bands, and it was concluded that for annealed TPBD lamellae a fold of three monomer units is present.

The method used in this work to determine the fold and the crystalline stem lengths in TPBD lamellae should be applicable to any system for which the monomer units in the folds, the units in the stems, and the "junction" points between folds and crystalline stems can be distinguished. Complete nondestructive chemical transformation of the fold without significant penetration of the crystalline portions should yield block copolymers that can be analyzed by ^{13}C NMR or some other sensitive method. This general type of approach using ^{13}C NMR is currently being applied to the study of folding in 1,4-*trans*-polyisoprene fractions; the results of that work will be reported at a later date.

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Registry No. Polybutadiene, 9003-17-2; 3,7-decadiene, 72015-36-2.

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^1H and ^{13}C NMR Investigation of Xanthan Gum

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ABSTRACT: ^1H NMR spectroscopy was used to determine the ratio of pyruvate/acetate groups in a sample of xanthan. In addition, a quantitative measurement of both substituents is given by the integral of the ^1H signals compared to the signal of H-1 α of the mannopyranosic unit. The results allowed us to conclude that in the xanthan investigated each side chain is substituted with one acetate and one pyruvate group. ^1H NMR spectroscopy is also used to investigate the chemical stability of the substituents. It is shown that the thermal stability is lower when the polymer is in the random coil state. The ^{13}C NMR spectra of initial and modified xanthans are given. Partial assignment of the carbon signals is proposed. The spectrum obtained for xanthan free of acetate and pyruvate groups confirms the regular chemical structure corresponding to five monomers per repeating unit. The NMR analysis was favored by partial enzymatic depolymerization of the xanthan to decrease the solution viscosity and the conformational melting temperature of the polymer.

Introduction

Xanthan gum solutions have been widely studied in recent years because of their commerical importance,[1,2] but also to establish the conformation and conditions of conformational transition of xanthan that can be driven by changes in temperature, ionic strength, pH, and polymer concentrations.[3-7] The ordered polymer chain conformation[8-11] and the molecular weight[12-15] remain a matter of discussion. However, some rheological investigations are consistent with a stiff conformation[11,14,16] that leads to a liquid crystalline solution.[17] The primary structure of the xanthan has been shown[18] (Figure 1) to consist of a (1→4)-β-D-glucose chain with a trisaccharide substituent on alternate glucose residues. This side chain is β-D-mannopyranosyl (1→4)-α-D-glucuropyranosyl-(1→2)-β-D-mannopyranoside 6-*O*-acetate. The terminal D-mannose residue of the side chain may have a pyruvic acid residue linked to the 4 and 6 positions. But the acetate and py-

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ruvate contents appear to vary due to the culture conditions and also to the post-fermentation processing.[19-21] Some properties of the xanthan depend on those contents.[19-22] So, the knowledge of the acetate and pyruvate degrees of substitution is important. However, in this way, few papers have dealt with NMR spectroscopy as an analytical technique. This is probably due to the fact that natural xanthan gum in solution is in an ordered and rigid conformation. That rigid conformation produces strong dipolar interactions between proton or carbon nuclei, causing such severe line broadening that the NMR spectra cannot be detected under the usual high-resolution conditions. This phenomenon has been noticed for xanthan gum solutions by Rees et al. (1977) using ^1H NMR[6] spectroscopy, by Hall[23] using ^{13}C NMR spectroscopy, and by Vincendon et al.[24,25] on polygalacturonate calcium salt solutions and κ-carrageenan potassium salt solutions.

This paper deals with NMR results obtained on a xanthan subjected to partial enzymatic hydrolysis in order to decrease the viscosity of the solutions and the melting temperature.[4] Chemically modified xanthan samples have also been prepared to allow the assignment of the carbon NMR signals of the substituents. The pyruvate groups were split under acidic conditions and the acetate groups under basic conditions. Proton NMR was used for the determination of acetate and pyruvate groups. Infrared spectroscopy was also used for acetate determination.

