(8) McNaughton, J. G.; Yean, W. Q.; Goring, D. A. I. *Tappi* **1967**, *50*, 548.
(9) Teller, D. C. *Methods Enzymol.* **1973**, *27*, 346–441.
(10) Kratzl, K.; Gratzl, J.; Claus, P. *Adv. Chem. Ser.* **1966**, *No. 59*, 157.
(11) Kratzl, K.; Schafer, W.; Claus, P.; Gratzl, J.; Schilling, P. *Monatsh. Chem.* **1967**, *98*, 891.
(12) The ordinate scales have been varied by using a constant multiplicative factor for each elution profile; the *relative proportions* of species throughout the accessible molecular weight range remain, of course, unchanged.
(13) The critical chain length, in general, is the minimum degree of polymerization necessary for participation in associated complex formation.[14] This chain length has been exceeded by the members of the lower molecular weight kraft lignin oligomeric subset since all of these components take part in the associative process.
(14) Tsuchida, E.; Abe, K. *Adv. Polym. Sci.* **1982**, *45*, 77.
(15) The degree of association for a kraft lignin sample can be assigned a formal value on the basis that the subset of components with molecular weight below 3500 interacts as a single stoichiometric unit with the complementary group of higher molecular weight species.
(16) Wallis, A. F. A. In "Lignins"; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley: New York, 1971; pp 345–372.
(17) Lindström, T. *Colloid Polym. Sci.* **1979**, *257*, 277.
(18) Manning, G. S. *Acc. Chem. Res.* **1979**, *12*, 443.
(19) Lenz, B. L. *Tappi* **1968**, *51*, 511.
(20) van der Klink, J. J.; Zuiderweg, L. H.; Leyte, J. C. *J. Chem. Phys.* **1974**, *60*, 2391.
(21) Kielman, H. S.; Leyte, J. C. *J. Phys. Chem.* **1973**, *77*, 1593.
(22) Meurer, B.; Spegt, P.; Weill, G. *Biophys. Chem.* **1982**, *16*, 89.
(23) Chu, P.; Marinsky, J. A. *J. Phys. Chem.* **1967**, *71*, 4352.
(24) Takahashi, A.; Kato, T.; Nagasawa, M. *J. Phys. Chem.* **1967**, *71*, 2001.
(25) Gardon, J. L.; Mason, S. G. *Can. J. Chem.* **1955**, *33*, 1491.
(26) Rezanowich, A.; Goring, D. A. I. *J. Colloid Sci.* **1960**, *15*, 452.
(27) Goring, D. A. I. *ACS Symp. Ser.* **1977**, *No. 48*, 273.
(28) Grubisic, Z.; Rempp, P.; Benoit, H. *J. Polym. Sci., Part B* **1967**, *5*, 753.
(29) Flory, P. J. "Principles of Polymer Chemistry"; Cornell University Press: Ithaca, New York, 1953; Chapter XIII.
(30) The reassociative process that occurs upon removal of the zwitterion through diffusion must have reached equilibrium during the time elapsed (0.1 h) between the introduction of the sample into the column and the initiation of size-exclusion chromatographic fractionation; no observable change in the degree of association in 0.10 M aqueous NaOH has been detected at component concentrations of 10 g L^{-1} during a period of 12 h, the time routinely required for elution of the sample through the column.
(31) Ivanov, M. A.; Nefedov, P. P.; Rusakov, A. E.; Sherbakova, L. D.; Lazareva, M. A.; Zakharov, V. I. *Khim. Drev.* **1979**, *No. 6*, 108.
(32) Gravitis, J.; Erins, P. *Appl. Polym. Symp.* **1983**, *37*, 421.
(33) Luner, P.; Kempf, U. *Tappi* **1970**, *53*, 2069.
(34) Hatakeyama, T.; Hatakeyama, H. *Rep. Prog. Polym. Phys. Jpn.* **1974**, *17*, 711.
(35) Preston, R. D. *Phys. Rep.* **1975**, *21*, 183.
(36) Adler, E. *Wood Sci. Technol.* **1977**, *11*, 169.
(37) Gierer, J. *Sven. Papperstidn.* **1970**, *73*, 571.
(38) Gierer, J.; Lindeberg, O. *Acta Chem. Scand., Ser. B* **1980**, *34*, 161.
(39) Garver, T. M.; Sarkanen, S. To be submitted for publication.

Evidence for the Existence of Associated Lignin–Carbohydrate Polymers As Revealed by Carbon-13 CPMAS Solid-State NMR Spectroscopy

Walter V. Gerasimowicz,* Kevin B. Hicks, and Philip E. Pfeffer

Eastern Regional Research Center,† Philadelphia, Pennsylvania 19118.
Received January 31, 1984

ABSTRACT: ^{13}C CPMAS NMR spectroscopy was used to examine the relaxation profiles in solid samples of *Piceae glauca* wood pulp. Proton spin–lattice (T_{1H}) and proton rotating-frame spin–lattice ($T_{1\rho H}$) relaxation techniques were utilized in conjunction with induced paramagnetic relaxation, ^{13}C–^{1}H interrupted-proton decoupling, and enzymatic degradation to infer details of structure in the wood pulp constituents. Divergent T_{1H}'s in this material were seen to converge toward a singular value as nonbound or remotely bound cellulose units were degraded and removed from the solid-state sample matrix. This observation suggests that the residual lignocellulosic heteropolymer was associated in such a manner that proton spin diffusion was constant for all components, providing evidence for the existence of naturally occurring lignin–carbohydrate structures.

Introduction

The nature of lignin–carbohydrate interactions in plant tissues has been a subject of increasing speculation in recent years. Mechanisms whereby lignin–carbohydrate bonds are formed have been proposed in synthetic model systems.[1-3] The understanding of both the characteristics and the stability of lignocellulosic linkages is an important scientific concern since lignocellulosic polymers are major constituents of vascular plants and are widely utilized organic materials. The lignin component of deciduous lignocellulosic plant cell wall matter is known to inhibit the enzymatic breakdown of bound cellulose.[4,5] The efficient disruption of these interactions is of prime importance in such diverse processes as paper pulping, biomass conversion, and in the digestibility of forage in ruminants.

