Secondary Isotope Multiplet Nuclear Magnetic Resonance Spectroscopy of Partially Labelled Entities (SIMPLE): ^{13}C Spectra of Stachyose and its Subunits

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^{13}C N.m.r. measurements have been made, in $(C^2H_3)_2SO$ solutions, of some carbohydrates (sucrose, raffinose, melezitose, and stachyose) in which exchangeable protons have been partially deuteriated (*ca.*50 : 50). Signals from a single carbon atom type are observed as a series of multiplets (singlets to octets, at least) resulting from observation of different isotopomers measured under conditions of slow exchange. The multiplets are analysed 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 vary, giving rise to characteristic multiplets which have been used to check the assignment of the disaccharide sucrose, and to provide the assignment of the ^{13}C n.m.r. spectra of the trisaccharides raffinose and melezitose and the tetrasaccharide stachyose in $(C^2H_3)_2SO$ solutions and in ^2H_2O solutions by using mixed-solvent studies. The SIMPLE n.m.r. method may be used to provide information on the composition, substitution, and glycosidic linkages of oligosaccharides in solution.

A fundamental problem in ^{13}C n.m.r. spectroscopy is the assignment of resonances to specific nuclear sites in the molecule. The problem is made difficult in carbohydrates because of the relatively small range of chemical shifts found for the non-anomeric carbon atoms and by the presence of more than one form of each sugar at equilibrium in aqueous solution. Attempts at assignment of spectra for carbohydrate oligomers based on chemical shift correlations from monomers are limited by the relatively large chemical shifts that can accompany formation of glycosidic linkages. Methods of assignment of ^{13}C n.m.r. spectra of carbohydrates have recently been reviewed.[1] Such methods as double resonance,[2] relaxation time measurements,[3,4] and isotopic substitution [5-14] may be used to aid the assignment of spectra, but each method has its own limitations. Double Fourier transform n.m.r. (2D n.m.r.) methods have led to a significant advance in the ability to assign signals of more complex carbohydrates.[15,16] For example, heteronuclear 2D n.m.r. spectroscopy has been used to assign both ^1H and ^{13}C spectra of the trisaccharide raffinose and its subunits (sucrose, melibiose, galactose, glucose, and fructose).[15] Although the carbon and hydrogen signals can be correlated, there is still the problem of assignment of signals to individual carbon atoms, especially for carbohydrates where the ^1H n.m.r. spectrum is more complex. Such correlations would be easier to apply if the carbon atoms could be assigned by an independent method that is straightforward to apply.

The use of isotope replacement to aid assignment of signals depends on the availability of appropriate isotopomers. Normally, carbohydrates specifically deuteriated at methine carbon atoms have been synthesized in order to identify the directly bonded carbon atom.[5-9] Methods based on isotopic substitution of hydroxy protons are advantageous because of the easy preparation of isotopomers. Early work by Vincendon and his co-workers [10] and Bock and his co-workers [11] indicated that carbon atoms substituted with hydroxy groups under conditions of slow exchange could be identified by the isotope shift shown by the deuterio- and the protio-isotopomer. Another development was introduced by Pfeffer and his co-workers,[12-14] who made ^{13}C n.m.r. measurements on carbohydrates in a dual coaxial cell containing equal concentrations of the molecule in H_2O and in D_2O. In this method the hydroxy protons are in fast exchange and the 'differential isotope shift' (DIS) observed for each carbon atom is the sum of all two-bond (β) and three-bond (γ) isotope effects. The method has been applied to the complete assignment of a number of mono- and di-saccharides.[13,14] Magnitudes of β and γ effects were averaged from measurements made on a series of molecules. Application of the method is difficult for unequivocal assignment of a number of carbon atoms where the sum of the isotope effects is similar, for cases where β and γ effects vary with configuration, and for cases where other effects contribute to the DIS, *e.g.* four-bond isotope effects.

It was recently shown that the ^{13}C n.m.r. spectra of $(C^2H_3)_2SO$ solutions of carbohydrates with partially deuteriated hydroxy groups are observed as series of multiplets (singlets to octets, so far) which can be analysed in terms of individual β and γ effects for each carbon atom.[17,18] The magnitudes of the β and γ effects vary with molecular structure and the characteristic multiplets can be used to assign the spectra of mono- and di-saccharides, *e.g.* cellobiose,[17] and maltose and melibiose,[18] with little or no prior knowledge of chemical shifts. In the present work the use of Secondary Isotope Multiplet ^{13}C n.m.r. of Partially Labelled Entities (SIMPLE n.m.r.) has been extended to the assignment of the tetrasaccharide stachyose and subunits whose structures are shown in Figure 1, *i.e.* stachyose [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→2)-β-D-Fru*f*], raffinose [α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→2)-β-D-Fru*f*], and sucrose [α-D-Glc*p*-(1→2)-β-D-Fru*f*] as well as the non-reducing trisaccharide melezitose [α-D-Glc*p*-(1→2)-β-D-Fru*f*-(3→1)-α-D-Glc*p*]. The ^{13}C n.m.r. spectrum recorded under conditions where the hydroxy groups are partially deuteriated enables the signs and magnitudes of individual β and γ effects to be determined from signal multiplicities. Consideration of the β and γ effects lost as the glycosidic bonds are made in the series sucrose, raffinose, stachyose enables the carbon atoms taking part in the linkages to be identified, and hence the structures of oligosaccharides may be determined.