Experimental Section

The sample of original xanthan gum (sample I) was kindly provided by the Institut Français du Pétrole (France). It was purified and partially depolymerized by using a crude cellulase (sample II) as previously discussed.[26] The weight-average molecular weight was 240 000. The decrease in molecular weight decreases the viscosity of solutions but also decreases the conformational melting temperature, T_m, at a given polymer concentration.[4]

As in the literature, pyruvate groups were split in the presence of oxalic acid (10^{-3} M) in 0.1 M NaCl at 95 °C for periods from 0.5 to 3 h.[22] Acetate groups were hydrolyzed in the presence of 2.5×10^{-2} M NaOH in 10^{-1} M NaCl under a nitrogen atmosphere at 20 °C for 3 h.[27] Under these conditions the polymer is in an ordered conformation. The rate of reaction should be greater for the coil form. In those conditions, from sample II, we prepared the xanthan free of acetate (sample III), pyruvate (sample IV), and acetate and pyruvate (sample V) groups. These chemical modifications do not modify the molecular weight but slightly modify the value of T_m determined by optical rotation (Table I) in agreement with previous data from Holzwarth.[22] Optical rotation was investigated on a solution of 1 g/L of xanthan in 0.04 M NaCl by using a Spectropol 1b spectropolarimeter from Fica. ^{13}C NMR spectra were obtained in D_2O solutions having a xanthan concentration of 30 g/L at 90 °C. The measurements were performed on a Brüker WM 250 spectrometer, operating at 62 MHz for ^{13}C. A 12.5-kHz spectral window was used and digitized in a 16K memory to give a digital resolution of 1.55 Hz. Generally, 80 000 scans were necessary. The pulse width was 20 μs, corresponding to a flip angle of 60°. Chemical shifts were referred to external HMDS (hexamethyldisiloxane), which is at +2.00 ppm on the Me_4Si scale.

Proton spectra were obtained at 90 °C in a D_2O solution at a concentration of 5 g/L on samples of xanthan previously exchanged once with D_2O. Twenty scans were accumulated with a pulse interval of 10 s and sweep width of 3000 Hz, digitized in 16K memory.

Infrared spectroscopy was performed on films cast from 0.50% aqueous solutions with a Perkin-Elmer infrared spectrophotometer (Model 598).

The chemical determination of acetate content was carried out by mild alkaline treatments, which remove all of the acetate groups without modification of the polymeric structure.[28] The average experimental conditions are 0.1 g of xanthan dissolved in 10 mL of 0.02 M NaOH. The titration of the remaining NaOH after 24 h at 25 °C under N_2 was performed with 0.01 M HCl. The acetate content is equal to the ratio between the quantity of NaOH used to neutralize the acetic acid formed and the number of pentasaccharide monomeric unit of xanthan. To improve the result it is necessary to take into account the approximate degree of pyruvate and acetate substitution.

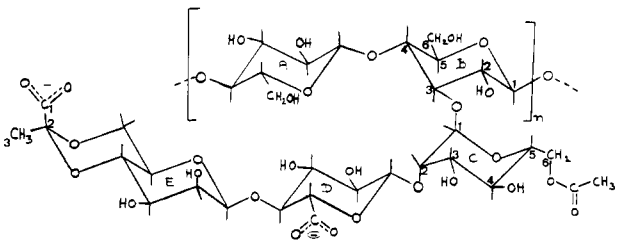

Figure 1. Chemical structure of xanthan.[18]

Table I
Melting Temperatures[a] as a Function of the Chemical Structure of Xanthan

samples	T_m, °C[a]
initial partially hydrolyzed (II)	76
acetate free (III)	65
pyruvate free (IV)	82
pyruvate and acetate free (V)	72.5

[a] Polymer concentration, 1 g/L; solvent, 4×10^{-2} M NaCl.

Results and Discussion

A. ^1H NMR Analysis. The chemical structure of xanthan proposed by Lindberg et al.[18] is shown in Figure 1. The samples of xanthan were characterized by IR and proton NMR. They are also characterized by their melting temperature, T_m, which varies exactly as predicted from electrostatic repulsion and solubility (Table I). The peaks corresponding to C=O at 1720 cm^{-1} in the IR spectrum are directly correlated with the yield of acetate and disappear during saponification.[29] No modification is observed for varying pyruvate content. Characterization of the different samples (I–V) by proton NMR is proposed. This method seems the best way to determine pyruvate and acetate yields by reference to an internal standard. In fact, to characterize a sample it is important to know the ratio of pyruvate/acetate as previously discussed by Smith et al.[19] and also the extent of acetylation of the C-6 OH of the mannopyranose unit C.

