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Note

Structural and biosynthetic studies on xanthan by ^{13}C-n.m.r. spectroscopy

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This report shows that the polysaccharide xanthan gives well resolved 75-MHz ^{13}C-n.m.r. spectra in aqueous solutions subjected to mild sonication and thermal denaturation. The spectrum of 5% xanthan in D_2O at 90° shows 30 discrete signals that may be correlated with the 35 signals anticipated for a regular sequence of the chemically established, pentasaccharide repeating-unit. Similar ^{13}C spectra were obtained for a high-pyruvate and a pyruvate-free xanthan from two *X. campestris* mutants, indicating that the latter two xanthans also have the same backbone repeating-unit. The metabolic utilization of D-[1-^{13}C] and D-[U-^{13}C]glucose by *X. campestris* was monitored by ^{13}C-n.m.r. spectroscopy. D-Glucose was shown to be a precursor for all structural subunits of high-pyruvate xanthan, including the acetate and pyruvate residues.

Xanthan gum, the extracellular polysaccharide of *Xanthomonas campestris*, has been the subject of extensive chemical and physical studies directed toward its structural and conformational characterization. Its primary sequence (**1**), as established[1] by chemical degradation, has a cellulose-like backbone to which is attached at alternate Glc residues an *O*-β-D-mannopyranosyl-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→2)-6-*O*-acetyl-α-D-mannopyranosyl side-chain; the terminal Man group may have a 4,6-pyruvic acetal substituent. The contents of acetate and pyruvic acetal appear to vary according to the culture conditions and to the post-fermentation processing. The five subunits in the repeating unit are denoted (**1**) as Glc, Glc′, GlcA, Man, and Man′.

The high viscosity of xanthan in solution impedes efforts to characterize it by n.m.r. spectroscopy, and most reports have been restricted to recognizing the methyl signals of the pyruvic and acetyl groups in ^{1}H-n.m.r.[2,3] and ^{13}C-n.m.r.[4] spectra of xanthan. Evidently xanthan requires partial depolymerization to decrease solution viscosity if well resolved spectra are to be obtained. Chemical degradation[5] permitted observation of the pyruvic methyl resonance in the ^{13}C-n.m.r. spectrum, and ultrasonic degradation[3] also allowed the acetyl and pyruvic methyl

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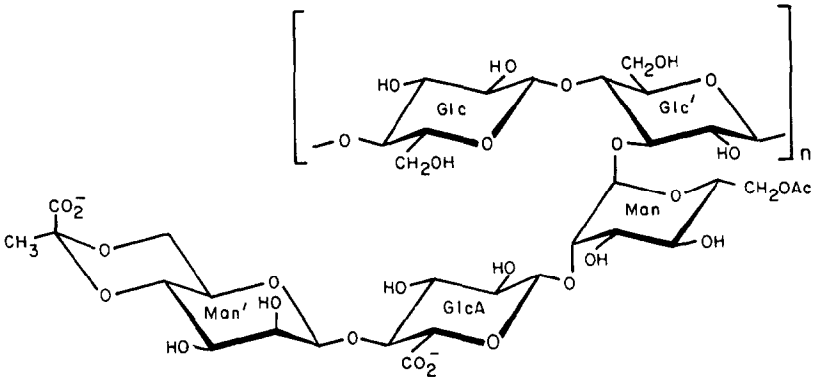

Xanthan (1)

resonances to be observed at room temperature in the ^{1}H-n.m.r. spectrum.

Since the communication of the initial results of our study[6], ^{1}H- and ^{13}C-n.m.r. investigations on a xanthan (apparently fully pyruvated) partially depolymerized by a crude cellulase have been published[7]. It had been earlier demonstrated[8] that in salt-free solution there was random breakdown of the main chain when the polysaccharide was in the *disordered* conformation, and the resultant product had M_w ~240,000. This procedure appears, however, to be effective only with highly pyruvated (>90%) xanthans.

We have developed a procedure of general value for solution n.m.r. studies on xanthans, including "pyruvate-free" xanthan and other highly polymeric (and viscous) polysaccharides, and demonstrate here its use in correlating by ^{13}C-n.m.r. the repeating primary structure of xanthan and in monitoring the metabolic utilization of D-glucose by *X. campestris*. Despite the promise offered by solid state n.m.r. techniques for the study of polymer structure, the capability for securing critical, detailed data for high polymers *in solution* remains of major importance.

