Carbohydrate Research, 164 (1987) 85–95
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

INVESTIGATIONS OF LIGNOCELLULOSIC MATERIALS BY THE CARBON-13 N.M.R. C.P.-M.A.S. METHOD*

HEINZ STERK,
Institute of Organic Chemistry, University of Graz, Heinrichstrasse 28, A-8010 Graz (Austria)

WOLFGANG SATTLER, AND HERMANN ESTERBAUER†
Institute of Biochemistry, University of Graz, Schubertstrasse 1, A-8010 Graz (Austria)

(Received October 14th, 1986; accepted for publication in revised form, January 20th, 1987)

ABSTRACT

High resolution solid state ^{13}C-n.m.r. spectra have been taken on several celluloses as well as on untreated and pretreated lignocellulosic materials (poplar, birch, beech, pine, spruce, wheat straw). The results indicate that milling and steaming have dramatic effects on the cellulose structure. Moreover it was found that sample preparation, particularly moisture content, markedly affects the features of the ^{13}C spectra.

INTRODUCTION

The availability of c.p.-m.a.s. probe units has led in the last few years to an increasing number of investigations on the solid state structure of natural products such as coal[1,2], wood[3,4], lignin[5,6] and different types of cellulose[7,8] and cellulose derivatives[9]. These investigations have broadened knowledge about molecular structure in the solid state, and have helped characterize the unit cells in ordered systems with crystal-like properties. The pioneering work of Freudenberg[10] has shown that celluloses from different sources are macromolecules with cellobiose as repeating unit. The arrangement of the cellulose chains in supermolecular structures was extensively studied by X-ray diffraction, infra-red spectroscopy and electron microscopy[11,12]. It is now assumed that cellulose chains are arranged longitudinally in elementary fibrils with an average diameter of about 3.5 nm, and that within the fibrils ordered, crystalline regions and less ordered, amorphous regions exist. Intra- and inter-molecular hydrogen bonds are responsible for the organisation of the cellulose chains in the elementary fibrils as well as for the formation of larger fibres. So far nearly all studies were performed on pure celluloses such as ramie, cotton, or pulp. In wood, cellulose is intimately linked with hemi-

*We dedicate this paper to Prof. Dr. K. Freudenberg on the centenary of his birth.
†To whom correspondence should be addressed.

0008-6215/87/$ 03.50

celluloses and lignin, and its isolation requires destructive methods that probably alter the hydrogen bonds and other structural parameters. So the findings on isolated celluloses do not necessarily give the true picture of cellulose in the native complex.

In our ongoing work on the enzymatic hydrolysis of cellulose and lignocellulosics[13–17] we have used the ^{13}C-n.m.r. c.p.-m.a.s. technique to study the effect of milling and steaming on the spectrum of cellulose in wood and straw. It is well established that the cellulose in untreated wood is attacked very slowly by cellulase enzymes, whereas proper pretreatments make the wood cellulose easily degradable by the enzymes. The crystallinity of cellulose, its shielding by the lignin and hemicellulose, and limited pore size, all resulting in a lack of accessible surface area, are considered as the major hindrances to the enzymatic degradation of cellulose in untreated wood[15]. The relative importance of these three factors is largely unknown, however, and difficult to define since the change of one parameter is always combined with alterations of one or two of the other parameters. The goal of our investigation was to show that solid-state n.m.r. could give valuable indications about the morphology and structure of the cellulose chains in pretreated wood, and thus contribute to our understanding of its enzymatic hydrolysability.

RESULTS AND DISCUSSION

Three different hardwoods (poplar, birch, beech), three softwoods (*Pinus nigra, Pinus radiata*, spruce), wheat straw, and a beechwood hemicellulose were used in this study. The main constituents of these samples, as determined by a previously described method[17], are listed in Table I. In all wood samples as well as in straw by far the most abundant component (38–50%) is glucan, which is of course mainly cellulose. From other analyses it is known that only softwoods contain minor

