Studies of Microstructure in Native Celluloses Using Solid-State ^{13}C NMR

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ABSTRACT: High-resolution solid-state ^{13}C NMR spectra have been taken on several native cellulosic materials as well as on a regenerated, low-DP cellulose I material. Resonance multiplicities are observed for several carbon positions in the anhydroglucose units. The C4 resonance is particularly notable in that both a broad resonance and a narrow-line multiplet can be distinguished. Arguments are presented for the assignment of the broad resonance feature to surface chains on crystallites as well as chains in three-dimensionally disordered regions. The narrow-line multiplets, which are assigned to chains in the interior of crystallites, show significant variations in relative multiplet intensities, implying that native celluloses exhibit heterogeneous crystal structures. On the basis of these spectra it is proposed that all native celluloses are a mixture of two crystalline modifications, cellulose I_α and I_β. All native celluloses examined represent mixtures of these two structures in various proportions. Similarities noted by other investigators, between *Valonia ventricosa* and *Acetobacter xylinum* celluloses on the one hand and cotton and ramie on the other hand, are reinforced by the NMR results. There is no indication in these samples that each elementary fibril must contain the mixture of the two forms typical of the bulk sample. Therefore, the possibility that native celluloses are biosynthetically tailored composites certainly exists. Finally, although the exact chain conformation of the I_α and I_β conformations cannot be determined by the NMR data, multiplicities can be interpreted as inequivalences within the unit cell. The cellulose I_α, I_β, and II NMR spectra are consistent with ideas, based on other spectroscopic evidence, that cellulose II and cellulose I_β (the major constituent in cotton and ramie) represent different chain conformations and have inequivalent successive glycosidic linkages along each chain.

Introduction

Current knowledge about the crystal structure of native cellulose, so-called cellulose I, is still in flux. Although a crystal structure has been proposed[1,2] on the basis of X-ray and electron diffraction data as well as chain packing energetics, others[3–6] would argue against the claim[1,2] that, aside from differing degrees of disorder, all native celluloses have the same crystal structure.

If different crystal structures exist, it is also not clear what differences exist. The question of parallel vs. antiparallel chains is still discussed.[6] Differences such as the relative rotation of neighboring chains about their respective chain axes and the conformation of the primary alcohol group are admittedly known with less precision.[2] Also questions still remain regarding the exact geometry of the glycosidic linkage,[6–9] although spectroscopic evidence has suggested nonequivalence of successive glycosidic linkages along the chain.[7–9]

One of the promising new experimental methods that can be applied to the study of cellulose crystal structure and morphology is solid-state ^{13}C nuclear magnetic resonance (NMR). Such ^{13}C spectra, acquired with the combined[10–13] techniques of proton–carbon cross polarization (CP),[14] high-power proton decoupling,[15] and magic-angle sample spinning (MAS),[16] not only contain unique signatures corresponding to the various recognized crystal forms of cellulose[17–21] but also distinguish different forms within a grouping such as cellulose I.[17] Some of these latter distinctions arise due to morphological features (e.g., varying degrees of disorder).[17,18,21] Other spectral features are believed to be associated with chains in the interior of crystallites.

It is the intent of this paper to argue for the assignment of certain ^{13}C resonance features to morphologically distinct regions in the cellulose I samples. Following this an interpretation will be given, based on those resonance features associated with the chains in the interior of crystallites, that all native celluloses consist of mixtures of at least two crystalline forms in varying proportions.

Experimental Section

The NMR spectrometer used to obtain these ^{13}C spectra is a Bruker CXP200[22] operating at 4.7 T, which corresponds to frequencies of 50.3 MHz for ^{13}C and 200 MHz for protons. Cross-polarization times were typically 1–2 ms. The respective radio-frequency amplitudes were set for a Hartmann–Hahn match[14] at a rotating-frame precession frequency of 60 kHz, although the amplitudes were slightly mismatched by the magic-angle spinning frequency to provide for greater magnetization-transfer efficiency.[23] The magic-angle spinning frequency was limited to the range 3100–3400 Hz because linear polyethylene (LPE) was usually added to the sample as a chemical shift reference. (The crystalline region of LPE shows a sharp resonance, 0.4 ppm full width at half-height, centered at 33.63[17] ppm with respect to tetramethylsilane.) The weak, first spinning sideband of LPE, which is displaced from its center band by the spinning frequency, is thus limited to a "window" in the cellulose spectra by this choice of spinning frequencies. The LPE also provides a reasonable monitor of the precision of the magic-angle setting, which is known to be critical if good resolution is to be obtained. The rotor material was deuterated poly(methyl methacrylate) (PMMA). This latter material has only weak resonances in the ^{13}C CP–MAS experiment since phase alternation was used to remove certain artifacts.[24] The weak PMMA resonances[25] do not overlap with the cellulose spectrum, although overlap is narrowly avoided (by a few ppm) on the upfield side of the cellulose resonance pattern.

The cellulose samples are described as follows:

(a) *Amorphous Cellulose.* The original material, Whatman CF1 powder, was ball milled for 24 h and kept under dry conditions prior to loading into the rotor.

(b) *Norway Spruce Kraft Pulp.* This material was pulped from a single Norway spruce log to a yield of 47.5% and a Kappa No. of 41.8. It had been stored in a sealed container in a freezer for a few years. This sample, along with the remaining samples, was equilibrated with ambient humidity (normally 40–50% relative humidity but limited to the range 35–60% relative humidity) before being loaded into the rotor.

(c) *Cotton Linters.* This sample, which was washed, bleached, and double cut (chemical cotton), was obtained from Hercules Chemical Co.[26]

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(d) *Hydrocellulose.* This sample was prepared from the cotton linters by the method of Rowland et al.[27] The sample was boiled for 30 min in 2.5 N HCl, neutralized, washed several times in distilled water, and finally dried under vacuum.

