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CP/MAS ^{13}C NMR Study of Cellulose and Cellulose Derivatives. 1. Complete Assignment of the CP/MAS ^{13}C NMR Spectrum of the Native Cellulose

Hiroyuki Kono,[†] Shunji Yunoki,[‡] Tamio Shikano,[‡] Masashi Fujiwara,[‡] Tomoki Erata,*[,‡] and Mitsuo Takai[‡]

Contribution from the Bruker Biospin Co., Ltd., Tsukuba, Ibaraki 305-0051, Japan, and Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, Hokkaido 060-8628, Japan

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Abstract: The precise assignments of cross polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectra of cellulose I_α and I_β were performed by using ^{13}C labeled cellulose biosynthesized by *Acetobacter xylinum* (*A. xylinum*) ATCC10245 strain from culture medium containing D-[1,3-^{13}C]glycerol or D-[2-^{13}C]glucose as a carbon source. On the CP/MAS ^{13}C NMR spectrum of cellulose from D-[1,3-^{13}C]glycerol, the introduced ^{13}C labeling were observed at C1, C3, C4, and C6 of the biosynthesized cellulose. In the case of cellulose biosynthesized from D-[2-^{13}C]glucose, the transitions of ^{13}C labeling to C1, C3, and C5 from C2 were observed. With the quantitative analysis of the ^{13}C transition ratio and comparing the CP/MAS ^{13}C NMR spectrum of the *Cladophora* cellulose with those of the ^{13}C labeled celluloses, the assignments of the cluster of resonances which belong to C2, C3, and C5 of cellulose, which have not been assigned before, were performed. As a result, all carbons of cellulose I_α and I_β except for C1 and C6 of cellulose I_α and C2 of cellulose I_β were shown in equal intensity of doublet in the CP/MAS spectrum of the native cellulose, which suggests that two inequivalent glucopyranose residues were contained in the unit cells of both cellulose I_α and I_β allomorphs.

Introduction

The crystal structure of the native cellulose (cellulose I) has continuously been investigated by CP/MAS ^{13}C NMR spectroscopic analysis. On the NMR spectra of cellulose I, the line splittings and the spectral features greatly differ from species to species,[1] and the number of the resonance lines of cellulose is much more than that of carbons of the glucopyranose unit composing cellulose chains because cellulose I is a composite of two distinct crystalline phases, namely cellulose I_α and I_β.[2,3] In similar polysaccharides such as chitin, a fibrous β-(1→4)-linked *N*-acetyl glucosamine polymer, the number of resonance lines of chitin agrees with the number of carbons in the *N*-acetyl glucopyranose residue. Each chitin resonance has already been assigned to its corresponding carbon. In the case of the CP/MAS ^{13}C NMR spectrum of cellulose I, the ^{13}C chemical shift ranges for C1, C4, and C6 have been well assigned;[4] however, the exact origin of each multiplet component is not well understood. For instance, I_α has a singlet in C1 and C6 and a doublet in C4, while I_β has all doublets. Cael et al.[5] explained the line splittings on the CP/MAS^{13}C NMR spectrum of the cellulose I as arising from the linear combinations of the CP/MAS^{13}C NMR spectra of two different allomorphs having either two-chain or eight-chain unit cells. On the other hand, recent electron diffraction studies[6−8] have revealed that cellulose I_α can be assigned to the allomorph having a 1-chain triclinic unit cell and that I_β can be assigned to another allomorph defined as the Meyer−Misch type[9,10] having a 2-chain monoclinic unit. Carbons C2, C3, and C5 show a cluster of resonances at 70−80 ppm in the CP/MAS ^{13}C NMR spectrum of cellulose I; the assignment of these resonances has not been made yet. For elucidating the structures of both allomorphs I_α and I_β, it is thus necessary to assign the CP/MAS ^{13}C NMR spectra of the allomorphs completely.

Recently, the site-specifically labeling technique with ^{13}C and ^{15}N has been widely applied for the structural analysis of biopolymers such as proteins by NMR spectroscopy. In the case

* To whom all correspondence should be addressed. Tel/Fax: +81-11-706-6566. E-mail: erata@dove-mc.eng.hokudai.ac.jp.
[†]Bruker Biospin Co., Ltd.
[‡]Hokkaido University.

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of cellulose, Kai et al.[11,12] and Arashida et al.[13] succeeded in the ^{13}C labeling of bacterial cellulose by using site-specifically ^{13}C labeled glucose as a carbon source of *A. xylinum* and elucidating the biosynthetic process of cellulose from glucose on the basis of the transition of ^{13}C labeling by NMR spectroscopic analysis. During the biosynthesis of cellulose from the site-specifically ^{13}C-labeled glucose, the migration of the ^{13}C labels was documented. It was shown that the cellulose is synthesized not only directly from glucose but also from resynthesized glucose, which is produced in some specific metabolic pathways such as the pentose phosphate cycle.