TABLE I

PERCENTAGE COMPOSITION OF THE WOOD SAMPLES, STRAW, AND HEMICELLULOSE

Component	*Poplar* (Populus tremuloides)	*Birch* (betula verucosa)	*Beech* (Fagus sylvatica)	*Pine* (Pinus nigra)	*Pine* (Pinus radiata)	*Spruce* (Picea abies)	*Wheat straw*	*Beech hemicellulose*
Glucan	43.0	38.2	50.4	47.1	41.4	41.9	40.9	9.1
Xylan	14.8	18.5	23.7	3.2	5.5	6.1	19.1	76.3
Mannan	0.2	1.1	0.0	9.1	8.4	14.3	0.2	9.7
Galactan	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Arabinan	0.0	0.0	0.0	0.0	0.2	1.2	2.5	0.0
Acetyl	3.4	7.0	5.3	—	2.0	—	5.0	0.0
Lignin	16.7	22.8	26.4	28.3	25.6	27.1	21.4	
Extractives	5.1	4.9	6.9	6.7	5.5	9.6	13.4	
Ash	0.4	0.6	1.1	0.3	0.1	0.7	2.3	

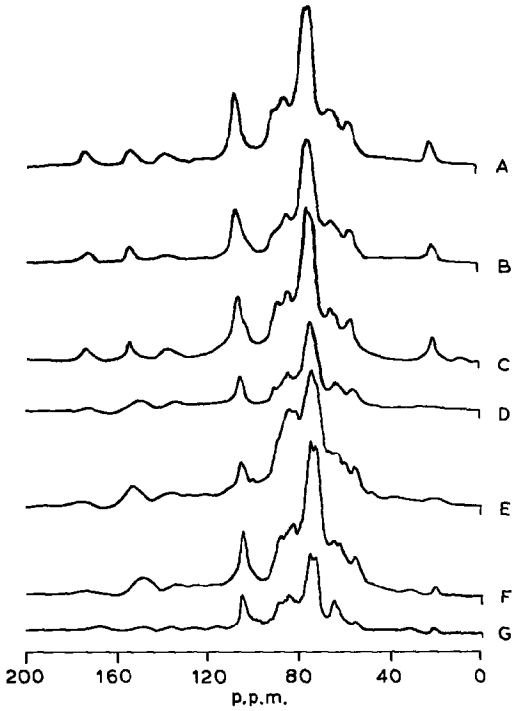

Fig. 1. ^{13}C-N.m.r. c.p.-m.a.s. spectra of various woods, and wheat straw. A, poplar, air-dried; B, birch, freeze-dried; C, beech, oven-dried; D, *Pinus nigra*, freeze-dried; E, spruce, freeze-dried; F, *Pinus radiata*, oven-dried; G, wheat straw, oven-dried. All samples were milled to a particle size of 0.25 mm. The distorted baseline in the vicinity of the signals was caused by the weighting function used.

amounts of glucose in their hemicellulose fractions as glucomannans and galactoglucomannans. The beechwood hemicellulose consisted mainly of xylan.

For recording the solid-state spectra the samples were either air dried, freeze dried, or dried at 100° for 12 h, and then milled to a particle size of 0.25 mm. The ^{13}C c.p.-m.a.s. spectra of the wood samples and wheat straw are shown in Fig. 1.

It is evident that the spectra are dominated by the carbohydrate signals resulting from cellulose. Following the interpretation of Atalla *et al.*[18] and Lindberg *et al.*[19] the cellulose signals can be assigned to C-1 at 105 p.p.m., C-4 at 83–89 p.p.m., and C-6 at 63–66 p.p.m. The largest signal in all samples is at 73 to 78 p.p.m. It is due to carbons 2, 3, and 5 and is partly resolved in the *Pinus radiata* and wheat straw spectra and clearly resolved in the spectra of pure celluloses (cotton, Avicel, Sigmacel) (Fig. 2).

Other characteristic signals from the lignocellulosic samples (Fig. 1) are at 56, 130–138, and 156–159 p.p.m., resulting from the methoxyl groups and the aromatic rings of lignin. The other structural elements of lignin, *e.g.*, CHOH, CH_2OH, and C–O–C groups, are hidden by the carbohydrate signals.

