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SOLID-STATE STRUCTURES OF KETO-DISACCHARIDES AS PROBED BY ^{13}C CROSS-POLARIZATION, "MAGIC-ANGLE" SPINNING N.M.R. SPECTROSCOPY*

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ABSTRACT

The ^{13}C cross-polarization, magic-angle spinning (CP-MAS) spectra of maltulose · H_2O and anhydrous lactulose were examined at different applied magnetic and proton-decoupling fields B_0 and B_1, respectively. Whereas the spectrum of lactulose was insensitive to these changes, that of maltulose showed significant responses. From selective-relaxation experiments, a mixture of three "forms" (based on the ratio of the C-2 resonances) of both lactulose and maltulose were shown to exist in the solid state. The 360-MHz ^1H spectra of the unmutarotated disaccharides in dimethyl sulfoxide solution were used to establish the tautomeric composition of these crystalline solids. The tautomeric composition of lactulose, as determined by the ^1H spectrum in dimethyl sulfoxide, correlated well with the CP-MAS data, but the spectrum of unmutarotated maltulose showed only a single, β-pyranose tautomer to be present. Based on the ratio of the lactulose tautomers determined from the ^1H spectrum in dimethyl sulfoxide (referenced to fructose), the C-2 resonances corresponding to each anomeric form of lactulose were assigned in the CP-MAS spectrum. A "crossover" in chemical-shift positions of the anomeric resonances was observed in going from solution to the solid state. Furthermore, a pronounced increase in the proportion of the furanoid anomers was noted for crystalline lactulose relative to its solution.

INTRODUCTION

Sugars usually exist as tautomeric mixtures in solution and in some instances

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in the solid state. When crystallization takes place from solutions containing an equilibrium mixture of molecules, one species, generally the least soluble, crystallizes preferentially. This is generally true for the reducing monosaccharides. Crystal structures of the monosaccharides 2-acetamido-2-deoxy-D-glucopyranose[1a] and 6-deoxy-L-sorbofuranose[1b] show α,β mixtures in the solid state, and co-crystallization of α,β anomers appears to be the rule, rather than the exception, for such reducing disaccharides as lactose monohydrate[1c], *O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose monohydrate[1d], maltose[1e], and melibiose monohydrate[1f]. In melibiose, α,β compositions of 17:3, 4:1, and 18:7 were reported by different investigators. These apparent differences may be real differences in content of the crystal. However, analysis of anomeric disorder by diffraction methods is difficult; differences in anomeric components of $<5\%$ may go undetected unless the crystal-structure analysis is exceptionally accurate. In each structure where anomeric disorder is observed in the crystal-structure analysis, alternative hydrogen-bonding schemes of comparable energy are possible for both anomers. In many instances it is important to establish the composition of these states, as sensory[2], biological[3], or water-binding properties[4] of such systems may be strongly influenced by a single preponderant or minor, highly active, tautomer.

Recent advances in the technology of Fourier-transform, solid-state, cross-polarization, magic-angle spinning (CP-MAS) ^{13}C-n.m.r. spectroscopy[5] have made it feasible to examine organic polymers[6], polysaccharides[7], humins and humic acids[8], intact microorganisms[9], and crystalline solids[10] in excellent detail. Although considerable progress has been made in refining this methodology to elucidate molecular structure at the level provided by neutron-diffraction studies, interpretations[10d] have thus far been restricted to small molecules. Nevertheless, solid-state n.m.r. spectroscopy can provide useful data for evaluating multicomponent solids.

In previous reports, we and others have examined in detail the solution properties of lactulose (**1**) and maltulose (**2**) by ^{13}C n.m.r. spectroscopy[11]. Our interest in the physical and chemical properties[4] of these materials has stimulated us to explore these structures by the solid state ^{13}C CP-MAS technique. In order to obtain high-resolution ^{13}C spectra in the solid state, the problems associated with intimate lattice-interactions must be overcome. These problems include extremely broad resonance-lines because of homonuclear and also heteronuclear dipole-coupling. The former problem may be eliminated by working with a magnetically dilute system such as ^{13}C, and the latter by the use of very high-powered decoupling at the resonance frequency of the heteronucleus[5a,b]. A second experimental problem arises from the very long relaxation-times (T_1 values) associated with magnetically dilute nuclei and the low signal-to-noise ratio that is a practical experimental result. This difficulty is overcome by the use of a magnetization transfer from the abundant proton-spins to the dilute ^{13}C nuclei. This cross-polarization technique results in much shorter times for obtaining high signal-to-noise ratios[5a,c]. A final problem arises because the position of a given resonance line is a function of the exact orientation of the molecule relative to the magnetic field (chemical-shift aniso-

tropy). In an amorphous or powder sample, this implies that the resonance line will be anisotropically broadened to a width of about 1–2 kHz for ^{13}C. To narrow the chemical-shift anisotropy, the sample is spun at ~2 kHz about the so-called "magic axis" 54.7° from the applied field-direction[5d], where the "magic angle" arises because the shift anisotropy contains a $1 - 3\cos^2\theta$ term that is equal to zero when $\theta = 54.7°$. The resulting high-resolution spectra contain lines that may correspond to physical as well as chemical differences in structure within the crystalline lattice.