(e) *Acetobacter xylinum Cellulose.* Two samples of different origin were investigated. One sample was grown in our laboratory in a static culture on a liquid glucose medium.[28] The pellicle of cellulose and bacteria that floated on the surface after 4 days of culture was boiled for 10 min in 3% NaOH followed by washing in distilled water and drying by manual pressure between absorbent towelling. The second sample, whose spectrum is displayed in this paper, was kindly supplied by Dr. Marc Vincendon of the Centre d'Etudes Nucleaires de Grenoble, France.

(f) *Valonia ventricosa Cellulose.* Algal cellulose was obtained from the cell walls of 10–15 *Valonia ventricosa* cells that had been purified and dried.[29]

(g) *Ramie.* This sample of ramie was obtained from Fischer, Dottikon, Switzerland.[29]

(h) *Low-DP Cellulose I.* This material was made by hydrolyzing Whatman CFl powder, dissolved in concentrated phosphoric acid at room temperature for 6 weeks. The cellulose was regenerated by adding water. After washing and neutralization, the material, in aqueous suspension, was placed in a pressure bomb and heated to 210 °C for 2 h. This low-DP material was recovered after the pressure bomb was cooled. The yield of this material is very small, typically 3–4% of the weight of the starting cellulose. Viscosity measurements indicate the DP is less than 30. The X-ray powder pattern (presented in the Results and Discussion section) and the Raman spectrum are very similar to those of ramie except that the low-DP material gives a more clearly resolved X-ray diffractogram and sharper Raman bands. Electron micrographs reveal no evidence of the original fibrillar structure.

Background

Historically NMR is not a primary probe of molecular structure although solid-state NMR methods have been used extensively either to establish internuclear distances (usually less than 0.3 nm) between specific pairs of magnetic nuclei or to test various proposed structures by means of a moment analysis. These determinations are possible because dipole–dipole interactions depend on geometry and in particular on the inverse cube of the internuclear separation. In contrast, however, the usual ^{13}C CP–MAS spectra contain no dipolar interactions since the strong interactions between the 1%-abundant ^{13}C nuclei and the neighboring protons are eliminated by high-power proton decoupling.[10] Furthermore, in order to achieve high-resolution spectra, the angular dependence of the chemical shift (chemical shift anisotropy) is also eliminated via the MAS.[16] The net result is that spectra obtained under CP–MAS conditions contain *isotropic* chemical shift information in analogy to liquid-state ^{13}C NMR with proton decoupling. The important distinction between solid-state CP–MAS spectra and liquid-state spectra is that in the latter, molecular motion averages over all states so quickly that usually all carbons in a given chemical site look identical. However, in solid-state CP–MAS spectra, because molecules tend to remain fixed, the isotropic chemical shift is often a sensitive probe of environment. The result is that differences in solid-state environment may be detected in the form of chemical shift differences for a given carbon site.

The ^{13}C MAS spectra of most of the materials to be discussed in this paper have been previously reported.[17-21,30,31] Included in these reports were arguments for the assignments of resonances to different sites in the anhydroglucose moiety. These assignments will be reviewed with the aid of the cotton linters spectrum in Figure 1. Starting on the upfield side of the spectrum, the region from 60 to 70 ppm is assigned to C6, the cluster of resonances from 70 to 81 ppm is assigned to C2, C3, and C5, the next region from 81 to 93 ppm belongs to C4, and finally the region from 102 to 108 ppm (and sometimes extending down to 96 ppm) is assigned to C1. Two of these resonance regions, namely those of C6 and C4, consist of sharper resonances partially overlapping broader wings. In the study[17] in which CP–MAS spectra of cotton linters, *Acetobacter xylinum* cellulose, and *Valonia ventricosa* cellulose were compared, it was noted that the broad wings of C4 and C6 (near 85 and 64 ppm, respectively) scaled in their relative intensities along with the crystallite surface areas, assuming that the typical lateral crystallite dimensions in these materials were respectively 3.5 × 3.5 nm,[32] 6 × 6 nm,[33] and 10 × 20 nm.[34] Thus it appeared reasonable to ascribe these wings to crystallite surfaces. Implicitly the need for invoking three-dimensional regions of disorder in addition to the crystal surface disorder seemed less obvious. Further comment on the nature of the disordered regions will be made in the Results and Discussion section.

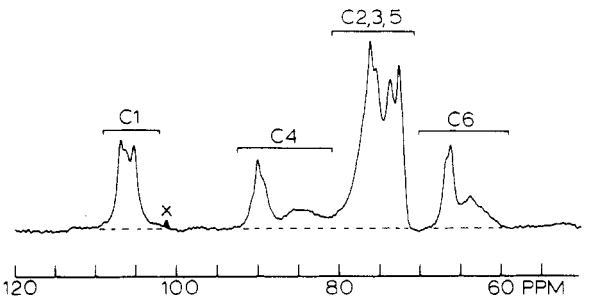

Figure 1. ^{13}C CP–MAS spectrum of cotton linters. The horizontal bars indicate the spectral ranges of the corresponding carbon sites in the anhydroglucose monomer unit of cellulose. The "X" marks the position of the small first spinning sideband of linear polyethylene, which was added as a chemical shift reference. The polyethylene center band (not shown) occurs at 33.63 ppm; the zero of reference for chemical shifts is liquid tetramethylsilane. Note the existence of both broad and narrow resonance features.

Two more points should be made about Figure 1. The first is that the sharper portions of the C4 and C6 resonances (at 90 and 66 ppm) seem to be made up of more than one component line even though the resolution is not good enough to distinguish these components well. The C4 resonance seems to consist of three closely spaced components while C6 contains at least two. These multiplicities have been interpreted as arising from carbons on chains in crystallite interiors and therefore should reflect heterogeneity of positions within the unit cell. On the basis of the C4 multiplicity and component intensities it was argued[17] that each of the four anhydroglucose moieties in the usually proposed unit cell might well be distinct, giving rise, via accidental degeneracy, to an apparent 1:2:1 multiplet intensity profile. The second point is that the C1 resonance region between 102 and 108 ppm also shows multiplicity and evidence of sharp resonance features. The difficulty in the interpretation of the C1 resonance is that the interior crystalline resonances overlap with the C1 resonances arising from the disordered surface as well as possible three-dimensionally disordered regions.

