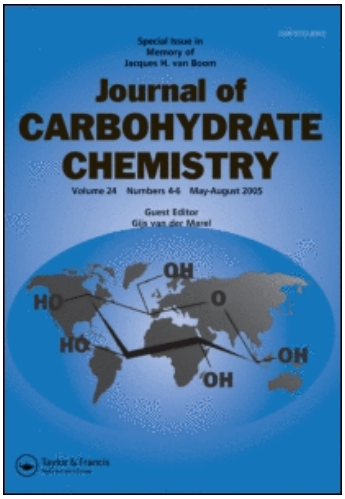

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ASSIGNMENT OF ^{13}C CPMAS NMR SPECTRA OF CRYSTALLINE METHYL β-D-GLUCOPYRANOSIDE AND SUCROSE USING DEUTERIUM LABELLING AND INTERRUPTED PROTON DECOUPLING

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ABSTRACT

The solid state ^{13}C cross polarization magic angle spinning (CPMAS) NMR spectra of partially deuterated samples of methyl β-D-glucopyranoside (1) and sucrose (2) were assigned using the spectral editing technique of interrupted proton decoupling (IPD). With the exception of the deuterium substituted CH_2OH each carbon resonance area of the IPD spectra, (after allowing for differences in magnetization) corresponded closely with the established level of deuteration at each site. A direct relationship between the level of deuteration and observed ^{13}C resonance intensity facilitated a number of ^{13}C CPMAS isotropic shift assignments without resorting to expensive and complex ^{13}C labelling. In general, the procedure is excellent for assigning deuterium exchangeable methine carbon resonances in solid state carbohydrate spectra, however, the methodology is problematic when applied to the identification of CH_2 and CH_3 resonances, which are not readily recognizable from the characteristic position of their chemical shifts.

INTRODUCTION

NMR methodology for examining the solution structures of carbohydrates has evolved to a highly sophisticated level. Two dimensional NMR can routinely give detailed information on chemical shift assignments,[1a,b] conformations and molecular structure of soluble carbohydrates,[2] however, solid state NMR is still unable to provide this level of information for oligo- and polysaccharides.[3] Our interest in cell wall biosynthesis using deuterium labelled precursors has created a need for us to establish the structure of

some of the elaborated oligosaccharide units. Such a task should be amenable to analysis with ^{13}C solid state NMR provided we could first assign the spectra. To this end we have begun to investigate convenient ways to identify isotropic ^{13}C chemicals shifts in the solid state.

In a previous study,[4a,b] we demonstrated that isotopic ^{13}C labelling could provide a means for making ^{13}C resonance assignments in cross polarization magic angle spinning (CPMAS) NMR spectroscopy. However, these experiments were extremely demanding, requiring excellent instrumental resolution and dynamic range, since the structural information was contained in the natural abundance portion of the ^{13}C spectrum. In addition, the studies were limited to simple monosaccharides such as glucose since the ^{13}C labelling methodology was very complex and costly.

In the present report we describe a novel and more convenient approach to assigning ^{13}C CPMAS spectra of carbohydrates using their partially deuterium exchanged counterparts and a spectral editing technique called interrupted proton decoupling (IPD).

RESULTS AND DISCUSSION

It is well known that protonated carbons, with the exception of methyl groups undergo dipolar dephasing, (extreme broadening) in ^{13}C CPMAS spectra when the proton decoupling is suspended, (IPD), for approximately 40 μs.[5a,b] However, carbons that are not protonated, such as quaternary carbons or carbonyls, do not interact with any directly bonded protons and therefore are not severely broadened or eliminated from the spectra.[5b] This spectral editing technique is quite useful for identifying non-protonated carbon resonances in complex ^{13}C CPMAS spectra,[6a,b] however, it still leaves many more assignments to be made. Another approach to solving the assignment problem would be to artificially introduce "non-protonation" into the molecule under study by substituting deuteriums for protons. Early work by Koch and Stuart[7a] and Balza *et al.*,[7b] and more recently detailed studies by Angyal *et al.*,[8a,b] have demonstrated that deuterium could be easily and inexpensively introduced into many different carbohydrates. In addition, it has been shown

that the amount of substitution, which varies from position to position is dependent on steric effects which control the rate of exchange at each site.[8b] We have exploited this variation in deuterium substitution as a means for identifying CPMAS-IPD generated ^{13}C resonances with corresponding intensities. Thus, by knowing the ^{2}H distribution in the carbohydrate, from standard proton solution NMR we have a reference from which to correlate and identify each solid state ^{13}C resonance.

