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Observation of the *keto* tautomer of D-fructose in D₂O using ¹H NMR spectroscopy

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Abstract

D-Fructose was analysed by NMR spectroscopy and previously unidentified ¹H NMR resonances were assigned to the *keto* and α -pyranose tautomers. The full assignment of shifts for the various fructose tautomers enabled the use of ¹H NMR spectroscopy in studies of the mutarotation (5 – 25 °C) and tautomeric composition at equilibrium (5 – 50 °C). The mutarotation of β -pyranose to furanose tautomers in D₂O at a concentration of 0.18 M was found to have an activation energy of 62.6 kJ.mol⁻¹. At tautomeric equilibrium (20 °C in D₂O) the distribution of the β -pyranose, β -furanose, α -furanose, α -pyranose and the *keto* tautomers was found to be 68.23%, 22.35%, 6.24%, 2.67% and 0.50%, respectively. This tautomeric composition was not significantly affected by varying concentration between 0.089 and 0.36 M or acidification to pH 3. Upon equilibrating at 6 temperatures between 5 and 50 °C there was a linear relationship between the change in concentration and temperature for all forms.

Keywords

D-Fructose; Carbohydrate structural analysis; Mutarotation; Tautomeric equilibrium

1. Introduction

The exact molecular structure that carbohydrates adopt in solution is an area of research that has received considerable investigation because of their relevance to biological systems.^{1–3} Nuclear magnetic resonance (NMR) spectroscopy is a valuable tool for this analysis, being used extensively in conformational analysis of both polysaccharides and simple sugars.^{1,3–5}

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Supplementary data

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In the latter case there is considerable complexity created by the existence of multiple tautomeric forms of these sugars in solution.^{1,5–7} These tautomers possess small ranges of shifts, leading to congested and strongly coupled spectra, which are particularly problematic in ^1H NMR spectroscopy.^{8,9} Additionally, the combination of congested spectra with the low concentrations of some of the tautomeric forms means that for several biologically important carbohydrates the resolution of ^1H NMR spectroscopy was insufficient to detect the minor forms in previous investigations.^{10,11}

Fructose as either a free sugar or in polysaccharide forms, such as inulin, is a highly valuable commercial product. As the free sugar, fructose is an example of a simple reducing sugar that has a complex ^1H NMR spectrum as a result of it existing in at least five tautomers in solution (Figure 1).^{4,12–14} At equilibrium in water β -D-fructopyranose (β -pyr) is the preponderant tautomers, followed by β -D-fructofuranose (β -fur), and then α -D-fructofuranose (α -fur). These tautomers have previously been determined to account for 69.6%, 21.1% and 5.7% of the solubilised sugar at room temperature, respectively.¹⁴ The minor tautomers of fructose are α -D-fructopyranose (α -pyr) and the linear *keto* form of fructose.^{12–16} The *keto* form of fructose has not been previously identified using ^1H NMR spectroscopy in D_2O .^{11,15,17–19} The α -pyranose tautomer has also been difficult to identify using ^1H NMR spectroscopy in aqueous solution, only being observed in experiments measuring the anomeric hydroxyl group conducted on samples equilibrated in water, flash frozen, and then melted into $\text{DMSO-}d_6$. This relies on the slow tautomeric equilibration in $\text{DMSO-}d_6$ to provide data for the tautomeric composition in water.¹⁸

Both of the minor tautomers of fructose have been detected using ^{13}C NMR spectroscopy.^{14,15,17,20} However, because of the low isotopic ratio of ^{13}C as compared to ^{12}C , these experiments are time consuming. This is particularly the case when trying to resolve components in low concentrations, and so ^{13}C NMR spectroscopy is not practical for determining changing aqueous tautomeric ratios, for example in the study of the mutarotation reaction.^{13,21} The speed of such ^{13}C NMR experiments can be increased through isotope labelling,^{9,22–24} but this increases the cost and inconvenience of the method.¹³ Fortunately, NMR technology and methods have progressed and herein we report the straightforward identification and quantitation of the *keto* form of fructose using ^1H NMR 1D and 2D techniques and subsequently investigate the mutarotation of fructose and the effect of temperature and pH on its tautomeric composition in solution.