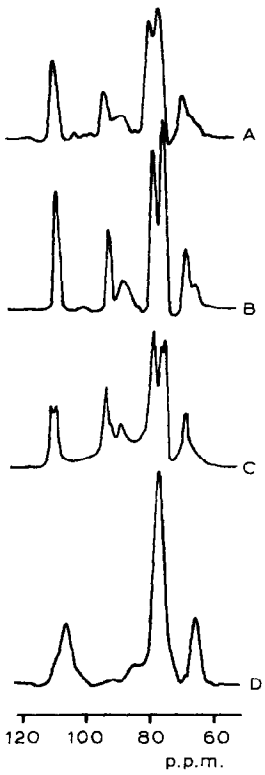

Fig. 2. ^{13}C-N.m.r. c.p.-m.a.s. spectra of various celluloses, and hemicellulose. A, Sigmacell; B, Avicel; C, cotton linters; D, hemicellulose.

Hemicelluloses, the third major constituent of lignocellulosics, are a very heterogenous group of polymeric carbohydrates. In hardwoods *O*-acetyl-4-*O*-methylglucuronoxylans comprise 80–90% of the hemicellulose fraction, and in these samples the acetyl group gives rise to the signals at 21 and 180 p.p.m. (Fig. 1A, B, C). The carbohydrate signals of the xylans should be at δ 103 (C-1), 84 (C-4), 72–75 (C-2, C-3), and 65 (C-5), as can be seen from the spectra of beechwood hemicellulose (Fig. 2D). In the c.p.-m.a.s. spectra of wood, however, these lines are hidden by the strong cellulose signals. It should be noted that the beechwood hemicellulose was prepared from beech pulp by extraction with 20% sodium hydroxide, a procedure which removes all the acetyl and most of the glucuronic acid residues. Wheat straw, which also contains acetylated xylans, gives only very weak acetyl signals. A possible explanation for this could be a strong line broadening that makes the signals indistinguishable from the baseline. In softwood, 60–70% of the hemicelluloses are glucomannans, and since the signals of

this polysaccharide overlap with the cellulose signals the spectra of softwoods do not show peaks that can be clearly attributed to hemicelluloses.

The resonance splitting of the C-1 signal in cotton linters and regenerated cellulose I has been ascribed[8,18] to two magnetically nonequivalent environments of the C-1 carbon atoms. Also in our measurements of cotton linters C-1 clearly gave two signals (Fig. 2C). Avicel and Sigmacell and more than 10 other pure celluloses (spectra not shown) did not show a splitting of the C-1 signal and in none of the wood samples could such a splitting be observed. This lack of splitting is due to the amorphous part of cellulose, which gives a single, broad C-1 signal that obscures the double peak of the crystalline cellulose[20].

For pure celluloses the C-4 signal at 89 p.p.m. was ascribed to the internal crystalline regions and the 83 p.p.m. signal to the amorphous regions and crystal surfaces of cellulose[7]. In the wood samples the C-4 signal is much less structured as compared to that of pure cellulose and in most cases the 83 p.p.m. signal is predominant. However it is not feasible to determine the degree of crystallinity of

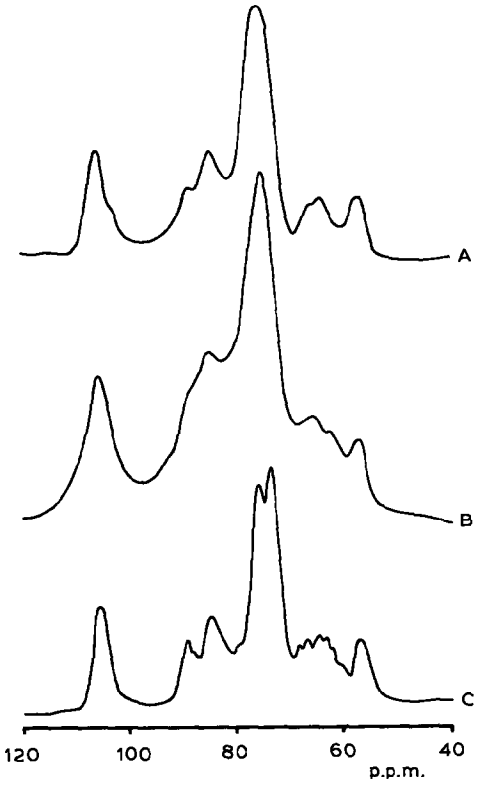

Fig. 3. Effect of moisture content on the ^{13}C-n.m.r. c.p.-m.a.s. spectrum of birchwood. A, oven-dried; B, freeze-dried; C, oven-dried and then brought to a moisture content of 20%. All samples were milled to a particle size of 0.25 mm.

cellulose in wood on the basis of the peak areas of the 89 and 83 p.p.m. signals because C-4 of hemicellulose also contributes to the signal intensity in this region.

