retains monooxygenase activity in vivo, its function could be distinct from the well-characterized role of intravesicular dopamine β-hydroxylase in norepinephrine biosynthesis.

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Solution Structure of 5-Keto-D-fructose: Relevance to the Specificity of Hexose Kinases†

John S. Blanchard, C. F. Brewer,* Sasha Englard,* and Gad Avigad

ABSTRACT: 5-Keto-D-fructose (5KF) is isolated from cultures of *Gluconobacter cerinus* growing on D-fructose as the sole carbon source. 5KF is a substrate for hexokinase, fructokinase, and several polyol dehydrogenases. ^1H and ^{13}C nuclear magnetic resonance studies show that 5KF exists in different forms in anhydrous dimethyl-d_6 sulfoxide and D_2O. In dimethyl-d_6 sulfoxide, 5KF exists as a spirane dimer with linked furanose and pyranose rings, similar to the structure reported for crystalline 5KF [Hassen, L., Hordvik, A., & Hove, R. (1976) *J. Chem. Soc., Chem. Commun.*, 572]. In D_2O, 5KF exists predominantly (>95%) in a β-pyranose form with the 5-keto group hydrated to form a *gem*-diol. ^{13}C–^1H coupling patterns, ^{13}C relaxation measurements, and ^{13}C deuterium-induced differential isotope shifts confirm this structure of 5KF. The phosphorylation of 5KF by fructokinase can be accounted for by an approximately 2% proportion of the β-furanose form in solution at 25 °C. Both the β-pyranose and β-furanose forms of 5KF are proposed to be substrates for yeast hexokinase.

5-Keto-D-fructose (5KF,[1] D-*threo*-2,5-hexodiulose) is produced by several strains of *Acetobacter* growing on D-fructose as the sole carbon source (Terada et al., 1960; Avigad & Englard, 1965; Ameyama et al., 1981). Specific NADPH-linked dehydrogenases have been isolated and purified from *Gluconobacter cerinus* (Avigad et al., 1966) and yeast (Englard et al., 1972) which reduce 5KF and 5KF-1-P to D-

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[1] Abbreviations: 5KF, 5-keto-D-fructose; 5KF-1-P, 5-keto-D-fructose 1-phosphate; Me_2SO-d_6, dimethyl-d_6 sulfoxide; NMR, nuclear magnetic resonance; DDS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; Me_4Si, tetramethylsilane.

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fructose and D-fructose-1-P or L-sorbose and L-sorbose-1-P, respectively. 5KF is a good substrate for yeast hexokinase (Avigad & Englard, 1968) and both rat and beef liver fructokinase (Englard et al., 1972; Raushel & Cleland, 1977), and the phosphorylated product has been identified as 5KF-1-P in both cases. 5KF is also a substrate for liver sorbitol dehydrogenase (Englard et al., 1965) and the polyol dehydrogenases from *Acetobacter melanogenum* (Sasajima & Isono, 1968) and *G. cerinus* (Englard & Avigad, 1965). No free ketone has been detected by infrared spectroscopy in KBr pellets of 5KF (Avigad & Englard, 1965). X-ray diffraction studies have shown that crystalline 5KF is a dimer of linked pyranose and furanose rings (Hassen et al., 1976).

In order to determine the structure and anomeric compn. of this symmetric hexose in solution, and to correlate this information with the known enzymatic reactivity of 5KF, we have obtained ^1H and ^{13}C NMR spectra of 5KF in aqueous and nonaqueous solutions.

Experimental Procedures

Materials. 5-Keto-D-fructose was isolated from *Gluconobacter cerinus* cultures as described previously (Avigad & Englard, 1965) and was a pure product (mp 172–174 °C uncor) as analyzed by cellulose thin-layer chromatography. Anhydrous Me_2SO-d_6 (99.5 atom % D) and D_2O (99.8 atom % D) were products of Stohler Isotope Co. Dihydroxyacetone phosphate and scylloinosose were from Sigma.

