of the reactions of carbon-centered radicals with oxygen have yielded k_{Ox} values that are lower, often considerably lower, than the correct values. Conclusions and any other rate constants that have been based on "low" k_{Ox} values may require substantial revision.

Note Added in Proof. A direct determination of the equilibrium constant and thermodynamic parameters for the gas-phase reaction, allyl + $O_2 \rightleftharpoons$ allylperoxyl, have recently been reported.[79]

(79) See: Morgan, C. A.; Pilling, M. J.; Tulloch, J. M.; Ruiz, R. P.; Bayes, K. D. *J. Chem. Soc., Faraday Trans. 2* **1982**, *78*, 1323–1330.

Acknowledgment. Thanks are due to Mr. S. E. Sugamori for technical assistance and to Dr. D. F. Williams for the loan, and advice on the usage, of the gas-mixing system. Two of us (B.M. and K.U.I.) acknowledge receipt of a NATO research grant without which the present work would not have been undertaken.

Registry No. $PhCH_2$·, 2154-56-5; Me_3C·, 1605-73-8; c-C_6H_7·, 12169-67-4; $(C_2H_5)N\dot{C}HCH_3$, 26374-14-1; $CH_3C(OH)CH_3$, 5131-95-3; *n*-Bu_3Ge·, 55321-84-1; *n*-Bu_3Sn·, 20763-88-6; O_2, 7782-44-7.

Supplementary Material Available: Tables II–XIII giving detailed kinetic data (7 pages). Ordering information is given on any current masthead page.

Secondary Isotope Multiplet NMR Spectroscopy of Partially Labeled Entities. Carbon-13 SIMPLE NMR of Carbohydrates

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Abstract: ^{13}C NMR measurements have been made in Me_2SO-d_6 solutions of some carbohydrates (methyl α-D-gluco- and galactopyranosides, melibiose, maltose, and β-cyclodextrin) in which exchangeable protons have been partially deuterated. Signals from single carbon atoms are observed as a series of multiplets (singlets to octets) with intensity ratios that vary quantitatively with OH:OD ratios. Partial deuteration of exchangeable protons in molecules permits direct observation of the different isotopomers measured under conditions of slow exchange and the resonance line separations can be analyzed in terms of the two-bond (β) and three-bond (γ) isotope effects that contribute to the deuterium-induced secondary isotope shift. Magnitudes of β and γ effects are found to vary with configuration of carbon atoms, and substitution and hydrogen bonding of hydroxyl groups. Signal multiplets and magnitudes of isotope effects are used to assign the spectra of carbohydrates as shown for both α- and β-forms of the α1-→6-linked (melibiose) and α1→4-linked (maltose) reducing disaccharides. The method also confirms the presence and direction of intramolecular hydrogen bonding in α1→4 glucosides (i.e., C2′–O2′···H–O–C3) by observation of an isotope effect on C2′ transmitted through a hydrogen bond. Measurements by NMR spectroscopy of secondary isotope multiplets of partially labeled entities (SIMPLE NMR) have widespread application for signal assignment and for studying isotope effects in molecules.

Isotope effects are well-established in NMR spectroscopy.[1] Primary isotope shifts have not been extensively studied because access to multinuclear NMR spectroscopy would normally be required; e.g., for the hydrogen atom the primary isotope shift [$\delta(^nH) - \delta(^1H)$, n = 2, 3] is determined by results of ^1H, ^2H, and/or ^3H NMR spectroscopy[2] of isotopically labeled species (isotopomers). A secondary isotope shift, $\delta(X^nH) - \delta(X^1H)$, is observed in isotopomers by NMR spectroscopy of the X nucleus (X = ^1H, ^{13}C, etc.). More work has been done with secondary isotope shifts because measurement, although depending on the availability of isotopomers, is achieved by observation of the secondary nucleus X. Use of the latter phenomenon is greatly facilitated if isotopic replacement is readily accomplished as with exchangeable protons, e.g., OH, NH, and SH groups, and application of the method depends on whether the protons are in fast or slow exchange.[3-7] For example, Feeney and co-workers[3] demonstrated that deuterium isotope effects could be observed by ^{13}C carbonyl resonances in peptides with slowly exchanging vicinal N–D bonds in a 50:50 H_2O–D_2O solution whereas Grant and co-workers[4] showed that the isotope effect can be used to differentiate between carbonyl groups associated with rapidly exchanging OH groups and those associated with slowly exchanging amide NH groups. Indeed, when the rate of proton exchange of amides in dipolar aprotic solvents is slowed down, resolution of individual $^{13}C{=}O$ signals corresponding to the $O{=}CNH_2$, O=CNHD, and $O{=}CND_2$ species is possible.[5] Similar work was attempted with carbohydrates by Vincendon and co-workers[6,7] where the hydroxyl group exchange rate was slowed down by working in dimethyl sulfoxide (Me_2SO-d_6) and the consequent broadening of carbon atoms substituted with hydroxyl groups served to identify them, though no information was derived to enable individual C–OH resonances to be assigned.

Assignment of ^{13}C NMR signals of carbohydrates[8] may be made by chemical substitution or empirical correlations[9-11] by

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Table I. Chemical Shifts of Carbohydrates in Me_2SO-d_6 Solution[a]

	C1	C2	C3	C4	C5	C6	OCH_3
glucose residues							
methyl α-D-glucopyranoside	99.87	72.18	73.54	70.49	72.66	61.25	54.56
β-cyclodextrin	101.94	72.38	73.01	81.57	72.00	59.90	
maltose (G = glucose)							
G′-Gα	100.85	72.68	73.47[b]	70.11	73.51	61.04	
G′-Gβ	100.81	72.57	73.45[b]	70.07	73.51	61.02	
G′-Gα	92.22	72.00	73.03	80.39	70.40	60.77	
G′-Gβ	96.89	74.53	76.57	79.86	75.16	60.88	
melibiose (G = glucose)							
Gal-Gα	92.29	72.26	73.12	70.94	70.32	67.29	
Gal-Gβ	96.91	74.79	76.72	70.51	74.85	67.29	
galactose residues							
methyl α-D-galactopyranoside	100.08	68.54	69.73	68.93	71.17	60.77	54.49
Gal-Gα	99.03	68.48	69.56	68.89	70.94	60.58	
Gal-Gβ	99.10	68.44	69.56	68.89	70.98	60.59	