The samples shown in Fig. 1 were measured in air-dried, freeze-dried, or oven-dried form and had residual water contents between 5% (air-dried) and <1% (oven-dried). A close inspection of the spectra shows that variable moisture contents up to 5% do not affect the chemical shifts to a measurable extent, and no influence on the peak widths is observed. That no significant spectral differences exist between air-dried, freeze-dried, and oven-dried samples is also evident from the spectra of birchwood (Fig. 3A, B) and pretreated poplar wood (Fig. 5A, B). However, if the moisture content is significantly increased a clear effect on the chemical shifts can be seen, *e.g.* in dry birchwood (Fig. 3A) C-2 and -3 give a single signal at 74 p.p.m. which splits into two signals (73 and 76 p.p.m.) when the moisture content of wood is ~20% (Fig. 3C). We assume that this splitting is mainly due to an effect of water on the chemical shift of C-2. A similar effect of the moisture content (0.5–16%) was described for cellulose and acetylcellulose[19].

All the spectra shown in Fig. 1 were recorded on samples milled to a particle size of 0.25 mm. Since the spectra were not very well resolved, we suspected that this could have resulted from inhomogeneities of the particles, and we expected that milling the wood to a finer particle size would give better resolved spectra. However, exactly the opposite was found. When wood was milled to 0.12 mm the resulting spectra were less well resolved than those from 0.25 mm or 0.50 mm samples, and all signals were significantly broadened, as can be seen from Fig. 4A, B and Fig. 5A, B, C.

This is in agreement with the observation by Kolodziejski *et al.*[21] that 24 h ball-milled pine wood, extractive-free wood, and holocellulose gave significantly broadened carbohydrate signals with a shift of C-1 to higher fields. The milling procedure used by us for obtaining the 0.12 mm particles (ultracentrifugal mill, 2 min) is certainly not as rigorous as 24 h ball-milling. Nevertheless the spectra clearly

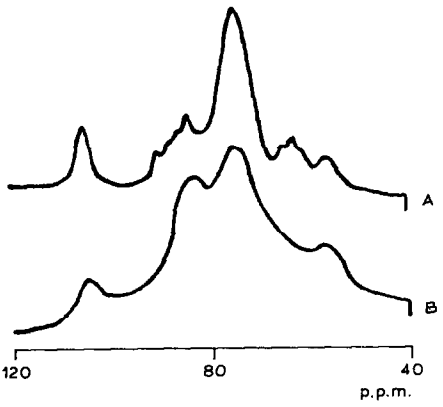

Fig. 4. ^{13}C-N.m.r. c.p.-m.a.s. spectra of *Pinus radiata* samples differing in particle size. A, 0.25 mm; B, 0.12 mm.

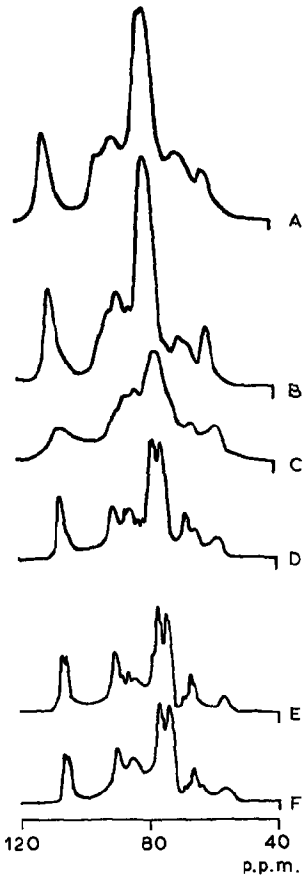

Fig. 5. ^{13}C-N.m.r. c.p.-m.a.s. spectra of variously prepared poplar wood samples. A, native wood, air-dried, 0.25 mm; B, native wood, freeze-dried, 0.50 mm; C, native wood, freeze-dried, 0.125 mm; D, steamed at 200°, then freeze-dried, 0.25 mm; E, steamed at 220°, then freeze-dried, 0.25 mm; F, steamed at 240°, then freeze-dried, 0.25 mm.