Standard ^{13}C Fourier-transform solution NMR techniques have been demonstrated to be very useful for the examination of isolated lignin and cellulosic compounds, as well as in studies of soluble lignin–carbohydrate complexes and model organic compounds.[6-9] However, the isolation of soluble lignocellulosic compounds is somewhat problematic in that chemical treatment and destructive methods result in structural modification of the components present in these systems as evidenced in their ^{13}C NMR spectra.[9]

The advent of solid-state ^{13}C NMR employing cross polarization and magic angle spinning (CPMAS) has generally overcome many of the difficulties associated with reduced molecular motion in solids: ^{13}C–^{1}H dipolar coupling, chemical shift anisotropy and ^{13}C sensitivity prob-

† Agricultural Research Service, U.S. Department of Agriculture.

Table I
Analytical Characteristics of *Piceae glauca* Pulp

	% C	% H	% N	% O	% methoxyl
untreated wood pulp	45.72	6.40	<0.1	40.62	4.77
cellulase-treated wood pulp	54.01	6.25	0.017	34.92	10.83

lems due to the low natural abundance, the relatively small magnetic moment, and long ^{13}C relaxation times.[10-14]

The question of the existence of lignin–carbohydrate complexes has recently been mentioned in a ^{13}C CPMAS NMR study of lodgepole pine wood, extracted residues, and derived lignin preparations.[15] In that study the spectra show that some lignin appears to be removed from the wood along with certain carbohydrates and cellulose during holocellulose sample preparation. According to the authors, such evidence suggests the existence of lignin–carbohydrate complexes in the solid state.[15]

In this contribution, we report a solid-state ^{13}C CPMAS NMR investigation of the nature of the lignocellulosic interactions seen in wood pulp from an Eastern White Spruce, *Piceae glauca*. Proton T_1 (spin–lattice relaxation time), proton $T_{1\rho}$ (rotating-frame spin–lattice relaxation time), paramagnetic-induced relaxation, and ^{13}C–^1H dipolar relaxation techniques have been used to assess the relationship between carbohydrates and lignin in these materials. Additional information has been derived from ^{13}C CPMAS studies of lignin-enriched samples of *Piceae glauca* pulp (PGP) which were prepared by enzymatic degradation and removal of degradable, nonbound, and peripheral cellulose.

Experimental Section

NMR Measurements. ^{13}C CPMAS measurements were obtained at a ^{13}C frequency of 15.0 MHz with a JEOL FX-60QS spectrometer.[53] The ^1H decoupling radio-frequency irradiation field strength was 11 G, thus cross polarizing with 44 G for 0.5 ms. A spectral width of 8000 Hz and a sampling of 2K data points zero filled to 4K was used for spectral acquisition. All chemical shifts were assigned relative to the hexamethylbenzene shift of 17.36 ppm for its CH_3 resonance based on the position of tetramethylsilane. Samples were spun at a rate of approximately 2.4 kHz at the magic angle of 54.7° in Kel-F bullet-type rotors.

Samples. Since the preparation of the *P. glauca* pulp/Fe^{3+} samples and metal determinations thereon have been previously described, only a brief summation will be provided here.[16] The PGP was obtained from a thermomechanical pulping process where the fibers were refined in a pressurized disk at a temperature exceeding 100 °C. These fibers were then ball-milled for 12 days. The ball-milled material was further ground in a Wiley mill. The material was passed consecutively through a 3-mm screen, a 20-mesh screen, and a 40-mesh screen. This freshly ground and sieved wood pulp was lyophilized overnight to constant weight. One-gram samples of the lyophilized wood pulp were added to each of five 250-mL glass-stoppered flasks. $FeCl_3 \cdot 6H_2O$ was added to each sample as follows: 0.00, 0.15, 1.50, 15.00, and 150.00 mg. Each of these samples was made up to 100 mL by the addition of H_2O. These samples were shaken at 100 rpm in a 25 °C water bath for 2 h. These mixtures were dried to constant weight on a lyophilizer. The total concentration of iron in these samples was determined by atomic absorption. Additional samples of PGP were enzymatically treated in the following manner. A 5.1-g sample of the mechanically exploded wood pulp was ground in a porcelain ball mill for 12 days. The powdered material was extracted with 200 mL of boiling H_2O, filtered on a vacuum funnel, and dried under N_2. The dry residue weighed 4.1 g. This dried residue was digested with a commercial preparation of cellulase (10 000 units) in 100 mL of acetate buffer (0.05 M, pH 4.8) by shaking at 45 °C for 72 h. The digest was filtered and the solid residue washed with 500 mL of hot H_2O. Upon drying under N_2 the final yield was 1.65 g. Microcrystalline cellulose (Avicel) was obtained from Hercules Powder Co., Wilmington, DE, and used without further treatment.

Analyses. C, H, N, O, and methoxyl analyses were performed by Galbraith Laboratories, Knoxville, TN (Table I).

Table II
Relaxation Values[a] of *Piceae glauca* Pulp[b]

$[Fe^{3+}]^c$	T_{1H}	$T_{1\rho H}$	$T_{CH}{}^d$
153[e]	78.8 (82.0)[f]	9.9	0.095
184	79.0 (82.6)	9.2	
419	53.2 (55.9)	7.8	
2860	13.1 (13.8)	6.8	
25400	2.9 (2.9)	2.7	

[a] ms ± 10%. [b] Carbohydrate region of ^{13}C spectra at ca. 75 ppm. [c] Concentration in ppm. [d] ±25%. [e] Iron content of zero-doped *Piceae glauca* pulp. [f] Values in parentheses represent T_{1H} values for anomeric carbohyhydrate resonances at ca. 105 ppm.