We have chosen methyl-β-D-glucopyranoside (1) and sucrose (2) as two examples to illustrate the use of this methodology for assigning ^{13}C chemical shifts in the solid state. Figure 1 shows 4 solid state CPMAS ^{13}C spectra of 1 with and without deuterium substitution, obtained under normal CPMAS conditions and with IPD (40 μs) prior to acquisition. In Fig. 1A we observe a standard CPMAS spectrum of 1 showing its seven well resolved ^{13}C resonances. Figure 1B shows the CPMAS-IPD spectrum of 1. Observe that only the resonance representing the OCH_3 has, to any significant extent survived the dipolar interaction introduced during the 40 μs suspension of proton decoupling. This is a consequence of rapid OCH_3 rotation which modulates the C-H dipolar interaction and renders it inefficient.[5a] Figure 1C depicts the CPMAS spectrum of partially deuterated methyl-β-D-glucopyranoside (1a). The somewhat broader resonances seen in this spectrum no doubt represent a slight chemical shift perturbation, (isotopic chemical shift effect of ^{2}H on ^{13}C) introduced by partial deuteration of different sites.[9] A splitting of each resonance due to the coupling of ^{2}H to ^{13}C is also evident.[10] Figure 1D exhibits only those resonances of 1b that that have survived the 40 μs IPD period. Table 1 gives the values of ^{2}H incorporation at different sites in 1a based on the integration of the well characterized 1a proton spectrum (not shown).

In addition, this table gives the values of remaining ^{13}C signal intensities obtained from Fig. 1D, normalized for differences in cross polarization efficiencies (see experimental). Interestingly enough, although the match of ^{13}C signal intensity with ^{2}H incorporation seems to be quite excellent for carbons 1-5, carbon 6 shows a poor correspondence. This is because, unlike the methine carbons the C-6 has three isotopic states, $C^{1}H_2$, $C^{1}H^{2}H$ and $C^{2}H_2$, two of

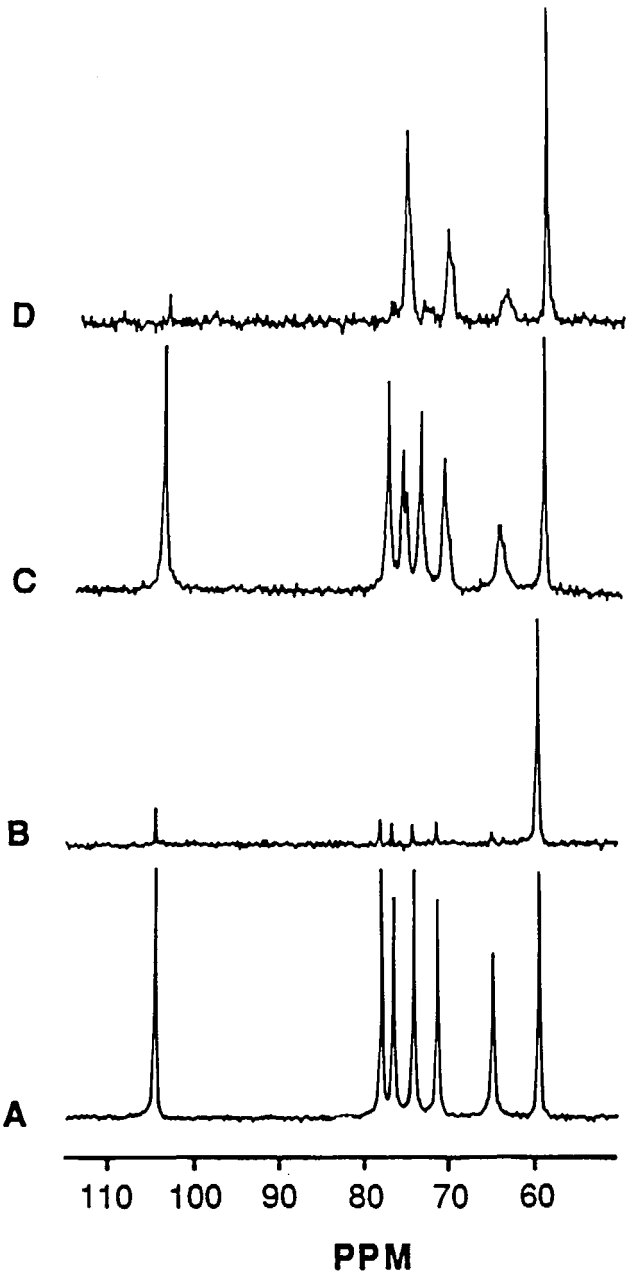

FIG. 1. A) 75.4 MHz ^{13}C CPMAS spectrum of 1, B) same as A except the pulse sequence contained a 40μs interrupted proton decoupling delay, C) same as A except the partially deuterated 1a was used, D) same as C with a 40μs interrupted proton decoupling delay.

