Measurement of Cross-Relaxation Effects in the Proton NMR of Water in Fibrous Collagen and Insoluble Elastin

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ABSTRACT: ^{1}H NMR relaxation measurements are reported for fibrous collagen and insoluble elastin hydrated by exposure to atmospheres of controlled humidity. Water proton magnetizations of these samples are monitored with the Goldman–Shen pulse sequence, which indicates fast and slow relaxation rates. These rates are analyzed for the spin–lattice relaxation parameters of the protein and water and the transfer rates between the water and protein protons. The cross-relaxation rates are shown to be related to the correlation times of the bound water molecules and the inverse of the sixth power of the distance between the water protons and the macromolecule protons.

Introduction

It is hardly necessary to emphasize the importance of water as a constituent of biological systems, but analyzing its behavior is complicated by the diversity of its interactions with the components of the cellular and extracellular matrix. Nuclear magnetic resonance relaxation is one of the direct methods that has been used to characterize the dynamics of water adsorbed on different tissues.[1] Recent research using this method has included magnetic interactions between the water protons and protein protons in an attempt to more completely describe the observations.[2,3] By studying a variety of tissues maintained at the same relative humidity, Renou et al.[4] concluded that the parameters derived from cross-relaxation measurements could distinguish collagens with different amounts of cross-linking. Here we systematically characterize the dynamics and structure of water bound to proteins. The present work asks two questions: first, are the dynamics of bound water dependent on protein composition and structure; second, can cross relaxation rates be related to the distances of bound water molecules to the protein surface? We chose proteins of diverse composition (collagen and elastin) to determine the effects of varying amounts of hydrophilic and hydrophobic side chains on the macromolecular surface. In addition, insoluble structural proteins were selected in an attempt to minimize the contributions of bulk molecular motion and thus allow us to concentrate on the dynamics of the water at the water–protein interface.

Materials and Methods

Sample Preparation. Fibrous collagen prepared by the method of Komanowsky et al.[5] was freed of residual lipid by extraction with 50/50 (v/v) chloroform/methanol in a Soxhlet apparatus for 48 h. The extracted material was dried in a vacuum oven at 313 K and stored in a dessicator over silica gel. Insoluble elastin was prepared from calf ligamentum nuchae by the procedure of Partridge et al.[6] Water was removed by exchanging with ethanol, followed by critical point drying. The material was stored in a dessicator over silica gel. Collagen and elastin samples for NMR measurement were hydrated by exposure to the same atmospheres of controlled humidity in closed containers. Water contents were determined gravimetrically after allowing the samples to come to constant weight. Portions of each sample were transferred to 5-mm NMR tubes and stored in the appropriate relative humidity chamber until the tubes were closed just before NMR measurement.

NMR Measurements. The NMR relaxation measurements were carried out at 399.78 MHz with a JEOL GX 400 spectrometer system that included a 9.4-T Oxford narrow bore magnet and a DEC LSI 11/23 data system. Measurements were carried out at the temperature of the probe, 294 K, which varied less than 1 K over any given day.

Relaxation time measurements were made on resonance with the $90°_x$-t_0-$90°_{\bar{x}}$-τ-$90°_x$ sequence of Goldman and Shen;[7] t_0 is a fixed delay permitting the protons in the macromolecule to dephase (70 μs), and τ is a variable delay. The free induction decay (FID) from the protons of the water was measured 70 μs after the end of a 6-μs 90° pulse, and 16 repetitions were accumulated for each τ value using 1024 data points and a 100-kHz observation window. The data were Fourier transformed with a 200-Hz broadening factor, and the area of the water peak was measured. Spin–spin relaxation measurements were made on resonance with the $90°$-τ-$180°$-τ pulse sequence of Hahn.[8] Examination of the T_2 values presented in Table I reveals that they are comparable to the 90° and 180° pulse lengths, which is expected to systematically lengthen the transverse relaxation times. If the effect of the longer pulses were taken into account, the maximum increase would be 18 μs, with the largest percentage change occurring for relaxation times at the lower water contents. This correction was not considered in calculating the values reported in Table I.