Results

Relaxation Measurements. Peak height measurements were employed for the calculation of relaxation times (T_{1H}'s and $T_{1\rho H}$'s). Each frequency-domain spectrum was obtained with 35 Hz of exponential computer-line broadening to yield spectra with satisfactory signal-to-noise ratios without a sacrifice in resolution. Values of T_{1H} were determined indirectly by observation of the ^{13}C magnetization via cross polarization in a 180°–τ–90° pulse sequence.[17] Optimal contact times of 0.5 ms necessary to obtain proper relative responses for all resolvable carbon types in these heterogeneous systems had been determined previously.[16] Double-exponential curve-fitting techniques were used for the analysis of all relaxation times. Recycling times were at least $10T_1$'s. The results of these T_{1H} measurements for the various PGP samples are given in Tables II and IV. Values of T_{CH} (carbon–proton cross-polarization time) and $T_{1\rho H}$ for PGP were calculated according to literature methods by measuring ^{13}C magnetization as a function of contact time.[18] The transfer of proton dipolar order from proton nuclei to carbon nuclei in the rotating Zeeman frame has been discussed in the literature.[19-21] In some crystalline materials, the observed ^{13}C magnetization is expected and has been shown to undergo transient oscillations due to the fact that vectors describing the ^{13}C–^1H bonds in such systems all have the same angle with the static magnetic field.[19-21] However, amorphous and polycrystalline materials generally do not exhibit such effects due to the random orientations of the ^{13}C–^1H bond angles which produce phase incoherence. Even in crystalline materials, this transient step rise has been shown to be over in approximately 20 μs.[21,22] However, in order to avoid the possibility of such effects in this work, we did not employ extremely short contact times as cited above. The results of these T_{CH} and $T_{1\rho H}$ measurements for PGP are given in Table II. Interrupted ^{13}C–^1H decoupling experiments were also performed to differentiate nonprotonated ^{13}C nuclei from protonated ^{13}C nuclei.[23] A 40-μs delay without proton decoupling was inserted into the pulse sequence prior to data acquisition. The indirect T_{1H} sequence was also combined with an interrupted ^{13}C–^1H decoupling in a 180°–τ–90°–delay pulse sequence to obtain relaxation data for nonprotonated and underlying ^{13}C resonances in our sample spectra. The delay used was a 40-μs pulse delay without proton decoupling prior to data acquisition in the 180°–τ–90° sequence.[17] The results of these measurements are given in Table III. T_{1H} relaxation time values, with and without interrupted decoupling, were checked on standard samples of poly(methyl methacrylate) to verify that both experiments give identical values of T_{1H} for any given peak in the spectrum.

Table III
Piceae glauca Pulp Component T_{1H} Relaxation Values[a] (Interrupted Decoupling)

	^{13}C chemical shift[b]			
[Fe^{3+}][c]	150	135	74	55
153	36.9	36.5	51.4	42.1
184	23.0	25.7	44.2	30.1
419	23.3	24.7	31.3	27.8
2860	14.1	12.3	17.2	14.3
25400	4.5	5.9	9.2	7.7

[a] ms ± 10%. [b] ppm. [c] Concentration in ppm.

Analytical Characteristics of the *Piceae glauca* Wood Pulp Samples. C, H, N, O, and methoxyl analyses for the various samples are presented in Table I. Additional analytical information for the PGP is also available.[24,25]

Discussion

^{13}C CPMAS Studies of *Piceae glauca* Pulp. The chemical composition of PGP consists predominantly of two major components: lignin and cellulose. Several ^{13}C CPMAS NMR studies of lignin and cellulose in the solid state have recently been published.[26-32] These studies have focused on the assignment of resolvable resonances for these wood constituents. The spectroscopic techniques of solid-state ^{13}C NMR have also been useful in the characterization of wood structure.[15,33] The assignments of the various peaks and signals from these reports on lignin, cellulose, and wood will be utilized in the interpretation of the ^{13}C CPMAS spectra acquired in this work. Although some of the referenced papers contain ^{13}C NMR solid-state spectra of lignin and cellulose from instruments operating at higher ^{13}C magnetic field strengths than that in our laboratories, the resolution obtained (upon careful examination of the published results) is not significantly enhanced over that observed here.

Figure 1A depicts the ^{13}C CPMAS NMR spectrum of the PGP. Several resonance lines are clearly resolved in this spectrum. However, these bands are relatively broad and reflect the complex chemical composition of the sample, as well as its amorphous, noncrystalline structure. The rather broad resonance line widths also reflect the thermomechanical treatment, subsequent grinding, and preparation of the samples. Amorphous samples, such as those examined here, generally produce fairly wide NMR lines in solid-state ^{13}C CPMAS spectra. The resonance at 105 ppm may be slightly narrower than similar resonances in ball-milled wood spectra.[15] This slight narrowing may reflect a small degree of crystallinity or nonamorphous regions in our samples. However, the extensive ball-milling as well as the spectral line widths observed essentially define the morphology of the PGP utilized in this work as amorphous. The spectrum shows that local molecular environments are nonequivalent within this polymeric network. The resolution achieved, however, is still adequate for identifying the major components of the PGP. The ^{13}C NMR spectrum is essentially dominated by resonances that are attributed to components arising from cellulose and hemicellulose. Carbon nuclei from these carbohydrate moieties give rise to signals between 60 and 110 ppm. The peak at approximately 105 ppm corresponds to the anomeric or C_1 carbon from β-D-glucopyranose repeating units of cellulose. This resonance is particularly well resolved. The broad signals from the cellulose are superimposed on those from the hexose and pentose units of the hemicellulose compounds.

The spectrum also contains features that are less intense. The shoulder at 56 ppm may be assigned to methoxyl groups from lignin and also from hemicellulose. The aromatic components of lignin are seen between 130 and 155 ppm. These less intense features and the ^{13}C CPMAS spectra cited and discussed so far only provide limited, qualitative information about the structure of these compounds. Other chemical and NMR methodologies are necessary to examine the chemical and structural relationship (connectivity) between lignin and carbohydrate in PGP and to enhance the underlying and unresolved components in the spectra obtained.

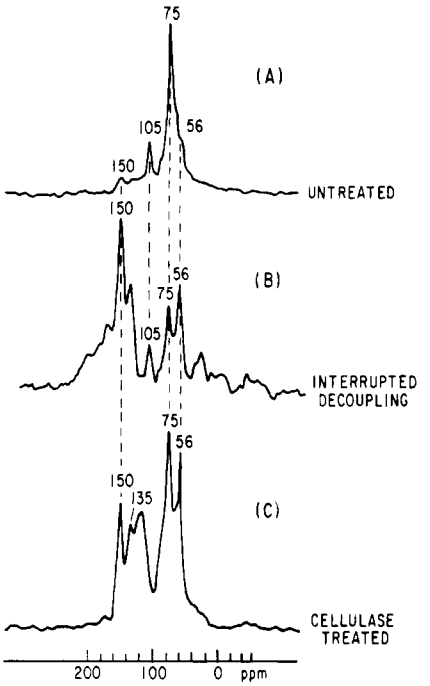

Figure 1. *Piceae glauca* pulp ^{13}C CPMAS spectra.