The ^1H NMR spectra are given in Figure 2; two peaks at 1.4 and 2.09 ppm are attributed to pyruvate and acetate groups as previously described by Rees[6] and Brant,[11] respectively.

For the first time, quantitative yields are calculated from the integral of ^1H signals corresponding to both substituents by reference to the equatorial anomeric proton, H-1α, of mannopyranosic unit C, which is known as the more deshielded proton located at 5.2 ppm (see Figures 1 and 2). This quantitative analysis can only be performed on the better resolved spectrum of the partially depolymerized samples (Table II). In this way, it is demonstrated that unit C is fully acetylated on C-6 OH within 10% precision. This conclusion is confirmed by chemical analysis in excess NaOH.

On the native xanthan the integrals for acetate and pyruvate protons are equal and of the same order as in the partially depolymerized sample. For the native xanthan, the precision of this ratio is better than 5%. For the xanthan gum investigated, no absolute determination can be obtained from ^1H NMR due to the low resolution of the spectrum; it is necessary to use the chemical determination of the acetate content as a reference. These results allowed

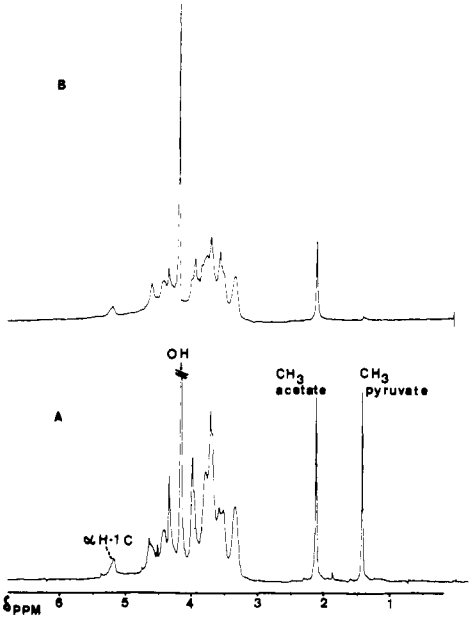

Figure 2. ^1H NMR spectra in D_2O of (A) partially depolymerized xanthan (sample II) and (B) modified xanthan (heated in D_2O under coil form) (polymer concentration 5 mg/mL, $T°$ = 90 °C).

Table II
Yield of Pyruvic Acetal (P) and Acetate (A) Groups as a Function of Heating Conditions[a]

	A, %	P, %
native xanthan (I)	100[b]	100
partially hydrolyzed (II)	100[b]	83
in D_2O under helical form[a]	100	23
in D_2O under coil form[a]	100	6
in H_2O under helical form[a]	100	10
in H_2O under coil form[a]	65	0

[a] Heating for 48 h at 90 °C. [b] Confirmed by chemical analysis.

us to conclude that in the xanthan investigated each side chain is substituted with one acetate group on unit C and one pyruvate on unit E. This assignment is based on the chemical structure proposed in the literature. In fact, ^{13}C NMR cannot distinguish between substituents on units C and E; the position for substitution may be reversed.

The value reported for acetate and pyruvate content is new, as generally the pyruvate yield found in literature is lower (around 50%, i.e., one per two side chains).[19-21] Handling of the polymer (purification, heating, ...) probably influences the amount of pyruvate group due to their low stability; nevertheless, differences between yields given in literature could also be due to differences in strains and culture conditions.

B. Chemical Stability. The observations of modification of ^{13}C NMR spectra depending on experimental conditions led us to discuss the chemical stability of xanthan. For this purpose ^1H NMR spectroscopy was also used. The native polymer (I) and partially enzymatically degraded polymer (II) were characterized by their pyruvate and acetate contents (percent, expressed per side chain with reference to the equatorial anomeric proton of the mannopyranose unit C); then sample II was heated in H_2O or D_2O in the presence or absence of external salt (NaCl) to control the conformation. The results are given in Table II. They allowed us to conclude that the stability is better in the helical conformation (NaCl) and that hydrolysis is less in D_2O than in H_2O. This last result is in agreement with kinetic data concerning hydrolysis of ester under neutral conditions.[30] The results also prove that enzymatic