^1H and ^{13}C NMR Spectroscoy. ^1H and ^{13}C nuclear magnetic resonance spectra were obtained at 99.99 and 25.15 MHz, respectively, on a JEOL PFT NMR spectrometer operating in the pulsed Fourier-transform mode and interfaced with a Nicolet 1080 series computer. Spectra were recorded with an internal deuterium lock. ^1H chemical shifts are expressed as parts per million downfield from DSS, using either internal Me_2SO or external DSS references. Each ^1H spectrum consisted of 64 free induction decays using 6-s repetition times. ^{13}C chemical shifts are expressed as parts per million downfield from Me_4Si, using either internal Me_2SO-d_6 (40.5 ppm) or external dioxane (67.4 ppm) as references. ^{13}C spectra were recorded with and without broad-band proton noise decoupling. Temperatures were controlled (±1 °C) with a thermostated probe. For ^1H and ^{13}C spectra, 5- and 10-mm sample tubes were used, respectively. T_1 measurements were performed by the spin-inversion, recovery method (Vold et al., 1968) using a sequence of $180°-\tau-90°-T$ pulses, where τ was varied from 20 μs to 8 s and T was 16 s. A total of 1000 scans were averaged, and the T_1 values were calculated by using a least-squares-fit computer program.

Sample Preparation. Rigorously anhydrous conditions were used to prepare samples of 5KF in MeSO-d_6 for ^1H and ^{13}C NMR analysis. All operations were performed in a dry box under nitrogen, and the sample tubes were sealed before removal. Solutions of approximately 0.8 M 5KF in Me_2SO-d_6 could be obtained. ^1H and ^{13}C NMR spectra of 5KF in D_2O were obtained at least 24 h after dissolution of the sugar to a final concentration of 0.6–1.5 M. Aqueous samples contained 1 mM EDTA to prevent line broadening due to paramagnetic impurities and were filtered through glass wool. Teflon plugs were used to prevent vortexing.

Results and Discussion

^1H NMR Spectra of 5-Keto-D-fructose. The ^1H NMR spectra of 5KF in anhydrous Me_2SO-d_6 and D_2O are shown in Figure 1 A,B. Although the peaks in Me_2SO-d_6 are well resolved, the splitting patterns are complex. In an attempt to identify hydroxyl and carbon-bound proton resonances, and thus simplify assignment, D_2O was added to the sample in 5-μL aliquots, and the spectra were recorded (data not shown). Although the downfield resonances of hydroxyl protons broadened and disappeared, there were also immediate changes in the upfield resonances, until at approximately 10% D_2O (v/v) the spectrum was identical with that obtained in 100% D_2O (Figure 1B). Thus, even small amounts of D_2O (or H_2O) in a solution of 5KF in DMSO-d_6 produce substantial changes in the ^1H NMR spectrum.

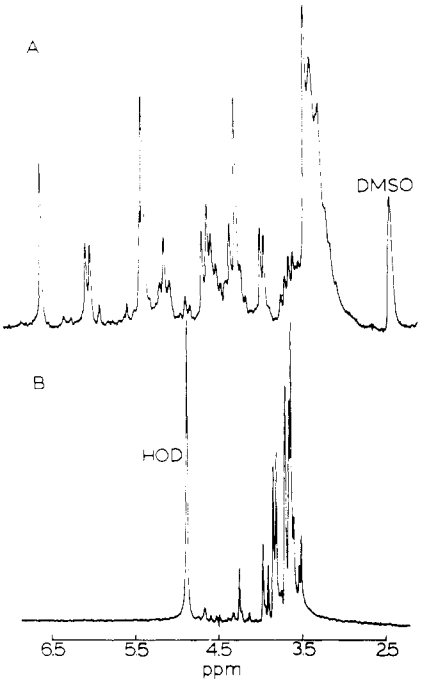


FIGURE 1: (A) ^1H NMR spectrum (100 MHz) of 0.6 M 5KF in anhydrous Me_2SO-d_6 at 30 °C (sweep width 750 Hz). (B) ^1H NMR spectrum (100 MHz) of 0.5 M 5KF in D_2O (sweep width 750 Hz).