Before discussing the results, we make a few general comments about the interpretation of ^{13}C CP–MAS spectra. First, for a system of rather rigid, hydrogen-bonded molecules like cellulose, in which all carbons have directly bonded protons, the CP–MAS spectra are expected to exhibit quantitative relative intensities; i.e., intensities arising from each of the six carbons are equal. The question whether intensity distortions exist between crystalline and noncrystalline resonances is more difficult. In cotton, for example, relative intensities within the sharp multiplet features are insensitive to changes in proton spin-locking times (prior to cross polarization) up to 30 ms,

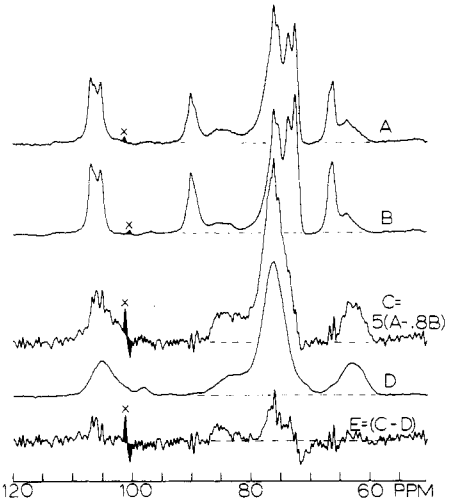

Figure 2. ^{13}C CP–MAS spectra that strongly suggest that three-dimensional regions of disorder exist in cotton linters. Spectra A, B, and D are respectively spectra of cotton linters, hydrocellulose (acid-hydrolyzed cotton linters), and an amorphous, ball-milled Whatman CF1 cellulose. Spectrum C is a linear-combination spectrum of (A) and (B), normalized to the same intensity as spectra A, B, and D. Spectrum C is the ^{13}C resonance profile of those anhydroglucose moieties preferentially lost in the acid hydrolysis. The similarity of spectra C and D, illustrated by the difference spectrum E, strongly indicates that acid hydrolysis attacks three-dimensional regions of disorder as modeled by the amorphous cellulose. See Figure 1 caption for other explanatory details.

although relative intensity changes are observed between the sharp and broad spectral features presumably because the broad features are identified with those regions in closest contact with labile water molecules. Therefore, we make the reasonable assumption that the sharp multiplet features represent true relative intensities. Second, broad, as opposed to sharp, spectral features are associated with either disorder in chain environment or significant molecular mobility with correlation times for motion in the 10^{-3}–10^{-7}-s region.[35] It has been shown previously[30] that dry cellulose displays little of the latter motion, although some enhanced mobility exists for C6 carbons whose resonances lie in the C6 wing. Therefore the broadness of the C4 and C6 wings primarily reflects molecular packing disorder. The spread of resonance frequencies in disordered regions may stem from several causes,[35] among which are conformational differences, slight differences in bond geometries, changes in hydrogen bonding, nonuniformities of neighboring chain environments, and broadening effects due to anisotropy in the bulk magnetic susceptibility.

The corollary to the statement that ^{13}C resonances associated with carbons in disordered regions are broad is that crystalline resonances are narrow. Because they are narrow, small shifts due to magnetic inequivalences within the unit cell are easier to detect. This sort of reasoning led to the interpretation[17] of the multiplicity of the C4 resonance in terms of unit cell inequivalences referred to earlier.

Results and Discussion

Nature of NMR-Detected Disordered Regions. Figure 2 shows the ^{13}C CP–MAS spectrum of cotton linters (A), that of hydrocellulose (B), the linear combination spectrum of (A) and (B), which represents the resonance profile of the anhydroglucose moieties lost by hydrolysis in the preparation of hydrocellulose (C), the CP–MAS spectrum of the amorphous, ball-milled cellulose (D), and the difference spectrum (E) = (C) – (D). Spectra 2A–D are normalized to the same intensity, and the first spinning sidebands of LPE are blackened and marked with an "×". The acid hydrolysis of cotton linters into hydrocellulose is thought to proceed by preferential attack in the disordered regions,[27] with the result that overall crystallinity increases and DP decreases for the hydrocellulose relative to the cotton linters. The spectrum of the amorphous cellulose (Figure 2D) is very similar to that published by other authors.[31] It has lost all evidence of crystalline resonances at C4 and C6; moreover, all sharper features typical of the parent material, Whatman CFI, whose spectrum is very similar to Figure 2A, have vanished. Thus the NMR spectrum qualitatively confirms that this sample has no detectable crystallinity. Obviously, the amorphous material is an example of a three-dimensional network of noncrystalline cellulose. Whether all disordered networks are the same is not entirely clear since one can partially recrystallize amorphous celluloses to either cellulose I or II in water. The crystalline form that returns depends on the extent of ball-milling.[36] Nevertheless, if it is assumed that Figure 2C represents the spectrum associated with all three-dimensionally disordered cellulose regions; than it seems quite clear from the similarity of spectra 2C and 2D that the spectral change in going from cotton linters to hydrocellulose reflects the loss of three-dimensional regions of disorder in the cotton linters. Thus the NMR supports the idea that three-dimensional regions of disorder do exist and that acid hydrolysis preferentially attacks these regions. The idea that three-dimensional regions of disorder exist in the cotton linters also follows from the logical inconsistency presented by the observation that the C4 and C6 wings decrease in spectrum 2B relative to spectrum 2A; this conflicts with the assumption that these wings arise only from carbons in surface chains, since hydrolysis should produce proportionately more, and not less, surface chains. Similar spectral changes have also been observed upon acid hydrolysis in rayon.[21]

The recognition that this cotton sample contains three-dimensionally disordered regions is a feature not explicitly recognized in an earlier study[17] of these materials, although this point has been considered more recently.[19,21] Indeed, the question that naturally follows is whether the spectral wings of C4 and C6 can be associated entirely with three-dimensionally disordered regions as opposed to a significant contribution from chains at crystallite surfaces. For if crystal surfaces do not contribute to the broad wings of C4 and C6, then the interpretation of the multiplicities within the *narrow* portions of these resonances would have to allow for surface chains as possible sources of the multiplicities observed.