Experimental

All carbohydrates (stachyose, raffinose, melezitose, and sucrose), were obtained commercially (Sigma Chemical Co.) and used without further purification. Samples were deuteriated and dried by lyophilising from ^2H_2O solution and dissolved in dry $(C^2H_3)_2SO$ (sealed vials from Merck, Sharpe and Dohme). The OH/OD ratio was adjusted by using appropriate

Table 1. Chemical shifts of stachyose and its subunits in $(CD_3)_2SO$ solution [a]

	β-D-Fructofuranoside						α-D-Glucopyranoside					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Methyl α-D-glucopyranoside							99.87	72.18	73.54	70.49	72.66	61.25
Sucrose	62.15	104.07	77.26	74.44	82.56	62.21	91.76	71.67	72.96	69.98	72.83	60.64
Raffinose	62.38	104.18	77.25	74.45	82.48	62.43	91.80	71.66	73.06	70.47	71.48	66.89
Stachyose	62.27	104.09	77.08	74.32	82.39	62.16	91.73	71.47	72.89	70.20	71.23	66.40
Melezitose	62.59	104.42	81.53	73.38	82.78	62.04	91.95	71.80	73.28 [c]	70.13	72.67 [c]	60.82
							98.22	72.07	73.66 [c]	70.13	72.54 [c]	60.82
Stachyose [d]	62.32	104.65	77.25	74.87	82.19	63.30	92.95	71.83	73.58	70.36	72.13	67.34
	α-D-Galactopyranoside											
Methyl α-D-galactopyranoside	100.08	68.54	69.73	68.93	71.12	60.77						
Raffinose	92.22	68.78	69.71	69.08	71.12	60.85						
Stachyose ext.	98.98 [c]	68.51 [c]	69.33 [c]	68.85 [c]	71.11	60.58						
Stachyose int.	98.82 [c]	68.55 [c]	69.72 [c]	69.03 [c]	68.72	66.40						
Melibiose [b] α	99.03	68.48	69.56	68.89	70.94	60.58	92.29	72.26	73.12	70.94	70.32	67.28 [c]
Melibiose [b] β	99.10	68.44	69.56	68.89	70.98	60.59	96.91	74.79	76.72	70.51	74.85	67.29 [c]
Stachyose [d] ext.	98.89 [c]	69.14 [c]	70.23 [c]	70.09 [c]	71.80	61.99						
Stachyose [d] int.	99.23 [c]	69.29 [c]	70.36 [c]	70.18 [c]	69.66	66.75						

[a] 100 MHz ^{13}C n.m.r. measurements at 295 ± 1 K; chemical shifts with respect to Me_4Si [$\delta(Me_2SO)$ +39.5 p.p.m.]. Assignments based on signal multiplets and magnitudes of β and γ isotope effects. [b] Ref. 18. [c] Assignments for the corresponding carbon atoms of two similar subunits which may be interchanged. [d] In ^2H_2O solution. Chemical shifts measured with respect to dioxane set to δ 67.4 p.p.m.

Table 2. Isotope shifts in carbohydrates ($\times 10^{-3}$ p.p.m.). All values have negative sign

	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}
(i) α-D-Glucopyranoside													
Methyl α-D-Glc*p*		105	104	105	116	15		32	42 [b]	42 [b]	36	28 [b]	28 [b]
Sucrose		100	96	104	113	*a*		33	41 [b]	41 [b]	37	25 [b]	25 [b]
Raffinose		96	100	97		*a*		29	39 [b]	39 [b]	35	27	
Stachyose		100	100	96		*a*		33	40 [b]	40 [b]	35	29	
Melezitose		102	102	103	115	15		35	44 [b]	44 [b]	39	27 [b]	27 [b]
		103	99	103	115	12		36	41 [b]	41 [b]	39	26 [b]	26 [b]
Melibiose α	97	106	102	100		15	38	38	42 [b]	42 [b]	33	27	
Melibiose β	105	102	102	99		20	66	36	43 [b]	43 [b]	35	30	
Average		102	101	101	115	15		34	42	42	36		
(ii) α-D-Galactopyranoside													
Methyl α-D-Gal*p*		108	105	110	118	16		39	42	15	21	26 [b]	26 [b]
Raffinose		105	107	108	118	*a*		38	39	16	20	26 [b]	26 [b]
Stachyose int.		105	106	107		18		37	37	16	20	*c*	
Stachyose ext.		107	106	107	119	15 [d]		32	42	15	22	26 [b]	26 [b]
Melibiose α		108	103	109	118	15		35	36	17	21	29 [b]	29 [b]
Melibiose β		106	103	109	118	18		33	36	17	21	30 [b]	30 [b]
Average		107	105	108	118	16		36	39	16	21		
(iii) β-D-Fructofuranoside													
Sucrose	105		108	98	94		17	0		47	48	28 [b]	28 [b]
Raffinose	106		106	103	97		15	0		51	49	29 [b]	29 [b]
Stachyose	108		108	103	93		18	0		48	48	27 [b]	27 [b]
Melezitose	109			97	100		15	0		*a*		38	25
Average	107		107	101	96		16			48	48		

[a] Signal not resolved. [b] Average value within experimental error of (±0.003 p.p.m.). [c] Cannot be determined owing to overlap of signals. [d] Approximate value (broad signal).

amounts of deuteriated and normal protiated carbohydrate. The extent of deuteriation was determined independently using ^1H n.m.r. spectroscopy.

Natural-abundance 50 MHz (JEOL FX200) and 100 MHz (Bruker WM 400) ^{13}C n.m.r. spectra were obtained at ambient temperatures (295 ± 1 K) under proton noise decoupling conditions. Spectra were calculated with adequate digital resolution (typically 0.25 Hz pt^{-1}) after zero filling and resolution enhancement.[19] Chemical shifts of the carbohydrates in $(C^2H_3)_2SO$ solution (summarised in Table 1) were measured with respect to the residual solvent signal and referenced with respect to tetramethylsilane *i.e.* $\delta = \delta_{obs} + 39.5$ p.p.m. Magnitudes of individual β and γ effects for each carbon atom are summarised in Table 2 in terms of the fructose, glucose, and galactose sub-units.