Data Analysis. The computer analysis reported in this study was carried out with a nonlinear regression program adapted for a Mod Comp computer.

Results and Discussion

Representative transformed data for hydrated elastin acquired after the third pulse of the three pulse sequence appear in Figure 1. In these spectra the amplitude of the water signal is moderate for the shortest delay, decreasing rapidly through cross-relaxation to a minimum at $\tau \sim 10$ ms. Subsequently the signal increases slowly as a result of spin–lattice relaxation.

A representative data set resulting from the $90°_x$-t_0-$90°_{\bar{x}}$-τ-$90°_x$ sequence is plotted in Figure 2 as h, the integrated water signal, vs. the delay τ. The initial area of the water signal is referred to as $h(0)$, $h(\infty)$ is the equilibrium value it would attain in the absence of spin–lattice relaxation, and $h(eq)$ is the value after a pulse separation that is long compared to the longitudinal relaxation time. The relaxation curves for all the triple-pulse experiments were fitted by means of a nonlinear least-squares program to eq 1, derived by Renou et al.[4]

$$h(\tau) = (h(0) - h(\infty)) \exp(-R_f\tau) + (h(\infty) - h(eq)) \exp(-R_s\tau) + h(eq) \quad (1)$$

The parameters R_f and R_s (where R_f and R_s are the fast and slow relaxation rates) and the T_2 values measured in the spin–echo experiments are presented in Table I. Before proceeding with analysis of the relaxation data, we first observe that the two proteins exhibit very different affinities for water and agree with commonly held notions that collagen is the more hydrophilic protein while elastin is the more hydrophobic.

Table I
Parameters Obtained from Least-Squares Fit of Relaxation Data[a]

rel humidity, %	collagen				elastin			
	wt % H_2O	R_f, s^{-1}	R_s, s^{-1}	T_2, μs	wt % H_2O	R_f, s^{-1}	R_s, s^{-1}	T_2, μs
15	4.12	734 (95)	0.84 (0.05)	43 (2)	2.10	1066 (112)	1.33 (0.05)	71 (1)
31	6.17	475 (40)	0.73 (0.04)	70 (2)	3.96	435 (30)	1.17 (0.05)	181 (4)
47	10.91	249 (9)	0.94 (0.03)	130 (3)	7.11	302 (16)	1.15 (0.04)	268 (5)
66	15.05	146 (11)	1.32 (0.08)	286 (5)	8.60	206 (8)	1.18 (0.04)	418 (11)
79	20.76	108 (8)	1.65 (0.12)	513 (7)	11.54	198 (16)	1.34 (0.09)	566 (18)
90	28.90	57 (5)	1.69 (0.14)	1066 (28)	17.29	142 (15)	1.42 (0.15)	893 (21)

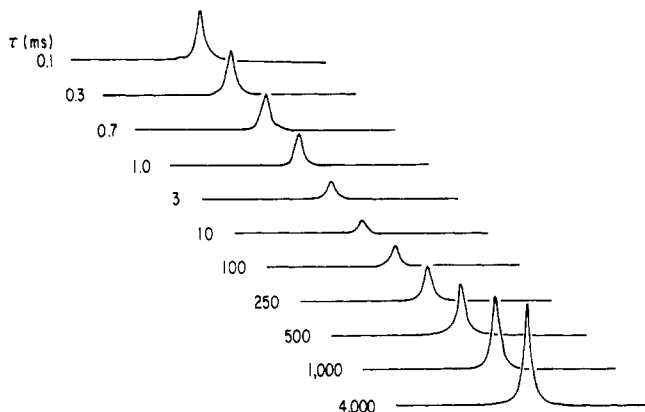[a] The estimated standard deviations are given in parentheses.