[a] 100-MHz ^{13}C SIMPLE NMR measurements referenced to Me_4Si by using the solvent peak as a secondary reference ($\delta_{Me_4Si} = \delta_{Me_2SO} + 39.5$ ppm). [b] Chemical shift difference taken from the ^{13}C spectrum of maltose prior to partial deuteration for the C3′ and C5′ signal overlap in the latter spectrum (Figure 5) masks precise determination of C3′ chemical shifts.

selective heteronuclear spin decoupling,[12-14] by selective spin labeling using ^2H or ^{13}C[15-17], by differential isotope shifts,[18,19] or more recently, by heteronuclear two-dimensional NMR spectroscopy.[20,21] Pfeffer and co-workers[18,19] measured the deuterium-induced ^{13}C differential isotope shift (DIS) of a number of mono- and disaccharides using a dual coaxial cell with equal concentrations dissolved in H_2O (inside tube) and D_2O (outside tube) in which secondary isotope shifts are manifested by exchangeable protons in the fast exchange condition. Results from a number of compounds were analyzed statistically to determine the magnitudes of the β and γ effects (i.e., H/D–O–C^{β}–C^{γ}–C) which were used in the assignment of the ^{13}C NMR spectra of many carbohydrates as well as to correct some assignments of a number of carbohydrates previously measured. The method has limited applicability because all hydroxy-bearing carbon atoms (and some others) will appear as doublets irrespective of their environment, because magnitudes of β and γ effects have to be made from measurements on a series of closely related compounds, and because other isotope effects such as those due to hydrogen bonding are not observed.

The present work describes an approach to measuring secondary isotope shifts that overcomes all the difficulties associated with the dual coaxial tube method and has the advantages of being able to determine the signs and magnitudes of β and γ effects of individual carbon atoms. The work essentially advances that of Gagnaire and Vincendon[6] under conditions where each carbon signal is observed as a series of multiplets (singlets to octets, so far); the number of lines corresponds to the number of possible isotopomers and their degeneracies, and the intensity distribution is characteristic of the deuteration ratio. It is found that magnitudes of β and γ effects vary with molecular structure and so are useful for assignment of signals. The method is demonstrated for two monosaccharides (methyl α-D-gluco- and galactopyranosides in Me_2SO-d_6 solution) where all carbon atoms exhibit characteristic multiplets and is used for the complete assignment of the ^{13}C NMR spectra of an α1→6-reducing disaccharide melibiose, α-D-galactopyranosyl-(1→6)-α-D-glucopyranose, and an α-(1→4)-reducing disaccharide maltose, α-D-glucopyranosyl-(1→4)-α-D-glucopyranose. The results are compared with the assignments in D_2O solution observed by heteronuclear two-dimensional NMR spectroscopy.[20,21] In α1→4 glucosides (β-cyclodextrin and maltose) an extra isotope effect is observed that is transmitted through a hydrogen bond. Observation of this effect on the C2 signals confirms not only the presence but also the direction of the intramolecular hydrogen bond in these molecules.

Experimental Section

All carbohydrates were commercial samples (maltose, BDH; methyl α-D-gluco- and galactopyranosides, melibiose, and β-cyclodextrin from Sigma Chemical Co.). The samples were deuterated and dried by lyophilizing from D_2O solution and dissolved in dry Me_2SO-d_6 (sealed vials from Merck Sharpe & Dohme). The OH:OD ratio was adjusted by adding appropriate amounts of the protiated and deuterated carbohydrate. The extent of deuteration was determined independently by comparing the intensities of the H1 and OH signals using ^1H NMR spectroscopy.

Natural-abundance 50-MHz (JEOL FX 200) and 100-MHz (Brucker WH 400) ^{13}C NMR spectra were obtained at ambient temperatures (ca. 295 ± 1 K) under proton noise decoupling conditions. Typically 5000 pulses were recorded for solution concentrations of about 100 mg/mL at a recycle time of 3 s (70° pulse, 32K data points, 8000-Hz sweep width) and were calculated with adequate digital resolution (0.25 Hz point^{-1} after zero filling) and resolution enhancement.[22] Using the solvent as a secondary reference peak, it was found that the chemical shifts of the protiated compound correspond to the downfield signal of each multiplet. Observed relative intensities of signals within one multiplet depend on the known OH:OD ratio though one might expect the intensities to be affected by such factors as the resolution enhancement calculation and possible NOE effects. Trial calculations showed that the resolution enhancement function[22] did not alter the relative intensities within one multiplet and ^{13}C NMR measurements on methyl α-D-galactopyranoside under proton noise or gated decoupling conditions indicated that secondary and tertiary isotope substitution had no discernible NOE effect. Chemical shifts of the fully protiated isotopomer (Table I) were measured with respect to the residual solvent signal and referenced with respect to Me_4Si ($\delta_{Me_4Si} = \delta_{Me_2SO} + 39.5$ ppm). Observed ^{13}C multiplets were analyzed in terms of two-bond (β) and three-bond (γ) isotope effects in which negative isotope effects correspond to lower frequencies by substitution with the heavier isotope. Hence, the magnitudes of β and γ isotope effects of carbohydrates measured in this work (Table II) are all negative.

Results and Discussion

Secondary Isotope Shift Parameters (β and γ Effects). The origins of the numbers of resonance signals and their relative intensities for carbon atoms with one, two, or three isotope effects