show that even this "mild" procedure dramatically decreases the crystallinity of the cellulose although no effects on the chemical shifts could be observed. A possible cause for the line broadening is the "freezing in" of stresses by the mechanical disruption of cellulose–cellulose hydrogen bonds[20], together with the formation of new intermolecular hydrogen bonds between cellulose, hemicellulose, and lignin. It should also be considered that the milling exerts strong shear forces on the wood cells, which could cause the internal generation of heat. Whatever the reasons for the effect of milling on the spectral features are, it is clear that an exact definition of the disintegration method should accompany any spectrum in order to make comparison of spectra meaningful.

We[15] and others[22–24] have reported that the hydrolysability of cellulose in

TABLE II

EFFECT OF STEAMING ON THE ENZYMATIC DIGESTIBILITY OF POPLAR WOOD[a]

Steaming temperature (°C)	*Yield of WIF[a] (%)*	*Yield of glucose (g/100 g WIF)*	
		48 h	*72 h*
160°	82.2	11.4	12.8
180°	81.6	15.2	17.1
200°	71.0	19.6	24.5
220°	64.4	35.0	44.3
240°	59.0	34.6	40.0

[a]Poplar wood sawdust was soaked for 3 h in sodium hydroxide (3 g NaOH/100 g wood) and then treated in an autoclave with saturated steam for 10 min at the temperatures shown. The steamed material was washed and the water insoluble fibers (WIF) were saccharified with *Trichoderma reesei* MCG 77 cellulase at a slurry density of 10%, 10 filterpaper units per g substrate, 50°.

wood and other lignocellulosics is markedly improved if the material is treated with saturated steam prior to enzymatic hydrolysis. An example of the effect of steaming is given in Table II for poplar wood over the temperature range 160–240°. The highest yield of glucose was obtained from material steamed at 220°. Steaming leads to a great variety of physical and chemical alterations in wood, of which the most significant are a temperature dependent extraction of hemicellulose, cleavage of acetyl groups, formation of furfural, partial solubilisation of lignin, and a dramatic increase in the surface area[15]. Which one of these factors is most important for the increase in enzymatic digestibility is largely unknown, but some clues may eventually be provided by the c.p.-m.a.s. technique which was used by Hemmingson and Newman[25] to study the effect of steam explosion on *Pinus radiata* and *Eucalyptus regnans*, and by us to examine poplar wood pretreated at 200, 220 and 240° (Fig. 5).

The differences between these spectra and those of untreated poplar wood are manifold. First, none of the steamed samples showed the signals for the acetyl groups of hemicellulose. This is in agreement with the chemical analysis showing that most of the acetyl groups are hydrolysed to give acetic acid in the steam condensate and wash water. Secondly, the spectra clearly show the resonance of the methoxyl group at 56 p.p.m., yet no signal for the aromatic carbons, although the material still contains most of the lignin originally present. Thirdly, the most remarkable effect is an apparent transformation of the cellulose towards a more structured, crystalline-like form, as evidenced by the clear, sharp signals for all six carbon atoms of the glucose units. Pretreatment temperatures of 220° and 240° lead to a clear splitting of the C-1 signal similar to that found in the spectrum of cotton. Moreover also the C-2, C-3, and C-5 resonances show a splitting which suggests that the crystallinity of the cellulose has increased and/or that internal stresses are released[20] by steaming at 220 and 240°. It is interesting to note that the enzymatic digestibility dramatically increases when the temperature is raised from 200 to 220°

and that exactly in this temperature range the spectra of the cellulose sharpen. This can also be clearly seen by the splitting of the C-1 signal.