Mixed-solvent studies were performed by adding suitable quantities of an aqueous (H_2O) solution of the molecule to that in $(C^2H_3)_2SO$ so that the concentration was kept constant

Table 3. Observed and expected isotope effects for stachyose and its subunits

	C-1	C-2	C-3	C-4	C-5	C-6
			(expected maximum no. of lines)			
			No. of lines observed			
(i) β-D-Fructofuranoside						
Sucrose	β	2γ	β + γ	β + γ	2γ	β
Raffinose	(2)	(4)	(4)	(4)	(4)	(2)
Stachyose	2	2 [a]	9—12 [b]	4	3 [c]	2
Melezitose	β	γ	γ	β	2γ	β
(3-Substituted)	(2)	(2)	(2)	(2)	(4)	(2)
sucrose)	2	2	3 [b]	2	3 [c]	2
(ii) α-D-Glucopyranoside						
Methyl α-D-Glc*p*	γ	β + γ	β + 2γ	β + γ	2γ	β
Sucrose	(2)	(4)	(8)	(4)	(4)	(2)
	2 [d]	4	6 [e]	4	3 [c]	2
Raffinose	γ	β + γ	β + 2γ	β + γ	γ	
Stachyose	(2)	(4)	(8)	(4)	(2)	(1)
(6-Substituted)	2 [d]	4	6 [e]	4	2	1
(iii) α-D-Galactopyranoside						
Methyl α-D-Gal*p*	γ	β + γ	β + 2γ	β + γ	2γ	β
Raffinose	(2)	(4)	(8)	(4)	(4)	(2)
Stachyose (ext.)	2 [d]	4	8 [f]	4	3 [c]	2
Stachyose	γ	β + γ	β + 2γ	β + γ	γ	
(Internal unit,	(2)	(4)	(8)	(4)	(2)	(1)
6-substituted)	2	4	6 [e]	4	2	1

[a] $\gamma_{23} = 0$. [b] Additional isotope effects observed. [c] $\gamma_{54} = \gamma_{56}$ within experimental error. [d] Isotope effect not always resolved. [e] $\gamma_{32} = \gamma_{34}$ and $(\gamma_{32} + \gamma_{34}) \neq \beta$. [f] $\gamma_{32} \neq \gamma_{34}$ and $(\gamma_{32} + \gamma_{34}) \neq \beta$.

(*ca.* 0.25M). The residual ^{13}C signal of the solvent $(C^2H_3)_2SO$ provided the internal reference for all solvent mixtures. The method leads to ^{13}C signal assignment of the molecule in H_2O which can be matched with the spectrum in ^2H_2O solution, making allowance for the upfield shifts of each carbon signal from the sum of appropriate isotope effects.

Results and Discussion

The summary of expected isotope shifts (β and γ) and numbers of isotopomer resonances (Table 3) for different subunits of stachyose provides an important tool for analysing the SIMPLE ^{13}C n.m.r. spectra of carbohydrates from monomer to tetramer level. The notation used for isotope effects (β,γ, *etc.*) denotes the ^{13}C signal being observed with a numerical subscript (*e.g.* β_3) and, when appropriate, a second numerical subscript for the hydroxy 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 C-3 signal resulting from deuteriation of the 2-OH and 4-OH groups, respectively). Substitution of ^2H for ^1H results in low-frequency shifts in the spectra of the observed nucleus; hence magnitudes of β and γ effects in this work are negative.

(1) *Assignment in* $(C^2H_3)_2SO$ *Solution.—Sucrose.* Sucrose consists of an α-D-glucose residue α(1→2)-linked to a β-D-fructofuranoside residue as shown in Figure 1. The ^{13}C n.m.r. spectrum has been completely assigned both in aqueous and in $(C^2H_3)_2SO$ solution by a variety of methods. Koch *et al.*[9] and Pfeffer and Valentine [12] have used *C*- and *O*-deuteriation methods, whilst Bock and Lemieux [2] have made a detailed analysis of both ^1H and ^{13}C n.m.r. spectra of sucrose; Smith *et al.*[20] have discussed the ^{13}C n.m.r. spectrum of sucrose in their studies of oligomers and polymers containing D-fructose. All these studies coupled with the 2D n.m.r. spectra [15,16] have

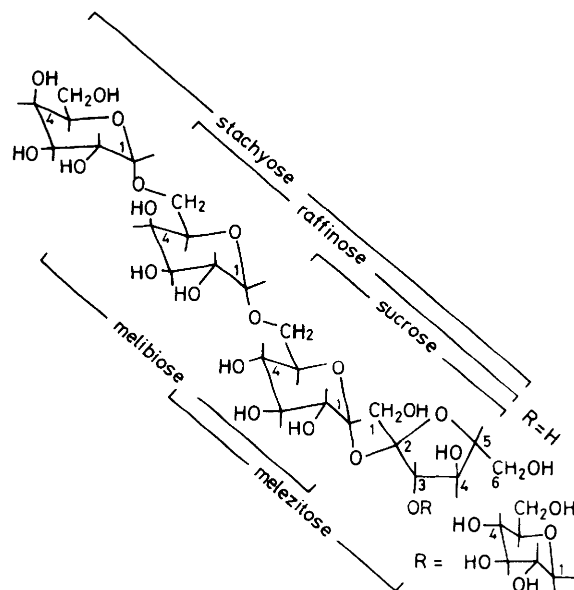

Figure 1. Structures of sucrose, raffinose, stachyose, and melezitose

allowed an unambiguous spectral assignment of sucrose to be made which provides a suitable model for investigating the use of the SIMPLE n.m.r. method in assignment of ^{13}C spectra of the sucrose, raffinose, and stachyose series.