Reasonably strong arguments can be made for the inclusion of surface-chain resonances in the wings of C4 and C6. First, the relative intensities of the wings compared with the narrow resonances in cotton linters indicate that about 40% of the chains are in disordered regions.[17] An implied crystallinity of 60% in cotton linters would be somewhat low compared with other measures of crystallinity.[37] Second, if spectrum 2D were subtracted from spectrum 2A until negative intensity were observed, the maximum content of spectrum 2D in 2A is approximately 20%. For example, the region near 81 ppm shows a minimum in the cotton linters and hydrocellulose spectra whereas no minimum exists in the amorphous cellulose spectrum. (Although the C4 wing narrows significantly at high moisture levels in any given cellulose sample,[19] these line shape differences in the 81 ppm region persist even when all of the samples, including the amorphous cellulose, are equilibrated at typical laboratory relative humidities of 35–60%.) This implies that the overall shape

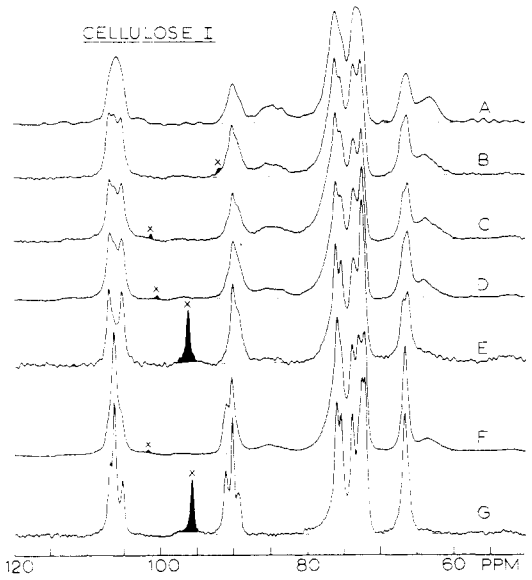

Figure 3. ^{13}C CP–MAS spectra of various cellulose I materials: (A) Norway spruce kraft pulp; (B) ramie; (C) cotton linters; (D) hydrocellulose made from cotton linters; (E) a low-DP regenerated cellulose I; (F) *Acetobacter xylinum* cellulose; (G) *Valonia ventricosa* cellulose. Note the varied fine structure, particularly at C1 and C4. Signal-to-noise varies because some samples were limited in amount. In that case more polyethylene was added so the side band intensity increased. No line broadening or resolution enhancement techniques were applied to these spectra. Approximately 2000–5000 transients were recorded before each spectrum was Fourier transformed. Other details are the same as in Figure 1.

of the C4 wing in the cotton linters and hydrocellulose spectra departs from the C4 resonance shape in the amorphous sample. Thus, a portion, on the order of 50%, of the C4 and C6 wing intensity in the cotton linters spectrum does not have the shape of the amorphous cellulose spectrum and for this reason may be attributed to carbons in chains on the surfaces of elementary fibrils. Thus perhaps 20% of the total observed NMR intensity is associated with cellulose chains at crystallite surfaces. This fraction is certainly reasonable even with a 3.5 × 3.5 nm elementary fibril since it is not clear a priori whether chains in the first chain layer only or in the first unit cell layer (two chains thick) should be considered surface chains. Furthermore, it is also not clear whether aggregation of elementary fibrils (in crystallographic register) occurs with any frequency to minimize surface area.

Two additional arguments to support inclusion of surface-chain resonances in the broad wings of the C4 and C6 resonances will be briefly mentioned. The first is that the surfaces of crystallites must, of necessity, be regions of packing disorder as well as enhanced, albeit limited, mobility. This situation should lead to measurably broader lines[35] for those carbons in surface cellulose chains compared with chains in a crystallite interior. Second, the multiplet intensities of the C4 resonance in the *Valonia* spectrum (see ahead to Figure 3G) are too intense to be ascribed to the surface resonances of crystallites whose lateral dimensions are typically 10 × 20 nm.[34] One would have to postulate that the carbons in *all* of the chains in the surface unit cell layer (two chains thick) contributed to a single sharp resonance having approximately 20% of the total C4 intensity. Although the outer multiplets each have intensities of this order, it is very unlikely that a single sharp resonace should describe chains on different crystal faces and at different distances from the surface.

For all of the above reasons, we believe that it is appropriate to proceed with the interpretation of the NMR

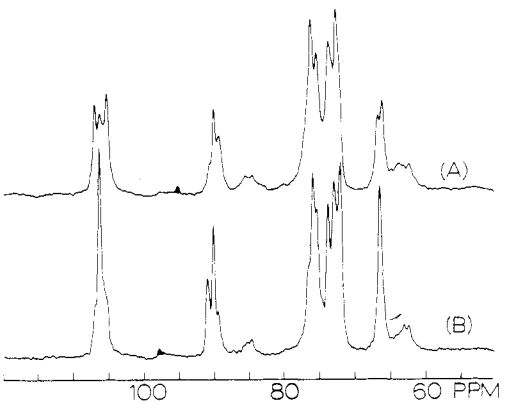

Figure 4. ^{13}C CP–MAS spectra of damp celluloses: (A) cotton linters and (B) *Acetobacter xylinum*. The damp samples contain approximately 40% water by weight. No line broadening has been applied. Cross-polarization times were 1.2 ms for each spectrum.

spectra under the assumption that the sharper resonance features for C4 and C6 arise from the interior of crystallites.