RESULTS AND DISCUSSION

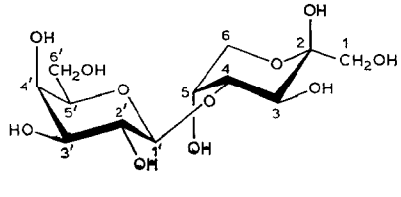
The solution properties of lactulose (4-*O*-β-D-galactopyranosyl-D-fructose, **1**), and maltulose (4-*O*-α-D-glucopyranosyl-D-fructose, **2**) as established by ^{13}C-n.m.r. spectroscopy are quite similar, namely, both sugars mutarotate rapidly (as does fructose) to tautomeric mixture in which the β-pyranose (**1a**) or (**2a**) form is predominant[11a] (Table I). Unlike **1**, which crystallizes exclusively in the anhydrous state, **2** exists as a stable monohydrate convertible into a hemihydrate and into the anhydrous form under various conditions[12]. Earlier workers[13] concluded from polarimetric

TABLE I

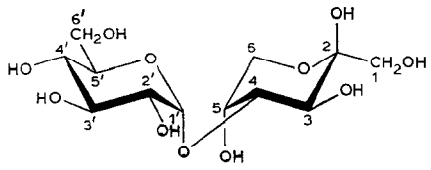
TAUTOMERIC COMPOSITION OF LACTULOSE (**1**) AND MALTULOSE · H_2O (**2**)

Compound	*State*	*Composition (%)*				
		βp	αp	βf	αf	keto
1	Solution (water)[a]	61.5		29.3	7.6	1.8
2	Solution (water)[a]	64.0		22.4	12.1	1.5
1	Solid[b]	15.0		74.5	10.0	
1	Solution (dimethyl sulfoxide)[c]	16.0		72.2	11.8	
1	Solution (dimethyl sulfoxide)[c] 48 h mutarotation	65.0		24.0	11.0	
2	Solution (dimethyl sulfoxide)[c]	100				
2	Solution (dimethyl sulfoxide)[c], 48 h mutarotation	34.6	4.2	39.6	21.6	

[a]Data taken from ref. 10a. [b]Values obtained from integration of the C-2 resonance. [c]Values obtained from integration of the 2-OH resonance.



1a



2a

data that **1** exists as a derivative (**1b**) of β-D-fructofuranose in the crystalline state. More-recent ^{1}H-n.m.r. studies[14] of **1** under non-mutarotating conditions in dimethyl sulfoxide agree in principle with these findings, but suggest that small amounts of the β-fructopyranose (**1a**) and α-fructofuranose (**1c**) tautomers may also be present in the crystal.

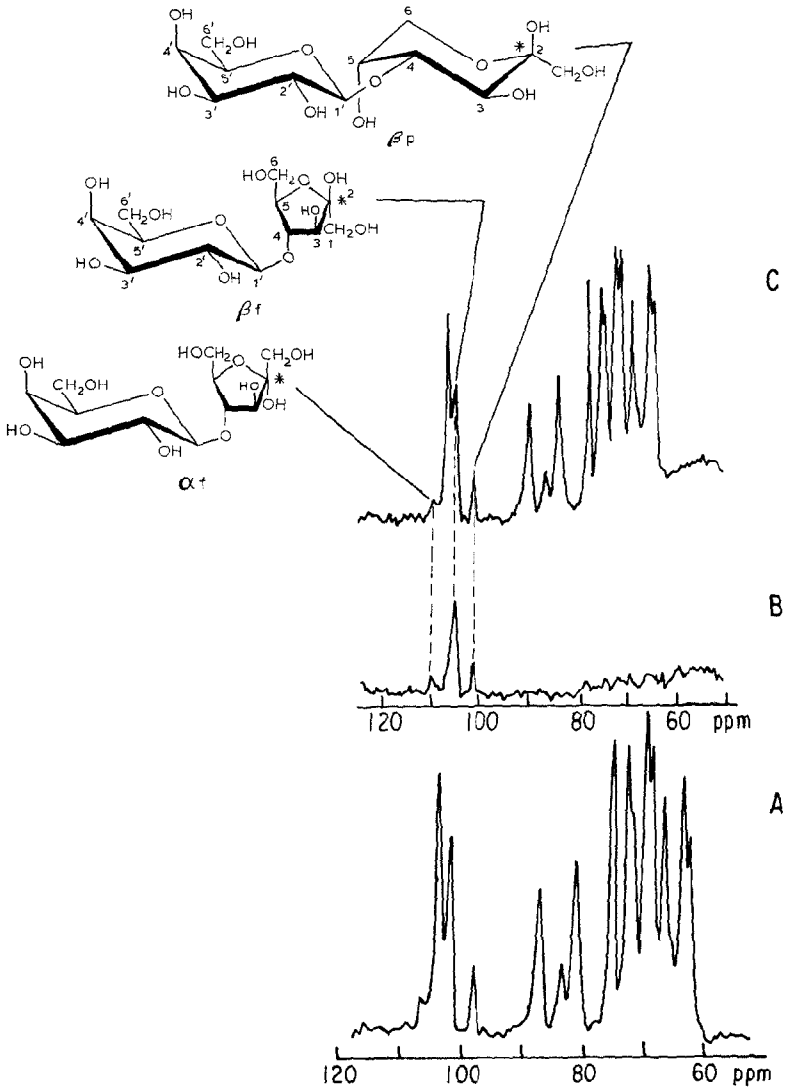
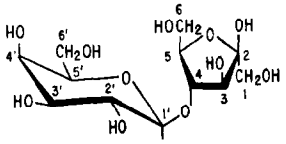