TABLE 1. Distribution of ^{2}H Label in 1a ^{1}H Solution NMR and ^{13}C CPMAS NMR with IPD

	Percent signal lost due to ^{2}H incorporation					
^{2}H substitution site	1	2	3	4	5	6,6'
	2	18	100	53	2	55
	Relative percent signal observed in ^{13}C CPMAS-IPD[a] spectra due to ^{2}H incorporation					
^{13}C shift (ppm)	104.6	74.2	76.4	71.4	78.1	64.9
	0	20	100	56	4	35

a. Ratios of ^{13}C areas are expressed as percent. The largest deuterated ^{13}C peak in the spectrum was assigned a value of 100 based on the corresponding ^{1}H spectrum.

which are protonated. Thus, excess proton dipolar broadening derived from the "mixed" $C^{1}H^{2}H$ isotope does not allow for a true measure of the presence of ^{2}H at the C-6 methylene position in the IPD experiment.

Even though the resonances at δ 104.6 and δ 78.1 show no ^{2}H incorporation, it is obvious that the low field resonance must be attributed to C-1 while the higher field in clearly the non-exchangeable C-5.[8] All of the other ring carbon resonance areas give excellent agreement with the deuteration ratios found in the proton spectrum. The C-6 resonance is identified by default since it must be realized that anything less than a 100% ^{2}H incorporation at this site, (a CH_2OH group) will produce a less than predicted CPMAS-IPD response.

Table 2 gives the corresponding chemical shifts for 1 in both solution and solid state. Note, that unlike α and β glucose,[4b] the relative chemical shift positions, (solution vs solid) appear to show a good correspondence, however, considerably better resolution between the C-3 and C-5 resonances is achieved in the solid state spectra. Also, the C-6 resonance is found at 3 ppm

TABLE 2. Chemical Shift Correspondence for ^{13}C Solution and CPMAS Spectra of 1

	Chemical Shift ppm					
	C-1	C-2	C-3	C-4	C-5	C-6
Solution[a]	104.0	74.1	76.8	70.6	76.8	61.8
Solid[b]	104.6	74.2	76.4	71.4	78.1	64.9

a. Chemical shift assignments from ref. 9.

b. Chemical shift assignments based on the CPMAS-IPD spectrum of 1a.

lower field in the solid presumably due to differences in inter vs intramolecular hydrogen bonding in the two states.[11]

In the case of sucrose 2, the assignment of resonances in the solid state becomes a bit more complicated because of the presence of three CH_2OH resonances and the lack of variations in the ^2H content at different positions. Figure 2 shows the same sequence of CPMAS spectra for 2 and the partially deuterated derivative 2a as given in Fig. 1. The analysis of the proton spectrum of 2a indicated that there were equal amounts of ^2H distributed at C-2 and C-4 of the glucose ring, (see Table 3). Unfortunately, such a situation made it impossible to distinguish between these two positions in assigning the ^{13}C resonances at δ 69.5 and δ 75.3 as seen in Fig. 2D. However, with analogy to the solution spectrum one might guess that the lower field resonance corresponded to C-2 glucose. The resonance at δ 74.6 with 14% ^2H incorporation is evidently the fructose C-4 while the peak at δ 84.4 unmistakably belongs to fructose C-3.