Deductions about Heterogeneity of Crystal Structure. In Figure 3 CP–MAS spectra of several different cellulose I samples are shown. They include Norway spruce kraft pulp (A), ramie (B), cotton linters (C), hydrocellulose (D), low-DP cellulose I (E), *Acetobacter xylinum* cellulose (F), and *Valonia ventricosa* (G). The sidebands associated with LPE are blackened and designated with an "×".

The intensity of the C4 and C6 wings is greatest for the Norway spruce pulp, indicating that this sample has the highest amount of disorder. The lack of sharpness in any spectral feature also reinforces the themes of disorder and small crystallite size in this pulp.

The most interesting feature in Figure 3 is the variability of the narrow patterns, primarily at C4 but also at C6 and C1. These narrow lines asserted to arise from chains in crystallite interiors (except for some overlap with disordered-chain resonances in C1) differ from sample to sample. At C6, for example, spectra 3C and 3E show a partially resolved doublet with a 0.5 ppm splitting while spectra 3B and 3D show definite asymmetry consistent with the presence of an unresolved doublet. Spectra 3F and 3G show only an apparent singlet at C6. For C4, spectra 3A–D have shoulders characteristic of a poorly resolved triplet whose downfield shoulder seems less intense than the upfield shoulder. In spectrum 3E the downfield shoulder of C4 is much more suppressed relative to the upfield shoulder; but then in spectrum 3F, the reverse is true. In the *Valonia* spectrum (spectrum 3G) the upfield and downfield shoulders are again more comparable in intensity except that the downfield shoulder is more intense (in contrast to the upper four spectra). The resonance positions of these three components of C4 stay constant within 0.2 ppm. Finally, at C1 a variety of changes also take place. The doublet, as strongly evidenced in the low-DP spectrum (spectrum 3E), can be recognized prominently in spectra 3B–D. In contrast, a strong, sharp central resonance dominates in the *Acetobacter* (spectrum 3E) and *Valonia* (spectrum 3F) spectra. The broader C1 resonance due to the disordered chains tends to dominate the shape of the Norway spruce kraft pulp spectrum (spectrum 3A).

Although the multiplets in spectra 3A–D are not well resolved, many of these samples have also been run damp, a condition that sharpens the multiplet line widths and reinforces the claims in the foregoing paragraph. Representative spectra of damp cotton linters and *Acetobacter xylinum* are shown in Figure 4 and may be compared with

the corresponding spectra (spectra C and F) of Figure 3.

The central point of this paper is the following: If the multiplicity observed at C4 is truly due to different sites in the interior of crystalline regions, then because the relative multiplet intensities are *not* constant and *not* in the ratios of small whole numbers (as would be true if the same unit cell prevailed throughout all cellulose I crystallites), heterogeneity in the crystal structure of native celluloses must exist. That is, native celluloses appear to be composites of more than one crystalline form.

Before interpreting the multiplicities in terms of crystal heterogeneities, we note that shifts can, in principle, be generated by anisotropic bulk magnetic susceptibility effects related to the geometrical assembly of the fibrils, which have nothing to do with unit cell inequivalences. This possibility can be readily dismissed on the basis of two arguments. First, on the basis of the assumption that the magnetic susceptibility anisotropy of sucrose is a good model for the susceptibility anisotropy of cellulose, maximum predicted susceptibility shifts in cellulose are much smaller than those observed.[17] Second, susceptibility shifts imply that each crystalline resonance shows the same pattern of multiplicity and this is clearly not true. Thus a very strong case can be made for ascribing the multiplicities observed in the C4 region between 89 and 91 ppm to site heterogeneity *within* the crystalline regions. In the following discussion focus will be maintained on the C4 resonance; however, the C6 and the C1 resonance profiles are also important to the arguments.

It was noted earlier that an inspection of the C4 narrow-line resonance region in the suite of samples of Figure 3 revealed that the relative ratios of the three components were not constant and were not obviously in the ratios of small whole numbers as one would expect if there was one crystal structure for all the materials and if the splitting reflected unit cell inequivalences. Significant differences were also noted in the narrow-line C6 and C1 resonance regions. If these features cannot be associated with one crystal structure, then how many are there? If each of the multiplets were associated with a different crystal modification, then there might be as many as three modifications required to produce the C1 and C4 patterns. Aside from how reasonable this assumption is, the spectral intensities are not consistent with three components, each of which gives rise to single resonances in the C1, C4, and C6 regions. This can be seen easily by comparing the C4 and C1 profiles of the low-DP (spectrum 3E) and *Acetobacter* (spectrum 3F) spectra. The most intense C4 peak in each spectrum occupies the same position at 90.1 ppm; however, there is no single most intense peak in the low-DP spectrum in the C1 region; moreover there is a strong minimum in the central region of this resonance. In contrast, in the *Acetobacter* spectrum the central peak of C1 is most intense. Thus, there is not a one-to-one correspondence between multiplet intensities at C1 and C4. Therefore, neither a unique crystal structure nor three simultaneous crystal modifications, each having a single resonance at C1 and C4, accounts for the multiplet intensities of C4 and C1.

A model featuring two independent crystal forms of native cellulose was found to be in satisfactory agreement with the spectra of all of these samples. The approximate component spectra of the two forms are illustrated in Figure 5 together with the spectrum of a high-crystallinity cellulose II sample. The spectrum of cellulose II has been included in order to distinguish the heterogeneity of crystalline forms here proposed for native celluloses from the well-recognized polymorphy of cellulose.