Paramagnetically Induced Relaxation in *Piceae glauca* Pulp. In pure organic compounds and in diamagnetic organic crystalline solids with abundant proton populations, rapid spin diffusion generally occurs due to the powerful ^{1}H–^{1}H dipolar interactions. All protons exhibit equivalent spin–lattice relaxation times regardless of the carbon-containing functional group to which they are attached in such compounds. However, previous studies of biological samples, heterogeneous mixtures, and plant materials indicate that proton spin–lattice relaxation may be inhomogeneous in complex matrices.[16,34,35] Different T_{1H}'s within a given sample indicate that components comprising the mixture are spatially separated such that their respective proton populations have distinct relaxation properties. General theory relating relaxation properties, magnetization transport, and spin diffusion has been adequately reviewed, and the reader is referred to the fundamental literature for further information.[36-39]

PGP was studied previously as part of a ^{13}C CPMAS examination of an amorphous, physical mixture consisting of lipid, lignin, keratin, and wood pulp.[16] Reductions in the proton T_1 and $T_{1\rho}$ values were seen for all of the components comprising the previously reported model mixture when comparing the proton relaxation values for the respective constituents upon addition of paramagnetic Fe^{3+}. However, the largest reductions in the proton relaxation values of the model components upon amendment with paramagnetic Fe^{3+} were observed in the carbohydrate components of PGP. Particularly interesting was the fact that its $T_{1\rho H}$ showed a marked decrease (7.0 to 2.2 ms) when the paramagnetic system was compared to the non-iron-containing mixture. None of the other compo-

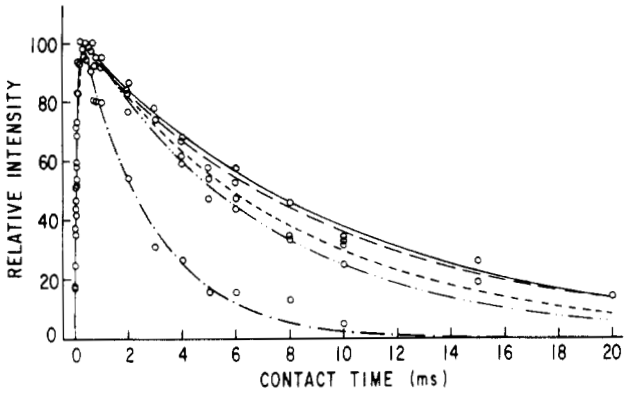

Figure 2. ^{13}C magnetization response of *Piceae glauca* pulp and Fe^{3+}-doped *Piceae glauca* pulp. (From top to bottom the curves correspond to 153, 184, 419, 2860, and 25 400 ppm Fe^{3+}, respectively.)

nents of that system exhibit such shortening of proton rotating-frame spin–lattice relaxation times when the same comparison is made. Proton T_{1H} values, however, proved to be a much more sensitive and general indicator of paramagnetically induced relaxation effects when ferric ion was added to the model system. Fe^{3+}-induced shortening of the T_{1H}'s was observed for all components with reductions ranging from 50% for the lipid to approximately 96% for the PGP. Furthermore, the variations in the T_{1H} and $T_{1\rho H}$ values of the physically mixed lipid–lignin–keratin–wood pulp systems indicated that spin diffusion was obviously nonuniform and demonstrated that structural domains were segregated for the different constituents therein. These observations prompted more thorough relaxation studies of the lignocellulosic polymers and their presumed internal linkages. Paramagnetically induced relaxation effects were utilized as a probe in conjunction with $T_{1\rho H}$ and T_{1H} measurements in order to investigate the structural connectivity of various polymeric domains and to determine whether or not lignin–carbohydrate complexes could be characterized in the solid state.

The data in Table II summarize the relaxation parameters determined for carbohydrate peaks in PGP. The macroscopic or apparent $T_{1\rho H}$ drops from 9.9 to 2.7 ms for these nuclei as the Fe^{3+} concentration increases from 153 to 25 400 ppm. Figure 2 also graphically illustrates the actual and calculated data for the T_{CH} and $T_{1\rho H}$ studies. These data are much more extensive and detailed than our previously published work.[16] We note that the calculated curves in Figure 2 are in excellent agreement with the experimental data points. Furthermore, the apparent proton rotating-frame relaxation observed as a function of increasing paramagnetic concentration in these systems may be described as a single, first-order relaxation phenomenon within experimental error. Although paramagnetically induced shortening of the observable $T_{1\rho H}$ is obvious, the paramagnetic contribution to proton relaxation appears to be uniform through the carbohydrate. Any non-first-order relaxation is not statistically significant. The T_{CH} or cross-polarization relaxation time is treated as a constant for these systems (within experimental error) even upon increasing the Fe^{3+} concentration as indicated. Similar trends (as those for the $T_{1\rho H}$ data presented above) are evident in Table II for paramagnetically induced shortening of the apparent T_{1H} for the carbohydrate portion of the PGP ^{13}C CPMAS NMR spectrum. The T_{1H} values included in parentheses in Table II indicate the proton spin–lattice relaxation times of the anomeric carbohydrate region (approximately 105 ppm) of the PGP ^{13}C CPMAS spectrum (see Figure 1A). All of the values listed for the 105 ppm resonance fall within the experimental error of the T_{1H} values shown for the 75 ppm resonances as expected. These data provide further evidence that proton spin–lattice relaxation is homogeneous throughout the observable carbohydrate region of the spectrum. Resonances from lignin, which may slightly underlie the carbohydrate peaks at approximately 75 ppm, are negligible and do not appreciably affect the relaxation measurements to this point.