Figure 1. ^1H NMR spectra from hydrated elastin (3.96 g H_2O/100 g elastin) obtained at 400 MHz and 294 K for $90°_x$-t_0-$90°_{\bar{x}}$-τ-$90°_x$ sequence.
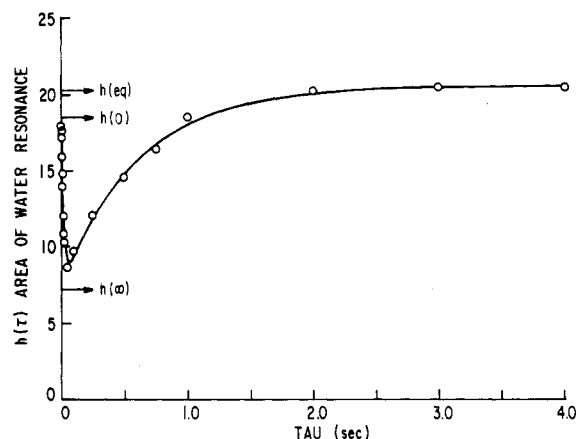

Figure 2. Areas of the water resonance plotted with respect to τ for hydrated collagen (28.9 g H_2O/100 g collagen).

The cross-relaxation model formulated by Edzes and Samulski[2] provides the most effective framework for analyzing proton spin–lattice relaxation in a heterogeneous system. In their model the water protons and macromolecule protons constitute two separate spin systems that interact magnetically. The normalized magnetizations, $m_i(t)$, of these spin systems behave according to the coupled differential equations

$$dm_w/dt = -(R_{1w} + k_w)m_w + k_w m_c \qquad (2a)$$

$$dm_c/dt = -(R_{1c} + k_c)m_c + k_c m_w \qquad (2b)$$

where R_{1w} and R_{1c} are the water macromolecule spin–lattice relaxation rates, while k_w and k_c are the rates of cross-relaxation for each component. The mole fractions of water and macromolecule protons are p_w and p_c, respectively, and satisfy the condition $p_w k_w = p_c k_c$. The solutions of eq 2 are given below and indicate that the relaxation behavior for either component is represented by a sum of two exponential decays characterized by the two apparent re-
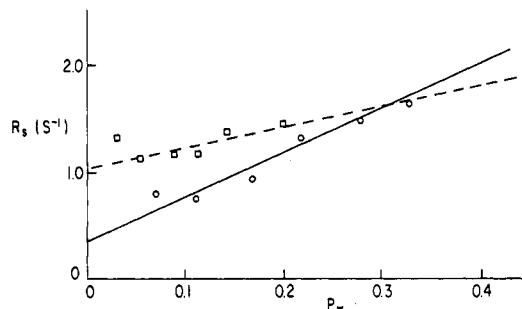

Figure 3. Dependence of the slow relaxation rates, R_s, for collagen (O) and elastin (□) upon the fraction of water protons, p_w. The molecular weights used for collagen and elastin were 2.89×10^5 and 7.20×10^4 Da, respectively. The number of protons per mole in each were 5.57×10^4 and 5.39×10^3.

Table II
Parameters Obtained in Linear Regression of R_s and p_w

protein	R_{1w}, s^{-1}	R_{1c}, s^{-1}
collagen	4.46 ± 1.56[a]	0.44 ± 0.39[a]
elastin	2.98 ± 1.38[a]	1.02 ± 0.20[a]

[a] Variances given at the 95% confidence interval.

laxation rates R_f and R_s. These rates are the same as those in eq 1.

$$m_i(\tau) = c_i^+ \exp(-R_f\tau) + c_i^- \exp(-R_s\tau) \qquad (3a)$$

$$2R_{f,s} = R_{1w} + R_{1c} + k_w + k_c \pm$$
$$[(R_{1w} - R_{1c} + k_w - k_c)^2 + 4k_w k_c]^{1/2} \qquad (3b)$$

where

$$c_i^\pm = \pm m_i(0)\frac{R_{1c} - R_{f,s}}{R_f - R_s} \pm \{m_i(0) - m_j(0)\}\frac{k_i}{R_f - R_s} \qquad (3c)$$

In eq 3b the second term is added for R_f and substracted for R_s.