In this study, we applied the ^{13}C-labeled cellulose biosynthesized in the culture containing D-[2-^{13}C]glucose or D-[1,3-^{13}C]glycerol as a carbon source by *A. xylinum* ATCC10245 for the complete assignment of CP/MAS ^{13}C NMR spectra of cellulose I_α and I_β. The final metabolic distribution of labeled cellulose sites that arise from singly labeled glucose was determined by quantitative solution-state ^{13}C NMR measurements of glucose. The latter was obtained by complete hydrolysis from the biosynthesized ^{13}C-labeled cellulose. A knowledge of this distribution enabled us to interpret the CP/MAS ^{13}C NMR spectra of celluloses. In the work presented herein, we use this information to assign completely the spectra of both the I_α and I_β allomorphs of cellulose I.

Experimental Section

^{13}C Labeling Sources. D-[2-^{13}C]Glucose and D-[1,3-^{13}C]glycerol (Isotec Inc., Ohio) were used as the labeling sources of bacterial cellulose. The isotopic purity of the both compounds was 99%.

Preparation of *Cladophora* and Tunicate Celluloses. Air-dried *Cladophora sp.* cut into small pieces was soaked with 1% aqueous HCl overnight at ambient temperature, washed thoroughly with water, heated at 60 °C for 1 h in a 1% NaOH solution, and finally washed thoroughly with water. The whole procedure was repeated twice. The lipid extraction from the cellulose specimen was carried out with acetone and followed by bleaching with 4% $NaClO_2$ solution under acidic condition at ambient temperature for 24 h. The completely white particles were sampled and washed thoroughly with water, followed by freeze-drying. The purified *Cladophora* cellulose was incubated in 40% H_2SO_4 solution at 60 °C for 1 h to hydrolyze the amorphous part of the cellulose sample. It was then filtered and washed thoroughly with a continuous stream of cold water, followed by freeze-drying.

The tunicate cellulose was prepared from commercially available tunicate (*Halocynthia sp.*) by removing the interior organs manually. Tunicate cellulose was obtained by purification of the outer skin cut into small pieces similar to *Cladophora* cellulose.

Preparation of ^{13}C-Labeled Cellulose. A 0.5 mL sample of preculture of *A. xylinum* ATCC10245 was added to 15 mL of HS medium[14] containing 7.5 mol % of D-[2-^{13}C]glucose in unlabeled D-glucose or D-[1,3-^{13}C]glycerol in place of D-glucose, and the culture was incubated at 28 °C for 7 days. The formed cellulose pellicle was purified by boiling in 1% (w/v) aqueous NaOH for 8 h, washed with distilled water, and freeze-dried. After the purified cellulose pellicle was cut with a scissors into small pieces, the cellulose sample was incubated in 40% H_2SO_4 solution at 37 °C for 8 h to hydrolyze its noncrystalline region. It was then filtered and washed thoroughly with a continuous stream of cold water, followed by freeze-drying.

Determination of ^{13}C-Labeling Intensity at Each Carbon of the ^{13}C-Labeled Cellulose. After the purification, 10 mg of the ^{13}C-labeled cellulose was hydrolyzed completely in 10 mL of 50 mM sodium acetate buffer, pH 5.0, containing 10 mg of *Trichoderma viride* (*T. viride*) cellulase ONOZUKA R-10 (Yakult Pharmaceutical Industry Co. Ltd., Japan) at 45 °C for 10 h. After the enzymatic reaction was stopped by heating at 100 °C for 5 min, the mixture was filtered off. d-Glucose in the filtrate was purified twice with HPLC using a TSK gel amide-80 column (25 × 300 mm, Tosoh Co., Japan) and dried under vacuum. The quantitative-mode ^{13}C NMR spectrum of the purified glucose in deuterium oxide was recorded at 23 °C with a Bruker MSL-400 spectrometer (Bruker Co., Germany) at 100.6 MHz for ^{13}C by a nonnuclear Overhauser effect gated decoupling with a pulse-repetition time 10.0 s, and with up to 5000 transients. The ^{13}C chemical shifts were referenced to internal dimethyl sulfoxide-d_6, 39.50 ppm.

Annealing Treatment. After the cellulose sample was annealed in glycerol at 260 °C for 30 min,[15] the sample was thoroughly washed with water and freeze-dried. The annealed specimen was used as I_β-rich cellulose for the CP/MAS ^{13}C NMR measurement.