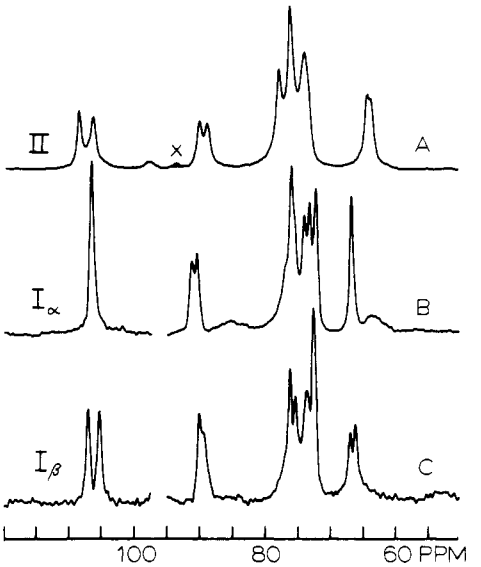

Figure 5. Comparison of the ^{13}C CP–MAS spectrum (A) of a low-DP cellulose II sample and the spectra (B and C) corresponding respectively to the two proposed crystalline forms of cellulose I, namely I_α and I_β. Spectra B and C were obtained by taking linear combinations of the low-DP and *Acetobacter* cellulose spectra (Figure 3E,F respectively). Discontinuities in spectra B and C occur where the polyethylene sidebands would have appeared. The I_α spectrum still contains a significant amount of non-I_α resonances as shown by the visible C4 and C6 upfield wings. Multiplicities of the C1, C4, and C6 narrower resonances ought to indicate unit cell inequivalences. Chemical shifts for the sharp resonance features are listed in Table I. See Figure 1 caption for other explanatory details.

Spectra 5B and 5C were obtained by taking appropriate linear combinations of the low-DP (spectrum 3E) and *Acetobacter* (spectrum 3F) spectra. These latter two spectra were judged to be most extreme in the variability of the C1, C4, and C6 resonance patterns; moreover their component line widths were very close, and their noncrystalline fractions were not large. Spectra (not shown) similar to spectra 5B and 5C and having identical narrow-line positions can be generated by using the cotton linters and *Acetobacter* spectra of Figure 4. The I_β spectrum generated in this manner is, however, inferior to spectrum 5C in that the stronger noncrystalline resonances in the former spectrum make the true intensity ratios of the crystalline peaks a bit less obvious, particularly at C1.

If we designate the two crystalline forms to be I_α and I_β, corresponding approximately to spectra 5B and 5C, then the following ranking of the materials investigated here can be given in decreasing order of I_α crystalline content (relative to total crystal content): *Acetobacter* $\approx$ *Valonia* > cotton linters $\approx$ ramie $\approx$ hydrocellulose $\approx$ Norway spruce kraft pulp > low-DP cellulose.

Spectra 5B and 5C are not intended to be the pure component spectra in every respect, primarily because we do not know the shape of the resonance associated with crystal surfaces. Spectrum 4B has a substantial component of intensity in the C4 and C6 wings, much of which presumably arises from crystalline surface resonances. Nevertheless, the narrow-component features in the C1, C4, and C6 regions are expected to be correct indicators of the unit cell magnetic inequivalences. Thus the I_α crystalline form, which is dominant in *Acetobacter* and *Valonia*, shows singlets at C1 and C6 vs. a closely spaced doublet at C4. On the other hand, the I_β crystalline form, which dominates in the cotton, ramie, and spruce pulp celluloses, has doublets at C1, C4, and C6. In spectra 5B and 5C the

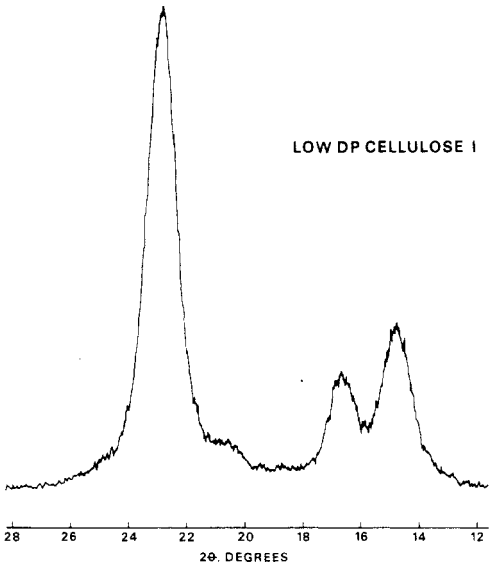

Figure 6. Wide-angle X-ray diffractogram of the low-DP cellulose sample. The diffractogram identifies this material as cellulose I.

C4 doublet components, particularly in spectrum 5C, may have unequal intensities. There is some indication, however, that line widths may not be equal so that these intensities may indeed be equal. In any case if the two crystalline forms exist as proposed, the criterion that component intensities should occur in the ratios of small whole numbers should prevail. For the narrow portions of the resonances of C1, C4, and C6, this seems true within experimental uncertainty.

This view of multiple crystal forms in native celluloses is new because it implies that none of the native celluloses studied represents a pure crystalline form. At the same time it substantiates earlier claims that *Acetobacter* and *Valonia* celluloses have similar crystal composition which is distinct from the "other" forms of native cellulose such as cotton and ramie. Estimates of 65% I_α content for *Acetobacter* and 25% I_α content for cotton linters were made, normalized by the total crystalline content. More accurate numbers were not sought, principally because of the uncertainty in the exact shape of the noncrystalline signal and because of the varied line widths. In this connection it is also interesting to note that the two samples of *Acetobacter* showed a minor variation in their I_α content. Spectra 3F and 4B correspond to the sample with the highest I_α content.