Fig. 1. CP-MAS ^{13}C-n.m.r. spectra of crystalline lactulose (**1**): (A) 15-MHz spectrum, 500 transients, 10-s repetitions, 1-ms contact time, 11-G ^{1}H decoupling, 2.2-KHz spinning; (B) same as C except for insertion of a 40-μs delay with no decoupling prior to acquisition; (C) 37.8-MHz spectrum, 250 transients, 10-s repetitions, 1-ms contact time, 20-G ^{1}H decoupling, ~3.2-KHz spinning.



1b

Structure of lactulose in the solid state. — To establish the nature of **1** in the solid state, it was examined as a crystalline powder at both 1.4 and 3.5 T by CP-MAS ^{13}C-n.m.r. with ^{1}H decoupling at r.f. field-strengths (B_1) of 11 and 20 G, respectively. At both field-strengths, identical spectra were obtained (Figs. 1a and 1c). Although the high-field portion (chemical shifts from 60–90 p.p.m.) is very complex, the low, field (anomeric) region may give insight into the composition. This low-field region of the spectrum contains the anomeric ^{13}C resonances (protonated C-1′ and non-protonated C-2) analogous to those observed in the ^{13}C solution spectrum[11]. However, to establish the ratio of the various tautomers of lactulose represented in this spectrum, it was first necessary to identify each anomeric resonance. A technique useful for simplifying complicated spectra and for assigning the resonance peaks of non-protonated carbon atoms, described by Opella and Frey[15], relies on the fact that, in the absence of proton decoupling, proton–carbon dipolar interaction is a powerful mechanism for relaxation. This proton–carbon interaction has a $1/r^3$ dependence and consequently is much stronger for carbon atoms having directly bonded protons ($\sim$1Å separation) than for carbon atoms that only have nearest-neighbor protons ($\geqq$2Å separation). By inserting a 40-μs delay with no decoupling between the cross-polarization pulse (during which time the carbon magnetization is generated) and the start of data acquisition, the relaxation of the protonated carbon atoms is greatly enhanced over that of non-protonated carbon atoms. The practical result of this particular pulse-sequence is that the resonance signals of protonated carbon atoms are greatly attenuated relative to non-protonated ones, albeit at the expense of a somewhat decreased signal-to-noise ratio for all signals. (One slightly confusing aspect of this pulse sequence is that methyl carbon resonances also remain in the spectrum, as rotation of the methyl group motionally reduces the proton–carbon dipole interaction for the directly bonded methyl protons. In practice this is not a serious limitation as methyl carbon signals may be readily recognized by their chemical shift.)

Fig. 1 shows both the ^{13}C selective, dipolar-relaxed spectrum, IB, and the normal, fully decoupled spectrum IC. The three resonances displayed in Fig. 1B correspond to three distinct "forms" of **1**, each bearing a non-protonated anomeric carbon atom. The ratio of these peaks, from low to high field, was 10:74.5:15.5. The structural nature of these three species is not immediately evident. Unlike n.m.r for solutions, solid-state n.m.r. spectroscopy is sensitive to all molecular orientations in the magnetic field, especially magnetic inequivalences in the unit cell[10a]. Consequently, the question arises as to whether the resonances are related to three, chemi-