CP/MAS ^{13}C NMR Measurements. The CP/MAS ^{13}C NMR measurements were performed at 23 °C with a Bruker MSL-300 spectrometer equipped with a solid-state high-resolution apparatus. The operating frequencies of proton and carbon are 300.13 and 75.47 MHz, respectively. The conventional CP/MAS method was used for high-resolution solid-state ^{13}C measurements. The rotors which contain the cellulose samples were spun at ca. 3.2 kHz, and the 90° pulse, contact time, and repetition time were 4 μs, 1.5 ms, and 4 s, respectively. ^{13}C Chemical shifts were calibrated through the carbonyl carbon resonance of glycine as an external reference at 176.03 ppm and converted to the values from tetramethylsilane (TMS). The obtained data were transferred to the PC for the line fitting. Nonlinear least-squares methods were engaged for line-fitting with the Lorentzian function previously described.[15]

For the quantitative discussion of ^{13}C resonances of the CP/MAS ^{13}C NMR spectra, the $T_{CH}-T_{1\rho(H)}$ measurements were also performed. The contact times in the CP/MAS measurements were varied from 100 μs to 100 ms. The obtained ratio of $T_{CH}/T_{1\rho}$ as from 0.01 to 0.1, which means that rather quantitative discussions are possible for cellulose when the contact time is properly chosen.

Results and Discussion

Derivation of Subspectra of Cellulose I_α and I_β Fractions. One of the reasons that makes the analysis of the solid-state NMR spectrum of the native cellulose confusing is that the native cellulose always appears as a composite of I_α and I_β allomorphs and noncrystalline regions.[2,3] The almost pure I_α and I_β celluloses can be obtained from the cell walls of *Glaucocystis*[16] and the outer skin of tunicate,[17] respectively; however, the completely pure I_α and I_β cellulose have not been obtained from any species of plants and animals. Although the $I_\alpha \rightarrow I_\beta$ transformation of cellulose could be performed by an annealing treatment in various organic solvents,[15] aqueous 0.1 N NaOH solution,[4,18] or helium gas,[15] a part of the I_α phase was unmodified. It is, however, necessary to obtain the respective CP/MAS ^{13}C NMR spectrum of the pure I_α and I_β allomorphs for the assignment of the CP/MAS ^{13}C NMR spectrum of cellulose I. In this experiment, therefore, we have derived the respective subspectra of I_α and I_β phases from the

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original spectrum with simple mathematical treatment such as a linear combination of spectra of I_α-rich and I_β-rich cellulose, as following:

$$\text{subspectrum } (I_\alpha \text{ phase}) = \text{spectrum } (I_\alpha\text{-rich}) - a \times \text{spectrum } (I_\beta\text{-rich})$$

and

$$\text{subspectrum } (I_\beta \text{ phase}) = \text{spectrum } (I_\beta\text{-rich}) - b \times \text{spectrum } (I_\alpha\text{-rich})$$

where constants *a* and *b* indicate the content of the I_α phase in I_β-rich cellulose and that of the I_β phase in I_α-rich cellulose, respectively. In general, the ^{13}C NMR spectra of cellulose I displays triplets at C1, C4, and C6 signals depending on the I_α/I_β ratio[2] and the C4 signals were used for line-shape analysis to measure the fraction ratio of the I_α allomorph.[19] The constants *a* and *b* were, therefore, determined by optimization of line-fitting at C4 signals. Parts a and b in Figure 1 show the CP/MAS ^{13}C NMR spectra of *Cladophora* cellulose (I_α-rich) and that of cellulose after the annealing treatment (I_β-rich), respectively. For the derivation of the subspectra of pure I_α and I_β phases, ^{13}C signals from the noncrystalline regions of cellulose must be separated because all native celluloses contain crystalline and noncrystalline phases.[20] However, since the intensity of C4 signals at 84−86 ppm[19] deriving from the noncrystalline phase is considerably lower than that at 87−92 ppm[19] deriving from the crystalline phase in the spectra of both I_α-and I_β-rich *Cladophora* celluloses (Figures 1), it could be considered that the ^{13}C signals arising from noncrystalline cellulose were negligible for the derivations of the pure I_α and I_β subspectra from the I_α- and I_β-rich *Cladophora* celluloses. Figure 2a,b shows the subspectra of pure I_α and I_β phases, respectively. Here the C4 resonance line of the I_α phase and the C1, C4, and C6 lines of the pure I_β phase are all doublets, whereas the C1 and C6 resonance lines of the I_α phase are singlets in accord with the previous proposals.[2] The subspectrum of the pure I_β phase and the CP/MAS ^{13}C NMR spectrum of the purified tunicate cellulose (Figure 2c, $I_\alpha/I_\beta = 8/92$) were equivalent to each other in all line shapes of resonances, suggesting that this mathematical treatment for the derivation of subspectra of I_α and I_β phases was adequate.