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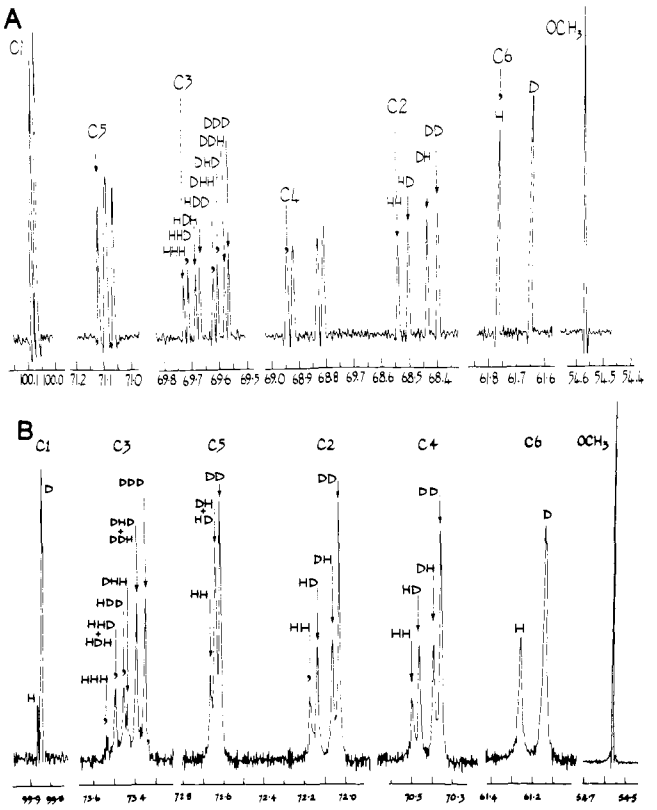
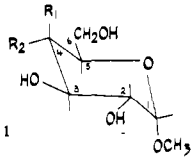


Figure 1. 100-MHz proton noise decoupled ^{13}C NMR spectra of monosaccharides with partially deuterated hydroxyl groups in dry Me_2SO-d_6 solution. (A) Methyl α-D-galactopyranoside (OH:OD, 1:1); (B) methyl α-D-glucopyranoside (OH:OD, 1:2).

of the same sign are discussed for the monosaccharides methyl α-D-galactopyranoside (I, R_1 = OH, R_2 = H) and methyl α-D-



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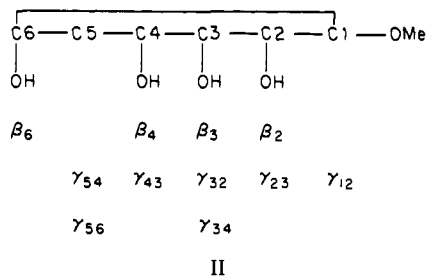
glucopyanoside (I, R_1 = H, R_2 = OH) shown in the $^4C_1$ conformation. The 100-MHz ^{13}C NMR spectra of partially deuterated (OH:OD, ca. 1:1) methyl α-D-galactopyranoside and methyl α-D-glucopyranoside (OH:OD, ca. 1:2) in Me_2SO-d_6 solutions are shown in parts A and B of Figure 1, respectively. The spectra consist of a series of multiplets with different numbers of lines and intensity ratios rather than the single resonance signals normally observed.[23,24] A summary of the possible β and γ isotope effects for each ^{13}C signal of both monosaccharides is shown diagrammatically in structure II. The notation used for individual



II

isotope effects in the present work describes the ^{13}C signal being

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Table II. Secondary Isotope Shifts (H/D) in ^{13}C NMR of Carbohydrates ($\times 10^{-3}$ ppm)[a]

	β_1	β_2	β_3	β_4	β_6	γ_{12}	γ_{21}	γ_{23}	γ_{32}	γ_{34}	γ_{43}	γ_{54}	γ_{56}	OCH_3	β'_{23}
literature[b]	110	⟵	140	⟶	150		60 (*f*)	⟵		30	⟶			60	
							30 (*e*)								
glucose units (G)															
methyl α-D-glucopyranoside		105	104	105	116	15		32	42[c]	42[c]	36	28[c]	28[c]	*d*	16
β-cyclodextrin		(93)	(89)		112	(41)		(46)	(31)		(27)		20[e]		
melibiose: Gal-Gα	97	106	102	100		15	38[c]	38[c]	42[c]	42[c]	33	27			
Gal-Gβ	105	102	102	99		20	66	36	43[c]	43[c]	35	30			
maltose: G′-Gα	96[e]	100	(95)		112[f]	16	50[c]	(50)[c]	42		40		20[e]		
G′-Gβ	105	100	(93)		108[f]	22	66	(53)	43	38[c]	34		22		
G′-Gα		100	103	102	115	(43)		36[c]	38[c]	38[c]	37	22[c]	22[c]		14
G′-Gβ		99	103	102	115	(40)		36[c]	38[c]	38[c]	38	22[c]	22[c]		14
average	97 α, 105 β	102 ± 2	103 ± 1	102 ± 2	114 ± 2	16 α, 21 β	38 α, 66 β	36 ± 1	41 ± 2	41 ± 2	37 ± 2	26 ± 3	22 ± 2		
(H bonding)		(93)	(92)			(41)		(50)							(15)
galactose units															
methyl α-D-Gal		108	105	110	118	16		39	42	15	21	26[c]	26[c]	*d*	
melibiose: Gal-Gα		108	103	109	118	15		35	36	17[e]	21	29[c]	29[c]		
Gal-Gβ		106	103	109	118	18		33	36	17[e]	21	30[c]	30[c]		
average		107 ± 1	104 ± 1	110 ± 1	118 ± 1	16 ± 1		36 ± 2	38 ± 3	16 ± 1	21 ± 1	28 ± 2	28 ± 2		

[a] 100-MHz ^{13}C NMR spectra in Me_2SO-d_6 solution; data resolution 0.003 ppm/point. All β and γ magnitudes are negative. [b] Determined in aqueous solution by using the dual coaxial cell method; accuracy ±0.004 ppm, ref 18 and 19. [c] Averaged value due to resonance degeneracies. [d] Not observed. [e] Larger error due to poorly resolved signal. [f] Error larger due to overlap of signals so omitted from average.

observed by a numerical subscript (e.g., β_3) and, when appropriate, a second numerical subscript is used for the hydroxyl group which gives rise to the isotope effect (e.g., γ_{32} and γ_{34} correspond to the three-bond isotope effects observed on the C3 signal from partial deuteration of the OH2 and OH4 groups, respectively). Each carbon atom (except C2 and C4) gives rise to a different pattern of β and γ effects which serves to assign the signals, taken in conjunction with a knowledge of the magnitudes of β (ca. 0.1 ppm) and γ (ca. 0.04 ppm) isotope effects.[18,19] In this work it is shown that variations in magnitudes of γ effects can also be used to assign the C2 and C4 signals of galactose derivatives. The behavior of carbon signals of isotopomers with zero, one, two, or three isotope effects is discussed in relation to the spectrum of methyl α-D-galactopyranoside (Figure 1A) at the 1:1 OH:OD ratio whereas the dependence of the relative intensities of resonance lines on the OH:OD ratio is discussed in relation to the spectrum of methyl α-D-glucopyranoside (Figure 1B) at the OH:OD ratio of 1:2.