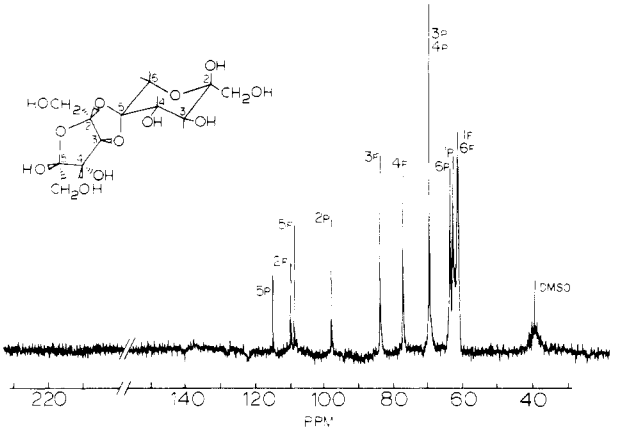


FIGURE 2: Proton-decoupled ^{13}C NMR spectrum (25 MHz) of 0.8 M 5KF in Me_2SO-d_6 at 30 °C (7.5-KHz sweep width, 18 192 transients). Me_2SO-d_6 is at 40.5 ppm. The numbering of pyranose (P) and furanose (F) carbons corresponds to the structure shown.

The spectrum of 5KF in D_2O is complex, with a number of overlapping resonances which do not permit simple assignment or measurement of coupling constants under these magnetic field conditions. We thus undertook an investigation of the ^{13}C NMR spectra of 5KF in order to resolve the obvious differences in the structure of the hexose in the two solvents.

^{13}C NMR Spectrum of 5-Keto-D-fructose in Me_2SO. The natural-abundance, proton noise decoupled ^{13}C NMR spectrum of 5KF in anhydrous Me_2SO-d_6 is shown in Figure 2. Ten well-resolved resonances are observed between 60 and 115

Table I: ^{13}Chemical Shifts and Assignments of 5KF in Me_2SO-d_6 and D_2O[a]

carbon	5KF in Me_2SO-d_6 pyranose ring	5KF in Me_2SO-d_6 furanose ring	5KF in D_2O[b]	β-fructo furanose[c] in D_2O	β-fructo-pyranose[c] in D_2O
1	63.9	62.5	65.9	64.7	63.6
2	98.9	110.6[d]	98.9	99.1	102.6
3	70.6	84.8	69.5	68.4	76.4
4	70.6	78.3	74.2	70.5	75.4
5	115.8	109.6[d]	92.7	70.0	81.6
6	64.8	62.5	64.6	64.1	63.2

[a] Chemical shifts are in parts per million. [b] Major species present at 30 °C. [c] From Angyal & Bethell (1976). [d] Assignments are tentative.

ppm; no resonance is observed in the carbonyl region of the spectrum even at long (>10 s) repetition times, flip angles of less than 10°, and long accumulations. Two of the resonances (70.6 and 62.5 ppm) each correspond in intensity to two carbons, and thus twelve carbon atoms are accounted for in essentially equal proportion. The ^{13}C–^{1}H splitting patterns observed in the absence of proton noise decoupling (not shown) revealed the presence of four methylene carbons at 62.5 (t, J_{C-H} = 140 Hz), 63.9 (t, J_{C-H} = 140 Hz), and 64.8 (t, J_{C-H} = 140 Hz) ppm, four methine carbons at 70.6 (d, J_{C-H} = 144 Hz), 78.3 (d, J_{C-H} = 155 Hz), and 84.8 (d, J_{C-H} = 167 Hz) ppm, and four quaternary carbons at 98.9, 109.6, 110.6, and 115.8 ppm, which were assigned to the C_2 and C_5 carbons of 5KF.