The problem in the assignment of a ^{13}C n.m.r. spectrum of an oligosaccharide is two-fold; first, there is the identification of the ^{13}C resonances of the constituent residues and, secondly, the correct assignment of the resulting ^{13}C spectrum of the oligosaccharide to individual carbon atoms. Both these

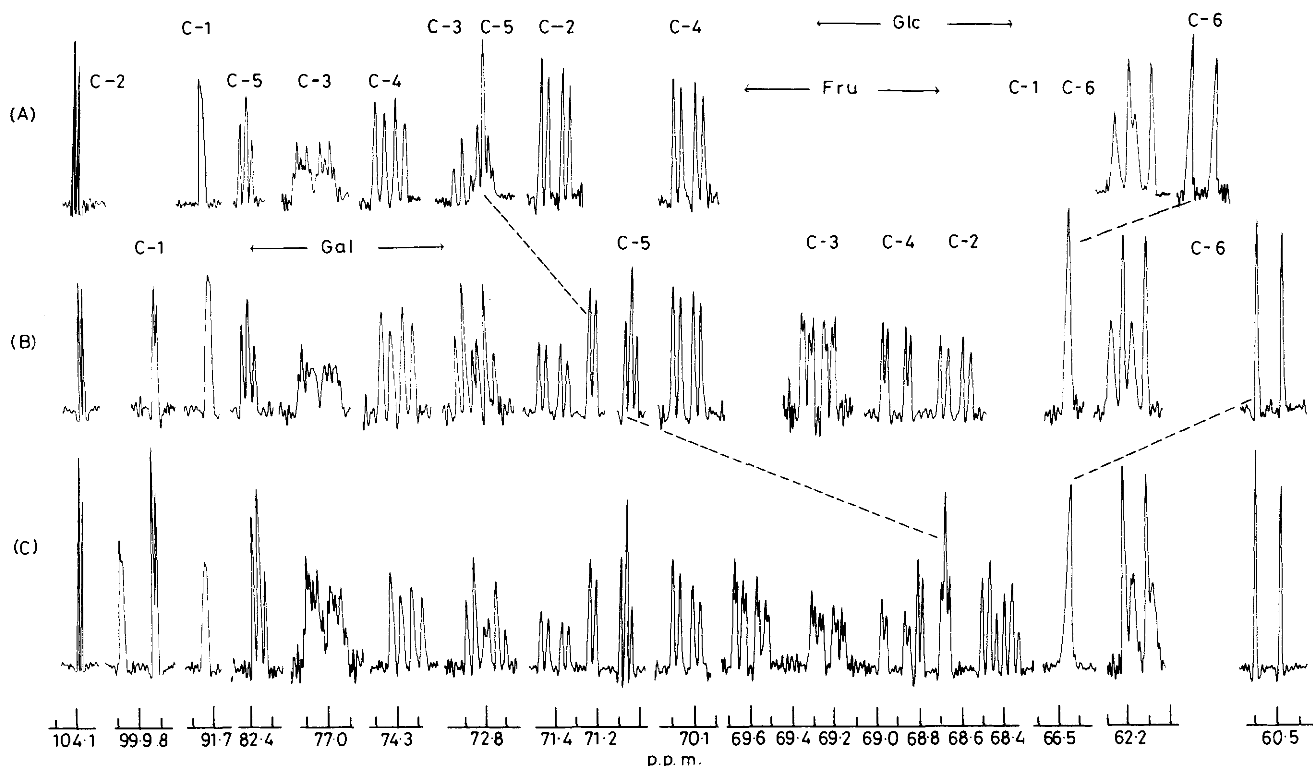

Figure 2. 100 MHz ^{13}C SIMPLE n.m.r. spectrum and signal assignment of (A) sucrose, (B) raffinose, and (C) stachyose in $(C^2H_3)_2SO$ solution. Chemical shift scale corresponds to stachyose; complete assignments in Table 1. Dashed lines indicate significant signal movement caused by appropriate glycosidic bond formation in the series of molecules

problems may be overcome by analysis of the ^{13}C n.m.r. spectra under conditions where the hydroxy groups are partially deuteriated.

The 100 MHz ^{13}C SIMPLE n.m.r. spectrum of sucrose (OH : OD, *ca.* 1 : 1) is shown in Figure 2(A). Twelve isotope multiplet patterns can be distinguished with overlap of signals at *ca.* 72.9 and *ca.* 62.1 p.p.m. The expected multiplicities for each carbon atom of the glucose and fructose units of sucrose, based on β and γ effects, are summarised in Table 3. Each carbon atom (except C-2 and C-4 of the glucose and C-4 of the fructose units and the C-5 atoms of the glucose and fructose units) gives rise to a different pattern of β and γ effects which serves to assign the signals. As the ^{13}C SIMPLE n.m.r. spectrum of methyl α-D-glucopyranoside has been analysed previously,[18] the carbon atoms of the glucose residue can be identified using approximate chemical shifts, multiplicities of carbon signals, and magnitudes of isotope effects as shown for this residue of sucrose in Figure 2. Each carbon signal can be analysed to give the magnitude and sign of the isotope shifts. For carbon atoms where only one isotope effect is expected (C-1, C-6) the observed doublets correspond to the H and D isotopomers and the chemical shift separation is the magnitude of the isotope effect, *i.e.* γ_{12} -0.015 p.p.m.; β_6 -0.113 p.p.m.