The dotted lines in Figures 3a and 4a represent the results of nonlinear least-squares line-fitting analyses of the 70−80 ppm region of overlapping resonances which belong to the C2, C3, and C5 of subspectra of the I_α and I_β phases, respectively. Table 1 lists chemical shifts and integrated fractions of the respective constituent lines of all resonance lines. In this region, the subspectrum of I_α has 5 resonance lines with the intensity ratios of ca. 1:1:1:1:2 starting from the downfield side. The subspectrum of I_β has 4 lines with ratios of ca. 1:1:1:3. Since three carbons of cellulose should appear in this region, this result of the line-fitting analysis suggests that some of the C2, C3, and C5 resonances in both subspectra of cellulose I_α and I_β should also be split similar to carbons such as C4 in the spectrum of the I_α phase and C1, C4, and C6 in that of the I_β phase.

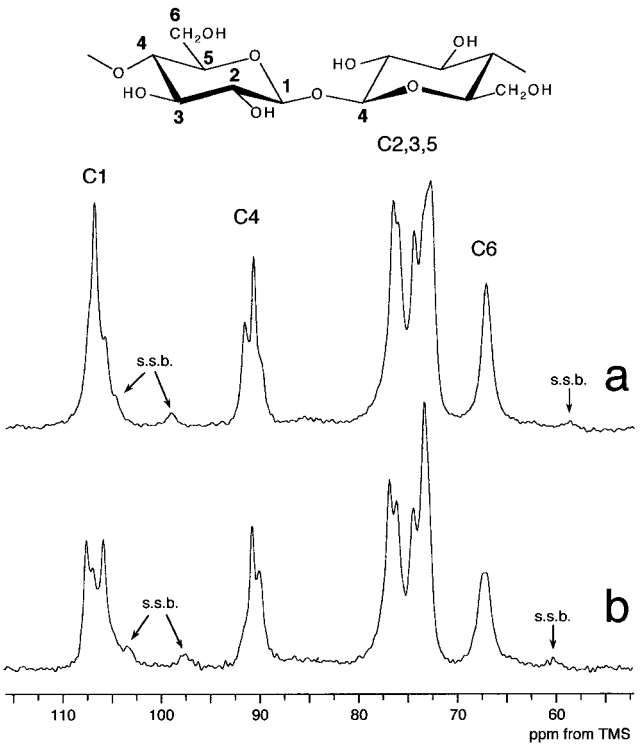

Figure 1. The CP/MAS ^{13}C NMR spectra of *Cladophora* celluloses at 75 MHz: (a) original I_α-rich cellulose; (b) I_β-rich cellulose annealed at 260 °C for 30 min. The abbreviation "s.s.b" in this figure indicates spinning sideband.

^{13}C-Labeled Cellulose Biosynthesized from D-[1, 3-^{13}C]-Glycerol. For the assignment of the C2−C3−C5 region of the CP/MAS ^{13}C NMR spectrum of cellulose I, ^{13}C-labeled cellulose was biosynthesized from D-[1,3-^{13}C]glycerol as a carbon sources for the *A. xylinum* ATCC10245 strain. Figure 5 shows the CP/MAS ^{13}C NMR spectrum of cellulose biosynthesized from the labeled glycerol (I_α-rich) and that of the labeled cellulose after the annealing treatment (I_β-rich). The resolution of the resonance lines in both the spectra is not so good; however, it is obvious that four main resonance lines were observed. With respect to the labeling of cellulose by D-glycerol, Greathouse[21] had already performed the same experiments with ^{14}C-labeled glycerol and they revealed that the introduced ^{14}C labeling was observed at positions of 1, 3, 4, and 6 of the synthesized cellulose. As shown in Figure 6, the transition of labeling was explained by neogenesis of glucose via the Embden−Meyerhof pathway, followed by formation of cellulose. In the spectra of both I_α-rich and I_β-rich cellulose biosynthesized from D-[1,3-^{13}C]-glycerol, because the main resonances observed at 106, 90, and 67 ppm have already been assigned to C1, C4, and C6, it was determined that the remaining line observed around 76 ppm should be assigned to C3 of cellulose I.