The crystal structure of 5KF has been reported to be a dimer of linked β-pyranose and β-furanose rings, as seen in Figure 2 (Hassen et al., 1976). This unusual spirane structure possesses no free ketone, and one would predict twelve ^{13}C resonances of nearly equal intensity, of which six would be assignable to a single β-pyranose form and six to a single β-furanose form. This structure is consistent with the J_{C-H} and chemical shift data for 5KF in Me_2SO-d_6. Assignments are based on the C–H coupling patterns and published assignments for D-fructose (Angyal & Bethell, 1976). Furanose methylene carbons appear upfield from β-pyranose methylene carbons, and we have assigned the resonances at 62.5 ppm to C_1 and C_6 of the furanose ring (Table I). Furanose methine carbons of ketohexoses resonate at lower field than pyranose methine carbons. Bond strain at the furanose C_3 atom lowers its resonance position, since it is 8.5-ppm downfield from the corresponding resonance in β-fructofuranose, while the furanose C_4 chemical shift is essentially unaffected. The resonance positions of the furanose quaternary carbons are 28- and 8-ppm downfield from the C_5 and C_2 resonances of β-fructofuranose, respectively. Although we have assigned the lowest field resonance to C_2 because of strain effects, we cannot be certain of these assignments at this time.

For the pyranose ring of 5KF in Me_2SO-d_6, we expect the presence of an additional oxygen atom at the pyranose C_5 to lower the C_6 pyranose resonance relative to the C_1 resonance. Hence, the resonances at 63.9 and 64.8 ppm are assigned to C_1 and C_6, respectively. The chemical shifts of the pyranose methinyl carbons correspond closely to the reported positions of β-fructopyranose. The quaternary pyranose resonance at 98.9 ppm is assigned to C_2 for similar reasons. The quaternary carbon resonance assigned to the pyranose C_5 at 115.8 ppm is unusually low. The C_5 pyranose carbon is a member of a spiro-hemiketal ring, and bond strain should deshield this atom relative to a *gem*-diol. The ^{13}C chemical shifts of similar isopropylidene carbons appear in this downfield frequency region (Lukacs et al., 1972; Omoto et al., 1973). Thus, 5KF

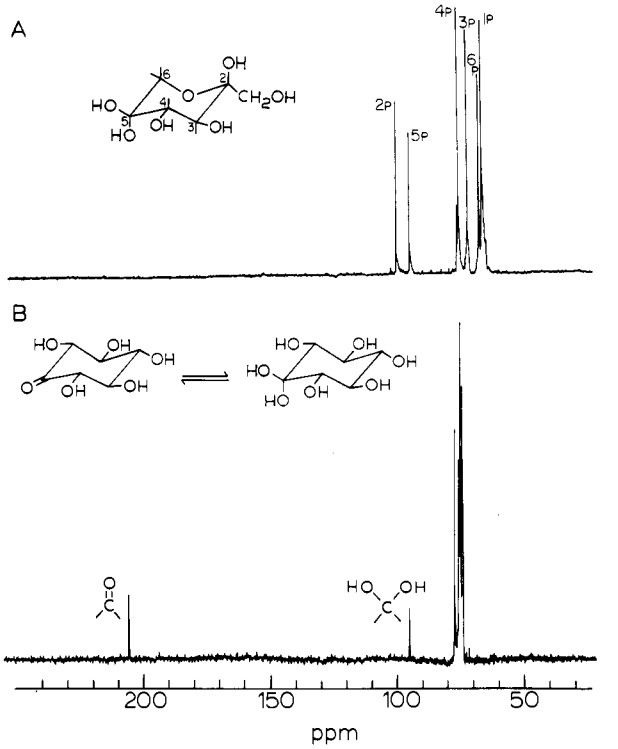


FIGURE 3: (A) Proton-decoupled ^{13}C NMR spectrum (25 MHz) of 0.6 M 5KF in D_2O at 30 °C (7.5-KHz sweep width, 18000 transients). The numbering of the pyranose (P) carbons corresponds to the structure shown. (B) Proton-decoupled ^{13}C NMR spectrum (25 MHz) of 0.3 M scylloinososse in D_2O at 30 °C (7.5-KHz sweep width, 42000 transients).