2. Results and discussion

2.1 NMR spectroscopic analysis

A ^1H NMR spectrum for fructose dissolved in D_2O and equilibrated at 20 °C is shown in Figure 2, and shifts for all tautomers are listed in Table 1. The largest resonances for β -pyranose, β -furanose and α -furanose have previously been assigned in the literature^{8,11,25} and our 2D NMR analyses confirm those assignments (see supporting information). Other smaller resonances were first observed by us in the ^1H NMR spectra created while monitoring the hydrolysis of inulin (a polysaccharide comprised of linear fructose chains having β -(2 $\rightarrow$ 1) glycosidic linkages and capped at the reducing end with glucose²⁶). These small peaks were not explained by the literature for the polymeric starting material or the fructose and glucose hydrolysis products and as such, an investigation was made to explain these resonances. A preliminary identification of the most obvious of these small resonances, occurring between 4.50 and 4.70 ppm, was made by comparison to ^1H NMR shifts reported for erythrulose, a linear tetrose that is analogous to the *keto* form of fructose at C1–C3.^{27,28} This enabled assignment of H1a, H1b and H3 of the linear *keto* form. The presence of this isomer in the equilibrated mixture was supported by Fourier-transform infrared (FTIR) spectroscopy (see Supplementary data). The FTIR results showed that

fructose equilibrated in D₂O (1 M) at room temperature was found to have a diagnostic peak for the *keto* tautomer at 1728 cm⁻¹,^{13,21} while freshly dissolved samples did not.

Confirmation of the ¹H NMR peak assignments for H1a, H1b and H3 was provided by heteronuclear 2D NMR techniques (HMBC and HMQC) for which correlations between the ¹H NMR shifts and ¹³C NMR shifts agreed with literature ¹³C NMR assignments for the *keto* tautomer.^{15,24} This analysis, combined with further 2D NMR experiments (including homonuclear DQF-COSY and NOESY spectra), allowed the full assignment of all resonances for the *keto* tautomer. Similarly, other previously unidentified ¹H NMR peaks were assigned to the α -pyranose form on the basis of 2D correlations with the literature values for the ¹³C NMR spectrum for this tautomer.^{15,17,20} The identification of the *keto* and α -pyranose tautomers in D₂O using ¹H NMR spectroscopy meant that an investigation of the mutarotation reaction and the influences on the tautomeric composition of fructose could be investigated with relative ease.

2.2 Mutarotation

The mutarotation of fructose from the exclusively β -pyranose conformation of the crystalline solid⁴ to the equilibrium composition of tautomeric forms in solution is a complex process due to the differing rates of transformations between tautomers. The pyranose to pyranose transformation is slow, occurring between two stable chair conformations,^{1,4,6,7,29} while the transformation between pyranose and furanose is rapid⁴ and the furanose-to-furanose transformation is very rapid, occurring essentially instantaneously between relatively high-energy envelope and twist forms.^{1,9,12,22,24} Nonetheless, the mutarotation of fructose can often be approximated as a simple first-order process,^{4,12} and it has been demonstrated that the kinetics can be represented by the conversion of β -pyranose to the furanose forms.³⁰

To investigate the mutarotation reaction, the change in the ¹H NMR integral for the combined peak occurring between 4.10 and 4.15 ppm was monitored for fructose freshly dissolved in D₂O at five temperatures between 5 and 25 °C. This peak is comprised of resonances attributable to β -furanose H-3 and H-4 as well as α -furanose H-3. Using the tautomeric composition determined by ¹H NMR spectroscopy (discussed further in the following section), the ratio of β -furanose to α -furanose of 4.1:1 was determined to be constant between 5 °C and 50 °C, in close agreement to values in the literature.¹² Consequently, it was possible to use this peak to establish the concentration of furanose forms relative to the total concentration of fructose. The relative concentration of the furanose forms over the course of the mutarotation was plotted and found to rise exponentially, confirming the validity of using first-order kinetics. KaleidaGraph scientific graphing software was used to provide exponential curve fits and determine the rate constant for each temperature. An Arrhenius plot (Figure 3) was then prepared from which the activation energy for the mutarotation of fructose was determined as 62.6 kJ.mol⁻¹ from the slope of the equation to the straight line [$\ln(k) = \ln(A) - E_a / RT$]

