2(b)). When acetate is present, this resonance (C_2E) is located at 79·40 ppm (Fig. 6, peak (c)). One would certainly expect a change of this nature at one of the key linkage points in the oligosaccharide.

The resonance at 78·04 ppm is as yet unassigned. Its chemical shift does not vary on pyruvate and/or acetate removal, and probably is due to a C_3 or a C_5 resonance. It correlates with a proton at 3·39 ppm. There is a large rather broad resonance at 77·29 ppm which is reduced in size on removal of acetate. It is probably a combination of C_3 and C_5 resonances (Usui *et al.*, 1973). The resonances at 76·44 and 73·16 ppm are assigned to the β- and α-anomeric carbon resonances respectively of C_3 in both residue A and residue C (cf. 76·3 & 73·1 ppm for cellobiose (Usui *et al.*, 1973). They do not vary in position or intensity with acetate/pyruvate removal, and are present in approximately the expected area ratio for free anomeric resonances. Similarly the resonances at 76·06 and 72·92 ppm do not vary from sample to sample and are assigned to the β- and α-resonances respectively of C_2 in both A and C (75·9 and 73·0 ppm for cellobiose (Usui *et al.*, 1973). Resonances at 75·53 and 75·49, 74·38 and 74·33 ppm are unassigned.

There are five resonances at 73·94, 73·64, 71·66, 71·41 and 68·51 ppm, of almost equal intensity and which do not vary in position in the various xanthan samples. They probably belong to C_3, C_4 and possibly C_2 and C_5 of the remaining unassigned ring resonances.

The resonance at 68·34 ppm is assigned C_2D and C_2B. The 68·34 ppm carbon resonance correlates with a 3·41 ppm proton resonance in the proton-carbon-13 correlation spectrum. In the HOHAHA spectrum, the 3·41 ppm proton shows a connectivity to the 4·56 ppm proton, which was shown above (Fig. 2(b)) to be the H_1 of residues B and D. Thus the 68·34 ppm carbon resonance is that of C_2 in residues D and B.

Peaks at 66·13 ppm and below belong to O-substituted and O-unsubstituted carbon-6 resonances. The 66·13 ppm resonance (Fig. 7, peak (a)) is assigned to C_6K as it appears in all pyruvylated samples, and shifts upfield to 62·93 ppm (C_6G) when pyruvate is removed. Though the latter resonance is further downfield than the C_6 resonance in methyl-β-D-mannopyranoside (61·6 ppm (Bock & Thøgersen, 1982)) various spectra with different amounts of pyruvate support the above assignment. Horton *et al.* (1985) quote 62·9 ppm at 90°C for polymeric xanthan. The resonance at 65·75 ppm (Fig. 7, peak (b)) attributed to C_6E, i.e. the acetylated α-linked mannose, shifts upfield to 62·05 ppm (C_6H) when acetate is removed. This compares with 62·2 ppm for C_6 of methyl-α-D-mannopyranoside (Bock & Thøgersen, 1982).

A resonance at 62·33 ppm is attributed to C_6 of the β-anomer of residues A and C. Their intensity is not altered during modification of the oligosaccharide. The C_6 resonance of methyl-β-D-glucoside occurs at 62·0 ppm (corrected) (Bock & Thøgersen, 1982). The large resonance at 62·05 ppm (C_6H), is also proposed to

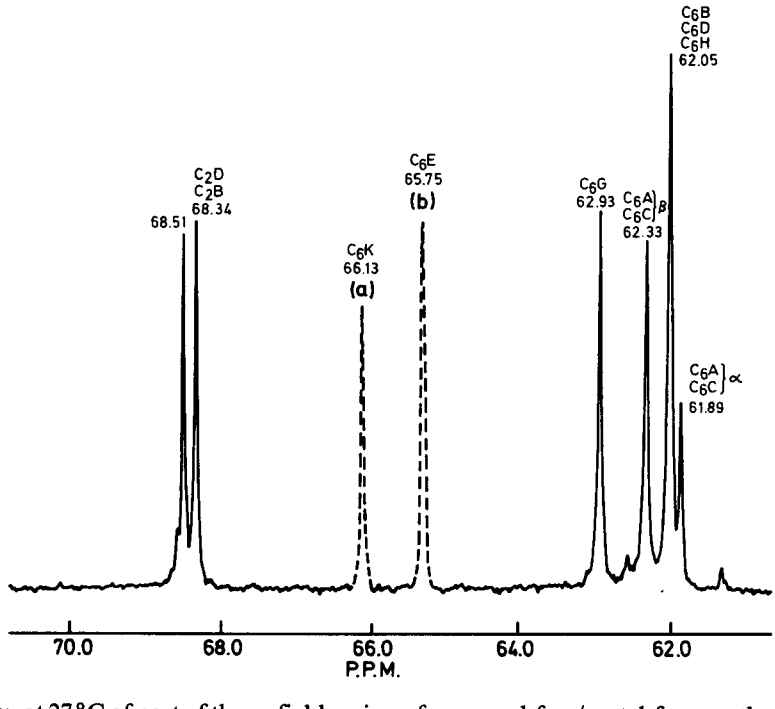

Fig. 7. Carbon-13 spectrum, at 27°C of part of the upfield region of a pyruvyl-free/acetyl-free xanthan-derived pentasaccharide. Peak (a): the C_6 resonance of the outer, -β-linked-D-mannosyl residue (66·13 ppm; C_6K in Fig. 4) when pyruvate is *present* (cf. 62·93 ppm; C_6G in Fig. 4, pyruvyl-free). Peak (b): the C_6 resonance of the inner, -α-linked-D-mannosyl residue (65·75 ppm; C_6E in Fig. 4) when acetyl is *present* (cf. 62·05 ppm; C_6H in Fig. 4, acetyl-free).

include C_6B and C_6D. (It decreases substantially in size when acetate is present.)