The role of the low-DP cellulose was critical in the development of our interpretation. It is recognized that this is the most unusual of our samples and the most difficult to prepare. Because of its unusual character some additional details about its characterization are in order. The X-ray diffractogram is given in Figure 6; it leaves little question that this low-DP sample is indeed a cellulose I. Since in the past some question has arisen concerning the possibility that this sample represents an insoluble residue that had survived the original dissolution in phosphoric acid, the sample was subjected to all of the tests previously described when the first regeneration of cellulose I was accomplished in our laboratory.[38] In addition to all of these tests, a sample similarly prepared was recently redissolved in phosphoric acid and regenerated at room temperature to give a cellulose II; such a result is inconsistent with the suggestion that these samples are insoluble residues that had previously resisted dissolution after 6 weeks in phosphoric acid.

It should be made clear what role this material plays in the arguments, particulary since it is not a native cellulose. The sharp resonance features of the low-DP spectrum (spectrum 3E) agree closely with a subset of the sharp resonance features of the other spectra of Figure 3 although the agreement is most obvious in comparison with the cotton, ramie, and hydrocellulose samples. This agreement and the close similarity of both the Raman spectra and the X-ray powder patterns of ramie and the low-DP cellulose are taken as proof that this material is closely related to native celluloses. More than that, however, the mere existence of a cellulose material yielding a spectrum like that of Figure 3E suggests that one can come close to isolating a pure component, in this case cellulose I_β. Proving that the spectral features can be preserved in a material whose content of cellulose I_β is high suggests that the two crystal forms need not be intimate mixtures. Thus, there is no reason to believe that each elementary fibril itself contains the mixture of the two crystal forms typical to the bulk material. One might speculate that the crystalline form present may be a function of the construction of each microfibril or a function of the various layerings of microfibrils in making up the fibers.

It is also hoped that the idea of two crystal structures in native celluloses may prove useful in resolving the questions outstanding[6] in establishing crystal structures in cellulose I. At this point there seems to be little evidence for more than two inequivalent sites for the anhydroglucose moieties within either unit cell. Why C4, and not C1 and C6, should show inequivalence in cellulose I_α is not known although it may suggest inequivalence at the glycosidic linkage[8,9] or possibly inequivalence of corner vs. center chains[2] in the two-chain-per-unit-cell structure. The existence of more than three sharp resonances in the C2,3,5 region indicates other sites of inequivalence besides C4.

It is not possible on the basis of the NMR spectra to suggest particular structural differences between the two crystal forms. Differences as subtle as the conformation of the primary alcohol group may play a role, but if this were true, one might expect more than a 0.5 ppm range of chemical shifts for the sharp components of C6.[39] In Table I the chemical shifts of the sharp components of the two crystalline forms of cellulose I are listed along with the corresponding peak positions for cellulose II. These

Table I
Comparison of the ^{13}C Chemical Shifts[a] of the Sharp Spectral Features in Cellulose I and Cellulose II

cellulose I_α	66.5	72.0 73.0 73.8 75.8	90.2[c] 91.0	106.3
cellulose I_β	66.1[c] 66.8	72.5 73.7 75.3 76.1	89.3[c] 90.1	105.2[c] 107.0
cellulose II[b]	63.6[c] 64.2	73.8 75.9 77.8	88.7[c] 89.9	106.0[c] 108.3

[a] Shifts are given in ppm and are measured with linear polyethylene as an internal chemical shift standard. Its crystalline resonance position is taken to be 33.63 ppm (see ref 25) with respect to tetramethylsilane at 0.0 ppm. Deconvolution of lines so as to establish resonance positions more accurately was not attempted. Errors in chemical shifts of well-resolved lines are of the order of 0.15 ppm, assuming that the LPE reference shift value is precise. Errors in positions of strongly overlapping lines are correspondingly greater. [b] These shifts were measured on a low-DP cellulose II sample that gave sharp lines. These shifts agree reasonably well with those reported in ref 20. [c] Doublets with approximately equal intensities.

latter shifts were measured on a low-DP cellulose II sample that gave sharp lines; these shifts are also in reasonable agreement with other published chemical shift data[20] on cellulose II. The shifts of Table I allow one to compare resonance differences between the cellulose I modifications with changes in going from cellulose I to cellulose II. In a very qualitative way the shifts in going from cellulose I to cellulose II are slightly larger than the spectral differences between the cellulose I forms, particularly for the C2,3,5 cluster and the C6 peaks.

It should be emphasized that our proposal that native celluloses are composites of I_α and I_β is distinct from, though complementary to, the structural model[8,9] proposed by one of us on the basis of spectroscopic studies. To place the present work in perspective in relation to that model, it should be noted that in that model, the crystalline chain conformation in cellulose I was considered to be different from that of cellulose II. Moreover, successive anhydroglucose units along a chain in both polymorphs occupied nonequivalent positions. In that model, each crystalline polymorph was thought to be characterized by a single chain conformation, and these were identified as k_I and k_{II}. Raman spectra corresponding to these components were also given. The correspondence between the k_I and k_{II} designation and the NMR spectra of Figure 4 would identify k_{II} with spectrum 5A and k_I with spectrum 5C although in the latter case it is now recognized that the sample on which the k_I Raman spectrum was taken did not represent a pure structure. Nevertheless, in recognition of the fact that this latter material contained I_β as its major constituent, the following statement can certainly be made: The NMR spectra 5A and 5C are consistent with, but do not in themselves prove, the two assumptions of the model, namely, that crystalline cellulose I is conformationally different from cellulose II and nonequivalent successive glycosidic linkages exist in both structures.

Finally, the level of our analysis of the fine structure of the NMR spectra is not sufficiently detailed to claim that our interpretation of *two* cellulose crystal structures, I_α and I_β, represents the only possibility.[40] Other, more complicated decompositions may provide better fits; however, the interpretation given herein is sufficient to explain the spectra of all of the native cellulose samples we have studied.