For carbon atoms with two possible isotope effects, four resonance signals are expected corresponding to HH, HD, DH, and DD isotopomers. If the two isotope effects are of different magnitudes all four signals are observed (C-2 and C-4) whereas three signals are observed for C-5 because the isotope effects (γ_{54} and γ_{56}) are equal in magnitude within the resolution of the experiment, and the observed signal intensity ratios (*ca.* 1 : 2 : 1) are accounted for by the degeneracy of the HD and DH isotopomers. For carbon atoms with three possible isotope effects (C-3) a maximum of eight lines is expected, corresponding to isotopomers with no D substitution (HHH), one D substitution (HHD, HDH, DHH), two (HDD, DHD, DDH), and three D atom substitution (DDD) of hydroxy groups. All eight are observed for the C-3 signal of the galactose residues of raffinose and stachyose, corresponding to three isotope effects with different magnitudes. On the other hand the C-3 signal of the glucose residue of sucrose is observed as six lines (complicated by overlap with the C-5 signal of the glucose residue), corresponding to degeneracy of the HHD/HDH and DHD/DDH isotopomers because the magnitudes of the two three-bond effects are approximately equal and their sum does not equal the largest effect, *i.e.* $\gamma_{32} \simeq \gamma_{34} \simeq 0.036$ and $(\gamma_{32} + \gamma_{34}) \neq \beta$.

Consideration of the isotopomer multiplicities for the fructofuranoside residue reveals that, because of the symmetrical substitution pattern, three pairs of carbon atoms are expected to exhibit similar effects, *i.e.* C-1 and C-6 should exhibit β effects, C-2 and C-5 2γ effects, and C-3 and C-4 $\beta + \gamma$ effects. Unambiguous assignment of all the resonances of the β-D-fructofuranoside residue in sucrose can be made by comparison with the SIMPLE n.m.r. spectrum of β-D-fructofuranose. The expected and observed isotope effects are now C-1 ($\beta + \gamma$), C-2 ($\beta + 2\gamma$), C-3 ($\beta + 2\gamma$), C-4 ($\beta + \gamma$), C-5 (2γ), and C-6 (β), which enables the C-1, C-2, and C-3 signals to be distinguished from those of C-6, C-5, and C-4, respectively.[21]

In constrast to the SIMPLE n.m.r. spectra of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside, which conform to the patterns expected from β and γ effects, the fructofuranoside residue of sucrose exhibits several unusual features. The C-2 signal of the fructose residue of sucrose (*ca.* 104 p.p.m.) is observed as two lines, whereas four resonance signals are expected from two γ effects ($\gamma_{21} + \gamma_{23}$); analysis

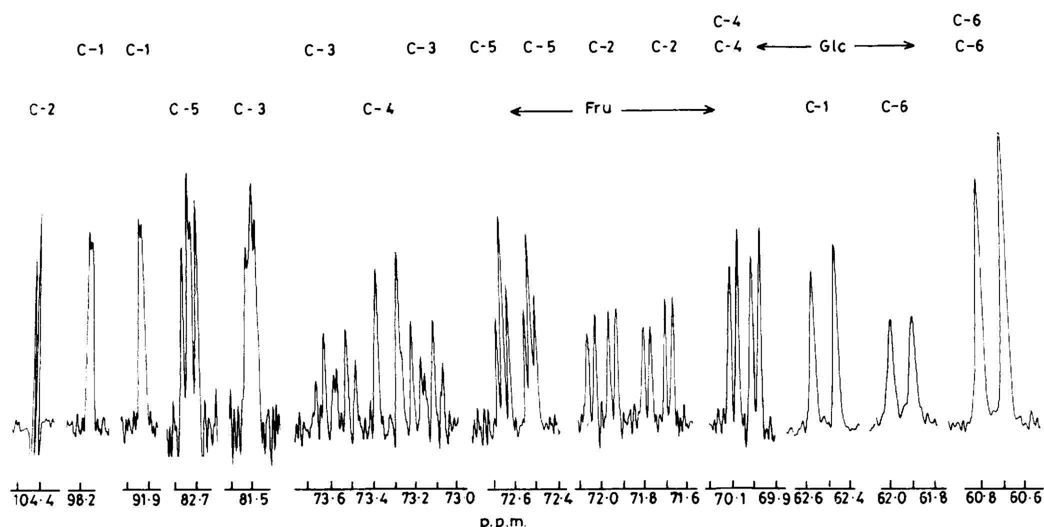

Figure 3. 100 MHz ^{13}C SIMPLE n.m.r. spectrum and signal assignment of melezitose in $(C^2H_3)_2SO$ solution

shows that the two signals come from the isotope effect due to 1-OH ($\gamma_{21} = -0.017$ p.p.m.) whereas that due to 3-OH is not observed (*i.e.* $\gamma_{23} \simeq 0$; see melezitose discussion later). The C-3 signal exhibits up to twelve lines, which is more than the four resonances expected from two isotope effects ($\beta_3 + \gamma_{34}$). Although the origin of the extra effects on C-3(Fru) is being investigated (long-range isotope effects and/or deuterium coupling), the appearance of similar extra effects on the C-3-(Fru) signal of sucrose, raffinose, and stachyose is used, in the present work, to aid the assignment of these signals. The C-4 signal appears as expected with normal β and γ effects ($\beta - 0.097$ and $\gamma_{43} - 0.044$ p.p.m.) and the three-line pattern of the C-5 signal (*ca.* 72.8 p.p.m., 2γ) is analysed to give an average magnitude of -0.028 p.p.m. for both γ_{54} and γ_{56}.