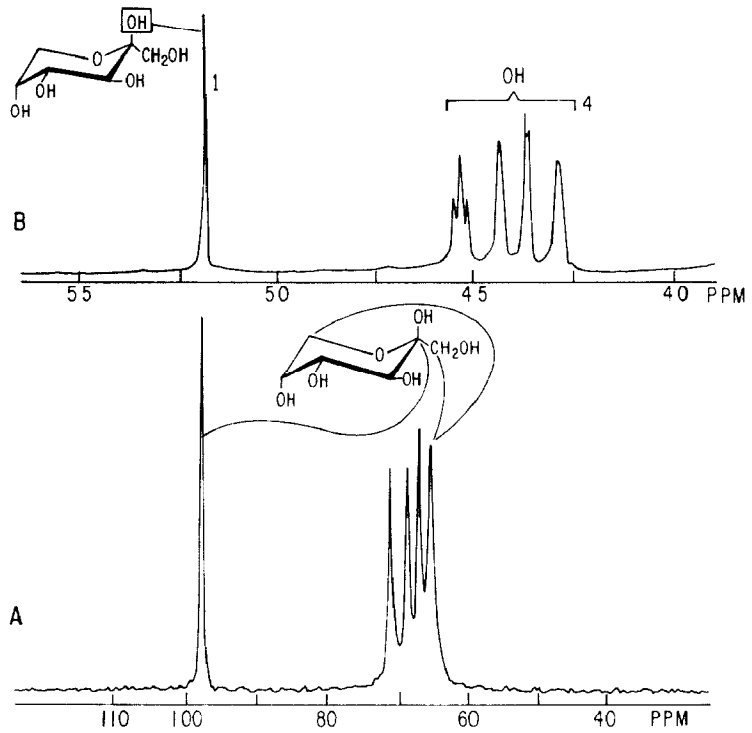


Fig. 2. (A) 15-MHz CP-MAS ^{13}C spectrum of crystalline D-fructose, 1000 transients, 20-s repetitions, 3-ms contact time, 11-G ^{1}H decoupling, 2.2-KHz spinning, displayed spectral width = 2K; (B) low-field OH region of the 360-MHz ^{1}H-n.m.r. spectrum of D-fructose (freshly dissolved crystals) in dimethyl sulfoxide, 16 transients, 10-s repetitions.

cally distinct entities or to inequivalences produced by physical differences between molecules in the crystal.

To answer this question, this system must first be examined without the restriction of the crystal lattice and prior to mutarotation. The tautomeric structures of sugars dissolved in anhydrous dimethyl sulfoxide may be evaluated[14] by ^{1}H-n.m.r. spectroscopy, as mutarotation is slow and the n.m.r. experiment can be completed before the onset of mutarotation[14]. Individual OH resonances corresponding to each tautomer may be assigned by comparison with the shifts of previously well characterized tautomers. Fructose was chosen as a reference compound for examining the related, fructose-containing disaccharides in this study. Fig. 2A shows the ^{13}C CP-MAS spectrum of crystalline fructose, established as the single β-pyranose tautomer by X-ray crystallography[16]. Fig. 2B shows the 360-MHz proton spectrum of fructose crystals freshly dissolved in dimethyl sulfoxide. The sharp singlet at δ 5.18 is attributable to the non-proton coupled 2-OH group of the β-pyranose tautomer. Fig. 3A shows the OH-proton spectrum of the four tautomers present in fructose pre-equilibrated in water prior to dissolution in dimethyl sulfoxide. The 360-MHz OH-proton spectrum of lactulose crystals used in the CP-MAS experiments was examined to