Previous determinations of the activation energy for fructose mutarotation in aqueous solutions are surprisingly rare. Grønlund and Andersen³¹ determined the activation energy in acetate buffer (pH 4) to be 72.0 kJ.mol⁻¹ using polarimetry. However, this research was conducted before all tautomeric forms of fructose had been identified and assumed that mutarotation was occurring between a single pyranose form and a single furanose form. Also, the investigation of the mutarotation of fructose by polarimetry, even when all tautomers are considered, relies on only one defined specific rotation for the β -pyranose. The specific rotation for the other tautomers is indirectly estimated with high uncertainty.⁴

Flood *et al.*³⁰ determined the activation energy of the mutarotation of fructose in aqueous ethanol solutions to be $53.0 \pm 5 \text{ kJ.mol}^{-1}$ using GLC. These authors claim that the activation energy of mutarotation is unaffected by solvent composition, but did not obtain their own data for the kinetics of the mutarotation in pure water. Their activation energy calculations were based on two points of data obtained from two different sources.

2.3 Tautomeric composition

The investigation of the concentrations of tautomeric components in equilibrium for dissolved fructose have been conducted previously using several techniques including ^{13}C NMR spectroscopy,^{14,15,17,20,24,25,32–34} ^1H NMR spectroscopy,²⁵ GLC and GLC–MS,^{12,35} polarimetry³⁶ UV–vis spectroscopy,³⁷ circular dichroism,³⁸ and FTIR spectroscopy.^{13,21} These investigations have provided inconsistent results in terms of tautomers identified and relative concentrations of each at equilibrium.^{12,13,15,18,19,32} The reasons for this inconsistency have typically been attributed to the shortcomings of the methods used,^{12,18,21,30} misreading of results by researchers¹⁸ and the complexity of the mutarotation of fructose.^{4,12}

Previously ^1H NMR spectroscopy has been considered inadequate to determine tautomeric composition of fructose due to the overlapping nature of the peaks and the lack of a separated anomeric proton resonance, such being useful in the anomer concentration determination for glucose.^{10,15,32,33,39} Despite this, Jaseja *et al.*²⁵ determined percentage concentrations of the three most abundant tautomers (β -pyranose 75%, β -furanose 21% and α -furanose 4%) of D-fructose in reasonable agreement to the rest of the literature. With the identification of the shifts for the α -pyranose and *keto* forms, specifically those for *keto* H-1 and *keto* H-3 separated from the overlapping peaks, determination of the concentrations D-fructose in equilibrium is easier and more accurate using ^1H NMR spectroscopy.

To determine the tautomeric ratios, fructose was equilibrated in D_2O at six temperatures from 5 °C to 50 °C for at least 48 hours. ^1H NMR spectroscopy was then conducted at the corresponding temperature for each sample. Subsequent analysis used either H-1 or H-1' for quantification of the concentration of the *keto* tautomer (the HDO resonance is mobile with temperature,⁷ interfering with one side of the *keto* peaks at 35 and 50 °C and totally obscuring them at 42.5 °C, thus explaining why this temperature was not evaluated), and subsequent peak integrals for other tautomers were normalised using this value.

The region of the spectrum between 3.93 and 3.97 ppm is a combination of resonances for *keto* H-4 and α -pyranose H-4. The value for the quantified *keto* form was subtracted from the integral for this combination of resonances to provide the relative concentration of α -pyranose. In turn the relative concentration for α -pyranose was subtracted from an integral covering the region from 3.88 to 3.92 ppm to provide a relative concentration for β -pyranose. Then the relative concentration for β -pyranose was subtracted from an integral covering the region from 3.54 to 3.6 ppm to provide the relative concentration of the β -furanose tautomer. Finally, depending on the temperature, α -furanose has an isolated shift for H-5 at 4.06 ppm from which its relative concentration was derived. This shift, however, is mobile with temperature and at low temperature it impinges on the peak for β -pyranose H-6. In this case the region between 3.99 and 4.09 ppm was integrated, and the relative concentration for β -pyranose was subtracted twice (to account for H-6 and H-5) and α -pyranose once (H-3), resulting in the calculated relative concentration for α -furanose.