Interrupted Decoupling T_{1H} Experiments. In order to enhance the spectral resolution of the noncarbohydrate components of the PGP, the ^{13}C–^{1}H dipolar dephasing interaction was utilized.[23] Many examples now exist in the literature where dipolar dephasing provides spectra consisting only of resonances from nonprotonated carbon nuclei and methyl groups.[23,40-43] Certain functional groups, such as methyl groups, undergo rapid, internal rotations, even in the solid state. Such motion attenuates the ^{13}C–^{1}H dipolar relaxation mechanism rather markedly, with the result that resonances from such carbon nuclei are attenuated but not entirely eliminated from the spectrum. Even though the internal motion of the methyl group reduces the dipolar effect because of angular averaging, such groups usually have distinct chemical shifts and are easily assigned in solid-state CPMAS ^{13}C NMR spectra. Although the interrupted decoupling technique is a somewhat phenomenological method and not totally quantitative, the method can effectively increase the resolution one may achieve in certain solid-state ^{13}C CPMAS studies.

Figure 1B depicts the spectrum of the PGP with the previously specified decoupling delay time. Almost all of the ^{13}C resonance responses from the carbohydrate portion of the sample have been attenuated or eliminated entirely from the spectrum. Some loss in the signal-to-noise ratio (approximately 25–30%) is apparent in such an experiment as would be expected due to some dipolar dephasing of the remaining nonprotonated and methyl resonance signal intensities. ^{13}C resonances are still evident at 105 ppm and some response is still seen at 75 ppm. When the magnitude of the signal at 75 ppm is compared to that at 150 ppm in Figure 1A, we notice the domination of the former. The 75 ppm resonance is only seen as a residual signal when compared with the 150 ppm resonance in Figure 1B. Therefore, this residual signal is probably from structures that are very low in concentration and does not adversely affect the values of the relaxation times determined by the standard T_{1H} methodology for the 75 ppm resonances in Figure 1A. In order to determine whether those residual signals at 75 ppm were from ordered cellulose molecules, a microcrystalline sample (Avicel) was examined under standard ^{13}C CPMAS conditions and under identical conditions but with interrupted ^{13}C–^{1}H dipolar decoupling (Figure 3). When the microcrystalline cellulose is examined with dipolar dephasing, no signals are evident either for the cellulose ring or for anomeric carbon nuclei. Thus, the peaks in Figure 1B at approximately 75 and 105 ppm are not from ordered, crystalline cellulose constituents in the PGP matrix. In several reports of the ^{13}C NMR solution spectra of lignins, investigators have proposed that ^{13}C signals between ca. 102 and 108 ppm have as much likelihood of being due to ^{13}C resonances from sinapyl structures as from anomeric carbon nuclei of glycosides.[44,45] Thus the ^{13}C resonance at 105 ppm in Figure 1B may be associated with lignin polymer, especially due to the corresponding strong intensity at 150–155 ppm. Another possible assignment for this resonance could be C-2 nonprotonated carbon nuclei of associated keto sugar polymers. However, the noncarbohydrate and other previously

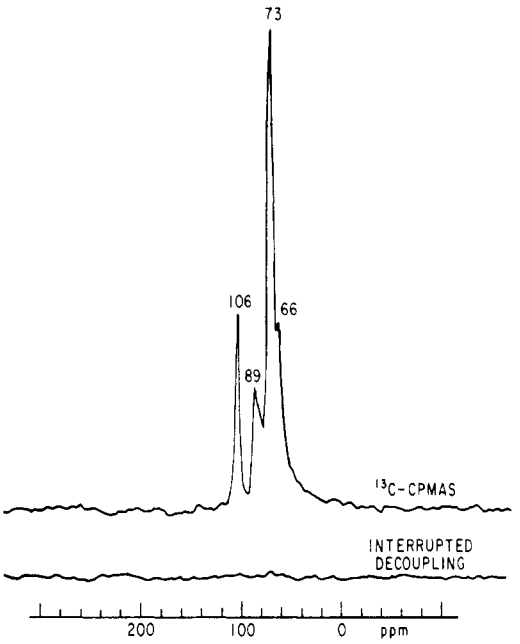

Figure 3. Microcrystalline cellulose ^{13}C CPMAS spectra.

unresolved components are no longer obscured as seen when Figure 1B is compared to Figure 1A. The features that were previously hidden, such as the lignin methoxy at 56 ppm and the aromatic carbon moieties between 130 and 155 ppm, now dominate the spectrum in Figure 1B.

In order to measure the T_{1H} values for these components, we have combined the T_{1H} inversion–recovery sequence with the ^{13}C–^{1}H dipolar dephasing mechanism. Under very careful Hartmann–Hahn matching conditions for spectral acquisition, the T_{1H} relaxation values were determined for these now-visible components. The results are listed in Table III. Several important features are apparent from this table. Unlike the homogeneous relaxation behavior observed for the wood pulp in Table II, the relaxation of the various resonances listed is heterogeneous. Proton spin–lattice relaxation effects are not uniform throughout the sample.