Although, we cannot distinguish between the two broadened upfield CH_2OH resonances at δ 61.8 and δ 63.5, we know that they represent the partially deuterated C-6 of fructose and glucose as opposed to the C-1 resonance of fructose. This is clear because the lower field broadened CH_2OH resonance at δ 67.9 showed no resonance under CPMAS-IPD conditions and therefore, contained little or no deuterium label, in agreement with the corresponding proton spectrum[8b] (see Table 3). As we observed with 1a the partially deuterated

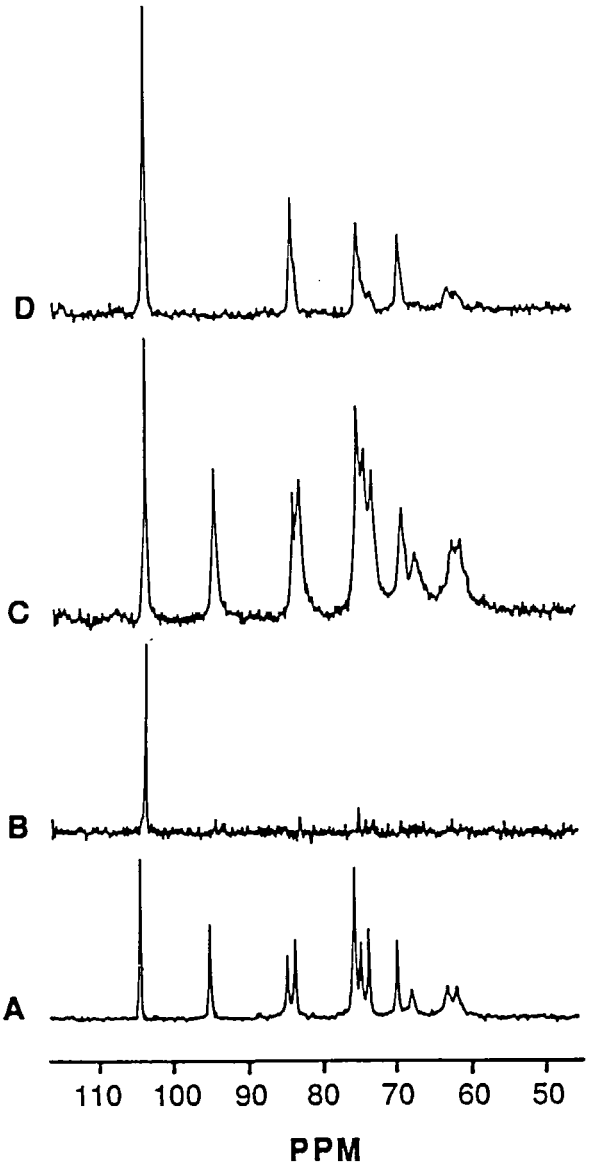

FIG. 2. A) 75.4 MHz ^{13}C CPMAS spectrum of 2, B) same as A except the pulse sequence contained a 40μs interupted proton decoupling delay, C) same as A except the partially deuterated 2a was used, D) same as C with a 40μs interrupted proton decoupling delay.

TABLE 3. Distribution of ^{2}H Label in 2a by ^{1}H Solution NMR and ^{13}C CPMAS NMR with IPD

	Percent signal lost due to ^{2}H incorporation											
	glucose						fructose					
^{1}H site	1	2	3	4	5	6,6'	1	2	3	4	5	6,6'
	0	60	0	60	0	27	2	0	82	14	0	27
	Relative percent signal observed in ^{13}C CPMAS-IPD[a] spectra due to ^{2}H incorporation											
	95.2	69.5	-	75.3	-	61.8	67.9	104.4[b]	84.4	74.6	-	63.1
$\delta^{13}C$		(75.3)		(69.5)		(63.1)						(61.8)
	0	64	0	60	0	17	0	0	82	15	0	17

a. Ratios of ^{13}C area are expressed as percent. The largest deuterated ^{13}C peak in the spectrum was assigned a value of 82 based on the corresponding ^{1}H spectrum.

b. A large signal is evident from this carbon because it is not protonated, no ^{2}H incorporation is observed at this site.

CH_2OH gave resonances at δ 61.8 and δ 63.5 which were equal but lower in response in the CPMAS-IPD spectra (Table 3) than anticipated (17% vs 27%), based on their ^{2}H content. The C-1 resonance of glucose and C-2 of fructose were readily assigned on the basis of their unique positions as well as dipolar dephasing properties, respectively in spectrum 2D.