Figure 4 shows a plot of the relative concentrations of each tautomer expressed as a percentage of total fructose for temperatures from 5 °C to 50°C. There was a linear relationship between the change in percentage concentration and temperature for all forms, matching previous studies.^{12,18,24} The changes in percentage concentration of each tautomer

also match the general results of the literature, with the percentage concentration of β -pyranose reduced with increasing temperature, while all other tautomers increased in concentration.^{4,5,12,14,17,18,20–22,24,25,32} More specifically, these results are a close match to those previously published using ^{13}C NMR spectroscopy in which at least four of the five tautomers were quantified (see Table 2).^{14,20,40} The results are also in reasonable agreement with an experiment using GLC to measure tautomeric composition in buffer solution (pH 4.4),¹² though the GLC measurements for the α -pyranose and *keto* forms are lower, and the one for β -pyranose is higher compared to the ^1H NMR results. The experimental procedure for these GLC measurements utilised relatively short equilibration times (e.g., 4 hours at 15 °C).¹² The equilibration times are sufficient based on the kinetics of the β -pyranose to furanose conversion, for which a half-life at 15 °C is 1159 seconds based on our kinetic data [$t_{1/2} = \ln(2)/k$]. The equilibration time was less convincing, however, for the β -pyranose to α -pyranose conversion, which is the slowest and has a half-life of 2793 seconds based on our results. However, given that the mutarotation occurs more rapidly in water than in D_2O ,²¹ our results obtained in D_2O may not be applicable. At this point it is constructive to also compare our results for the concentration of the *keto* tautomers to other results that did not necessarily quantify all of the tautomers of D-fructose in solution. Table 3 shows that generally measurements are reasonably consistent, when temperature is taken into account. Amongst the largest variances include the low measurements using GLC reported by Cockman *et al.*,¹² discussed previously. Also, the UV-vis measurement of Avigad *et al.*³⁷ appears too high for this temperature, and indeed subsequent research suggested that some data may have been in error.³⁸ The FTIR measurements of Yaylayan *et al.*¹³ are an anomaly; at 25 °C the concentrations of the *keto* form are consistent with other results, but they are much higher at 80 °C. The authors explain this discrepancy by comparing the linear relationship between relative concentration and temperature found for ^{13}C NMR studies between 20 °C and 50 °C¹⁴ and the quadratic relationship they found using FTIR from 25–80 °C.²¹ However, at 80 °C their result (13.1%) is much higher than that found using ^{13}C NMR spectroscopy (3%)¹⁵ and even at 50 °C their measurement (4.9%) is elevated compared to the consistent results produced by ^{13}C NMR spectroscopy¹⁴ and our current ^1H NMR experiments (1.3%).

2.4 Effect of fructose concentration and pH on tautomeric equilibrium

Mutarotation and tautomeric equilibrium experiments previously have often been conducted at a range of different conditions in terms of the concentration of fructose (0.2 M–4 M)^{12,17,18,20} and the pH (2–9).^{12,13,31} These factors have been identified as influential to the tautomeric equilibrium,^{4,13,24} though the effect is known to be low compared to temperature and relatively extreme conditions required to observe the effect.^{12,13,32} To verify that our results can reasonably be compared to the previous literature, tautomeric equilibrium experiments were conducted in which these parameters were varied.