^{13}C-Enriched Cellulose Biosynthesized from D-[2-^{13}C]-Glucose. In the previous reports on the ^{13}C labeling of bacterial cellulose by use of the culture medium containing D-[2-^{13}C]-glucose, which was described by Kai et al.[11] and Arashida et al.,[13] the introduced ^{13}C labeling was observed at C1, C3, and C5 as well as C2 of cellulose synthesized by *A. xylinum* and the amounts of the ^{13}C labeling intensity markedly differed with the position of the cellulose. Figure 7 shows the CP/MAS ^{13}C

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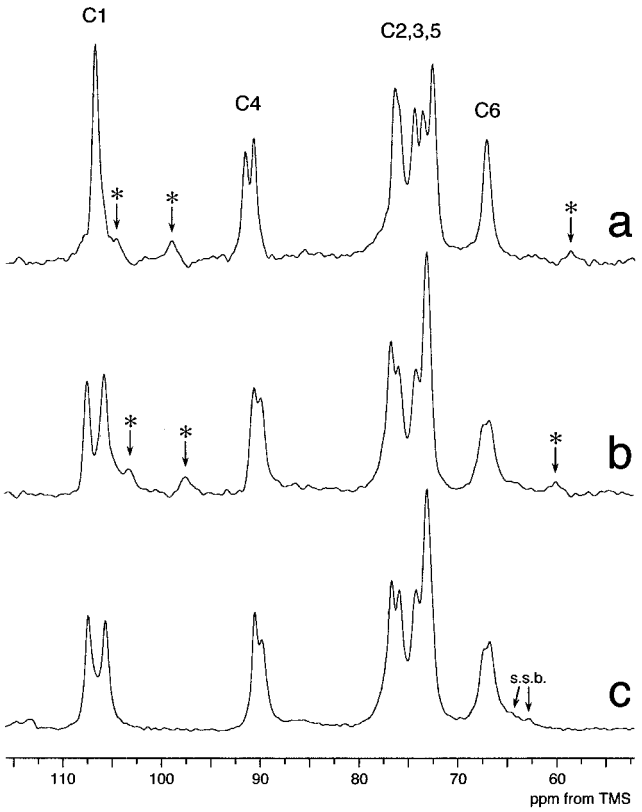

Figure 2. The CP/MAS ^{13}C NMR subspectra of celluloses samples at 75 MHz: (a) the pure I_α phase of *Cladophora* cellulose; (b) the pure I_β phase of *Cladophora* cellulose; and (c) tunicate cellulose. The abbreviation "s.s.b" in this figure (c) indicates spinning sideband. Resonance lines indicated by asterisks are artifacts, which arise from the spinning sidebands in the spectra of I_α-rich and I_β-rich celluloses (Figure 1).

Table 1. ^{13}C Chemical Shifts (δ) and Integrated Fractions (I) of the Respective Lines for the CP/MAS ^{13}C NMR Spectra of Cellulose I_α and I_β

allomorph	δ/ppm	I^a	atom
cellulose I_α	106.9	2.05	C1
	91.6	0.84	C4
	90.8	0.96	C4
	76.5	1.23	C3
	76.2	1.00	C3
	74.4	1.10	C5
	73.6	1.08	C2
	72.6	2.05	C2,5
	67.1	1.69	C6
cellulose I_β	107.6	1.07	C1
	105.9	1.16	C1
	90.6	1.09	C4
	90.0	0.93	C4
	76.8	1.20	C3
	76.0	0.95	C3
	74.2	1.14	C5
	73.0	2.73	C2,5
	67.5	0.74	C6
	66.9	0.99	C6

[a] Total of each I value observed in the respective spectrum was 12.

NMR spectra of I_α- and I_β-rich cellulose from D-[2-^{13}C]glucose synthesized by *A. xylinum*. In both spectra, the expected unenriched resonances at both C4 (90 ppm) and C6 (67 ppm) regions are insignificant, in good agreement with the previous reports,[11,13] and the spectral features at the C2−C3−C5 region (70−80 ppm) of I_α- and I_β-rich cellulose biosynthesized from D-[2-^{13}C]glucose were obviously changed in comparison with

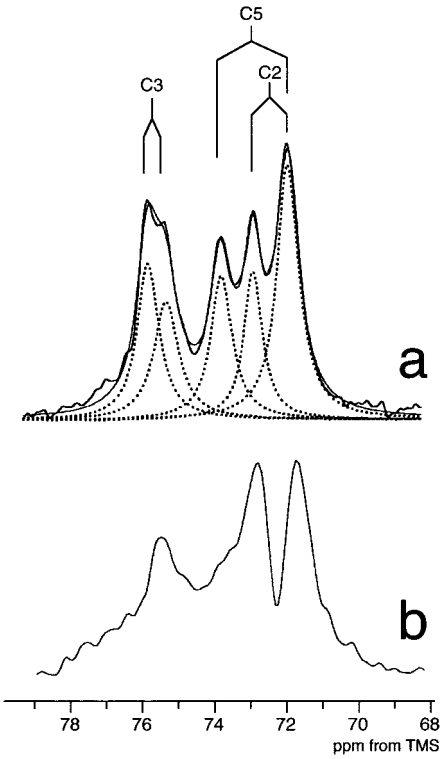

Figure 3. Expansion of C2, C3, and C5 regions of the CP/MAS ^{13}C NMR spectra of pure I_α phases of *Cladophora* cellulose and ^{13}C-enriched cellulose biosynthesized from D-[2-^{13}C]glucose: (a) I_α subspectrum of *Cladophora* cellulose and (b) I_α subspectrum of ^{13}C-enriched cellulose biosynthesized from D-[2-^{13}C]glucose. Dotted lines in this figure (a) indicated the result of the line-fitting analysis of the spectrum.