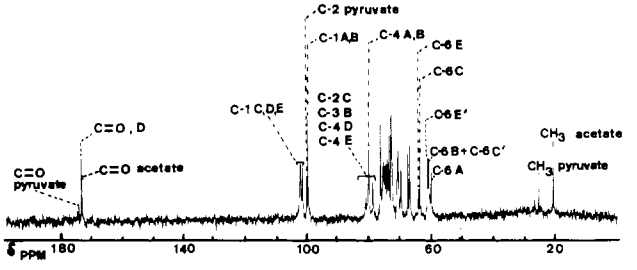

Figure 3. ^{13}C NMR spectrum of initial xanthan (sample II) in D_2O (polymer concentration 30 mg/mL, $T°$ = 90 °C).

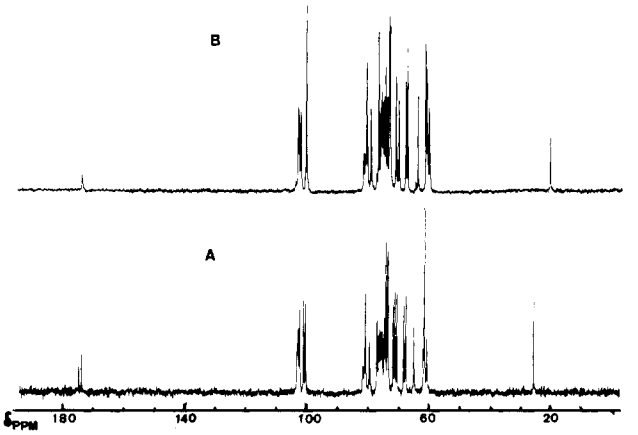

Figure 4. ^{13}C NMR spectra of chemically modified xanthans: (A) acetate free (sample III) and (B) pyruvate free (sample IV) (same experimental conditions).

treatment modifies only slightly the chemical structure of the polymer: comparison of the native (I) and enzymatically treated sample (II) shows that the yield of pyruvate is lowered to 83% (Table II). These results allowed us to conclude that whatever the purification or pretreatment, even in neutral medium, the initial xanthan may be modified.

C. ^{13}C NMR Investigation. The ^{13}C NMR spectra of initial (II) and chemically modified xanthans (III–V) are given in Figures 3–5. Comparison between these spectra allows us to assign the peaks of the carbon atoms due to the two substituents split off. Assignment of signals in Figure 3 is as follows: carbon atoms of the acetate group are located at 20.6 ppm for the methyl and 174.4 ppm for the carbonyl; carbon atoms of the pyruvate are located at 25.3 ppm for the methyl C-3, at 100.9 for the quaternary carbon C-2 and at 175.3 ppm for the carbonyl C-1. These values are in agreement with those previously given;[31] they allow us to confirm that the absolute configuration of the pyruvic acid acetal is *S* with an equatorial methyl group. Other signals are strongly affected by the chemical modifications. These are the skeleton carbon atoms bearing these substituents. For example, the C-6 carbon atom of the $\alpha(1\rightarrow2)$-manopyranosyl (unit C) unit bearing the acetate group is shifted upfield from 64.1 ppm in the spectrum of the initial xanthan (Figure 3) to 61.3 ppm on the spectrum of the deacetylated sample (Figure 4A), this signal (C′) being then superimposed with one of the C-6 hydoxymethyl signals of the main chain (unit A or B). The same phenomenon is observed for the carbon atoms (C-4 and C-6 of the $\beta(1\rightarrow4)$-mannopyranosyl unit bearing the pyruvate group (unit E becoming E′ when free of pyruvate). They are shifted upfield by splitting this group from 64.6 ppm (C-6 E) to 61.6 ppm (C-6 E′) while a modification is observed in the unresolved signal at 81.2 ppm in the spectrum of Figure 3, which becomes a doublet on the spectrum of the depyruvated xanthan (Figure 4B). As