Fructose concentrations between 0.5 M and 1.5 M have often been used for experiments investigating tautomeric equilibrium,^{12,17,20} higher concentrations frequently benefiting the method of determination. In contrast, quantification using ^1H NMR spectroscopy can suffer broadening of resonances using high concentrations, and consequently concentrations of 0.089 M, 0.18 M and 0.36 M were investigated in our research. Tautomeric ratios were determined from 5–50 °C for each of these concentrations, and there was no significant difference between results. Similarly, Hyvönen *et al.*³² found the difference in tautomeric composition for solutions containing 20–80% fructose were not measureable. However, it cannot be ruled out that high concentrations of fructose can affect mutarotation and tautomeric equilibrium. Indeed, the tautomeric ratios determined for fructose at a concentration of 4 M, while roughly correlated to our results, do have different rates of

change in tautomer concentration (illustrated by different slopes for plots of tautomeric composition at equilibrium for different temperatures).¹⁸

The effect of pH on the mutarotation of fructose was evaluated by determining tautomeric ratios from 20 °C to 50 °C in a D₂O solution adjusted to pH 3 with acetic acid. Again, no significant difference was found in the tautomeric ratios with or without the acid, which is in agreement with previous ¹³C NMR studies for mildly acidic pH.¹⁴

2.5 Conclusion

Previously unidentified shifts in the ¹H NMR spectrum of D-fructose at equilibrium in D₂O were assigned to *keto* and α -pyranose tautomers, completing the assignment of all detected tautomers. This has enabled the use of ¹H NMR spectroscopy in investigations of mutarotation and the equilibrium composition of D-fructose. The benefits of using ¹H NMR spectroscopy for these types of investigation are many. Firstly, ¹H NMR spectroscopy is fast, comparable to the fastest techniques employed for this work, such as polarimetry, FTIR, CD and UV–vis spectroscopic methods. Compared to these techniques ¹H NMR spectroscopy can easily give accurate concentrations for all tautomers, without requiring complicated calculations or indirect estimations. The speed of the ¹H NMR experiment makes it suitable for investigating mutarotation experiments in which concentrations of tautomers are changing over time, something ¹³C NMR spectroscopy cannot achieve for the time scales relevant for D-fructose mutarotation. ¹H NMR spectroscopy is also convenient, not requiring chemical modification of the sugar prior to analysis, such as gas chromatographic techniques and isotope enrichment for ¹³C NMR studies. Given these analytical advantages further investigation the complex mutarotation of D-fructose using ¹H NMR spectroscopy may reveal more information on this important biological species.

3. Experimental

3.1 General methods

D-(-)-fructose and trifluoroacetic acid were purchased from Sigma–Aldrich Pty. Ltd. (Australia), acetic acid was purchased from Ajax Finechem Pty. Ltd. (Australia), and deuterium oxide (D₂O) was obtained from Novachem Pty. Ltd. (Australia). All were used as received.

3.2 Mutarotation

Mutarotation experiments were conducted using ¹H NMR spectroscopy at temperatures from 5 °C to 25 °C in 5 °C increments on D-fructose freshly dissolved in D₂O (0.18 M).

3.3 Equilibrium Composition of D-fructose

D-Fructose was dissolved in D₂O (0.089 M, 0.18 M and 0.36 M) and equilibrated in a water bath at six different temperatures from 5 °C to 50 °C (5, 12.5, 20, 27.5, 35 and 50 °C) for at least 48 h. The effect of pH on tautomeric equilibrium was evaluated by dissolving D-fructose D₂O (0.089 M) acidified with acetic acid (0.25% v/v, ~ pH 3) and equilibrated in a water bath at 20 °C, 35 °C and 50 °C for at least 48 h. Subsequent to equilibration, the tautomeric composition of D-fructose was determined using ¹H NMR spectroscopy.

3.4 NMR spectroscopy

NMR spectra were recorded on a Bruker Avance III 600 operating at 600 MHz for ¹H. 1D and 2D spectra were collected using standard gradient-based pulse programs. The 1D ¹H NMR data were obtained over 64 scans with a 30° flip angle (90° pulse = 8.4 μ s), an acquisition time of 2.7 s, a relaxation delay of 2 s and 65k data points. The temperature for

all experiments was held constant using an in-built heater and was calibrated using ethylene glycol. All experiments were conducted in D₂O at concentrations of 0.089, 0.18 and 0.36 M with chemical shifts reported in parts per million (ppm) downfield from 3-(trimethylsilyl)propionic acid sodium salt (TPS). Calibration of fructose shifts to TPS was achieved in separate experiments using an acetic acid internal standard.⁴¹ Subsequently, experiments were conducted without acetic acid where it was undesirable due to potential influence on mutarotation.²²