those of I_α- and I_β-rich *Cladophora* cellulose, respectively (Figure 1). To determine the precise transition of the ^{13}C labeling to each position of cellulose, the ^{13}C-labeled cellulose biosynthesized from D-[2-^{13}C]glucose was completely hydrolyzed to glucose by *T. viride* cellulase and the quantitative ^{13}C NMR measurement of the regenerated glucose was performed. Table 2 shows the ^{13}C-labeling intensities at each position of the labeled cellulose which were estimated by integrating the signal area of the resonances on the ^{13}C NMR spectrum of the regenerated glucose. Here, the amount of ^{13}C at C4 of the labeled cellulose was assumed to be only natural abundance because there are no metabolic pathways of *A. xylinum* which cause transition of labeling from C2 of glucose to C4 of cellulose.[11−13] According to this table, 13 and 52% of the introduced ^{13}C labeling was observed at the C1 and C2 positions of the biosynthesized cellulose, respectively, C3 has less population (10%), and C5 has slight population (6%). The remaining ^{13}C labeling (19%) was metabolized in the TCA cycle of the microorganism and converted to carbon-13 dioxide.[11−13] The ^{13}C intensities of each carbon resonance line of the CP/MAS ^{13}C NMR spectrum of cellulose biosynthesized from D-[2-^{13}C]-glucose should reflect the ^{13}C population ratio. It was, therefore, expected that the intensity order in the C2−C3−C5 region should be C2 > C3 > C5 in the cellulose biosynthesized from D-[2-^{13}C]glucose. Figures 3b and 4b show the subspectra of the I_α and I_β phase derived from the CP/MAS ^{13}C NMR spectra (Figure 7) of I_α- and I_β-rich cellulose from D-[2-^{13}C]glucose. In both subspectra, resonance lines around 76 ppm could be assigned already to C3 of cellulose in the experiments with D-[1,3-^{13}C]glycerol, so only C2 and C5 should exist from 70 to

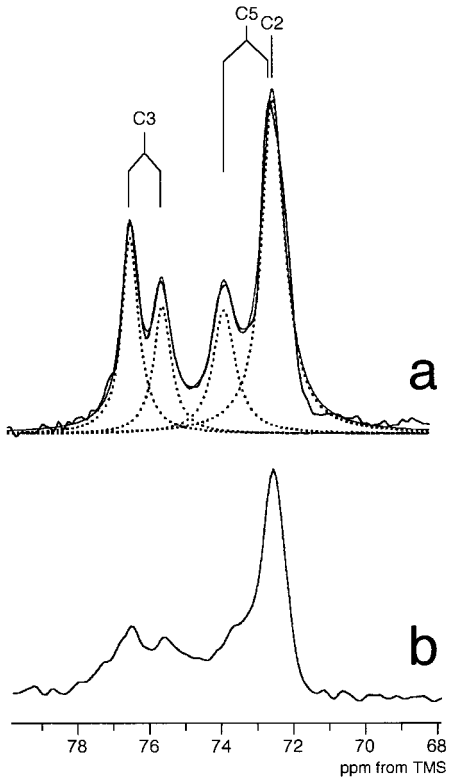

Figure 4. Expansion of C2, C3, and C5 regions of the CP/MAS ^{13}C NMR spectra of pure I_β phases of *Cladophora* cellulose and ^{13}C-enriched cellulose biosynthesized from D-[2-^{13}C]glucose: (a) I_β subspectrum of *Cladophora* cellulose and (b) I_β subspectrum of ^{13}C-enriched cellulose biosynthesized from D-[2-^{13}C]glucose. Dotted lines in this figure (a) indicated the result of the line-fitting analysis of the spectrum.