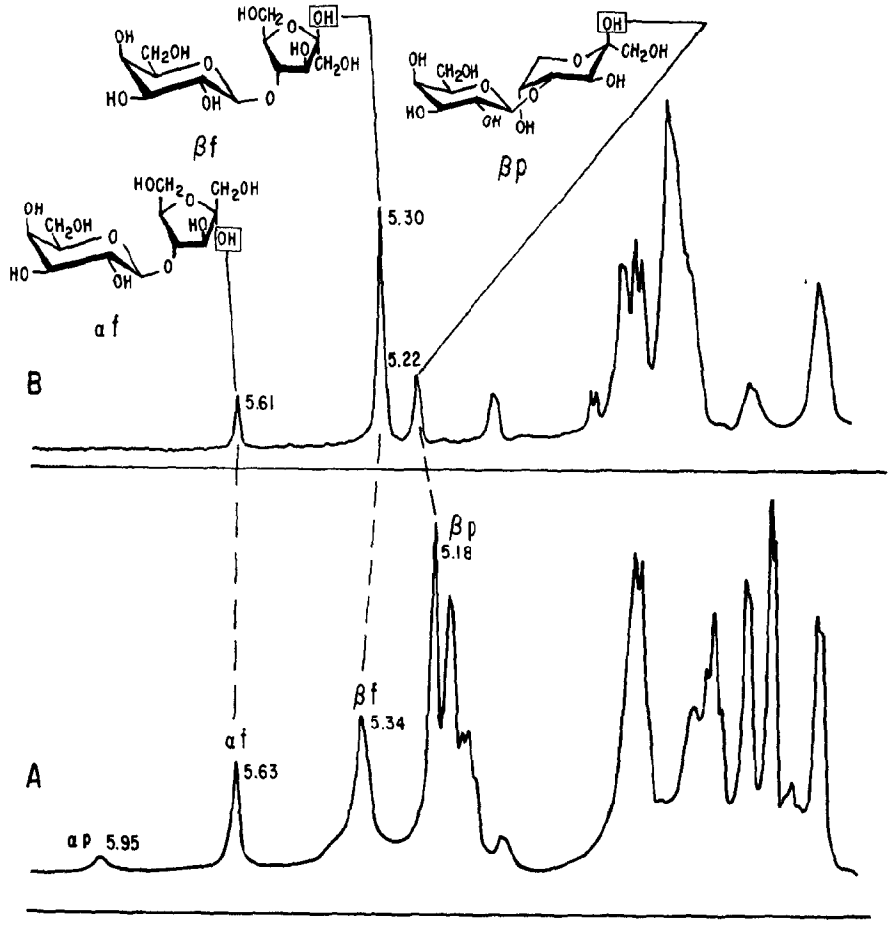


Fig. 3. (A) Low-field OH region of the 360-MHz ^{1}H-n.m.r. spectrum of water-pre-equilibrated D-fructose (freshly dissolved) in dimethyl sulfoxide, 16 transients, 10-s repetitions, spectral section taken from displayed spectral width of 1152 Hz; (B) low-field OH region of the 360-MHz ^{1}H-n.m.r. spectrum of crystalline lactulose, (freshly dissolved crystals) in dimethyl sulfoxide, 16 transients, 10-s repetitions, spectral section taken from a displayed spectral width of 1050 Hz.

determine the correspondence between tautomer distribution as observed by CP-MAS and in dimethyl sulfoxide solution. Fig. 3 shows the correspondence between the three 2-OH singlets attributable to the three major tautomers of pre-equilibrated fructose (spectrum 3A) and three singlet resonances given by crystalline lactulose dissolved in dimethyl sulfoxide (spectrum 3B).

The percentages of the three lactulose tautomers determined from the peak areas in spectrum 3B are the same as those determined from the non-protonated C-2 resonances in Fig. 1B and given in Table I. The proportion of each tautomer did not vary according to the method of crystallization used, for instance, either low- or high-temperature crystallization from methanol (see Experimental section). Clearly,

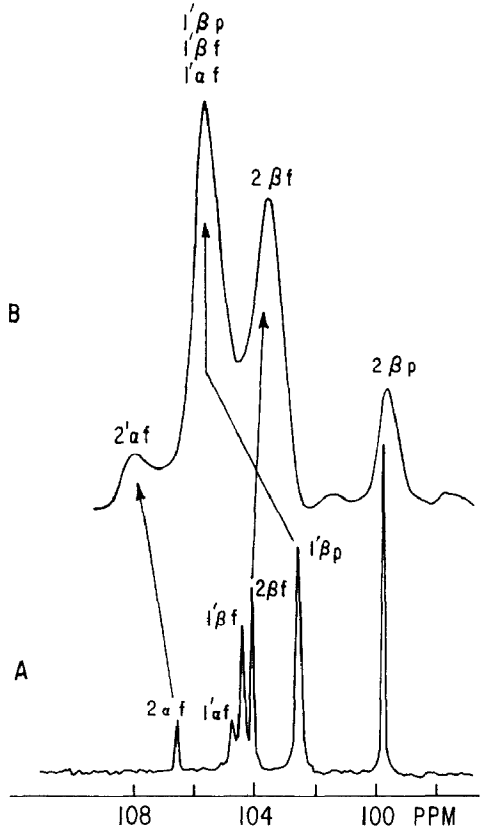


Fig. 4. (A) 15-MHz ^{13}C-n.m.r. solution spectrum of the anomeric region of lactulose (**1**) equilibrated in 4:1 H_2O–D_2O. Chemical shifts are given relative to internal 1,4-dioxane (δ 67.4). The displayed spectral width is 500 Hz, 500 transients, 2-s repetitions; (B) 15-MHz ^{13}C CP-MAS n.m.r. spectrum of the anomeric region of crystalline lactulose, 500 transients, 10-s repetitions, 1-ms contact time, 11-G ^{1}H decoupling, 2.2-KHz spinning, displayed spectral-width 500 Hz.

the fructofuranose tautomers **1b** and **1c** (total 84%) preponderate in crystalline lactulose relative to equilibrated fructose, where the major tautomer is the β-pyranose. As noted later, lactulose differs from crystalline fructose and maltulose in that its ketose moiety exists mainly in the five-membered ring forms in the solid state. Long-time equilibration of solutions of lactulose in dimethyl sulfoxide led to a mixture of tautomers whose composition approximated that of the aqueous, equilibrated mixture described earlier[11a].