MATERIALS AND METHODS

Biosynthesis of xanthan. — Normal (FLOCON 1035, Pfizer), high-pyruvate (FLOCON 4800), and pyruvate-free[9] xanthans were produced by growing, on a defined medium containing D-glucose, appropriate strains of *X. campestris*. The use of D-[1-^{13}C]glucose or D-[U-^{13}C]glucose (both of ~90% isotopic purity, MSD Isotopes, St. Louis, MO) gave, after post-fermentation processing, samples of ^{13}C-labeled high-pyruvate xanthan.

Preparation of xanthan samples for n.m.r. spectroscopy. — Samples were initially dispersed in water by means of a tissue homogenizer and then mildly sonicated (10–15 min) at 0° (Bronwill Biosonic IIA sonicator, standard 1-cm diameter probe), with subsequent ultracentrifugation of the solution at 144,000 × *g* for 1–4 h

at 0°. The solutions of xanthan (mol. wt. 500,000–2,000,000 according to the starting sample, determined by molecular sieving on Sephacryl S-500) were then dialyzed and lyophilized, and the samples were redissolved in D_2O (or 20mM NaCl–D_2O to prevent aggregation)[10] to give 1–5% solutions.

^{13}C-N.m.r. spectra. — Spectra were recorded at 75 MHz with a Bruker WM-300 spectrometer operating in the Fourier-transform mode. Spectra were obtained at 90° with a 30° pulse and a digital resolution of ±1.8 Hz; 1,4-dioxane (67.4 p.p.m.) was used as the external reference. Gated decoupling (time 1 s) was used for fully proton-coupled spectra.

RESULTS AND DISCUSSION

Normal xanthan. — The ^{13}C-n.m.r. spectrum of a 5% solution of **1** in D_2O (Fig. 1) shows 30 discrete signals (plus one additional signal corresponding to C-6 of the depyruvated Man′ terminal group) that may be correlated with the chemical shifts of the 35 signals anticipated on the basis of a regular sequence of the established[1] pentasaccharide repeating-unit. A different commercial xanthan (Keltrol, Kelco Division of Merck, Sharp & Dohme, Inc.), processed similarly gave a spectrum indistinguishable from that shown. Both traces closely resemble the published spectrum of a fully pyruvated xanthan[7]; because of the relatively low pyruvate

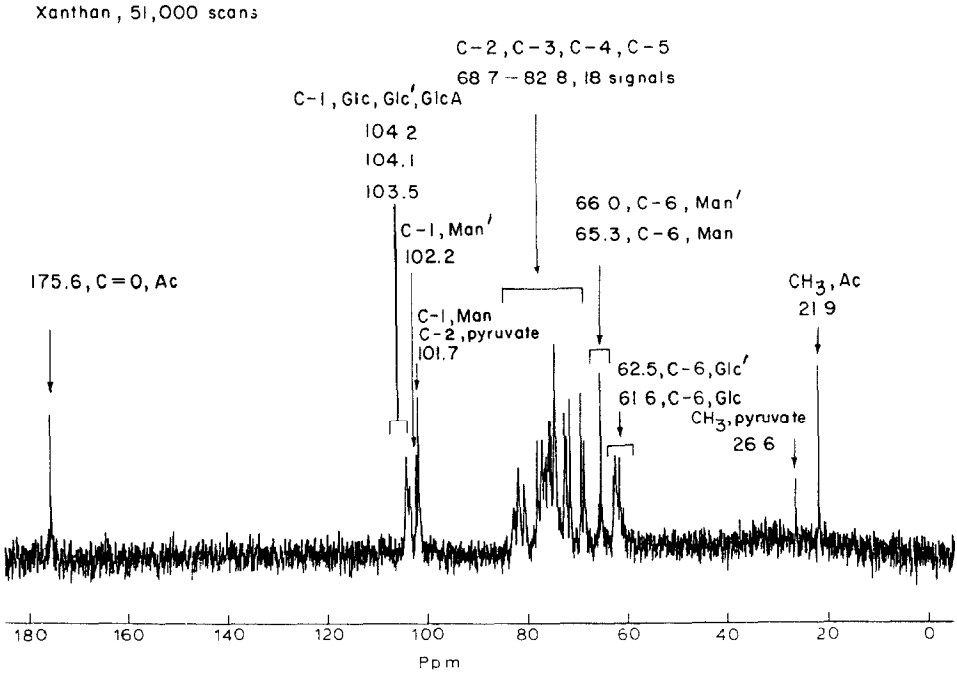

Fig. 1. The ^{13}C-n.m.r. spectrum of normal xanthan (D_2O, 90°), after accumulation of 51,000 scans.

content of the present xanthan samples, the pyruvate carbonyl signal is not clearly differentiated in Fig. 1.