CONCLUSIONS

1. The predominant signals in the ^{13}C spectra of native lignocellulosics (wood, straw) are due to the carbon atoms of the homopolymer cellulose. The other two major constituents, lignin and hemicellulose, are heteropolymers which give, due to the great diversity of nonequivalent carbon atoms, only weak and mostly hidden signals.

2. The strongly increased line width of all cellulose signals in the spectra of native wood, as compared to pure cellulose, could in principle result from a significantly enhanced molecular motion having correlation times in the range of 10^{-3} to 10^{-7} sec. It is known from the literature[26] that the most mobile group in cellulose is CH_2OH, which should therefore give the broadest signal. However, in all wood samples measured an increased line width was observed for all carbon atoms, whence it is unlikely that molecular motion significantly contributes to the line broadening. A reasonable explanation could be that the cellulose in its native environment is structurally less ordered or forms crystallites having smaller lateral sizes[20], compared with cellulose in cotton. Additionally it should be considered[20] that some anisotropic magnetic-susceptibility broadening[27] could exist due to the close proximity of the cellulose to domains of well oriented lignin molecules.

3. Milling of the wood to very fine particles strongly changes the spectral features. Most remarkable in this respect is an additional line broadening of all signals which is probably due to an increased disorder of the cellulose chains and to the formation of new intermolecular hydrogen bonds. In addition, milling might render the aromatic rings of lignin more oriented and thereby enhance line broadening through anisotropic magnetic susceptibility. It is also significant that the peak area of C-4 (at 83 p.p.m.) significantly increases in the milled sample. A plausible explanation would be that this is due to a strong downfield shift of the C-2 signal caused by the formation of hydrogen bonds involving the OH group at C-2. This assumption is supported by the finding[8] that in amorphous cellulose new hydrogen bonds affecting C-2 and to a lesser extent also C-3 and C-4 are formed when the moisture content is increased.

4. Treatment of wood with steam at 220 and 240° leads to very remarkable changes in the cellulose spectrum, characterized by line narrowing of all signals and resonance splitting of the C-1 signal. The causes for this could be manifold, *e.g.*, increase in the crystallinity of the cellulose, loss of orientation of the aromatic rings, aggregation of lignin, and others.

5. Finally we want to emphasize that the features of the cellulose spectrum of wood are very much dependent on the method of sample preparation. The most important factors are the moisture content and the grinding procedure. Comparable and reproducible spectra can only be obtained if sample preparation is standardized.

MATERIALS AND METHODS

Solid-state ^{13}C-n.m.r. measurements were made with a Varian XL 200 spectrometer equipped with a solid state accessory. Cross-polarization times were typically 1–2 ms. The respective radio frequency amplitudes were set for a Hartmann–Hahn match at a rotating-frame precession frequency of 50 kHz. The magic-angle spinning frequency was 3100–3400 Hz. The rotor material was Kel-F. An external reference was used to calibrate the carbon spectra. The weighting procedure used involved a combination of an exponential, $\exp(t/0.06)$, and a Gaussian function, $\exp[-(t/0.1)^2]$, together with zero filling up to 8K data points. The acquisition time was 0.128 s, the delay between pulses was 3 s.

The cellulose samples were from Sigma (Sigmacell) and Merck (Avicel). The beechwood hemicellulose was a byproduct in the production of dissolving pulp (Chemiefaser Lenzing A.G., Austria); it was extracted with 20% sodium hydroxide and purified by ultrafiltration.

The wood of *Populus tremuloides* (poplar) was obtained from Forintek, Ottawa, Canada, and *Pinus radiata* from the Forest Research Institute, Rotorua, New Zealand. All other samples were from local sources.

The wood samples were milled with an ultracentrifugal mill (model ZM1, Retsch, F.R.G.) equipped with 0.5, 0.25, and 0.12 mm sieves.

The chemical analysis of the various samples was carried out as previously described[17]

ACKNOWLEDGMENTS

We gratefully acknowledge financial support by the Bundesministerium für Wissenschaft und Forschung, Vienna, and by the "Fonds zur Förderung der Wissenschaftlichen Forschung", Projekt Nr. 3929.

We also thank D. L. VanderHart (National Bureau of Standards, Polymer Division, Washington, D.C.) for valuable comments regarding the interpretation of the spectra.