Conclusion

Various samples of cellulose I have been examined by high-resolution solid-state ^{13}C NMR methods. The spectra show substantial variation between different native forms of cellulose. Some of the differences are due to varying degrees of disorder in the samples. In particular, broad and rather intense resonance wings associated with the C4 and C6 carbons in the anhydroglucose moieties in a material like cotton linters are interpreted as arising both from chains in three-dimensional regions of disorder and from crystallite surfaces. Other narrow spectral features are identified with crystallite interiors. Certain assignable resonances show multiplicities for given carbons, notably for C4, C6, and C1. On the basis of the variability of the intensity patterns in going from sample to sample it is argued that two crystalline forms of cellulose I exist. These two forms are designated cellulose I_α and cellulose I_β. The crystalline component of every native cellulose studied represents a corresponding mixture of these two forms as opposed to a pure form. The nomenclature of I_α and I_β is used in distinction from earlier IR work[3b] that classified *Valonia* and *Acetobacter* celluloses as I_A and all the other native celluloses, including cotton and ramie, as I_B. The NMR results reinforce the similarity of crystal composition within these groups but go on to suggest that the I_A form is only dominated by I_α while the major constitutent of the I_B form is I_β. It is also noted that there is no particular ratio in which these two crystalline forms must exist so that each elementary fibril need not be a typical mixture of the two forms. This raises the possibility that through the biosynthetic control of the crystal structure a tailored composite may be formed with, perhaps, tailored mechanical properties.

The precise structural differences between the two crystalline forms is an area of speculation. Chemical shift differences between these forms are compared with changes in going from cellulose I to cellulose II. The conversion to cellulose II seems to involve slightly larger chemical shift changes, particularly for the C2,3,5 resonance cluster as well as for C6.

It is hoped that this work will help to resolve some of the questions outstanding concerning the crystal structure of native celluloses.

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(40) **Note Added in Proof**: After examining more native celluloses and after trying harder to isolate experimentally those resonances associated with the crystalline interior, we substantiated the theme of multiple crystalline forms. However, the exact component spectra may depart slightly from those given. In particular, the spectrum of I_β seems to indicate more complexity than a doublet at C1 and C4, thereby suggesting that the number of magnetically inequivalent sites in that unit cell exceeds two. The general features of the I_α spectrum remain the same.

Conformational Characterization of Solid Polypeptides by ^{13}C NMR Recorded by the Cross Polarization–Magic Angle Spinning Method: Conformation-Dependent ^{13}C Chemical Shifts of Oligo- and Poly(γ-benzyl L-glutamates) and Sequential Copolymers of γ-Benzyl and γ-Methyl L-Glutamates and Qualitative Evaluation of Side-Chain Orientation

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ABSTRACT: We have recorded ^{13}C NMR spectra of oligo(γ-benzyl L-glutamates), poly(γ-benzyl L-glutamates) ([Glu(OBzl)]$_n$), and copolymers of γ-benzyl and γ-methyl L-glutamates in the solid state by the cross polarization–magic angle spinning method, in order to elucidate conformational features as viewed from the conformation-dependent ^{13}C chemical shifts. It is found that the relative ^{13}C chemical shifts of the α-helix with respect to those of the β-sheet form are 5.2, −3.4, and 4.6 ppm for the C_α, C_β, and amide carbonyl carbons, respectively, for [Glu(OBzl)]$_n$. For the sequential copolymers consisting of γ-benzyl L-glutamate (B) and γ-methyl L-glutamate (M), we found that the α-helix conformation is achieved when the proportion of B is over 50% regardless of their sequences. On the contrary, the β-sheet form is obtained when M is dominant. There appears no significant displacement of the ^{13}C chemical shifts for the sequential copolymers from those of [Glu(OBzl)]$_n$. Observation of differential line broadening among carbons in the backbone and side-chain moiety was found to be an excellent means to examine the orientation of the side chains. In particular, we found that considerably disordered side chains, as viewed from the selective line broadening, are characteristic of the β-sheet conformation for both the homopolypeptides and the sequential copolymers.

Introduction

It is now obvious from our previous studies[1-4] as well as others[5-10] that ^{13}C chemical shifts of solid polypeptides, as determined by the cross polarization–magic angle spinning (CP–MAS) technique, exhibit substantial extents of conformation-dependent ^{13}C chemical shifts (up to 7 ppm) depending on particular conformations such as α-helix and β-sheet forms. It appears that such conformation-dependent ^{13}C shifts mainly arise from the local conformation of individual amino acid residues characterized by the dihedral angles (Φ and Ψ) as well as the manner of intra- or intermolecular hydrogen bonding, as suggested by the previous theoretical calculation of the ^{13}C chemical shifts utilizing the FPT–INDO molecular orbital method.[11] Tonelli previously noted that the ^{13}C NMR chemical shifts of polypeptides should be and are sensitive to their local conformations.[12] Peptide residue sequence effects on the ^{13}C NMR chemical shifts, when they occur, are caused by the dependence of the local conformation on the sequence, as, for example, in those residues that precede proline in the polypeptide sequence. Such dependence of the ^{13}C chemical shifts upon the local conformation is one of the major advantages of ^{13}C NMR as a tool for conformational characterization of complicated peptides and proteins,[3,7,13,14] although prior knowledge with regard to how and to what extent the ^{13}C chemical shifts are displaced by particular conformations is required. For this purpose, it is essential to utilize the ^{13}C chemical shifts of a variety of polypeptides or peptides with known dihedral angles as determined by X-ray diffraction for a source of reference data.

As a continuation of our effort to relate the conformation-dependent ^{13}C chemical shifts of polypeptides to their particular conformation in the solid state, we aimed, in this paper, to analyze ^{13}C NMR spectra of monodisperse molecular weight oligo(γ-benzyl L-glutamates), poly(γ-benzyl L-glutamates) ([Glu(OBzl)]$_n$ or (B)$_n$), and sequential copolymers of γ-benzyl and γ-methyl L-glutamates. α-Helical

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