The model parameters R_{1w}, R_{1c}, and k_w are extracted in the following manner based on the conclusions of Zimmerman and Brittin,[9] which is subject to the condition $k_w > R_{1w}$ and $k_c > R_{1c}$:

$$R_s \simeq p_w R_{1w} + p_c R_{1c} \qquad (4a)$$

$$\simeq p_w R_{1w} + (1 - p_w)R_{1c} \qquad (4b)$$

Therefore, when R_s is plotted with respect to the fraction of water protons, R_{1w} and R_{1c} can be obtained from the intercepts. Manipulation of eq 3b and application of the condition $p_w k_w = p_c k_c$ yield

$$k_w = [R_f + R_s - (R_{1w} + R_{1c})]/(1 + p_w/p_c) \qquad (5)$$

The slow rate data for collagen and elastin were submitted to linear regression analysis and spin–lattice relaxation parameters determined. The parameters are presented in Table II, and the plots along with the linear fits in Figure

Table III

rel humidity, %	collagen			elastin		
	wt % H_2O	k_w, s^{-1}	τ_c, ns	wt % H_2O	k_w, s^{-1}	τ_c, ns
15	4.12	677	1530	2.10	1030	922
31	6.17	418	934	3.96	407	362
47	10.91	203	506	7.11	270	244
66	15.05	111	229	8.60	170	156
79	20.76	75	128	11.54	165	116
90	28.90	35	61	17.29	110	73

3 show differences that could be a reflection of the diverse nature of the two proteins. First, consider the relaxation rates for the protein component, R_{1c}. Andrew, Bryant, and Cashell[10] carried out a proton NMR spin–lattice relaxation investigation of several well-characterized proteins in powder form and concluded that methyl group reorientation is the major source of proton relaxation (about 70%), with the remaining 30% attributed to side-chain reorientations, segmental motions, NH_3 rotations, and whole body motions. As a test of the significance of methyl group reorientation in determining the proton spin–lattice relaxation in elastin and collagen, the measured values have been compared to ones computed on the basis of the model of Kalk and Berendsen.[11] Their model attributed the spin–lattice relaxation rate R_{1c} of all protein protons (in the limit of long molecular correlation time and high frequency) to the average R_1 for the methyl protons multiplied by the ratio of the number of methyl protons to the total number of protons.

Using their model and assuming a τ_r of 10^{-10} s for methyl group reorientation, we computed an average R_1 of 4.03 s^{-1} for the methyl protons. Multiplying this rate by the fraction of protons in methyl groups yields calculated proton relaxation rates of 0.47 s^{-1} and 1.26 s^{-1} for collagen and elastin, respectively. These values compare favorably with the measured rates reported in Table II and demonstrate the usefulness of extrapolation at R_s values to p_w = 0 for estimating protein proton relaxation times. Although the variances, calculated at the 95% confidence limit, do not permit us to distinguish between the collagen and elastin experimental values, we feel they are representative.

In addition to the spin–lattice relaxation rates for the protein, proton relaxation rates for the water, R_{1w}, are also provided by this method of analysis. Beyond the observation that the R_{1w} values for the water in elastin and collagen are made up predominantly of contributions from protein-associated water molecules, any attempt to interpret the measurements in terms of any of the proposed models[12] seems inappropriate.

The cross-relaxation rates, k_w, are the final parameters obtainable for this model. They were computed from eq 5 and are presented in Table III for the two proteins studied. Since cross-relaxation involves the transfer of spin energy across the phase boundary between water and macromolecules, it is expected that the measured k_w values would provide direct information on the molecular dynamics of water molecules bound at the macromolecular surface. At low water contents, water molecules are expected to be more tightly bound, to facilitate spin diffusion, and thus to lead to large cross-relaxation rates. At higher water contents cross-relaxation is expected to be inhibited because the water is more loosely bound. These conclusions are borne out by the data in Table III.