Fe^{3+} induces shortening of proton spin–lattice relaxation times across the board. The residual resonances seen at a ^{13}C chemical shift of 74 ppm at an Fe^{3+} concentration of 153 ppm do not have the same T_{1H} as observed for the overall wood pulp carbohydrate seen in Table II under the same conditions. This result suggests that these structures may be isolated or different in nature than the majority of the cellulose or hemicellulose previously observed under the normal noninterrupted decoupling conditions. Furthermore, these structures may be somewhat more mobile in the solid than more rigid cellulose-like domains resulting in localized attenuation of the ^{13}C–^{1}H dipolar interaction. Such differences in native wood carbohydrate, lignin, and aromatic relaxation times would not be inconsistent with the fact that molecular weight distributions of polysaccharides in typical wood and plant polymers point to their polydisperse nature, as well as their very large size.[46] Obviously, configurations and conformations of native cellulosic units in native wood polymers, which consist of approximately 10 000–15 000 glucose residues alone, may be such that spatial communication and proton spin diffusion are segregated or nonhomogeneous in different, widely separated polymeric regions. An additional implication (based upon the proton spin–lattice relaxation times in Table III) is that these structural domains where heterogeneous proton T_{1H}'s have been detected are separated by distances in the solid of at least 80–100 Å. Similar polydispersity in lignin structure is well documented.[46] Lignin is known to undergo linear aggregation and endwise polymerization.[46] These relaxation properties are somewhat dependent upon the dimensions of the structural domains comprising the PGP. Wood cell microfibrils have been studied by electron microscopy. Wood cellulose microfibrils tend to range from 100 to 200 Å in width. Furthermore, lignin as well as disordered cellulose and hemicellulose molecules are located interstitially between the microfibrils.[46–51] If pure wood has domain sizes of 100–200 Å, the T_{1H} data reported here are in reasonable agreement with both the fibrillar nature and microscopic estimates of the molecular diameters to be expected of ball-milled PGP constituents. These T_{1H} relaxation data also show that although long-range order exists among the PGP constituents, phase separation of lignin and carbohydrate has been demonstrated. The data in Table III demonstrate the spatial proximity of the components attributable to lignin in PGP. The T_{1H} values for the 150, 135, and 55 ppm lignin resonances are equivalent within the experimental uncertainty, indicating rapid spin diffusion among the components comprising this domain of the PGP. However, the lignin T_{1H} values are significantly different from the T_{1H} data for the resonance at 75 ppm. The proton T_{1H} differences, as seen in Table III, indicate that in large heteropolymers, relaxation parameters may provide information concerning the gross morphology of the material, as well as data regarding the boundaries to effective spin–lattice relaxation ranges in such samples. Zumbulyadis has recently shown that multicomponent proton relaxation behavior is useful in examining crystalline and amorphous domains in poly(ethylene) terephthalate, and has suggested that proton spin–lattice relaxation times may be used to estimate domain sizes in heterogeneous systems, particularly polymers.[52] Our work extends the utility of this concept to naturally occurring solid heteropolymers.

Finally, the T_{1H}'s for all of the ^{13}C resonances listed in Table III are shortened as a function of increasing paramagnetic concentration. When pure lignin was examined as part of our previously described model mixture, the proton spin–lattice relaxation time changed from 164.0 to 73.0 ms when the Fe^{3+} concentration was increased from 0 to 1.9%.[16] Thus, the T_{1H} fell by approximately 55%. The lignin in that system, however, was part of a physical mixture and was not influenced by directly bonded cellulose or carbohydrate. In stark contrast, the data in Table III of this paper portray the relaxation profile of lignin which is part of an overall matrix dominated by cellulose and other carbohydrates at the molecular level. The T_{1H} values of the lignin resonances listed decrease not by a factor of approximately 2, but rather by a factor of approximately 10 when comparing the systems at the highest and lowest levels of Fe^{3+}. In the earlier work, the differences in proton relaxation values between the lignin and PGP could be attributed to the fact that these constituents exhibited differential affinity for iron.[16] The Fe^{3+} was more strongly complexed by the carbohydrate, leaving very little iron in the lignin portion of the mixture, thereby resulting in less rapid relaxation of this component. The particle size in the mixtures used in the earlier study precluded rapid spin diffusion from lignin to PGP. The lignin examined in this report is an actual portion of the PGP structural matrix at the molecular level. Thus, the iron affecting the carbohydrate comprising the PGP must be closer in proximity to the lignin and aromatic wood pulp substituents that are being examined. Additionally, the presence of the carbohydrate appears to communicate

Table IV
Cellulase-Treated *Piceae glauca* Pulp Component T_{1H} Relaxation Values[a]

	^{13}C chemical shift[b]			
	150	135	75	56
T_{1H}	41.2	40.6	42.8	40.1

[a] ms ± 5%. [b] ppm.

paramagnetic relaxation to the lignin components of the PGP. In the absence of associated carbohydrate, the transmission of paramagnetic relaxation to lignin is markedly diminished. Lignin–carbohydrate association appears to be evident in these solid-state studies, and paramagnetic Fe^{3+} is an excellent probe in examining interactions between such structures, particularly as a function of proton spin diffusion and relaxation mechanisms. However, even at the highest concentrations of Fe^{3+}, the proton spin–lattice relaxation times are still somewhat inhomogeneous, indicating that ferric binding or association to the wood pulp matrix is localized and site specific, corroborating our previous report.[16]

^{13}C CPMAS of Enzymatically Treated *Piceae glauca* Pulp. Figure 1C depicts the standard ^{13}C CPMAS NMR spectrum of cellulase-treated PGP. A remarkable, qualitative similarity is noticeable in the resolvable ^{13}C chemical shifts when Figure 1B is compared with Figure 1C. Due to their nonprotonated nature (OCH_3 excepted), the interrupted decoupling technique seems to differentiate the noncarbohydrate components without any chemical or enzymatic degradation of the cellulose. Table IV lists the proton spin–lattice relaxation times determined for the cellulase-treated sample. Note the convergence of the T_{1H} values for all of the measurable carbon types. The T_{1H} values are homogeneous in all cases based on their calculated uncertainties. These results show that all of the components of the treated wood are in close proximity in the solid state. Another very interesting observation centers around the fact that the proton spin–lattice relaxation times in Table IV have converged to the T_{1H} value for the methoxyl carbon nuclei as presented in Table III. This result suggests the possibility that phenylmethoxy functional groups are associated with the site of bond formation and stabilization of lignin–carbohydrate complexes. Further investigation, however, is necessary to clarify this point. However, the analytical data (Table I) indicate that the methoxyl concentration more than doubles when comparing the untreated PGP to the cellulase-treated PGP. Methyl or methoxyl groups, which can undergo rapid propeller-like rotation even in motionally rigid solids, can readily dominate the proton relaxation times of a material. Consequently, the convergence of T_{1H}'s (Table IV) may indicate that the methoxyl groups act as the primary source of proton relaxation in lignin. As the concentration of such groups increases with a simultaneous decrease in nonbound cellulose, the residual material becomes more and more physically homogeneous, as do the proton spin–lattice relaxation times. Additionally, since the enzymatically degraded PGP was extracted with boiling H_2O and filtered, the homogeneity of the residual material was further enhanced in that holocellulose and small molecules were removed at that point. The question arises as to whether the convergence of the T_{1H}'s as a function of enzymatic degradation is merely a fortuitous occurrence. Such an event is unlikely in that our previous report showed that in physical, nonconnected mixtures, the T_{1H} values of lignin and PGP carbohydrate did not coalesce due to large particle size geometry within the matrix, and the respective proton spin–lattice relaxation times were significantly different (lignin T_{1H} = 164 ms, carbohydrate T_{1H} = 78.8 ms).[16] Furthermore, although the signal-to-noise ratio was low for the low-shielding region of the spectrum at approximately 150 ppm (Figure 1A), the T_{1H} value was calculated to be 40.6 ± 20% ms. Comparison of this T_{1H} value with those for the 150–155 and 55 ppm spectral regions in Table III (at the lowest Fe^{3+} concentration of 153 ppm) and Table IV indicates that all of the relaxation times are within experimental error. Thus, the proton spin–lattice relaxation times in the cellulase-treated sample approach a uniform value which is in agreement with the relaxation properties of the nondigestible portion of the PGP. These data also further support the viability of examining complex materials by combining the interrupted decoupling experiment with proton spin–lattice relaxation. Previously cited studies have shown that enzymatic degradation of lignin-bound cellulose is inhibited by the carbohydrate's association with lignin.[4,5,15] The prominent peak in Figure 1C at 75 ppm shows that residual carbohydrate is present even after treatment of the wood pulp with enzyme. The exhaustive digestion of the PGP samples with cellulase resulted in the removal of nonbound cellulose and the enzymatic degradation of its internal β-1,4 linkages. The NMR spectrum (Figure 1C), however, clearly shows evidence of carbohydrate which is most likely not linked in a β-1,4 manner, but rather is connected by some other means to the aromatic or lignin matrix. The unique T_{1H}'s of this material, as seen in Table IV, are consistent with the premise that upon removal of nonbound or peripheral cellulose, proton spin diffusion between the lignin and carbohydrate is complete and homogeneous. The results provide evidence for the first, direct observation of lignin–carbohydrate complexes in the solid state, and corroborate previous indirect evidence for the existence of such species in nature. Furthermore, the single-valued T_{1H}'s of Table IV do indeed show that association of lignin with carbohydrate contributes to the stability and structure of lignocellulosic polymers.