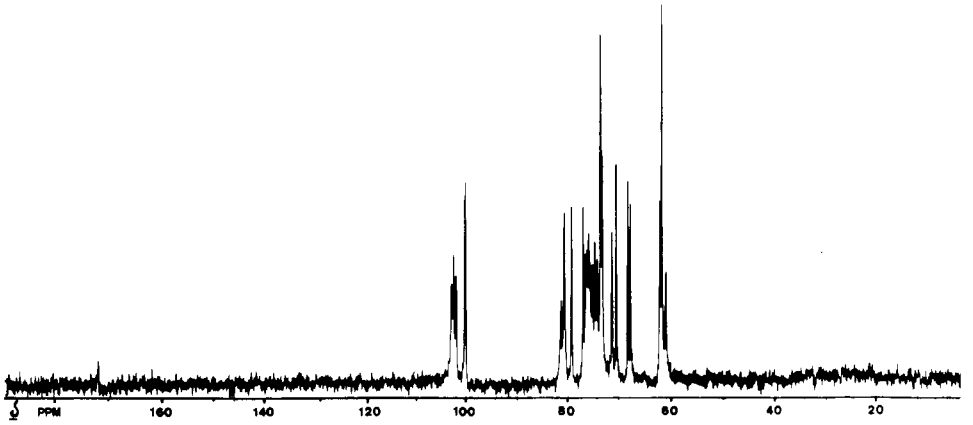

Figure 5. ^{13}C NMR spectra of xanthan free of acetate and pyruvate groups (sample V, same experimental conditions).

previously mentioned, ^{13}C NMR, does not allow us to give the position of the acetate and pyruvate groups (units C and E can reversed).

Figure 5 gives the ^{13}C NMR spectrum of sample V of xanthan. Only one carbonyl signal is visible at 172.4 ppm due to the D-glucuronic unit D. A total of 26 resolved signals can be detected in this spectrum. Three of these correspond to two carbon atoms: the signals at 80.6 ppm due to two C-4 of the (1→4)-β-D-glucopyranosyl units of the main chain (C-4 A and B), at 79.3 corresponding to the two remaining carbon atoms involved in the glycosidic linkages, and at 61.2 ppm for the two C-6 hydroxymethyl groups (C-6 B and C-6 C'). The ^{13}C NMR spectrum of this derivative of xanthan is consistent with a regular copolymer having a constitutive unit of five sugars, for which 30 signals are to be expected. Comparison of the ^{13}C NMR spectrum of the initial xanthan (Figure 3) with that of the derivative (Figure 5) shows that the main differences are due to the disappearance of the signals of the substituents. The other major observable modification is the upfield shift of the signals of the C-6 carbons bearing the acetate (from 64.1 (C-6 C) to 61.3 ppm (C-6 C')) and the pyruvate (from 64.6 (C-6 E) to 61.6 ppm (C-6 E')). The number of signals in the ^{13}C NMR spectrum of the backbone of the initial xanthan (Figure 3) is basically the same as those of the unsubstituted xanthan (Figure 5). Thus native xanthan has to be regarded as a copolymer having a repeating unit of five sugars, with a lack of pyruvate groups on each side chain not exceeding 10%.

Recording of the ^{13}C NMR spectra requires long accumulation times and high temperature (12 h at 90 °C) in order to ensure sufficient chain mobility. However, as previously seen, this may induce pyruvate hydrolysis. Figure 3 thus gives the ^{13}C NMR spectrum of a partially depyruvated xanthan.

Conclusion

This paper deals with the quantitative analysis of xanthan samples for acetate and pyruvate contents from ^{1}H NMR spectroscopy. This analysis is only possible on a partially depolymerized sample to reduce viscosity and increase the resolution of the spectrum. Thus, quantitative determination is obtained with reference to the equatorial anomeric proton of the mannopyranosic unit C. Contrary to the literature[19-21] our results lead to the conclusion that each side chain of the xanthan is substituted with an acetate and a pyruvate group.

On the basis of the ^{1}H NMR spectrum the low chemical stability of both substituents, particularly the pyruvate group, is discussed, and it explains the variability of yields given for different samples in the literature. A preliminary ^{13}C NMR spectrum analysis. using chemically modified samples is given. The spectra confirm that the xanthans free of acetate and pyruvate groups are regular polymers corresponding to five monomers as repeating unit; this conclusion can be extended to the native polymer.

Registry No. Xanthan gum, 11138-66-2.

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