Carbon atoms with no isotope effects are observed as single resonance signals as exhibited by the methyl signals of both methyl α-D-gluco- and methyl α-D-galactopyranosides in Figure 1. For carbon atoms where only one isotope effect is expected, the observed doublets for C1 and C6 corresponding to H and D isotopomers are readily analyzed to give the magnitude and sign of the isotope shift, i.e. β_6 −0.118 and γ_{12} = −0.016 ppm. For carbon atoms with two possible isotope effects (C2, C4, C5), four resonance signals are expected corresponding to the HH, HD, DH, and DD isotopomers. If the two isotope effects have different magnitudes, all four resonance signals are observed (C2 and C4) and the magnitudes of the separate isotope effects are determined from appropriate line separations, i.e., for C2, β_2 = −0.108 ppm and γ_{23} = −0.039 ppm and, for C4, β_4 = −0.110 and γ_{43} = −0.021 ppm in Figure 1A. If the two isotope effects are of similar magnitude within experimental error, the carbon atom is observed as three signals (C5, γ_{54} ca. γ_{56} ca. −0.026 ppm) and the observed signal intensities are accounted for by the degeneracy of the HD and DH isotopomers. The C5 signal is readily distinguished from the C2 and C4 signals because of the difference in magnitudes of the β and γ effects.

For carbon atoms with three isotope effects of different magnitudes, a total of eight lines are expected corresponding to isotopomers with no D substitution (HHH), one D substitution (HHD, HDH, DHH), and two (HDD, DHD, DDH) and three D atom substitution of hydroxyl groups (DDD). Depending on the magnitudes of the isotope effects, it is also possible to observe signals with seven, six, five, and a minimum of four lines where each effect has the same magnitude. The origins of the resonance signals and their possible degeneracies are shown in Figure 2 for the three isotope effect case often observed in carbohydrates in which the carbon atom has one relatively large effect (labeled β) and two relatively small effects [labeled γ and δ (the use of δ does not signify a four-bond isotope effect but rather that the third isotope effect can be distinguished from the β and γ effects)]. Examples of cases 1–4 are observed for molecules in this work. The magnitudes of the isotope effects are determined by analysis of signal separations: i.e., δ = (1–2) = (3–4) = (5–6) = (7–8), γ = (1–3) = (2–4) = (5–7) = (6–8), and β = (1–5) = (2–6) = (3–7) = (4–8). The C3 signal of methyl α-D-galactopyranoside consists of eight lines corresponding to three isotope effects of different magnitudes (i.e., Figure 2, case I: β_3 −0.105 ppm, γ_{32} = −0.042 ppm, γ_{34} = −0.015 ppm) whereas the C3 signal of methyl α-D-glucopyranoside consists of six lines because the magnitudes of γ_{32} and γ_{34} are approximately equal, resulting in degeneracies of the HHD/HDH and DHD/DDH isotopomers (case 3, Figure 2).

The relative intensity of any resonance signal is proportional to the product of the probabilities of hydroxyl groups being observed as OH or OD. For one isotope effect the intensities of the H and D isotopomer signals are in the ratio of the OH:OD deuteration as shown for the C6 signals in parts A (1:1) and B (1:2) of Figure 1. The relative intensities of signals resulting from two isotope effects depend on their relative magnitudes as well as on the deuteration ratio; e.g., the C2 and C4 signals in Figure 1B exhibit four lines in the ratio 1:2:2:4 because $\beta \neq \gamma$ whereas the C5 signal exhibits three lines in the approximate ratio 1:4:4 because the two γ effects have similar magnitudes within experimental error.

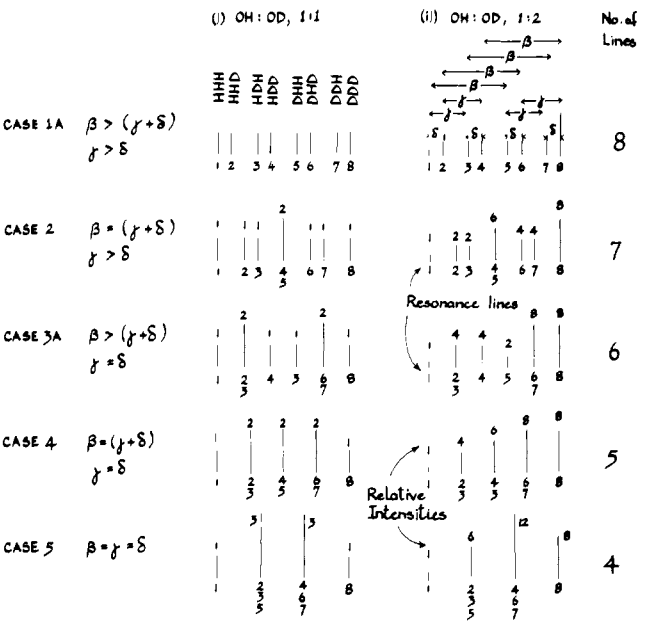

Figure 2. Origins of the numbers of lines, intensities, and possible degeneracies for carbon atoms with three negative (β, γ, δ) isotope effects at OH:OD ratios of (i) 1:1 and (ii) 1:2. A different designation of signals occurs for cases (1B, 3B) where $\gamma + \delta > \beta$. NB: The use of δ does not signify a four bond isotope effect but rather that the third isotope effect can be distinguished from β and γ.

For three isotope effects the relative intensities of signals depend on the deuteration ratio in the following manner:

$$p(HHH) = p(OH)^3$$

$$p(HHD) = p(HDH) = p(DHH) = p(OH)^2 \cdot p(OD)$$

$$p(HDD) = p(DHD) = p(DDH) = p(OH) \cdot p(OD)^2$$

$$p(DDD) = p(OD)^3$$

A summary of the expected intensities of signals at two deuteration ratios (OH:OD, 1:1 and 1:2) is given in Figure 2 for different magnitudes of isotope effects where it is seen that all relevant information (i.e., signs, magnitudes, and number of isotope effects) can be determined from one experiment in which deuteration best lies in the range 40–60% as long as it is not 50%. For example, the C3 signal of methyl α-D-glucopyranoside in Figure 1B consists of six lines with observed relative intensities (1:4:4:2:8:8) which can be predicted at the OH:OD ratio of 1:2, i.e., case 3 in Figure 2.