The resonance at 61·89 ppm in Fig. 7 remains relatively constant, in shift and intensity, in all derivatives. It is assigned to C_6 of the α-anomer of residues A and C (cf. 62·0 ppm for methyl-α-D-glucopyranoside (Bock & Thøgersen, 1982) and 62·0 ppm for α-cellobiose (Usui *et al.*, 1973).

CONCLUSIONS

Both the proton (Fig. 1) and carbon-13 NMR (Fig. 8) spectra of xanthan derivatives show significant shifts in the anomeric resonances as the molecular weight decreases. These largely reflect the decrease in the level of ordered conformation as the molecule 'unwinds' and the 1 → 4-β-D-glucopyranosyl backbone moves from the 5/1 helical towards the 2/1 ribbon conformation. At intermediate molecular weights it is possible to obtain (rather poor) room temperature spectra (e.g. Fig. 8). Here the anomeric carbon resonances from Fig. 5(c) have been superimposed on those of a partly-depolymerised sample obtained from native xanthan. At this level of polymerisation, the anomeric resonances from the backbone 1 → 4-β-D-glucose residues are in virtually the same position as they are in the pentasaccharide repeat-unit (104·06 ppm). This differs from the corresponding value for cellopentaose (103·57 ppm) reflecting that even at the pentasaccharide level, the xanthan side-chain influences the backbone conformational angles. The major difference between the pentasaccharide spectrum and that of the polymeric sample is in the position of the β-D-glucuronic acid anomeric resonances (Fig. 8; 102·75 and 102·51 versus 103·2 and 103·0 ppm respectively). This no doubt is due to the presence of long enough sections of backbone in the polymer for interactions with the side-chain. Such interaction has been proposed (Morris *et al.* 1977) to stabilize the ordered xanthan conformation, but is not possible in the pentasaccharide, which has only two backbone glucosyl residues present. The large chemical shift differences in the glucuronic acid proton anomeric resonances in oligomer versus polymer were mentioned earlier in the discussion on the proton spectra.

The effects of temperature on the anomeric proton resonances (Figs 2 and 3) of the pentasaccharide could be indicative of conformational changes.

Other significant conformational changes appear to be caused by the removal of the pyruvyl and/or acetyl substituents. These are best illustrated in the carbon-13 spectra (Figs 4 and 5). Further conformational analysis of the pentasaccharide, e.g. by NOESY and long range carbon-13–proton coupling NMR experiments is planned.

A continuing debate exists concerning the conformation of xanthan when it interacts synergistically with certain galactomannans to form gels (Morris *et al.*, 1977; Cairns *et al.*, 1986, 1987). It was hoped that NMR might be able to contribute some information. Preliminary experiments on mixtures of the pentasaccharide repeating unit of xanthan, and mannohexaose show no evidence for conformational changes either by NMR or optical rotation experiments. This is perhaps not surprising, as the pentasaccharide is not truly representative of the structure of the polymer. Interaction between polymeric derivatives of xanthan (such as that in Fig. 8) and galactomannans at room temperature

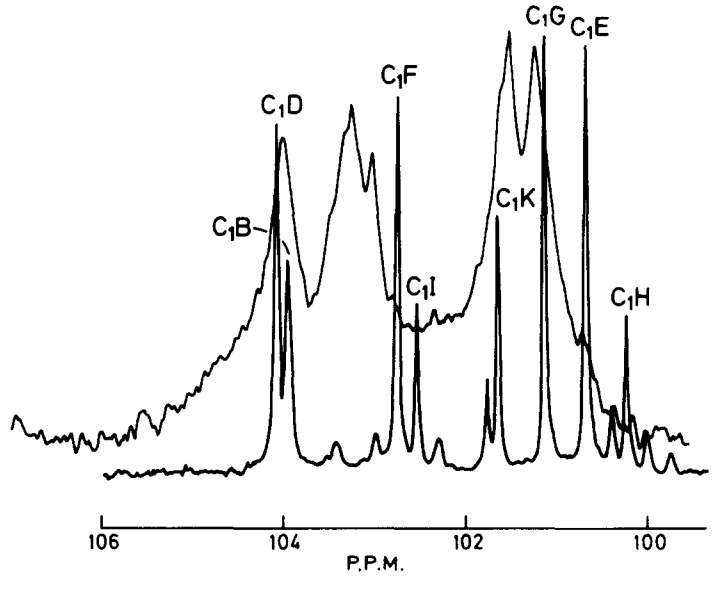

Fig. 8. The anomeric resonances of the carbon-13 spectrum of a partially-depolymerised sample of xanthan. The identified, well-resolved peaks are those from Fig. 5(c) (i.e. the pentasaccharide), which have been superimposed for comparison. C_1B, etc., relate each peak to the structures in Fig. 4.

has been demonstrated by optical rotation and gel-permeation experiments (Cheetham & Punruckvong, 1989).

Preliminary interaction studies using NMR spectroscopy, between xanthan derivatives and carob galactomannan have been inconclusive. Proton NMR studies are hampered by the presence of the water peak in a critical region of the spectrum. Carbon-13 spectra have been of poor quality at room temperature (Fig. 8). Further work is in progress.

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