The relaxation of a system of coupled water protons and coupled protein protons is too complex for rigorous treatment, so one is required to rely on approximations based on the behavior of a spin pair. Using Solomon's theory[13] of spin pairs, Kalk and Berendsen were able to

show that the cross-relaxation rate between spins i and j is given by

$$k = \frac{1}{10} \frac{\gamma^4 \hbar^2}{r_{ij}^6} \left[\tau_c - \frac{6\tau_c}{1 + 4\omega_0^2 \tau_c^2} \right] \qquad (6)$$

where r_{ij} is the distance between spins i and j and τ_c is the effective correlation time of the i–j interproton vector. If i is taken to be a proton of a bound water molecule and j a proton at the macromolecular surface, eq 6 can be used to compute the cross-relaxation rate, k_w, knowing the distance of the water molecule from the biopolymer surface and the effective correlation time. Alternatively, the effective correlation times could be determined by an independent procedure and eq 6 used to determine r_{ij}. This latter idea is pursued in this paper, using the transverse relaxation times of the bound water for estimating the correlation times.

We have used the BPP[14] theory rather than some of the more complicated models to extract correlation times from the transverse relaxation times. In this model the equation relating the molecular reorientation time of the water to its transverse relaxation rate is

$$R_2 = \frac{1}{T_2} = \frac{3}{20} \frac{\gamma^4 \hbar^2}{r^6} \left[3\tau_c + \frac{5\tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{2\tau_c}{1 + 4\omega_0^2 \tau_c^2} \right] \qquad (7)$$

where all the parameters are as previously described. With r = 1.60 Å (determined from the O–H distance in ice) and ω_0 = 2.5 × 10^9 s^{-1}, it is found that $\omega_0 \tau_c \gg 1$ for all transverse relaxation times in Table I; therefore T_2 is inversely proportional to τ_c alone. The correlation times are therefore easily calculated and are given in Table III. It can be seen that the water correlation times are always longer in collagen than in elastin although the free energy of the water is the same in both proteins (samples on the same line in the table were equilibrated with water at the same partial pressure). The systematic lengthening of the transverse relaxation times mentioned in Materials and Methods would result in comparable systematic decreases in correlation times, but as their relative differences are unaffected the observations made above are unaltered. Thus the entropy of the water is lower in collagen than in elastin. It is also to be noted, based on the work of Bone and Pethig[15] that the first two lines of Tables I and III provide data on the first presumed BET monolayer of water.

In the following analysis the water correlation times are taken equal to the effective correlation times of the interproton vectors between the protons of the water and the protein protons at the biopolymer surface, an assumption we justify on the grounds that the water molecules move relative to a large fixed protein matrix. This results in $\omega_0 \tau_c$ again being much greater than 1 and k_w being a linear function of τ_c only. This behavior is illustrated in Figure 4 for the two proteins studied. Determining the slopes of these two lines leads to interproton distances between water and protein of 2.25 and 1.93 Å
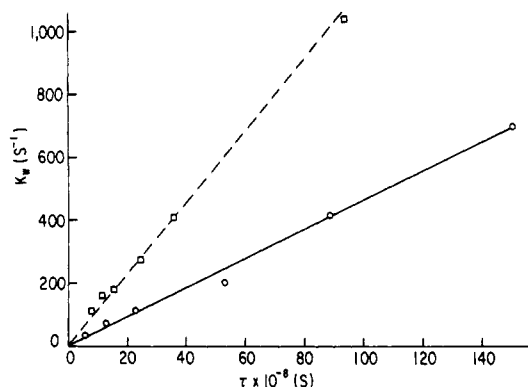
Figure 4. Dependence of the cross-relaxation rates, k_w, for collagen (O) and elastin (□) upon the correlation times of the bound water.

for collagen and elastin, respectively. If the systematic shortening of the correlation times resulting from pulse widths comparable to the spin–spin relaxation times is considered, the slopes increase but the interproton distances decrease only marginally. The recalculated distances become 2.12 and 1.86 Å. A second factor that may modify these results is the influence of the protein protons on the transverse relaxation times of the water. Edzes and Samulski[2] show that this contribution cannot exceed $^5/_2$ k_w. Including this further increases the T_2 of the water and consequently decreases the correlation times, shortening the calculated protein–water distances to 2.10 and 1.77 Å for the collagen and elastin. The effects of these corrections are neither to alter the relative slopes of the cross-relaxation curves of water on the two proteins nor to significantly change the values of the interproton distances.