From the foregoing analysis of the low-field, anomeric region of the CP-MAS spectrum of lactulose, it is apparent that the resonances at δ 99.8, 103.7, and 107.9 are the C-2 signals of the β-pyranose (**1a**), β-furanose (**1b**), and α-furanose (**1c**) tautomers. The combined area of these three resonances is equal to that of the large resonance at δ 104.9, which corresponds to the sum of the C-1′ resonances of the galactosyl group of each lactulose tautomer, **1a–c**. The ^{13}C solution spectrum dis-

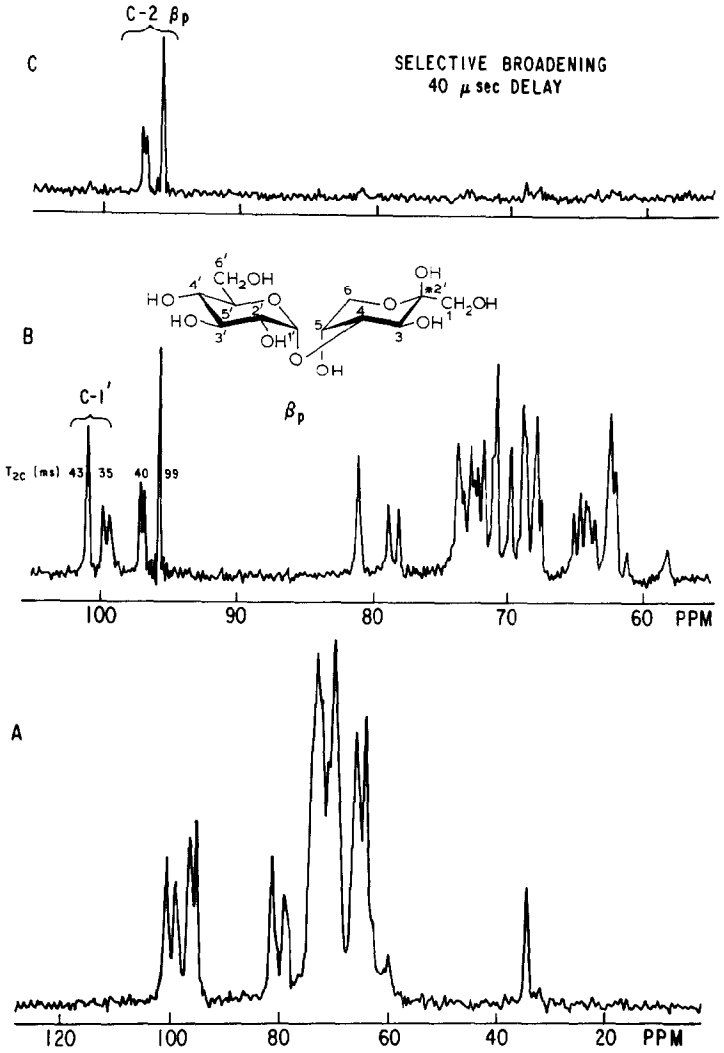


Fig. 5. CP-MAS ^{13}C-n.m.r. spectra of crystalline maltulose (**2**): (A) 15-MHz spectrum, 500 transients, 10-s repetitions, 1-ms contact time, 11-G ^{1}H decoupling, 2.2-KHz spinning; (B) 37.8-MHz spectrum, 250 transients, 10-s repetitions, 1-ms contact time, 25-G ^{1}H decoupling, 3.2-KHz spinning; (C) Same as B except for insertion of a 40-μs delay with no decoupling prior to acquisition.

played in Fig. 4A shows clear separation of all three C-1′ resonances, as discussed in an earlier assignment study using the differential isotope-shift method[11a]. Furthermore, we observed that the C-1′ resonance of the β-pyranose tautomer **1a** was found 2 p.p.m. to higher field than the corresponding C-1′ resonances of **1b** and **1c** (Fig. 4A). This is not the case in the CP-MAS spectrum of lactulose (Fig. 4B), in which all of the C-1′ resonances are found together at lower field (δ 105.0). Essentially what we