REFERENCES

1 V. J. BARTUSKA, G. E. MACIEL, J. SCHÄFER, AND E. O. STEJSKAL, *Fuel*, 56 (1977) 354–358.
2 W. T. DIXON, J. SCHÄFER, M. D. SEFCIK, E. O. STEJSKAL, AND R. A. MCKAY, *J. Magn. Reson.*, 45 (1981) 173–178.
3 J. SCHÄFER AND E. O. STEJSKAL, *J. Am. Chem. Soc.*, 98 (1976) 1031–1033.
4 J. HAUR AND G. E. MACIEL, *Holzforschung*, 38 (1984) 327–331.
5 J. SCHÄFER, M. D. SEFCIK, E. O. STEJSKAL, R. A. MCKAY, AND P. L. HALL, *Macromolecules*, 14 (1980) 557–559.
6 J. A. HEMMINGTON, *J. Wood Chem. Technol.*, 3 (1983) 289–312.
7 D. L. VANDERHART AND R. H. ATALLA, *Macromolecules*, 17 (1984) 1465–1472.
8 R. L. DUDLEY, C. A. FYFE, P. J. STEPHENSON, Y. DESLANDES, G. K. HAMER, AND R. H. MARCHESSAULT, *J. Am. Chem. Soc.*, 105 (1983) 2469–2472.
9 D. T. CLARK AND A. H. K. FOSTER, *Polym. Commun.*, 24 (1983) 140–142.

10 K. FREUDENBERG AND C. BLOMQVIST, *Ber. Dtsch. Chem. Ges.*, 68 (1935) 2070–2082.
11 H. J. MARRINAN AND J. MANN, *J. Polym. Sci.*, 21 (1956) 301–311.
12 R. H. MARCHESSAULT AND P. R. SUNDARARAJAN in G. O. ASPINALL (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, 1983, pp. 12–95.
13 H. ESTERBAUER, G. JUNGSCHAFFER, AND J. SCHURZ, *Holzforschung*, 35 (1981) 129–135.
14 H. ESTERBAUER, M. HAYN, G. JUNGSCHAFFER, E. TAUFRATZHOFER, AND J. SCHURZ, *J. Wood Chem. Technol.*, 3 (1983) 261–287.
15 H. ESTERBAUER AND A. JANOSI, *Das Papier*, 38 (1984) 599–606.
16 H. ESTERBAUER, J. SCHURZ, AND A. WIRTL, *Cellul. Chem. Technol.*, (1985) 341–355.
17 H. ESTERBAUER, M. HAYN, AND H. TUISEL, *Das Papier*, 36 (1982) 589–595.
18 R. H. ATALLA, J. C. GAST, AND G. MACIEL, *J. Am. Chem. Soc.*, 102 (1980) 3249–3251.
19 J. J. LINDBERG AND B. HORTLING, *Adv. Polym. Sci.*, 66 (1984) 1–22.
20 D. L. VANDERHART, personal communication.
21 W. KOLODZIEJSKI, J. S. FRYE, AND G. M. MACIEL, *Anal. Chem.*, 54 (1982) 1419–1424.
22 J. N. SADDLER, H. H. BROWNELL, C. P. CLERMONT, AND N. L. LEVITIN, *Biotechnol. Bioeng.*, 24 (1982) 1389–1402.
23 J. N. SADDLER AND H. H. BROWNELL, presented at the International Symposium on Ethanol from Biomass, spon. Royal Society of Canada, Winnipeg, Canada, Oct. 13–15, 1982.
24 J. PULS, K. POUTANEN, AND H. KÖRNER, *Appl. Microbiol. Biotechnol.*, 22 (1985) 416–423.
25 J. A. HEMMINGSON AND R. H. NEWMAN, *J. Wood Chem. Technol.*, 5 (1985) 159–188.
26 W. L. EARL AND D. L. VANDERHART, *J. Am. Chem. Soc.*, 102 (1980) 3251–3252.
27 D. L. VANDERHART, W. L. EARL, AND A. N. GARROWAY, *J. Magn. Reson.*, 44 (1981) 361–401.