The magnitudes of the isotope effects of the two monosaccharides summarized in Table II indicate that β effects differ slightly for a primary (ca. −0.117 ppm) or secondary (ca. −0.106 ppm) hydroxyl group whereas the variation in γ effects is greater (−0.015 to −0.042 ppm) and accounts for the different appearance of analogous signals of the galacto and gluco derivatives in parts A and B of Figure 1, respectively. In the present work the differences in magnitudes of β and γ effects and the variability in the magnitude of the γ effect are used diagnostically for assignment purposes, though it can be shown[25] that magnitudes of γ(CCOD) depend on stereochemistry analogous to that recently observed for isotope effects in CCCD molecular fragments.[26]

Assignment of Disaccharides: Melibiose. Melibiose is a reducing disaccharide, α-D-galactopyranosyl-(1→6)-α-D-gluco-

(25) J. C. Christofides and D. B. Davies, to be published.
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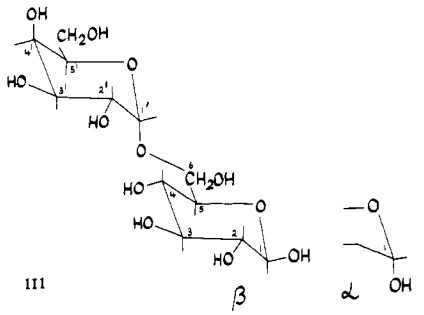
pyranose, III, which when dissolved in aqueous solution isomerizes



III

to give both α- and β-forms at the glucose unit. Both anomers are present (in a ratio α:β of 60:40 as determined by ^1H NMR spectroscopy) when melibiose was deuterated by lyophilization from D_2O solution. The ^{13}C NMR spectrum of melibiose has previously been assigned in D_2O solution by heteronuclear two-dimensional NMR spectroscopy.[20] The assignment of melibiose in Me_2SO-d_6 solution provides a good test of the SIMPLE NMR method because the results can be compared with those obtained in D_2O solution and checked by mixed-solvent studies.

Assignment of the 100-MHz ^{13}C SIMPLE NMR spectrum of melibioise (OH:OD, ca. 1:1) shown in Figure 3 was made by consideration of the isotopomer multiplicities expected for each carbon atom as summarized in Table III together with a knowledge of which signals correspond to the glucose and galactose residues. The latter information was determined in a separate ^{13}C NMR experiment by following the isomerization of melibiose in Me_2SO-d_6 solution and assuming that signals from the non-reducing residue (galactose, C'α and C'β) exhibit similar chemical shifts to each other whereas signals from the reducing residue (glucose, Cα and Cβ) exhibit different chemical shifts due to the effect of axial or equatorial hydroxyl groups at C1.[10]

The C1, C4, C5, and C6 signals of the glucose residue were assigned by inspection of their isotopomer multiplicities (4, 4, 2, and 1, respectively) resulting from their expected isotope effects ($\beta_1 + \gamma_{12}$, $\beta_4 + \gamma_{43}$, γ_{54}, and none, respectively) and by comparison of the magnitudes of the effects with those for methyl α-D-glucopyranoside. Both the C2 and C3 signals are expected to exhibit a maximum of eight lines corresponding to three isotope effects ($\beta + 2\gamma$, Table III). The six-line signals at 76.72 and 73.12 ppm exhibit three effects (−0.102, −0.042, −0.042 ppm) with magnitudes typical of the C3 signal of methyl α-D-glucopyranoside (case 3, Figure 2) and are assigned by signal intensities to C3β and C3α, respectively. The remaining two signals of the glucose residue at 74.79 and 72.26 ppm are assigned to C2β and C2α, respectively. The C2β signal is observed as a seven-line multiplet corresponding to case 2 (Figure 2) and the C2α signal is observed as a six-line multiplet corresponding to case 3 (Figure 2). The difference in appearance of the two signals is accounted for by the variation in γ_{21} magnitudes with the configuration of the hydroxyl group at C1.

The carbon signals of the galactose residue of melibiose can be completely assigned by consideration of the expected isotopomer multiplicities (Table III), e.g., C1', C3', C5', and C6'. The C2' and C4' signals have the same expected multiplicities, i.e., four lines from $\beta + \gamma$ effects, but can be assigned by comparison of the magnitudes of the γ effects (−0.021 and −0.034 ppm) with those for γ_{43} (−0.021 ppm) and γ_{23} (−0.039 ppm) of methyl α-D-galactopyranoside. The signals of melibiose at 68.48 and 68.44 ppm are assigned to C2'α and C2'β, respectively, and the signal at 68.89 to C4'α and C4'β. The C3' signal of the galactose residue at 69.56 ppm also has similar chemical shifts for the α- and β-anomers which might account for the fact that the smallest of the expected three isotope effects is observed as a line broadening rather than as a clear line separation as in the monomer (ca. −0.015 ppm).

Chemical shift differences of the α- and β-anomers of melibiose in Me_2SO-d_6 are compared with those determined previously in D_2O solution[20] (Table III). Most pairs of signals behave in a similar manner in the two solvents except for $\Delta\delta$ of C1'(Gal) which