appears to exist in Me_2SO-d_6 as the dimer[2] reported for crystalline 5KF (Hassen et al., 1976). Alternative structures for 5KF in Me_2SO-d_6 have been considered but deemed unlikely. The presence of equal proportions of a single anomeric pyranose and a single anomeric furanose ring form would be extremely unusual for a ketohexose (Perlin et al., 1973). Furthermore, the resonance at 115.8 ppm is without precedent in either pyranose or furanose ring forms of unmodified ketohexoses.[3]

^{13}C NMR of 5-Keto-D-fructose in D_2O. The natural-abundance, proton-decoupled ^{13}C NMR spectrum of 5KF in D_2O at 30 °C is shown in Figure 3 and is distinctly different from that observed in Me_2SO-d_6. Six major peaks and several weak resonances are observed from 60 and 100 ppm (Table I). No free ketone resonances are observed under a variety of experimental conditions at this temperature. The spectrum recorded under proton-coupled conditions (not shown) was used to make assignments of methylene carbons at 64.6 (t, J_{C-H} = 145 Hz) and 65.9 (t, J_{C-H} = 145 Hz) ppm, methine carbons at 69.5 (d, J_{C-H} = 136 Hz) and 74.2 (d, J_{C-H} = 146 Hz) ppm, and quatenary carbons at 92.7 and 98.9 ppm. At the repetition rates used for this experiment, the intensities of the four upfield resonances are approximately equal, and the quaternary resonances are less intense (see T_1 measurements below). The

[2] When ascorbate is oxidized in nonaqueous solution, a dimer of dehydroascorbate is isolated (Albers et al., 1963) whose symmetrical crystal structure has been determined (Hvoslef, 1972). The ^{13}C NMR spectrum of a freshly dissolved aqueous solution of the dimer consists of six resonances (Berger, 1977) assignable to the dimer. After 3 days, six resonances assignable to the monomer are observed.

[3] The solid-state high-resolution ^{13}C NMR spectrum of 5KF is nearly identical with that obtained in Me_2SO-d_6 (Brewer et al., 1982) and provides additional evidence for the dimeric structure of 5KF in Me_2SO-d_6.

Table II: Relaxation Rates, Chemical Shifts, and Deuterium-Induced Differential Isotope Shifts (DIS's) of Aqueous Solutions of 5-Keto-D-fructose at 30 °C

carbon	T_1 (s)	chemical shift (ppm)[a]	DIS exptl[b]	DIS calcd[c]
1	0.24 ± 0.02	65.9	0.19	0.18
2	5.49 ± 0.61	98.9	0.18	0.17
3	0.45 ± 0.04	69.5	0.28	0.20
4	0.44 ± 0.05	74.2	0.26	0.26
5	5.09 ± 0.59	92.7	0.26	0.31
6	0.24 ± 0.02	64.6	0.10	0.06

[a] Values in 100% D_2O. [b] Downfield shifts of ^{13}C resonances in H_2O. [c] Calculated from the data of Pfeffer et al. (1979).

resonances observed at 64.6, 98.9, and 69.5 ppm (Table I) are assigned to the C_1, C_2, and C_3 atoms of the pyranose form of 5KF, respectively, on the basis of the reported postions of C_1, C_2, and C_3 of β-fructopyranose. The C_6 resonance appears 1.8-ppm downfield from the resonance in fructose as a result of the presence of an additional oxygen atom at C_5. This same electronic effect shifts the C_4 resonance downfield from 70.5 ppm in β-fructopyranose to 74.2 ppm in 5 KF. The resonance at 92.7 ppm we assign to a *gem*-diol hydrate of the C_5 ketone.