Some assignments of signals in Fig. 1 are as follows: acetyl-methyl group at 21.9 and carbonyl at 175.6 p.p.m.; pyruvate methyl at 26.6, and quarternary carbon (C-2) at 101.7 p.p.m. These values accord with published data[5,7] and the *S* configurational assignment[5] at C-2 (equatorial methyl group). The signals at 104.2, 103.7, and 103.5 p.p.m. are attributable to C-1 of Glc, Glc′, and GlcA, respectively, the signal at 102.2 to C-1 of Man′, and that at 101.7 to C-1 of Man superposed on the C-2 signal of the pyruvate acetal; these assignments, including that for the pyruvate quarternary carbon atom, differ from those proposed in ref. 7. The present assignments were confirmed by a gated decoupling experiment (see later), and they are in agreement with literature data[11] for corresponding residues in various oligo- and poly-saccharides. The assignments of the 18 signals in the region 68.7–82.8 p.p.m. will be discussed elsewhere.

The C-6 signals are observed at 66.0, 65.3, 62.5, and 61.6 p.p.m. for the Man′, Man, Glc′, and Glc residues, respectively. Shifts in these signals are related to depyruvation and/or deacetylation (see later).

Pyruvate-free xanthan. — The absence of pyruvate groups from this product[9] is evidenced by the lack of ^{13}C signals at 176.6 and 26.6 p.p.m. (Fig. 2), and these signals are likewise absent in the spectrum of chemically depyruvated[7] **1**; furthermore there are no signals at 102.2 and 66.0 for C-1 and C-6, respectively, of pyru-

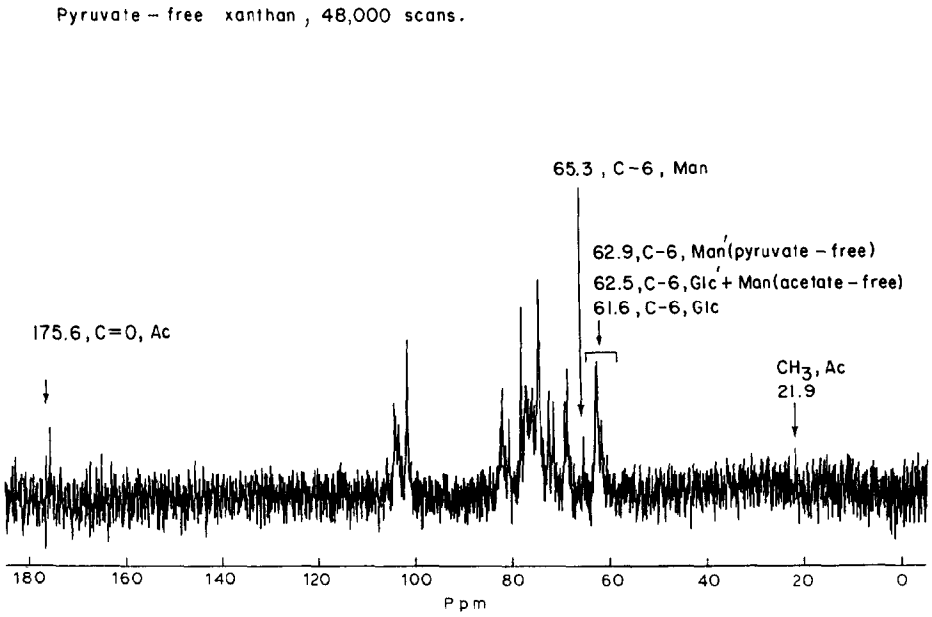

Fig. 2. The ^{13}C-n.m.r. spectrum of pyruvate-free xanthan (D_2O, 90°), after accumulation of 48,000 scans (see **1** and Fig. 1 for detailed assignments).