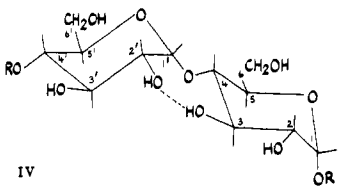
Table III. Characteristics of Carbon Signals of α1→6 Glycosides: Melibiose[a]

carbon atom	isotope effects	no. of lines: expected	no. of lines: obsd	δ(Cβ) − δ(Cα): expected	δ(Cβ) − δ(Cα): obsd[a]	δ(Cβ) − δ(Cα): D_2O[b]
C1α, C1β	$\beta_1 + \gamma_{12}$	4	3, 4	different	4.62	3.88
C1'α, C1'β	γ_{12}	2	2, 2	similar	0.07	−0.04
C2α, C2β	$\beta_2 + \gamma_{21} + \gamma_{23}$	8	6, 7	different	2.53	2.62
C2'α, C2'β	$\beta_2 + \gamma_{23}$	4	4, 4	similar	−0.04	0
C3α, C3β	$\beta_3 + \gamma_{32} + \gamma_{34}$	8	6, 6	different	3.6	2.94
C3'α, C3'β	$\beta_3 + \gamma_{32} + \gamma_{34}$	8	4,[c] 4[c]	similar	0	0
C4α, C4β	$\beta_4 + \gamma_{43}$	4	4, 4	different	−0.43	−0.15
C4'α, C4'β	$\beta_4 + \gamma_{43}$	4	4, 4	similar	0	0
C5α, C5β	γ_{54}	2	2, 2	different	4.53	4.25
C5'α, C5'β	$\gamma_{54} + \gamma_{56}$	4	3, 3	similar	0.04	0
C6α, C6β		1	1, 1	different	0	−0.09
C6'α, C6'β	β_6	2	2, 2	similar	0.01	0

[a] 100-MHz secondary isotope multiplet ^{13}C NMR spectra of partially labeled entities observed in Me_2SO-d_6 solution. [b] Signal assignment in D_2O solution accomplished by heteronuclear two-dimensioned NMR spectroscopy (ref 20). [c] The smallest isotope effect is observed as line broadening.

are of opposite sign and for cases where small chemical shift differences are observed in one solvent but not the other, e.g., for C2', C5', and C6' in Me_2SO-d_6 but not D_2O and for C6 in D_2O but not Me_2SO-d_6 solution. The different behavior is entirely accounted for by the solvent effect on signals because the assignment of melibiose in D_2O solution was confirmed by following the chemical shift changes of signals assigned in Me_2SO-d_6 solution in this work by using Me_2SO-d_6–H_2O mixtures. Small differences in chemical shifts of melibiose measured in H_2O and D_2O solutions can be accounted for by the expected upfield shifts in the latter solvent because the fully deuterated isotopomer is being observed.

Hydrogen-Bonding Effects. Cyclodextrins and maltose are examples of α1→4-linked glucosides in which intramolecular hydrogen bonding has been observed in the crystal by X-ray[27-29] and neutron[30] diffraction and in solution by analysis of NMR parameters.[31,32] The structure of α-maltose (IV, R = H) and partial structure of β-cyclodextrin (IV, R = glucose residue) is shown with the intramolecular hydrogen bond between the OH3 group of one residue and the OH2 group of the preceding residue.



IV

In the solid state both OH3···O2 and OH2···O3 hydrogen bondings have been observed in cyclodextrin with the former being favored. For cyclodextrins in solution early ^1H NMR work by Casu et al.[31] indicated the presence of an intramolecular hydrogen bond between OH2 and OH3 which was later confirmed by Marchessault and co-workers,[32] who concluded from the analysis of a number of NMR parameters that there was an overwhelming preference for the OH3 group being the donor and the O2 atom the acceptor in the hydrogen-bonding scheme as shown in structure V. Although the presence of the intramolecular hydrogen bond in cyclodextrin in Me_2SO-d_6 solution has been confirmed by

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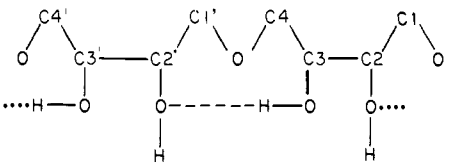

V

observation of OH2 and OH3 deuterium-induced isotope shifts by ^{1}H NMR spectroscopy,[33] it is shown in this work that the donor and acceptor atoms are confirmed by observation of isotope-induced shifts in the ^{13}C NMR spectra of both cyclodextrin and maltose.

Part of the ^{13}C SIMPLE NMR spectrum of cycloheptylamylose (β-cyclodextrin) in which the hydroxyl groups are partially deuterated (ca. 60%) is shown for the C2, C3, and C6 signals in Figure 4. The remaining C1, C4, and C5 signals appear as doublets resulting from the appropriate isotope effect (i.e., γ_{12}, γ_{43}, and γ_{56}, respectively). The C3 signal is observed as the expected quartet resulting from two isotope effects (β_3 and γ_{32}) whereas the C2 signal is observed as an octet resulting from three isotope effects although only two (β_2 and γ_{23}) are expected. As the unequivocal assignment of the ^{13}C NMR spectrum of β-cyclodextrin in Me_2SO-d_6 has been made previously,[34] it is known that the extra isotope effect is observed on the C2 signal. The hydrogen bonding model (V) in which OH3 acts as the donor atom can account for the extra isotope effect on C2 being transmitted through the hydrogen bond from OH3. The two-bond isotope effect (labeled β'_{23} to indicate a hydrogen bond in the C2′–O2′···H–O3 pathway) is more likely to be observed than the four bond effect (i.e., δ'_{23}) that might occur if the hydrogen-bonding scheme corresponded to C2′–O2′–H···O3–H.

Analysis of the C2 signal reveals that the β_2 effect is smaller (−0.093 vs. −0.105 ppm) and γ_{23} is larger (−0.046 vs. −0.032 ppm) than the corresponding effects in methyl α-D-glucopyranoside (Table II) and that the β'_{23} effect (−0.016 ppm) transmitted through a hydrogen bond is much smaller than a β effect transmitted through a covalent bond (−0.09 to −0.11 ppm).