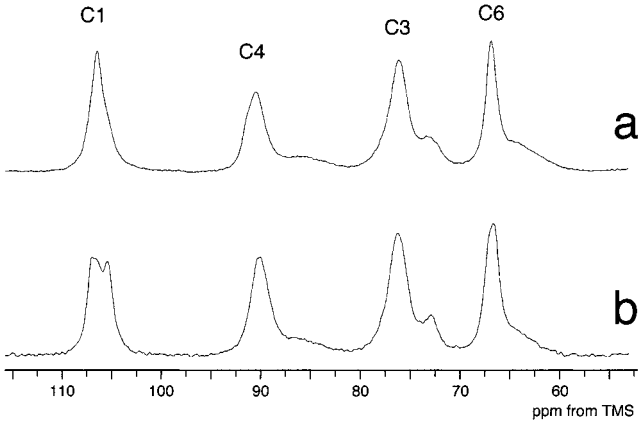

Figure 5. The CP/MAS ^{13}C NMR spectra of ^{13}C-enriched cellulose biosynthesized from D-[1,3-$^{13}C_2$]glycerol at 75 MHz: (a) original I_α-rich cellulose and (b) I_β-rich cellulose annealed at 260 °C for 30 min.

75 ppm. As already shown in Figure 3a, there are three resonance lines at 74.4, 73.6, and 72.6 ppm with the intensity ratio of ca. 1:1:2 in the region from 70 to 75 ppm in the I_α subspectrum derived from *Cladophora* cellulose, while there are two resonance lines at 74.2 and 73.0 ppm with the intensity ratio of ca. 1:3 in the I_β subspectrum. In the I_α subspectrum of cellulose from D-[2-^{13}C]glucose (Figure 3b), the resonance line at 74.4 ppm is missing and the 72.6 ppm line is losing intensity to nearly half in comparison with that of *Cladophora* cellulose, which suggested that resonance lines for C5 are split into a doublet at 74.4 and 72.6 ppm. Therefore, the remaining lines observed at 73.6 and 72.6 ppm should be assigned to C2. On

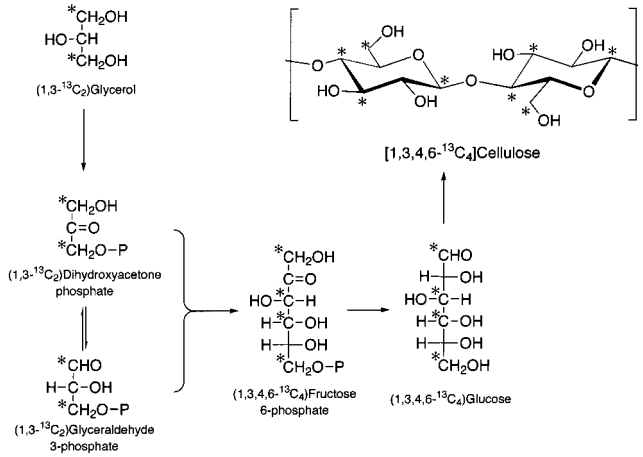

Figure 6. Transfers of ^{13}C labeling from C1 and C3 to C4 and C6 via the Embden−Meyerhof pathway.

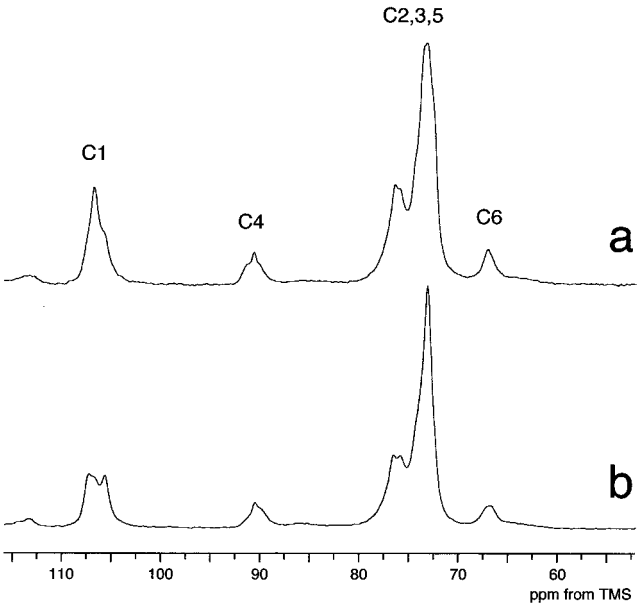

Figure 7. The CP/MAS ^{13}C NMR spectra of ^{13}C-enriched cellulose biosynthesized from D-[2-^{13}C]glucose at 75 MHz: (a) original I_α-rich cellulose and (b) I_β-rich cellulose annealed at 260 °C for 30 min.