Registry No. Lignocellulose, 11132-73-3.

References and Notes

(1) Hemmingson, J. A.; Leary, G. J.; Miller, I. J.; Thomas, W. A.; Woodhouse, A. D. *J. Chem. Soc., Chem. Commun.* **1978**, 92–93.
(2) Leary, G. J.; Sawtell, D. A.; Wong, H. *Holzforschung* **1983**, *37*, 11–16.
(3) Brežny, R.; Kovač, P. *Chem. Zvesti* **1980**, *34*, 136–143.
(4) Schaefer, J.; Sefcik, M. D.; Stejskal, E. O.; McKay, R. A.; Hall, P. L. *Macromolecules* **1981**, *14*, 557–559.
(5) Pew, J. C. *Tappi* **1957**, *40*, 553–558.
(6) Košiková, B.; Polčin, J.; Joniak, D. *Holzforschung* **1973**, *27*, 59–64.
(7) Merewether, J. W. T.; Samsuzzaman, L. A. M.; Calder, I. C. *Holzforschung* **1972**, *26*, 180–185.
(8) Merewether, J. W. T.; Samsuzzaman, L. A. M.; Cooke, R. G. *Holzforschung* **1972**, *26*, 193–197.
(9) Bartuska, V. J.; Maciel, G. E.; Bolker, H. I.; Fleming, B. I. *Holzforschung* **1980**, *34*, 214–217.
(10) Pines, A.; Gibby, M. G.; Waugh, J. S. *J. Chem. Phys.* **1973**, *59*, 569.
(11) Lowe, I. J. *Phys. Rev. Lett.* **1959**, *2*, 285.
(12) Kessemeier, H.; Norberg, R. E. *Phys. Rev.* **1967**, *155*, 321.
(13) Andrew, E. R. *Prog. Nucl. Magn. Reson. Spectrosc.* **1971**, *8*, 1.
(14) Schaefer, J.; Stejskal, E. O. *J. Am. Chem. Soc.* **1976**, *98*, 1031.
(15) Kolodziejski, W.; Frye, J. S.; Maciel, G. E. *Anal. Chem.* **1982**, *54*, 1419–1424.
(16) Pfeffer, P. E.; Gerasimowicz, W. V.; Piotrowski, E. G. *Anal. Chem.* **1984**, *56*, 734–741.
(17) Sullivan, M. J.; Maciel, G. E. *Anal. Chem.* **1982**, *54*, 1606.
(18) Mehring, M. "High Resolution NMR Spectroscopy in Solids"; Springer-Verlag: Berlin, 1976; p 138.
(19) Demco, D. E.; Tegenfeldt, J.; Waugh, J. S. *Phys. Rev. B* **1975**, *11*, 4133–4151.
(20) Cheung, T. T. P.; Yaris, R. *J. Chem. Phys.* **1980**, *72*, 3604–3616.