The 1- and 6-carbon atoms both exhibit a β effect (-0.105 and -0.094 p.p.m.) and have very similar chemical shifts, which makes it difficult to differentiate between them. One of the C-1/C-6 signals is considerably broader than the other; the broadening is probably due to a small long-range isotope effect and this effect manifests itself in the spectra of sucrose, raffinose, and stachyose (Figure 2) and melezitose (Figure 3). The assignment of the C-1/C-6 signals in melezitose is straightforward because that of C-1 is shifted downfield by 0.55 p.p.m. as compared with sucrose, owing to the proximity of the glucose residue at C-3. Therefore the extra isotope effect appears on C-6 of the β-D-fructofuranoside residue, and hence it is possible to distinguish the two signals (C-1 and C-6) in the spectra of sucrose, raffinose, and stachyose where the chemical shift separation is very small.

Raffinose. The 100 MHz ^{13}C n.m.r. spectrum of raffinose shown in Figure 2(B) can be readily assigned by consideration of the spectrum of sucrose in Figure 2(A) and that for methyl α-D-galactopyranoside.[18] No changes in isotope multiplicities of the galactopyranosyl unit in raffinose are expected as compared with methyl α-D-galactopyranoside (Table 3), whereas the only changes expected in the sucrose unit are those caused by attachment of galactose at C-6 of the glucose unit, *i.e.* a chemical shift change for C-6 and the loss of its β effect (readily assigned at 66.81 p.p.m.) and a likely chemical shift change for C-5 of the glucose unit which can be readily identified at 71.48 p.p.m. as a doublet ($\gamma_{54} - 0.027$ p.p.m.) having lost the isotope effect from 6-OH. Consideration of the expected isotope multiplicities of glucose and galactose residues in Table 3 suggests that it is not possible to differentiate the C-2 and C-4 of galactose or glucose units because they all have ($\beta + \gamma$) effects or to differentiate the C-3 signals ($\beta + 2\gamma$) in the same residues. On the contrary it was found for the methyl gluco- and galacto-pyranosides that magnitudes of istope effects vary with configuration of hydroxy groups such that the C-3 signal of galactose unit appears as 8 lines [*i.e.* $\beta_3 > (\gamma_{32} + \gamma_{34})$ and $\gamma_{32} > \gamma_{34}$] whereas the C-3 signal of glucose units appear as 6 lines [$\beta_3 > (\gamma_{32} + \gamma_{34})$ but $\gamma_{32} = \gamma_{34}$] so the two signals can be distinguished however close they may be.[18] A similar situation applies to the C-2 and C-4 signals of glucose and galactose residues which have similar magnitudes of γ effects in glucose derivatives ($\gamma_{23} = -0.032$, $\gamma_{43} = -0.036$ p.p.m.) but different magnitudes in galactose derivatives ($\gamma_{23} = -0.039$, $\gamma_{43} = -0.021$ p.p.m.). The complete assignment of the raffinose spectrum is shown in Figure 2(B) and the magnitudes of the isotope effects are summarised in Table 2.

A limitation of the SIMPLE n.m.r. method for *complete* assignment of the ^{13}C spectra of molecules is exemplified by melezitose, α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(3→1)-α-D-glucopyranose), which consists of two α-D-glucopyranosyl units linked to the fructofuranoside residue as shown in Figure 1. Except for isotope effects involving 3-OH of the fructose residue, the spectrum of melezitose is similar to that of sucrose with the glucose carbon signals appearing twice. The individual C-2 to C-6 signals of the two glucose residues can be identified but they cannot be unequivocally assigned to specific glucose residues by the SIMPLE n.m.r. method alone. For analogous signals in the two glucose residues the chemical shifts are similar (C-2, C-3, C-5) or the same (C-4, C-6) and only the C-1 signal of the glucopyranosyl-(1→3) unit is significantly downfield (6.25 p.p.m.) of that for the glucopyranosyl(1→2) unit by comparison with sucrose. The magnitudes of β and γ effects for both glucopyranosyl residues are the same within experimental error (Table 2). Changes are observed in the SIMPLE n.m.r. spectrum of the fructose residue of melezitose as compared with sucrose caused by substitution of 3-OH. Owing to the loss of the γ_{43} effect the C-4 signal appears as a doublet (73.38 p.p.m.) whereas the C-2 signal of melezitose (104.42 p.p.m.) is observed as a doublet similar to that of sucrose (104.07 p.p.m.) despite the expected loss of a γ_{23} isotope effect; the latter result shows that $\gamma_{23} \simeq 0$, and the isotope effect observed on C-2 of sucrose, raffinose, and melezitose is then γ_{21}. As summarised

in Table 3, only one isotope effect is expected on C-3 of the fructofuranoside unit of melezitose (γ_{34}) whereas extra effects occur analogous to those observed for sucrose.

Stachyose. The structure of stachyose, *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-α-D-fructofuranoside, is shown in Figure 1. The 100 MHz ^{13}C SIMPLE n.m.r. spectrum in $(C^2H_3)_2SO$ solution [Figure 2(C)] consists of 24 ^{13}C multiplet patterns with little signal overlap. The assignment of the signals due to the fructose and glucose residues of stachyose can be readily made by comparison with the spectra of sucrose and raffinose. Signals of C-1—6 of the two galactose residues can be identified, but each analogous pair of carbon atoms (except C-5 and C-6, Table 3) is expected to exhibit the same isotope multiplicities, which leads to ambiguities in assignment of C-1 to C-4 to individual galactose residues. Signals due to C-5 and C-6 of the internal galactose residue are readily identified and assigned because substitution at C-6 (in stachyose) causes loss of isotope effects involving 6-OH (*i.e.* C-6 gives a singlet, and C-5 a doublet). Hence the C-5 and C-6 signals of the terminal galactose residue are also readily identified as they retain the isotope effects involving 6-OH. It should be noted that C-5 of the internal galactose unit (68.72 p.p.m.) is observed adventitiously as a triplet, owing to overlap with one part of the galactose C-4 signals.