Table 2. ^{13}C Carbon Intensity Ratio (I.R.) and Labeled Ratio (L.R.) for Each Carbon of Cellulose Obtained from D-[2-^{13}C]Glucose

	atom						
	C1	C2	C3	C4	C5	C6	total
I.R.	1.95	4.91	1.76	1.00	1.48	0.95	
L.R.[a] (%)	12.7	52.2	10.1	0	6.37	−0.69	80.7

[a] The L.R. values of each carbon in cellulose obtained from D-[2-^{13}C]glucose were calculated assuming that L.R. values of the C-4 carbon are zero, i.e., only natural abundance.

the other hand, in the case of the I_β subspectrum of cellulose from D-[2-^{13}C]glucose (Figure 4b), the resonance line at 74.2 ppm is missing compared with that of the I_β subspectrum derived from *Cladophora* cellulose (Figure 4a). In addition to this, because the ^{13}C labeling at the C5 position (6%) was less than that at the C2 position (52%), it is expected that the resonance line at 74.2 ppm should be C5 and that the remaining line at 73.0 ppm should be assigned to C2. However, since the ratio of ^{13}C labeling intensity of the resonance lines at 74.2 and 73.0

ppm is ca. 1:3 in the I_β subspectrum derived from *Cladophora* cellulose, the resonance lines for C5 of cellulose I_β are split into a doublet at 74.2 and 73.0 ppm and the latter resonance line is composed of a C2 singlet and a weaken C5 doublet. Those assignments of the CP/MAS ^{13}C NMR spectra of I_α and I_β phases are indicated in Figures 3 and 4, respectively, and summarized in Table 1.

With respect to the assignments of the complicated C2−C3−C5 region, Teeäär and Lippmaa[22] had tried indirectly to assign the C2, 3, 5 region of the CP/MAS ^{13}C NMR spectrum of cotton cellulose (I_β-rich cellulose) based on a consideration of the ^{13}C spin−lattice relaxation times. They assigned the low-field doublet at 76.8 and 76.0 ppm to C2 as the closest neighbor to the C1 doublet. They also assigned the singlet line at 73.0 ppm to C5 and the remaining line at 74.2 ppm to C3 because the 74.2 ppm relaxed faster than the other lines in the cluster region. In our assignment of I_β subspectra, on the other hand, the doublet line at 76.8 and 76.0 ppm is directly assigned to C3 by using the ^{13}C-labeled cellulose biosynthesized from D-[1,3-^{13}C]glycerol, which was also supported by the experiment of the ^{13}C-labeled cellulose from D-[2-^{13}C]glucose, and it was proved that the resonance lines for C5 were split into a doublet in both subspectra of the I_α and I_β phases.

For the assignment of the ^{13}C signal of cellulose in the solid state, Bardet et al.[23] applied the two-dimensional spin-exchange solid-state NMR technique to ^{13}C-enriched wood chips from aspen (*Populus euramericana*) grown under a 20% $^{13}CO_2$ atmosphere and observed the two broad signals around 76 and 74 ppm in the region of signals corresponding to C2, C3, and C5. They assigned the signal around 76 ppm to C2 and that at 74 ppm to C3 and C5 by comparing the intensities of the signals as a function of increasing mixing time. However, it is very difficult to assign ^{13}C signals of C2, C3, and C5 of cellulose by using the uniformly ^{13}C-enriched aspen chips because the signals of C2, C3, and C5 of cellulose and those of C2 and C3 of the poly-β-(1→4)-D-xylopyranosyl polymer, the major compounds of hemicellulose, were completely overlapped.[24] On the other hand, since bacterial cellulose used in this experiment is known to have high crystallinity and be of exceptionally high purity, no resonance lines derived from the impurities such as hemicellulose and lignin were observed. Thus, we could directly assign the signals of C2, C3, and C5 considering the intensity of the signals in the CP/MAS ^{13}C NMR spectra of the ^{13}C-enriched *Acetobacter* celluloses with selectively ^{13}C-labeled carbons.

In conclusion, the complete assignment of NMR spectra of both cellulose I_α and I_β was directly performed for the first time through the analysis of ^{13}C-labeling intensity. In the NMR spectra of both the I_α and I_β allomorphs, the majority of resonances are doublets of equal intensity. Exceptions are singlets associated with C1 and C6 in the I_α allomorph and C2 in the I_β allomorph. Hence, both the I_α and I_β phases are characterized by two equally distributed inequivalent glucose residues. If the I_α phase has only one molecule per unit cell, there must be inequivalent anhydroglucose units. This indicated that there is an inequivalence of glycosidic linkages in the I_α phase, which were confirmed by this study. With respect to the I_β phase, Atalla and VanderHart[2,20] proposed that the splitting of the C1 and C4 signals into two in the I_β phase may be due to inequivalent anhydroglucose resigues along the cellulose chain based on Raman, IR, and NMR spectroscopies. The existence of two kinds of glucose residues with different conformations in the unit cell of the I_β phase is indicated because most carbons of cellulose I_β are doublets in the solid-state NMR spectrum. Such inequivalence could possibly arise from an inequivalence of the glycosidic linkage in the I_β phase, which is in agreement with the proposal by Atalla and VanderHart.

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