We now examine these data in light of a model proposed by Ramachandran and Chandrasekharan,[16] in which tightly bound water molecules constitute an integral part of the three-dimensional structure of collagen. In this model, in addition to hydrogen bonds between the polypeptide strands, adjacent strands of the triple helix are held by two types of bridging water molecules. Both of these bridges occur on the surface of the triple helix: one joins carbonyl groups of adjacent strands; the other bridges carbonyls and NH groups of neighboring strands. The contact distances between the protons of these water molecules and other protons in the collagen strands were reported in ref 16, and the average distance is 2.22 Å. This is in surprisingly good agreement with the value of 2.25 Å determined from the cross-relaxation rates and correlation times of the present study.

Structural information with regard to the location of water in elastin is virtually nonexistent. The only data available are derived exclusively from single-crystal studies of oligopeptides[17] representative of sequences present in elastin. Water molecules have been located in these model systems, but the coordinates of their hydrogens have not been determined. Therefore it is impossible to calculate contacts between these protons and those in the peptide as was done in collagen. From our data we calculate a protein–proton distance of 1.93 Å. This is less than twice the van der Waals radius of a H atom, but distances as small as 1.90 Å have been inferred from the collagen fiber structure.[16] It seems paradoxical that collagen binds water more tightly than elastin, yet the water–protein distance is greater than elastin. We suggest that the tight binding of water to the collagen restricts it to positions more distant from the protein protons. In this view the water around elastin is less rigidly structured and can sample regions

nearer to the protein protons.

The model proposed here for interpreting cross-relaxation rates leads to physically reasonable water proton–protein proton distances and correlation times and might therefore be of use in determining the consequences of cross-linking in collagen fibrils. Subject to the restriction that the water correlation times satisfy the condition $\omega_0\tau_c \gg 1$, cross-relaxation rates of partially hydrated proteins will be proportional to the correlation times of the bound water and inversely proportional to the distance between the protons on the surface of the protein and the water. Therefore decreases in k_w reflect either decreases in τ_c or increases in the bound water–protein distance. In their study of epimysial tissues taken from animals of increasing ages (and increasingly cross-linked) Renou et al.[4] observed decreases in water cross-relaxation rates, implying either that the water was more mobile (decreasing τ_c) or its average distance from the protein surface became greater. A priori it is impossible to predict how cross-linking, which can be both intermolecular as well as intramolecular in collagen, will effect the dynamics of bound water.

Conclusions

Cross-relaxation plays a very important role in the proton relaxation of heterogeneous two-component spin systems, the magnetization of each component exhibiting two apparent relaxation rates of widely differing magnitudes. This phenomenon is conveniently investigated with the Goldman–Shen pulse sequence, which clearly separates the relaxation processes of the water into its fast and slow components.

The fast component of the relaxation process is dominated by the rate of transfer of magnetization between the bound water molecules and the protons of the protein surface and is termed the cross-relaxation rate. This rate is determined by both dynamic and structural aspects. The dynamic aspect is established by the correlation times of the water molecules and the structural one by the interaction between the water protons and biopolymer protons. By measuring this structural factor it is possible to calculate the average interproton distance between the water and protein surface.

When cross-relaxation is rapid the slow component of relaxation is found to correspond to an average of the proton relaxation rates for the protein and water components weighted by the fraction of protons in the two components. Thus measurement of the slow relaxation rates at varying fractions of water protons allows the determination of the protein relaxation rate. These rates were measured for collagen and elastin and are shown to be dependent on the number of methyl protons that act as relaxation sinks for the remainder of the protein protons.

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Effect of Water in the ^{13}C CP/MAS NMR Spectrum of White Spruce Wood

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ABSTRACT: Improved resolution and signal-to-noise ratio were observed for the ^{13}C CP/MAS NMR spectra measured for white spruce wood saturated with water, in comparison to that measured for dry wood. Variable contact time experiments illustrated that the cross-polarization step of the CP/MAS NMR technique was more efficient for the wet sample and that the presence of water had a larger effect on the carbohydrate component than the lignin component of the wood.