(2) *Assignment in* D_2O *Solution*.—Assignments of the ^{13}C n.m.r. signals of sucrose in both $(C^2H_3)_2SO$ and ^2H_2O have been made by Bock and Lemieux [2] and by Smith and his co-workers; [19] it was shown that the order of chemical shifts is the same in both solvents and there is complete agreement between these assignments. The results are also in agreement with the assignment in $(C^2H_3)_2SO$ by the SIMPLE n.m.r. method in this work and the assignment in ^2H_2O solution using heteronuclear 2D n.m.r. methods.[15] On the other hand, the assignment of raffinose in $(C^2H_3)_2SO$ (Table 1) differs from that in D_2O solution, *i.e.* in Me_2SO solution C-2(Glc) > C-5(Glc) > C-5(Gal) whereas in ^2H_2O solution C-5(Glc) > C-5(Gal) > C-2(Glc). These results can be rationalised by following the chemical shifts of signals in $(C^2H_3)_2SO$–H_2O solvent mixtures (Figure 4): there is a crossover of the C-2(Glc) and C-5(Glc) signals. At the same time the C-2(Glc) and C-5(Gal) signals in H_2O solution are close together with C-2(Glc) only 0.06 p.p.m. downfield of C-5(Gal). For raffinose measured in ^2H_2O solution the C-2 and C-5 signals would experience an upfield isotope shift with the effect on C-2(Glc) ($\beta_2 + \gamma_{23}$ *ca.* 0.125 p.p.m.) greater than on C-5(Gal) ($\gamma_{54} + \gamma_{56}$ *ca.* 0.05 p.p.m.), causing these signals to cross over from H_2O to ^2H_2O solution. With such closely spaced resonances deuterium-induced shifts become important in determining the chemical shift order and must therefore be taken into consideration when signal assignment is made. The mixed-solvent study also shows that C-4(Glc) and C-3(Gal) signals are well separated in Me_2SO solution but are coincident in H_2O solution; with similar magnitudes of isotope effects these signals are also coincident in ^2H_2O solution as observed by Morris and Hall.[15] Signal assignments for raffinose and melezitose in ^2H_2O solution made in the present work are in complete agreement with those made by Morris and Hall [15] and by Seymour *et al.*,[22] respectively. The 50 MHz ^{13}C n.m.r. spectrum of the tetramer stachyose is shown in Figure 5. There are 11 of the 24 carbon signals in the region 68—72 p.p.m. as shown in the expanded spectrum which poses a formidable problem in assignment. The previous study of stachyose in D_2O solution using partially relaxed Fourier transform spectra [3] provided assignment for only a few of the resonances. Assignment of the 50 MHz ^{13}C n.m.r. spectrum of stachyose in ^2H_2O solution has been made by mixed-solvent studies starting from the assignment of the molecule in $(C^2H_3)_2SO$ solution as described for raffinose. Several crossovers are observed from $(C_2H_3)_2SO$ to ^2H_2O and some of the signals become coincident in ^2H_2O solution, *e.g.* the signals for C-2 and C-4 of the glucose residue become coincident with the signals for C-5 and C-3 of the external galactose residue, respectively. Chemical shifts for stachyose in ^2H_2O are summarised in Table 1.

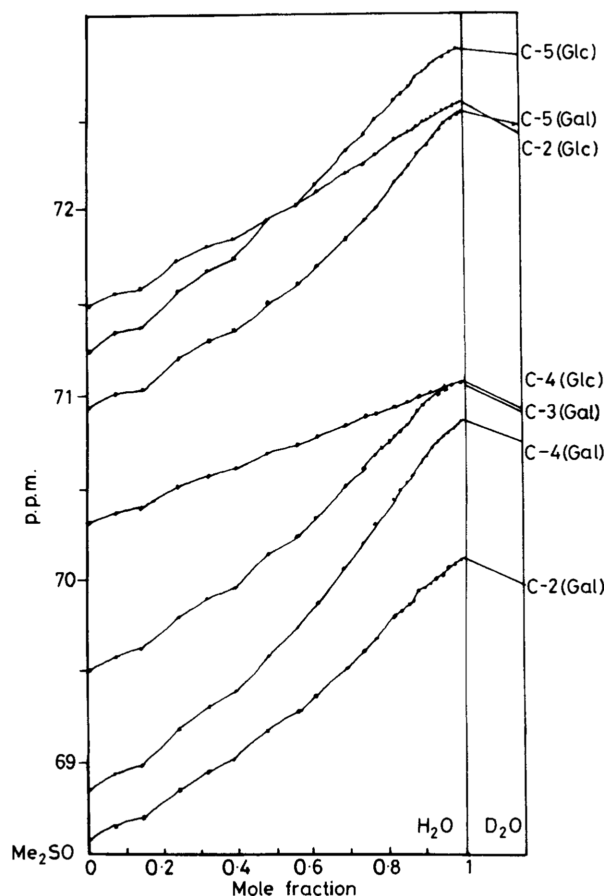

Figure 4. Variation of chemical shifts of closely spaced signals of raffinose in $(C^2H_3)_2SO$–H_2O solvent mixtures. Assignment of signals in ^2H_2O solution given by consideration of the sum of isotope effects on each carbon atom as compared with the assignment in H_2O solution

(3) *Magnitudes of Isotope Effects*.—The magnitudes of the β and γ effects for stachyose, raffinose, and melezitose (Table 2) agree well with the analogous effects for the constituent residues of methyl α-D-gluco- and galacto-pyranosides [18] and β-D-fructofuranoside (as part of sucrose). The β effects for tertiary carbons (C-1 to C-4) vary from −0.096 to −0.108 p.p.m. (12% variation), whereas those for secondary carbon atoms [C-1(Fru) and C-6] exhibit greater variation (−0.094 to −0.118 p.p.m.). The latter variation appears more marked because magnitudes of β_6 for the β-D-fructofuranoside residue (−0.094 to −0.100 p.p.m.) are much smaller than those of β_6 for the gluco- and galacto-pyranosides (−0.113 to −0.118 p.p.m.).