The complete ^{13}C NMR spectrum of maltose (OH:OD, 1:1) in Me_2SO-d_6 solution is shown in Figure 5. Signals from both α- and β-forms are observed with the α:β ratio of 40:60 being determined by ^{1}H NMR spectroscopy. The ^{13}C NMR signals of the reducing and non-reducing glucose residues in maltose were differentiated by following the anomerization of β-maltose in Me_2SO-d_6 solution by using the criterion that signals from the reducing residues are different from each other and signals from the non-reducing residues are similar to each other. From a summary of the expected isotopomer multiplicities for an α1→4 glucoside in Table IV, it is seen that the assignment of most signals in both residues is straightforward as found for melibiose. Although analysis of most signals with three isotope effects conforms to the cases outlined in Figure 2, it should be noted that the C2β signal corresponds to isotope effects in which $\gamma + \delta, > \beta$, i.e., case 1B. One might expect to differentiate between the C2′($\beta + \gamma$) and C3′($\beta + 2\gamma$) signals on the basis of their isotopomer multiplicities, but both signals are observed to exhibit three isotope effects. In this case assignment was made by comparing the magnitudes of the isotope effects with those of methyl α-D-glucopyranoside and noting that the magnitude of the extra isotope effect on the C2′ signals is about the same as that observed in β-cyclodextrin. Hence, the signal at 73.45 ppm is assigned to C3′ with isotope effects β_3 = −0.103 ppm and $\gamma_{32} = \gamma_{34}$ = −0.038 ppm and that at 72.4–72.7 ppm to C2′α (72.68 ppm) and C2′β (72.57 ppm) with isotope effects β_2 = −0.100 ppm, γ_{23} = −0.036 ppm, and β'_{23} = −0.014 ppm. These ^{13}C NMR measurements confirm, for the first time, the presence and direction of intramolecular hydrogen bonding in maltose in solution according to structure V as suggested by Marchessault and co-workers.[32]

Table IV. Characteristics of Carbon Signals of α1→4 Glycosides: Maltose[a]

carbon atom	isotope effects	no. of lines expected	no. of lines obsd[a]	δ(Cβ) − δ(Cα), ppm expected	δ(Cβ) − δ(Cα), ppm obsd[a]	δ(Cβ) − δ(Cα), ppm D_2O[b]
C1α, C1β	$\beta_1 + \gamma_{12}$	4	3, 4	different	4.68	4.0
C1′α, C1′β	γ_{12}	2	2, 2	similar	−0.04	0
C2α, C2β	$\beta_2 + \gamma_{21} + \gamma_{23}$	8	5, 6	different	2.53	2.7
C2′α, C2′β	$\beta_2 + \gamma_{23}$ (+β'_{23})	4 (8)	7, 7	similar	−0.11	−0.1
C3α, C3β	$\beta_3 + \gamma_{32}$	4	4, 4	different	3.54	3.0
C3′α, C3′β	$\beta_3 + \gamma_{32} + \gamma_{34}$	8	6, 6	similar	−0.02[c]	0
C4α, C4β	γ_{43}	2	2, 2	different	−0.53	−0.3
C4′α, C4′β	$\beta_4 + \gamma_{43}$	4	4, 4	similar	−0.04	0
C5α, C5β	γ_{56}	2	2, 2	different	4.78	4.6
C5′α, C5′β	$\gamma_{54} + \gamma_{56}$	4	3, 3	similar	0[c]	0
C6α, C6β	β_6	2	2, 2	different	0.11	0.2
C6′α, C6′β	β_6	2	2, 2	similar	−0.02	0

[a] Observations by SIMPLE NMR in this work. [b] Reference 35; signal assignment in D_2O solution accomplished by using a combination of selective proton decoupling, ^{13}C selective labeling, and β isotope shifts induced by deuterium. [c] Overlap of C5′ and C3′ multiplets masks any small chemical shift difference between α- and β-anomers, whereas prior to partial deuteration a small chemical shift difference (−0.02 ppm) may be observed for the C3′α and C3′β signals.

The ^{13}C NMR spectra of maltose (α- and β-forms) in D_2O and in Me_2SO-d_6 solution have previously been assigned by using selective proton decoupling, ^{13}C selective spin-labeling, and β isotope shifts induced by deuterium[35] and in D_2O solution by using heteronuclear two-dimensional NMR spectroscopy.[21] The present assignment in Me_2SO-d_6 solution using only the SIMPLE NMR method is in general agreement with the previous ^{13}C assignment of maltose,[35] except for the C3′ and C5′ signals which have nearly identical chemical shifts ($\Delta\delta$ ca. 0.02 ppm). The origin of the small discrepancy in assignment is due to the different experimental conditions used. Having followed the solvent dependence of chemical shifts, we found that the results are in complete agreement with the assignment in D_2O solution.[21,35] In the present work it was also possible to detect small chemical shift differences between the α- and β-anomers for the nonreducing residue of maltose in Me_2SO-d_6 (Table IV).

Conclusions

(1) Observation of secondary isotope multiplet ^{13}C NMR of partially labeled entities (SIMPLE NMR) enables both the signs and magnitudes of various isotope effects to be determined directly in one experiment at deuteration ratios close to but not equal to 50:50.

(2) Each carbon atom gives rise to a characteristic multiplet which can be used to assign most signals with little prior knowledge of expected chemical shifts. The number of resonance lines in each multiplet depends on the number of isotope effects, their signs, and relative magnitudes, whereas the intensities of the lines depend on the isotope ratio. Different substitution patterns of hydroxyl groups give rise to different isotopomer multiplicities of carbon atoms as shown for 1→6-linked (Table III, melibiose) and 1→4-linked (Table IV, maltose) disaccharides. The method can be extended to other oligosaccharides and assignment of signals of a tetrasaccharide is feasible.[25]

(3) Magnitudes of β and γ effects depend on structural features such as configuration, substitution, and hydrogen bonding, and characterization of such magnitudes will be of great importance when using SIMPLE NMR for signal assignment. It can be seen from the data in Table II that a β effect for a secondary hydroxyl group (β_6 ca. −0.116 ppm) is greater than that for C2′–O2′···H–O3). primary hydroxyl group (−0.092 to −0.110 ppm). A large variation in magnitudes of γ effects (−0.015 to −0.066 ppm) is observed. In cases of isotope effects being associated with