vated Man′. A new signal appears at 62.9 p.p.m. for C-6 of the pyruvate-free Man′ group, and the C-1 signal of the non-pyruvated Man′ residue is superposed on that of C-1 of Man. Pyruvate-free **1** appears to be less acetylated than normal **1**, as the signals of the acetyl group (175.6 and 21.9 p.p.m.) and of C-6 of acetylated Man are weaker than those in normal **1**. The C-6 signal of the nonacetylated Man residue (at 62.5 p.p.m.) is superposed on that of C-6 of Glc′.

High-pyruvate xanthan. — The ^{13}C-n.m.r. spectrum of this material was similar to that of normal **1** except that the pyruvate carbon signals were of greater relative intensity. Fig. 3a shows the ^{13}C-n.m.r. spectrum (600 scans) of **1** labelled *in vivo* with D-[1-^{13}C]glucose. The five signals observed in the spectrum correspond to the five C-1 resonances of the repeating unit of **1** and thus D-glucose appears to serve as a precursor for all five subunits of the repeating unit of xanthan.

The gated decoupled spectrum of the same sample is shown in Fig. 3b. Even though there is considerable overlap of signals, it may be determined that the four C-1 signals attributed to Glc, Glc′, GlcA, and Man′ are split into doublets displaying one-bond C–H couplings of ~160 Hz, while the C-1 signal of Man is split into a doublet having a one-bond coupling of 176 Hz, thus confirming the anomeric

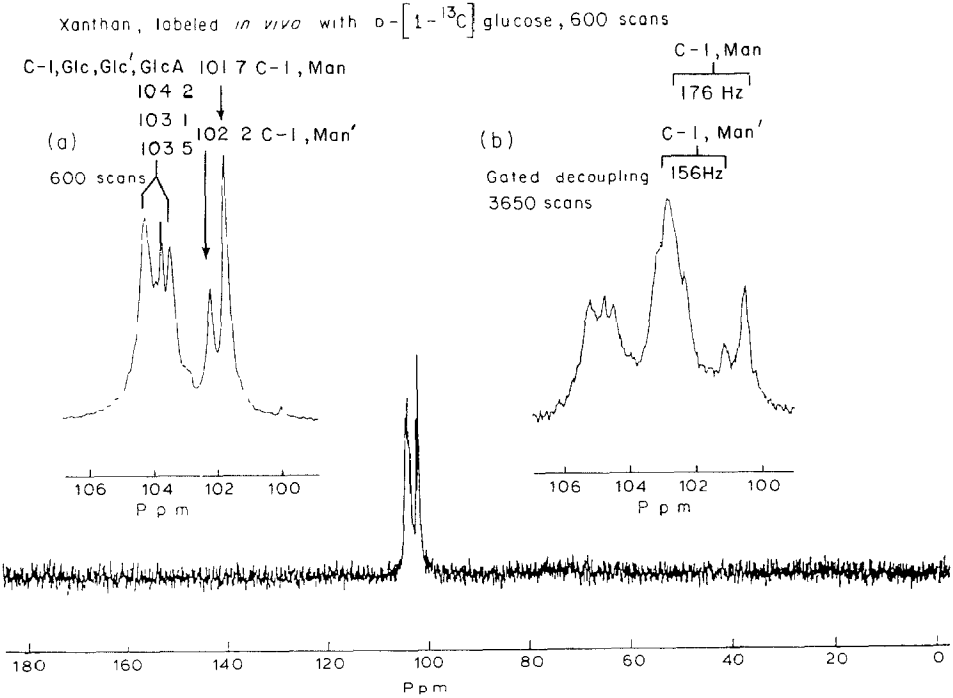

Fig. 3. The ^{13}C-n.m r. spectra of high-pyruvate xanthan labeled by biosynthesis from D-[1-^{13}C]glucose (D_2O, 90°), after accumulation of 600 scans with broad-band decoupling. Inset (a) is a scale expansion, and (b) shows the spectrum with gated decoupling after 3,650 scans (see **1** and Fig. 1 for detailed assignments)

configurations of these residues[12], as well as the assignments of the signals. After the accumulation of 43,000 scans, the anticipated signals of all carbon atoms in the repeating unit appeared in the spectrum.