The magnitudes of γ effects in these molecules vary from 0 (γ_{23} of the fructofuranoside residue) to about −0.050 p.p.m. (γ_{34} for the fructofuranoside residue). Where the stereochemical disposition of the hydroxy group is the same relative to the carbon atom on which the γ effect is observed, the

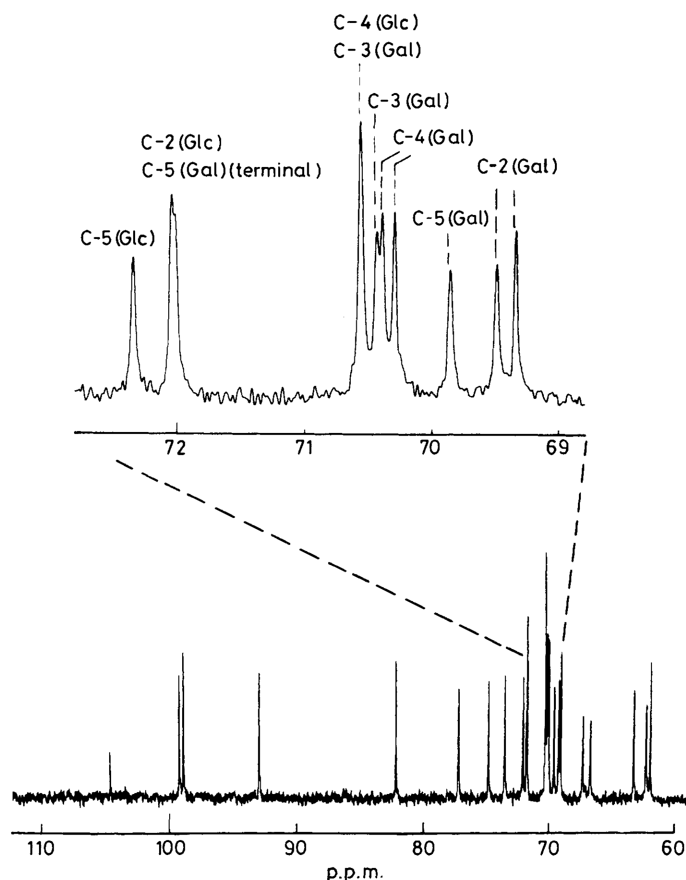

Figure 5. 50 MHz ^{13}C N.m.r. spectrum of stachyose in ^2H_2O solution with expansion of closely spaced signals (69—72 p.p.m.) to show assignment

magnitude of the isotope effect is approximately the same, *e.g* the γ_{23}, γ_{32}, γ_{34}, and γ_{43} effects for methyl α-D-glucopyranoside and methyl α-D-galactopyranoside are the same as the corresponding γ effects for these residues in all compounds.

Differences between the glucose and galactose residues occur for those γ effects involving the 4-hydroxy group (*i.e.* γ_{34}) and also γ effects observed on C-4. Thus for methyl α-D-glucopyranoside, $\gamma_{32} = \gamma_{34} = -0.042$ p.p.m. and $\gamma_{43} = -0.036$ p.p.m., whereas for methyl α-D-galactopyranoside $\gamma_{32} = -0.042$, $\gamma_{34} = -0.016$, and $\gamma_{43} = -0.021$ p.p.m.

The magnitudes of γ effects involving 2-, 3-, and 4-OH in glucose derivatives also exhibit some variation ranging from -0.028 (γ_{54}) to -0.042 p.p.m. (γ_{34}, γ_{43}). These and the magnitudes of γ isotope effects for the galactose and fructose residues (as well as the additional effects on the C-3 and C-6 signals of the fructose residue) can be used to aid signal assignment as shown for the sucrose–stachyose series (Figure 2).

Comparison of the spectra of sucrose, melezitose, raffinose, and stachyose under conditions where the hydroxy groups are partially deuteriated (to the same extent) shows that even before assignment is made structural information on the composition of the oligosaccharide can be obtained. For example, comparison of the sucrose and melezitose spectra shows that melezitose consists of two residues which have the same isotope effects as the glucose residue of sucrose and comparison of the raffinose and stachyose spectra shows that stachyose has two residues which have the same isotope effects as the galactose residues of raffinose. Therefore, melezitose must contain two glucose units and stachyose must contain two galactose residues in addition to the sucrose unit present.

Conclusions

(1) Observation of SIMPLE ^{13}C n.m.r. spectra of stachyose and its subunits raffinose and sucrose in $(C^2H_3)_2SO$ solution enables both the signs and magnitudes of various isotope effects to be determined directly in one experiment at deuteriation ratios about 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 their relative magnitudes, whereas the intensities of the lines depend on the isotope ratio. Although the analysis of some signals is incomplete where extra isotope effects [C-3(Fru)] or line broadening [C-6(Fru)] occur, the appearance of similar effects in the stachyose series of molecules may also be used to aid assignment of the spectra.

(3) Magnitudes of β and γ effects are found to vary with such structural features as configuration of hydroxy groups and carbon atom hybridisation. Characterisation of such magnitudes is of importance when using SIMPLE n.m.r. for signal assignment.

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