(21) Schaefer, J.; Sefcik, M. D.; Stejskal, E. O.; McKay, R. A. *Macromolecules* **1981**, *14*, 280-283.
(22) Schaefer, J.; Stejskal, E. O.; Steger, T. R.; Sefcik, M. D.; McKay, R. A. *Macromolecules* **1980**, *13*, 1121.
(23) Opella, S. J.; Frey, M. H. *J. Am. Chem. Soc.* **1979**, *101*, 5854.
(24) Dorris, G. M.; Gray, D. G. *J. Colloid Interface Sci.* **1979**, *71*, 93-106.
(25) Dorris, G. M.; Gray, D. G. *Cellulose Chem. Technol.* **1978**, *12*, 721-734.
(26) Atalla, R. H.; Gast, J. C.; Sindorf, D. W.; Bartuska, V. J.; Maciel, G. E. *J. Am. Chem. Soc.* **1980**, *102*, 3249.
(27) Earl, W. L.; VanderHart, D. L. *J. Am. Chem. Soc.* **1980**, *102*, 3251.
(28) Earl, W. L.; VanderHart, D. L. *Macromolecules* **1981**, *14*, 570.
(29) Robert, D.; Gagnaire, D. *Int. Symp. Wood Pulping Chem.* **1981**, *1*, 86-88.
(30) Maciel, G. E.; Kolodziejski, W.; Bertran, M.; Dale, B. *Macromolecules* **1982**, *15*, 686.
(31) Horii, F.; Hirai, A.; Kitamaru, R. *Polym. Bull.* **1982**, *8*, 163-170.
(32) Nimz, H. H.; Nemr, M. *J. Wood Chem. Tech.* **1982**, *2*, 371-382.
(33) Taylor, M. G.; Deslandes, Y.; Bluhm, T.; Marchessault, R. H.; Vincendon, M.; Saint-Germain, J. *Tappi* **1983**, *6*, 92-94.
(34) Sullivan, M. J.; Maciel, G. E. *Anal. Chem.* **1982**, *54*, 1615.
(35) Haw, J. F.; Maciel, G. E. *Anal. Chem.* **1983**, *55*, 1262-1267.
(36) Abragam, A. "The Principles of Nuclear Magnetism"; Oxford University Press: London, 1961.
(37) Spiess, H. W. "Rotation of Molecules and Nuclear Spin-Relaxation in NMR: Basic Principles and Progress"; Springer-Verlag: Berlin, 1978; p 55.
(38) Goldman, M. "Spin Temperature and Nuclear Magnetic Resonance in Solids"; Oxford University Press: London, 1970.
(39) VanderHart, D. L.; Garroway, A. N. *J. Chem. Phys.* **1979**, *71*, 2773-2787.
(40) Opella, S. J.; Frey, M. H.; Cross, T. A. *J. Am. Chem. Soc.* **1979**, *101*, 5856-5857.
(41) Alemany, L. B.; Grant, D. M.; Pugmire, R. J.; Alger, T. D.; Zilm, K. W. *J. Am. Chem. Soc.* **1983**, *105*, 2133-2141 and references therein.
(42) Alemany, L. B.; Grant, D. M.; Pugmire, R. J.; Alger, T. D.; Zilm, K. W. *J. Am. Chem. Soc.* **1983**, *105*, 2142-2147 and references therein.
(43) Alemany, L. B.; Grant, D. M.; Alger, T. D.; Pugmire, R. J. *J. Am. Chem. Soc.* **1983**, *105*, 6697-6704 and references therein.
(44) Lüdemann, H.-D.; Nimz, H. *Biochem. Biophys. Res. Commun.* **1973**, *52*, 1162.
(45) Himmelsbach, D. S.; Barton, F. E., II. *J. Agric. Food Chem.* **1980**, *28*, 1203-1208.
(46) Sjöström, E. "Wood Chemistry Fundamentals and Applications"; Academic Press: New York, 1981; pp 56-81 and references therein.
(47) Preston, R. D. "The Molecular Architecture of Plant Cell Walls"; Wiley: New York, 1952; pp 71-90.
(48) Hodge, A. J.; Wardrop, A. B. *Nature (London)* **1950**, *165*, 272-273.
(49) Mark, H. *J. Phys. Chem.* **1940**, *44*, 764-787.
(50) Frey-Wyssling, A.; Mühlethaler, K.; Wyckoff, R. W. G. *Experientia* **1948**, *6*, 475-476.
(51) Rånby, B. G. *Acta Chim. Scand.* **1949**, *3*, 649-650.
(52) Zumbulyadis, N. *J. Magn. Reson.* **1983**, *53*, 486-494.
(53) Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

A New Method To Characterize Curing of Epoxy with Aromatic Diamines by Azochromophore Labeling†

In-Joo Chin and Chong Sook Paik Sung*

Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received April 17, 1984*

ABSTRACT: When diepoxide is cured with aromatic diamine, primary amine reacts with the epoxide ring to form a secondary amine and eventually a tertiary amine. Due to the significant differences in the reactivities of primary and secondary amines, the major reaction products will be the four species H_2NRNHR'' (A), $R''HNRNHR''$ (B), $R''HNRNR''_2$ (C), and $R''_2NRNR''_2$ (D), where $R'' = CH_2CH(OH)\sim$. In order to obtain the quantitative composition of these four species during cure, we used a very small amount of *p,p'*-diaminoazobenzene (DAA) as a label in the DGEBA–DDS epoxy (diglycidyl ether of bisphenol A–diaminodiphenyl sulfone). The reactivities of DDS and DAA appear to be similar so as to allow us to follow the curing as manifested by the UV–vis spectral changes of DAA. As epoxy is cured, λ_{max} of the $\pi \rightarrow \pi^*$ transition corresponding to the azo bond of DAA shows bathochromic shifts in a way that provides spectral discrimination for the products A–D. On the basis of predicted λ_{max} positions and with the assumption of equal extinction coefficients among the four products, deconvolution of the spectra shows increasing concentration of C and D (branch points and cross-links, respectively) after gelation and increasing concentration of cross-links after extensive curing.

The structure and properties of epoxies are known to strongly depend on the extent of cure and of physical aging which has taken place after the cure cycle is completed. A number of physicochemical techniques have been used or developed toward a better characterization of cure and physical aging phenomena in epoxies. Among them are such techniques as FT-IR spectroscopy,[1] thermal analyses,[2] GPC[3,4] (size exclusion chromatography), microdielectrometry,[5] torsional braid analyses,[6] ^{13}C solid-state NMR,[7] thermally stimulated current measurement,[8] fluorescence,[9] and ESR spectroscopy.[10] While these experimental techniques provide useful information on the extent of cure and on epoxy structure, there are certain limitations and disadvantages associated with each technique. For example, FT-IR fails to monitor later stages of cure when the epoxy peak disappears.[11] The use of GPC and of size exclusion chromatography is limited to the early stages of the curing reaction. Fluorescence and ESR techniques mainly measure decreasing mobilities of the label with increasing cure and viscosity but can shed some light on the reaction products. For example, when a nitroxide monoamine is reacted with epoxy, Brown and Sandreczki[12] recently observed different ESR spectra which may allow spectroscopic monitoring of the initial addition products, if the reactivity of the nitroxide is similar to that of a diamine.

^{13}C MAS–CP (magic angle spinning–cross polarized)

† This paper is dedicated to Professor W. H. Stockmayer on the occasion of his 70th birthday with admiration and gratitude.

* Present adress: Department of Chemistry, Institute of Materials Science, University of Connecticut, Storrs, CT 06268

0024-9297/84/2217-2603$01.50/0