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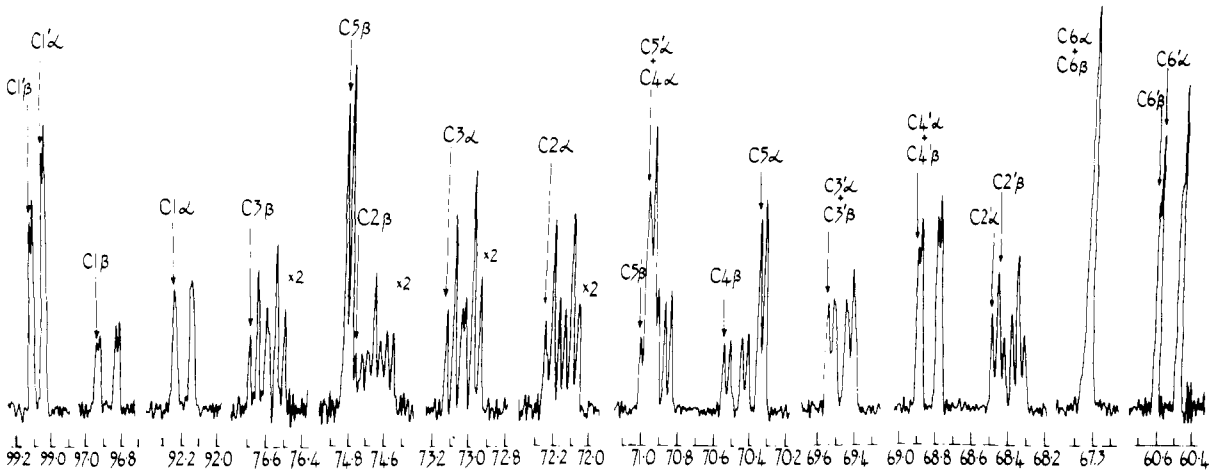

Figure 3. 100-MHz proton noise decoupled ^{13}C NMR spectrum of melibiose (α:β, 60:40) with partially deuterated hydroxyl groups (OH:OD, 1:1) observed in dry Me_2SO-d_6 solution.

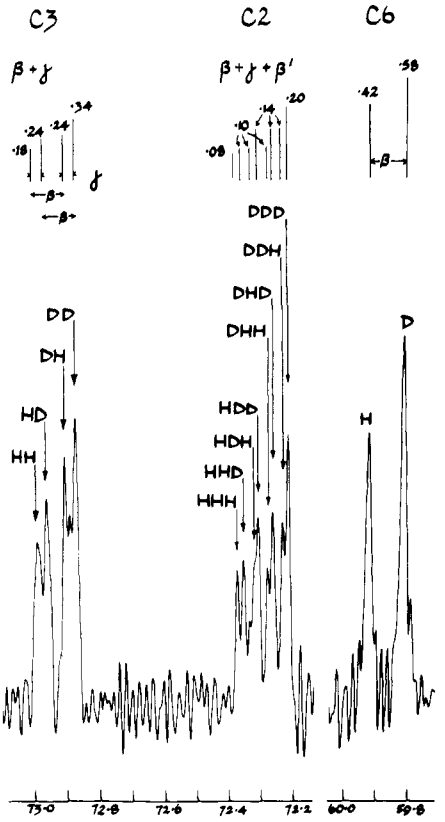

Figure 4. Part of the 100-MHz noise decoupled ^{13}C NMR spectrum of β-cyclodextrin with partially deuterated hydroxyl groups (OH:OD, 42:58) observed in dry Me_2SO-d_6 solution. The C1, C4, and C5 signals appear as doublets with appropriate γ effects.

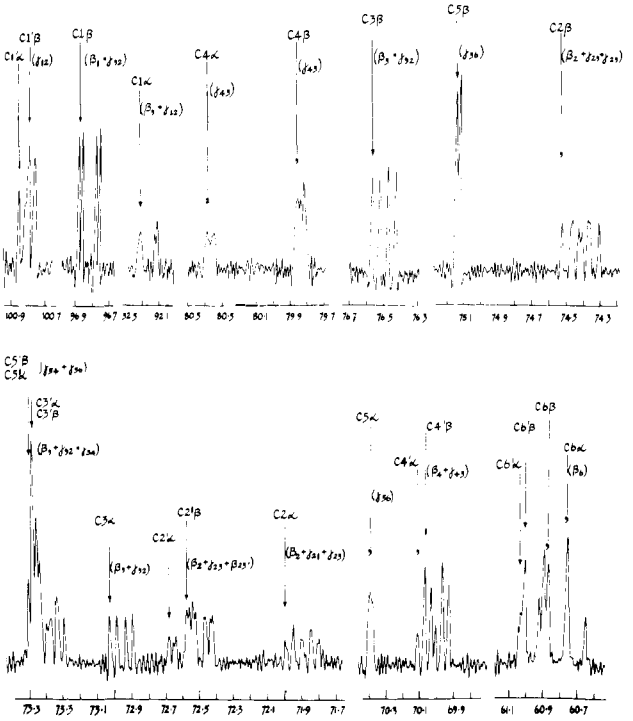

Figure 5. 100-MHz proton noise decoupled ^{13}C NMR spectrum of maltose (α:β ratio, 40:60) with partially deuterated hydroxyl groups (OH:OD, 50:50).

equatorial hydroxyl groups, magnitudes of γ effects are similar (range −0.036 to −0.041 ppm as observed for γ_{23} and γ_{32} of glucose and galactose units and for γ_{34} and γ_{43} of glucose units) whereas for axial hydroxyl groups magnitudes are smaller, e.g., γ_{34} (Gal) = −0.016 ppm and γ_{43}(Gal) = −0.021 ppm. There is also a large variation in magnitudes of γ isotope effects involving the hydroxyl group at the anomeric carbon atom of the glucose units, i.e., γ_{21} = −0.038 ppm (α) and γ_{21} = −0.066 ppm (β).

(4) SIMPLE NMR observations of β-cyclodextrin and maltose in Me_2SO-d_6 also revealed the presence of an isotope effect for hydroxyl groups involved in hydrogen bonding. The isotope effect (β'_{23} ca. −0.015 ppm) was transmitted through the hydrogen bond and was only manifested for one direction of the bond between adjacent glucose units (i.e., C2′−O2′···H−O3).

The SIMPLE NMR method has been shown to be a useful technique for assignment of ^{13}C signals of molecules with exchangeable protons, such as carbohydrates, by straightforward proton noise decoupled measurements. The method can be extended to other carbohydrates and to other molecules with exchangeable OH, NH, SH groups, etc. Being a secondary isotope shift, measurements are not confined to ^{13}C NMR observations but may be made on any nucleus as long as the magnitudes of the isotope effects are greater than the line widths. The added attraction of the method is for the study of hydrogen bonding not only in carbohydrates but also in peptides, nucleotides, etc. where both the presence and direction of hydrogen bonding may be determined in favorable cases.

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