In the ^{13}C-n.m.r. spectrum of high-pyruvate xanthan biosynthesized from D-[U-^{13}C]glucose (Fig. 4), the C-6 signals, and also those of carbonyl and methyl groups, appear as doublets because of ^{13}C–^{13}C coupling; other signals appear as doublets of doublets or as complex multiplets. The magnitudes of these vicinal ^{13}C–^{13}C couplings served to confirm earlier assignments of the signals in the ^{13}C-n.m.r. spectrum of **1**. Thus, for example, the value of 58.5 Hz is found for the coupling between the methyl and carbonyl carbon atoms of the acetyl group. The methyl and carbonyl carbon atoms of the acetyl group appear, after the accumulation of only 1,700 scans, as doublets (at 21.9 and 175.6 p.p.m., respectively; J = 58.5 Hz) in the spectrum shown in Fig. 4, and thus D-glucose also serves as the precursor for pyruvate and acetyl groups in **1**.

Notwithstanding the minor differences here noted in the ^{13}C-n.m.r. spectra of xanthans produced by various strains of *X. campestris*, the basic ^{13}C signal pattern for these and also for chemically depyruvated and/or deacetylated xanthans[7] affirms the general architecture of **1** as a regular repeating sequence of the pentasaccharide building unit[1] deduced chemically.

Furthermore, ^{13}C-n.m.r. spectroscopy provides an excellent tool for monitoring the biosynthesis of **1**. Previous studies have shown[13] that the *in vitro* biosynthesis of xanthan involves the initial assembly of a lipid diphosphate of the pentasaccharide repeating unit from the precursors UDP-D-Glc, GDP-D-Man, and UDP-D-

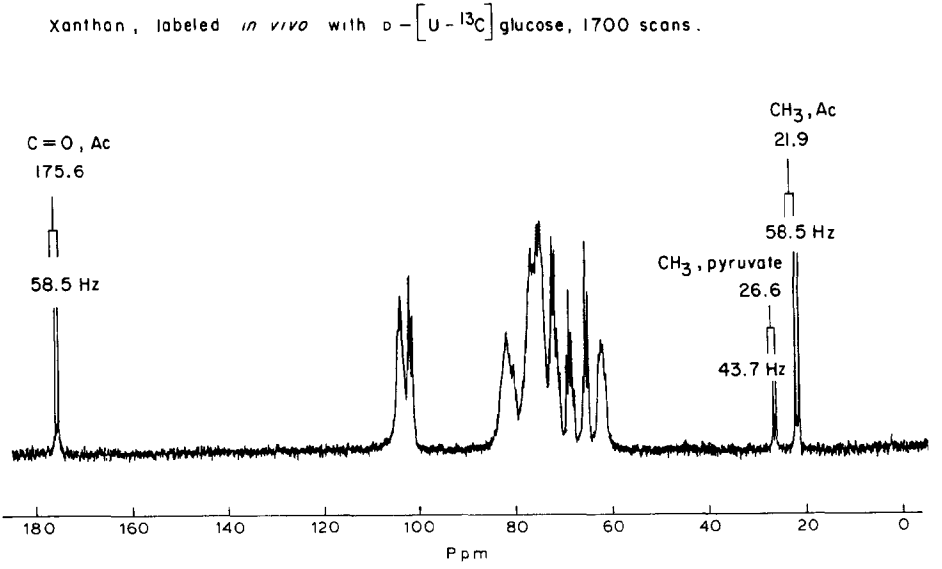

Fig. 4. The ^{13}C-n.m.r. spectrum of high-pyruvate xanthan produced from D-[U-^{13}C]glucose (D_2O, 90°), after accumulation of 1,700 scans.

GlcA, followed by the polymerization of the repeating units. It has also been demonstrated[14] that incorporation of the pyruvic acetal groups occurs at the "lipid intermediate" stage, and that the donor is enolpyruvate phosphate. Similarly acetylation takes place at the "lipid intermediate" stage with acetyl-CoA as the donor, and not at the macromolecular level[15].

These experiments with ^{13}C-enriched xanthan indicate that D-glucose acts *in vivo* as a precursor for all subunits of the pentasaccharide repeating unit of xanthan, including the acetate and pyruvate groups.

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