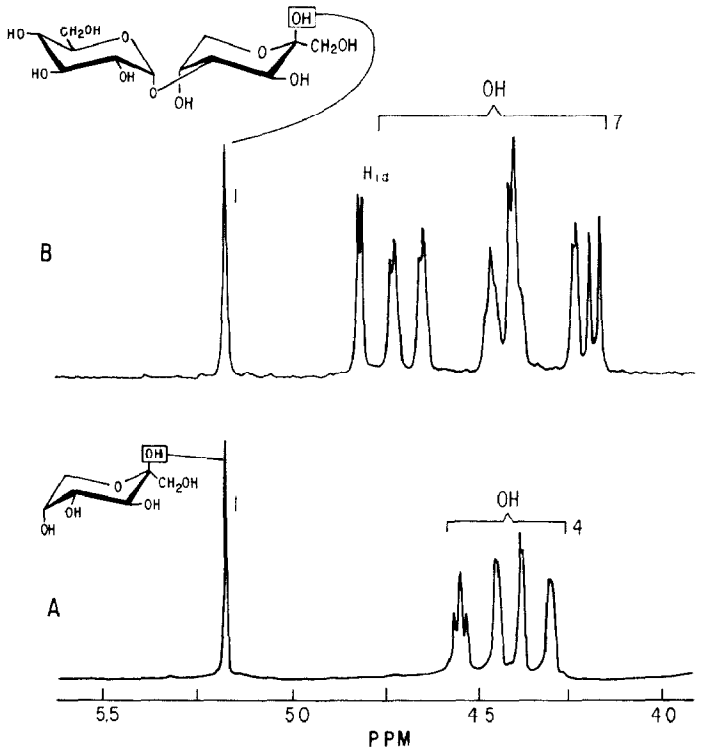


Fig. 6. (A) Low-field OH region of the 360-MHz ^{1}H-n.m.r. spectrum of fructose (freshly dissolved crystals) in dimethyl sulfoxide, 16 transients, 10-s repetitions; (B) low-field OH region of the 360-MHz ^{1}H-n.m.r. spectrum of crystalline maltulose (**2**, freshly dissolved crystals) in dimethyl sulfoxide, 16 transients, 10-s repetitions.

observe is a "cross-over" in the chemical shifts of the $2\beta f$ and $1\beta p$ carbon resonances in comparing the solution spectrum with that in the solid state.

These results emphasize the point that strict correspondence of chemical shifts between solution and the solid state cannot be assumed. This is not surprising for sugars, as the aqueous environment has an overriding effect on the solution conformation of the molecules. Although the conformation of lactulose in solution has been established previously by ^{1}H-n.m.r. spectroscopy[17], there are no definite X-ray crystallographic data that would suggest that it is the same in the solid state. As we have observed that the ratio of the three tautomeric forms **1a–c** is independent of the crystallization technique used here, it is conceivable that lactulose crystallizes as a discrete mixture of the three tautomers within each unit cell, as suggested[18] for the molecular complex of five α- and three β-lactose molecules. Detailed X-ray analysis of lactulose is at present in progress.

Structure of maltulose in the solid state. — The low-field anomeric region of the 15-MHz ^{13}C CP-MAS spectrum of crystalline maltulose · H_2O (**2**, Fig. 5A) suggests that there are two non-equivalent "forms" of this sugar in the solid state. However,

examination at a higher magnetic field (37.8 MHz) with more powerful decoupling (25 G *vs.* 10 G) gives better resolution and suggests that there are three "forms" of this sugar in the ratio of 1:1:2 (Fig. 5B). This supposition is borne out by the results of selective-relaxation experiments (Fig. 5C), which show three non-protonated anomeric carbon resonances, in the ratio of 1:1:2, remaining after the insertion of a 40-μs delay time in the pulse sequence. Further investigation of crystalline maltulose after dissolution in anhydrous dimethyl sulfoxide however, clearly demonstrates that the three "forms" apparent in the CP-MAS spectrum do not constitute three tautomers of maltulose as had been the case with lactulose (**1**). This point is made in Fig. 6, which shows that maltulose exists as the single β-pyranose tautomer (2a) prior to its mutarotation in dimethyl sulfoxide (Table I). Only one 2-OH resonance is observed, at δ 5.18 (Fig. 6b), and this signal coincides in shift with the 2-OH signal in β-fructopyranose (Fig. 6a). Thus the three "forms" of maltulose indicated in the CP-MAS spectrum cannot arise from tautomeric mixtures in the crystal. In principle, small differences in the crystal structure will produce magnetic inequivalences in the molecules, resulting in chemical shifts in the solid. As yet, we have not established whether the chemical shifts observed in maltulose arise from small crystal-packing effects, hydrogen bonding in the solid, or from a complicated crystal-structure having three or more maltulose molecules per unit cell. Evidence that only one tautomer of **2a** is present is substantiated in Fig. 6 by the fact that there are only seven additional resonances corresponding to exchangeable OH protons in the spectrum. The only other low-field proton resonance (non-exchangeable), observed at δ 4.83, is the 3.6-Hz doublet assigned to H-1′ (of the non-reducing glucosyl group). Long-term mutarotation (48 h) of **2a** in dimethyl sulfoxide yields a mixture of tautomers, mainly the β-pyranose and β-furanose (Table I). A small percentage ($\sim$4%) of the α-pyranose tautomer is also present, whereas this tautomer is not observed in equilibrated lactulose (Table I).