Although thorough investigations have sought the presence of ketone hydrates in ketohexoses, only several examples of such *gem*-diol ketone hydrates have been reported. Dehydroascorbate exists in aqueous solution with both the C_2 and C_3 ketones hydrated (Berger, 1977), with resonances at 95.6 and 97.4 ppm (assignments of individual resonances not made by author). D-*threo*-2,5-Hexodiulosonic acid also exists in aqueous solution with the 5-keto group hydrated (97 ppm; Andrews et al., 1979). We have also examined the ^{13}C spectrum of dihydroxyacetone phosphate (DHAP), which exists as a mixture of free ketone and hydrated forms as determined by ^1H NMR (Gray, 1976; Midelfort et al., 1976). The proton-decoupled ^{13}C NMR spectrum of DHAP consists of six peaks at 211.1, 95.3, 64.5, 66.2, 67.6, and 68.6 ppm. The resonance at 95.3 ppm was assigned to the hydrated ketone on the basis of peak intensities, positions, and ^{31}P coupling patterns. To obtain additional information on the resonance position of a hydrated ketone in a six-membered ring, we obtained the ^{13}C spectrum of scylloinosose (2,4,6/3,5-pentahydroxycyclohexanone) (Figure 3B). This compound exists as a mixture of 63% keto and 27% hydrated forms (by integration), with the *gem*-diol appearing at 95.3 ppm. 3-Ketolactose has also been proposed to exist predominantly as the *gem*-diol on the basis of ^1H NMR studies (DeBruyn et al., 1975). Our assignment of the peak at 92.7 ppm observed in aqueous solutions of 5KF is in accord with the data for these acyclic and cyclic *gem*-diol resonances. To confirm and extend these observations, we have performed relaxation measurements on 5KF in aqueous solution.

T_1 Measurements. The measurement of ^{13}C spin-relaxation times, T_1, has been used to assist in structural assignments (Levy, 1973; Breitmaier et al., 1975). The T_1 values measured for 5KF in D_2O at 30 °C are presented in Table II. These measurements were made on saturated solutions of 5KF (approximately 2.6M) to reudce the time required, and thus, the absolute value of T_1 are shorter than they would be in a less concentrated solution (Bock & Hall, 1975) due to the higher viscosity of the solution. The two carbon resonances with T_1 values of 0.24 s are assigned to the C_1 and C_6 methylene carbons, the two resonances with T_1 values of 0.44 s are assigned to the C_3 and C_4 methinyl carbons, and the two resonances with T_1 values of approximately 5 s are assigned

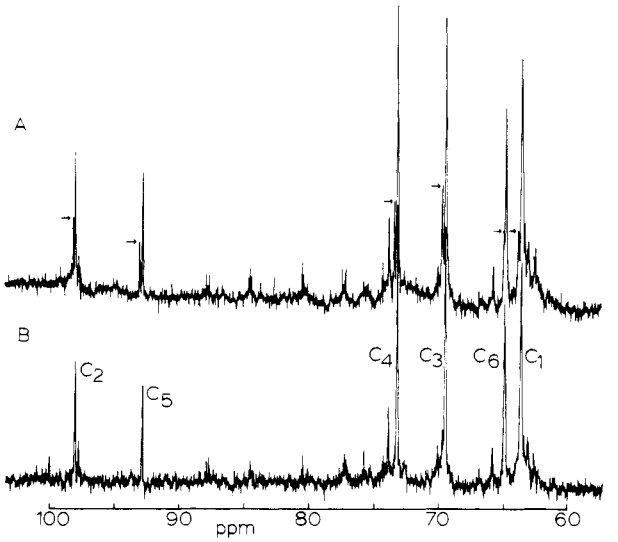


FIGURE 4: (A) Proton-decoupled ^{13}C NMR spectrum (25 MHz) of 1.7 M 5KF in H_2O and 0.6 M 5KF in D_2O in a coaxial NMR tube at 30 °C (1.25-KHz sweep width, 24000 transients). Arrows indicate carbon resonances of 5KF in H_2O. (B) Proton-decoupled ^{13}C NMR spectrum (25 MHz) of 0.6 M 5KF in D_2O at 30 °C (conditions the same as those described above).

to the C_2 and C_5 carbons. These results are consistent with a dipole–dipole relaxation mechanism for the methylene and methinyl resonances. The T_1 results clearly indicate that C_5, like C_2, has no directly attached protons, in agreement with the proposed *gem*-diol structure.