3.5 FTIR

FTIR spectroscopy was conducted with a Thermo Electron Corporation Nicolet Nexus 870 spectrophotometer using the transmission Smart Collector attachment and data generated was manipulated using OMNIC[®] software. Experiments were conducted on fructose samples dissolved in D₂O (1 M) in a liquid cell with barium fluoride windows and a path length of 0.025 mm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Tautomeric forms of D-fructose in solution⁴

Figure 2.
 ^1H NMR spectrum (600 MHz) for D-fructose equilibrated in D_2O (0.18 M) at 20 °C

Figure 3.
Arrhenius plot for the mutarotation of D-fructose in D₂O (0.18 M)

Figure 4.
Equilibrium tautomeric composition of D-fructose in D₂O (0.18 M)

Table 1

¹H NMR shifts for D-fructose equilibrated in D₂O

Tautomer	Chemical shift (ppm) ^a						
	H-1	H-1'	H-3	H-4	H-5	H-6	H-6'
β-pyranose	3.71	3.56	3.80	3.90	4.00	4.03	3.71
α-pyranose	3.69	3.65	4.03	3.95	3.88	3.87	3.70
β-furanose	3.59	3.55	4.12	4.12	3.85	3.81	3.68
α-furanose	3.67	3.64	4.11	4.00	4.06	3.82	3.70
<i>keto</i>	4.65	4.54	4.64	3.94	3.78	3.85	3.67

^aFor D-fructose equilibrated in D₂O (0.18 M) at 20 °C (fructose shifts calibrated to TPS using internal AcOH).

Table 2Equilibrium tautomeric composition of D-fructose tautomers in D₂O

Source	Method	Temp. (°C)	Tautomeric composition (%)			
			<i>keto</i>	<i>α-pyr</i>	<i>α-fur</i>	<i>β-pyr</i>
Wolff ⁴⁰	¹³ C NMR	31	0.8	2.65	6.5	25.25
Horton ²⁰	¹³ C NMR	20	-	2	5	23
Mega ¹⁴	¹³ C NMR	21	0.5	3	5.7	21.1
Cockman ¹²	GLC	25	0.36	0.52	5.49	22.25
This research	¹ H NMR	20	0.50	2.67	6.24	22.35
						68.23

Table 3Equilibrium composition of the *keto* tautomer of D-fructose

Source	Method	Temp. (°C)	Solvent	Conc. (M)	pH	<i>keto</i> (%)
Avigad ³⁷	UV-vis	25–27 ^a	H ₂ O	0.04–0.3 ^a	<i>b</i>	2
Hayward ³⁸	CD	20	D ₂ O	0.2–1 ^a	5.2–7.0 ^a	0.7
Wolff ⁴⁰	¹³ C NMR	31	D ₂ O	4	<i>b</i>	0.8
Funke ¹⁵	¹³ C NMR	80	D ₂ O	3.7	<i>b</i>	3
Cockman ¹²	GLC	10–55	H ₂ O	0.5	4.4	0.22–0.36
Mega ¹⁴	¹³ C NMR	21, 40, 50	30% D ₂ O	0.081	7	0.5, 1.2, 1.3
Mega ¹⁴	¹³ C NMR	21, 50	30% D ₂ O	0.081	4.9	0.6, 1.3
Yaylayan ¹³	FTIR	25	D ₂ O	1.1–2.8 ^a	2–9	0.9–0.8
Yaylayan ¹³	FTIR	25–80	D ₂ O	1.1–2.8 ^a	7	0.7–13.1 ^c
This research	¹ H NMR	20	D ₂ O	0.089, 0.18, 0.36	<i>b</i>	0.49, 0.50, 0.50
This research	¹ H NMR	5–50	D ₂ O	0.18	<i>b</i>	0.25–1.30
This research	¹ H NMR	20	D ₂ O	0.18	3	0.5

^a Specific value not specified, but range given;^b pH not determined, but should approximate neutrality;^c Relationship between change in concentration and temperature determined to be quadratic