Introduction

Extensive studies have been performed on the analysis of cellulose macromolecules of varying crystallinity and conformation by using the ^{13}C CP/MAS (cross-polarization/magic angle spinning) NMR technique.[1-9] In some cases,[10-12] it was noted that the hydration of cellulosic materials resulted in line narrowing of the NMR spectra. The recent study by Horii et al.[10] illustrated how the presence of excess water in samples of cotton increased the resolution of some of the lines in the ^{13}C CP/MAS NMR spectrum. The decrease in the line widths was attributed to relaxation effects where a more ordered structure was possible due to the presence of water. Similar hydration effects were noted in the ^{13}C CP/MAS NMR spectra of 1,3-β-D-glucans[11] and various native cellulosic materials.[12]

There were two objectives for the present study. Firstly, the effect of hydration on the ^{13}C CP/MAS NMR spectrum of a sample of white spruce wood was investigated to determine if the lignin component would undergo the same line narrowing as that observed for cellulose. Secondly, a variable contact time analysis of wet and dry white spruce was performed to give further insight to the observed hydration effects.

Experimental Section

Sample Preparation. In preparation for the NMR measurements, air-dried white spruce (*Picea Glauca*) chips were ground to pass 40 mesh in a Wiley mill. Samples saturated with water were prepared by introducing approximately 0.5 mL of distilled water to 250 mg of ground wood. The effect of the extent of hydration was not investigated in the present study. Such a study was performed by Horii et al.,[10] who had observed a maximum effect of hydration using saturated samples. As a result, only saturated samples were used in the present investigation.

NMR Measurements. The ^{13}C CP/MAS NMR spectra were obtained by using a Bruker CXP 200 NMR spectrometer, operating at resonance frequencies of 200 and 50.3 MHz for protons and carbon-13 nuclei, respectively. Spin-locked cross-polarization was used to establish the single contact Hartmann-Hahn con-

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dition.[13] A delay time of 1 s was used between successive sampling pulses. For a 20-kHz spectrum, between 8000 and 10000 scans were accumulated for each experiment. Phase alternation was used throughout the signal acquisition to minimize base-line and intensity artifacts.

The magic angle setting was adjusted by monitoring the ^{79}Br spectrum of potassium bromide.[14] This was facilitated by adding 20–50 mg of KBr to each sample before packing the Kel-F rotor. A spinning rate of 2.5 ± 0.5 kHz was used in all NMR measurements. The chemical shifts were measured relative to tetramethylsilane (Me$_4$Si) using benzene as an internal reference.

Variable contact time experiments were performed by using an automation program available on CXP NMR software. The contact times ranged from 0.01 to 6 ms.

Results

Figure 1 illustrates the ^{13}C CP/MAS NMR spectra obtained for dry and wet white spruce at a contact time of 1 ms. The peaks at 150 and 56 ppm correspond to the C$_3$, C$_4$ aromatic carbons and methoxyl groups of lignin (**1**), respectively. The peak at 105 ppm corresponds to the C$_1$ anomeric carbon of cellulose (**2**). For both cellulose and
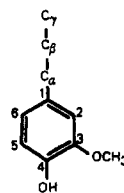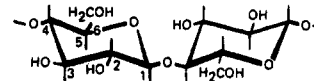
1   **2**

hemicellulose, the C$_4$ carbons appear at 85 and 89 ppm, the C$_6$ carbons at 62 and 66 ppm, and the C$_2$, C$_3$, C$_5$ carbons at 74 and 77 ppm. The acetyl methyl carbon of hemicellulose appears at 22 ppm.

By comparing the two spectra in Figure 1, it can be seen that the resolution of the peaks is far better for the wet sample than the dry one. This is particularly true for the carbohydrate peaks. Due to the convolution of these peaks, it is not possible to accurately measure the line widths. As a result, an assessment of the resolution enhancement can only be made qualitatively.