With regard to the ^{13}C solid vs solution spectra of 2 we note in Table 4 that there are two resonances whose relative chemical shift positions do not correspond to each other. We refer specifically to the C-1 of fructose, found downfield with the C-6 of fructose in the solution spectrum. This C-1 fructose resonance is observed at a much lower field (δ 67.9) in the solid state while both C-6 resonances of glucose and fructose are found together at higher fields in the solid state. Again, differences may be due to different bond rotamer distributions in the solid and liquid states.[11] In addition, the fructose C-3 resonance is also somewhat out of place relative to its position in solution (δ 84.4 vs δ 77.5, respectively). We have left spaces in Table 4 where no

TABLE 4. Chemical Shift Correspondence for ^{13}C Solution and CPMAS Spectra of 2

	Chemical Shift ppm											
	glucose						fructose					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Solution[a]	92.9	72.6	73.5	70.1	73.3	61.0	62.2	104.4	77.5	74.9	82.2	63.2
Solid[b]	95.2	69.5	-	75.3	-	61.8	67.9	104.4	84.4	74.6	-	63.1
		(75.3)		(69.5)		(63.1)						(61.8)

a. Chemical shift assignments from ref. 9 and 12.

b. Chemical shift assignments based on the CPMAS-IPD spectrum of 2a.

assignment other than those from extrapolation of solution shifts could be made.

The ^{13}C shift assignment method described in this paper depends heavily on the ability to produce partially deuterated carbohydrates in which the deuterium is unevenly distributed throughout the molecules. In essence the identification of each ^{13}C resonance requires that it have a unique amount of label associated with it. Evidently, in choosing 2 as an example, deuterium exchange was not extensive enough as it was for 1 to provide a sufficient number of signals of unique intensity for a complete ^{13}C shift assignment. Nevertheless, this methodology can be effective for making solid state ^{13}C shift assignments of carbohydrates providing sufficient label with enough variation in its distribution is incorporated. Our future studies will concentrate on optimizing the amount and variation of deuterium incorporation in different simple as well as complex carbohydrates to clearly delineate their corresponding ^{13}C CPMAS spectra.

EXPERIMENTAL

Preparation of Methyl-β-D-glucopyranoside-d 1a and Sucrose-d 2a. Deuterium exchange over the catalyst (Raney nickel) was conducted as described

by Koch and Stuart.[7a] Commercial Raney nickel (W.R. Grace & Co., No. 2800 Raney Active Nickel Catalyst) was used; kept in the refrigerator under water, this catalyst retained its activity for at least three years. After completion of the exchange the reaction mixture was filtered, the filtrate concentrated, the residue redissolved in D_2O and the ^1H NMR spectrum was recorded.

NMR Spectroscopy. All ^1H spectra were obtained on a JEOL GX-400 NMR spectrometer operating at 9.4 T (400 MHz for ^2H). Sixty four transients of 32 K data points were recorded for each spectrum. The pulse width was 6.0 μs (90° pulse angle), the spectral width was 10 KHz and there was a delay of 5.6 s between pulses. Each spectrum was Fourier transformed after applying a 0.2 Hz line-broadening function. Chemical shifts are given in ppm relative to the TMS resonance at 0.0 ppm. Chemical shift positions were based on previous characterization.[1a,12]

The ^{13}C CPMAS spectra were obtained on a wide bore Brüker MSL-300 NMR spectrometer operating at 7.0 T (75.473 MHz for ^{13}C). The samples were spun at approximately 4K Hz (5 mm rotor). Each spectrum was obtained from 8 to 1024 transients with a 1.5 ms contact time 4k data points, a spectral width of 10 KHz and a pulse delay of 60s for 1 and 3-5s for 2. The proton 90° pulse was 4-5 μs. For the IPD spectra an inserted delay of 40 μs without proton decoupling was inserted following the cross polarization pulse for each transient.[5a] Typically, 2-3 times the number of transients were used to obtain the IPD spectra since there is a general 25-30% S/N loss relative to the normal CPMAS spectra.

In the solid state experiments the amount of deuterium incorporation was calculated by obtaining the area of each ^{13}C resonance in the IPD spectrum after normalizing it to the magnetization responses observed for the resonances in the normal CPMAS spectrum. This is an important point to remember since the cross polarization maxima varies for each carbon, depending on its T_{C-H} value.[5b] Thus, carbon resonance areas, each corresponding to a single carbon may have different values for a given selected contact time.[5b] In these experiments 5 ms was selected as the optimum contact time (see

above). No account was made for the areas of ^{13}C resonances associated with the residual protonated carbons, obtained in the CPMAS-IPD experiments since they were barely measurable relative to the observed deuterated carbon responses.

The amount of deuterium incorporated in the various positions of 1a and 2a were calculated from the loss in proton intensity of the well-established 400 MHz ^{1}H solution spectra.[1a,12] Instrumentally measured areas or cut-out and weigh methods were used to establish resonance intensities in all spectra.

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