It is apparent from the experiments with both lactulose and maltulose that the interpretation of CP-MAS ^{13}C-spectral data is not trivial. It is important to have criteria that permit differentiation between shifts corresponding to physical differences induced by the crystalline state (maltulose) *vs.* structural differences resulting from tautomeric mixtures (lactulose). Clearly, verification of these differences must be made before spectral information from the solid state can be used to its fullest advantage.

CONCLUSIONS

1. ^{13}C CP-MAS spectroscopy may be used to define the tautomeric composition of carbohydrates in the solid state.

2. Chemical-shift multiplicities arising from crystal effects *vs.* chemical structural differences in sugars may be differentiated by means of ^{1}H spectra in dimethyl sulfoxide.

3. The assignment of chemical shifts and tautomeric composition in the solid state may be verified by a combination of selective broadening experiments and spectra recorded in dimethyl sulfoxide solution.

EXPERIMENTAL

N.m.r. spectra. — The CP-MAS ^{13}C n.m.r. spectra were recorded with a JEOL* FX-60Q-S spectrometer operating at 15 MHz. Chemical shifts are reported relative to the upfield CH_3 signal of hexamethylbenzene taken as 17.36 p.p.m. from tetramethylsilane. Prior to each experiment, the field was calibrated and the irradiation offsets were established with hexamethylbenzene. Samples (350–500 mg) were examined in an 8-mm (i.d.), 0.7 cm^3 poly(trichlorofluoroethylene) rotor, spinning at ~2.2 KHz. Contact times of 1–3 ms were used with 10-sec repetitions and 10–11 G ^1H decoupling. The proton relaxation-times (T_{1H}) for fructose, lactulose, and maltulose were 10, 6, and 3 s, respectively.

The higher-field CP-MAS ^{13}C n.m.r. spectra were recorded with a Bruker CXP-200 spectrometer operating at 50 MHz with ^1H decoupling of 11 G, spinning rate of 3.2 KHz, and contact time of 1 ms; and with a home-built 150-MHz n.m.r. spectrometer operating at 37.8 MHz, sample spinning at 3.5 KHz, and 25-G ^1H decoupling.

The ^1H spectra were recorded with a Bruker WH 360 n.m.r. spectrometer equipped with a 5-mm probe. Each 10-mg sample was dissolved in 0.5 mL of freshly dried dimethyl sulfoxide (distilled from calcium hydride) and the spectrum recorded immediately. All shifts are reported relative to internal dimethyl sulfoxide assigned at δ 2.49. All OH resonances were verified by their subsequent exchange and disappearance upon addition of D_2O. Each spectrum was produced from 16 transients (90° pulses) with 10-s repetition times to ensure complete relaxation. Spectral widths were 5000 Hz with 16,000 data points.

Low-temperature crystallization of lactulose. — Pure lactulose[19], (5 g), freshly dissolved in water (5 mL), was evaporated *in vacuo* at 45°. The dry residue was dissolved in abs. methanol (5 mL) and placed in a 25-mL round-bottom flask. The flask was incubated at 40–50° and kept in gentle motion for several h until crystallization was complete. The crystals were filtered off and dried in air. The white, translucent microcrystals had a melting range of 168–171°. X-Ray powder patterns indicated that this material has three crystalline phases.

High-temperature crystallization of lactulose. — A mixture of pure lactulose (0.5 g) and methanol (3.0 mL) was sealed in a 3-mL high-temperature reaction vial. The vial was immersed in an oil bath at 100° and shaken periodically until all of the lactulose had dissolved (~1 h). During the next 2 h, the bath was cooled to 60° and then maintained at that temperature for 24 h. During this time, lactulose crystal-

*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

lized spontaneously from the solution. The transparent crystals were isolated on a filter and air-dried. The crystalline lactulose recovered (~350 mg) had m.p. 166–168°. Elemental analysis showed the crystals to be anhydrous.

Crystallization of maltulose · H_2O. — Maltulose was prepared by isomerization of maltose with dilute sodium hydroxide or triethylamine in the presence of boric acid. The resultant product was purified by preparative liquid-chromatography. Details of the isomerization and chromatography will be published later[20].

The chromatographically pure maltulose (1 g) was dissolved in water (4 mL) and the solution slowly warmed to 40°. The solution was stirred and acetone (26 mL) was added dropwise. The cloudy mixture was allowed to reach room temperature over the course of 2 h. After 1 h, the solution was seeded with crystals of maltulose monohydrate. Crystals began to form after several h and grew slowly for about a week. When crystallization had ceased, the mixture was filtered and the fluffy white crystals were washed with cold acetone and dried in air. The crystals (700 mg) had m.p. 116.5–118.5°. The state of hydration was verified by Karl Fischer determination of water and by elemental analysis. X-Ray powder patterns indicated the material to be crystalline.

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