Deuterium-Induced Differential Isotope Shifts (DIS's) for 5-Keto-D-fructose. To further substantiate the proposed structure of 5KF in aqueous solution, we measured the deuterium-induced differential isotope shifts exhibited by 5KF (Pfeffer et al., 1979). Isotope-dependent shifts are observed in the ^{13}C resonance positions of carbons to which hydroxyl groups are directly attached (β shifts) and of carbons adjacent to a hydroxyl-bearing carbon (γ shifts). The magnitudes of these shifts are additive and can be predicted for any sugar structure. Figure 4A shows the ^{13}C spectrum obtained when a coaxial tube containing 1.7 M 5KF in H_2O is placed inside a 10-mm tube containing 0.6 M 5KF in D_2O. The spectrum in D_2O is shown for comparison in Figure 4B. The ^{13}C resonances in H_2O are smaller than those in D_2O due to the smaller cross-sectional area of the inner coaxial tube, and when this experiment is run with 1.7 M 5KF in D_2O in the inner tube, with 0.6 M 5KF in H_2O outside, exactly reverse peak heights are observed, but the chemical shifts remain the same. The expected shifts calculated from the data of Pfeffer et al. (1979) and the observed deuterium shifts for C_1–C_6 are shown in Table II. The DIS's at C_1 and C_2 are within experimental error of the predicted shifts and those reported for β-fructopyranose (Pfeffer et al., 1979). As expected for a *gem*-diol, the C_5 shift is large, and the presence of an additional hydroxyl group at C_5 causes predictably larger shifts to be observed at C_4 and C_6 compared to fructose. The shift at C_3, however, is larger than predicted, and we do not have an explanation for this result at present.[4] The DIS results lend considerable support to the proposed structure.

Minor Forms of 5-Keto-D-fructose. At 70 °C, the natural-abundance, proton noise decoupled ^{13}C NMR spectrum of 5KF reveals the presence of a number of new peaks due

[4] Similar unusual DIS effects have recently been reported (Pfeffer et al., 1980).

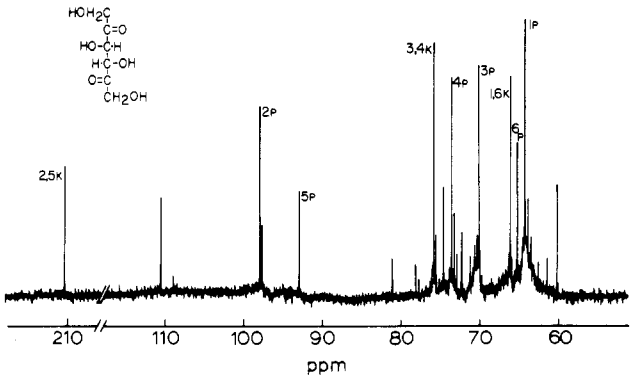


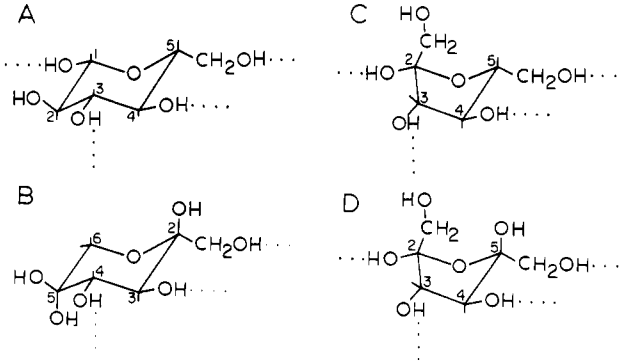
FIGURE 5: Proton-decoupled ^{13}C NMR spectrum (25 MHz) of 1.5 M 5KF in D_2O at 70 °C (7.5-KHz sweep width, 32 000 transients). The numbering of pyranose (P) carbons corresponds to the structure shown in Figure 3A, and the numbering of open-chain keto (K) carbons corresponds to the structure shown in the inset.

to minor forms of the sugar (Figure 5). The symmetric open-chain form accounts for three new resonances at 215 (C_2 and C_5), 75.4 (C_3 and C_4), and 65.9 (C_1 and C_6) ppm, which were assigned on the basis of the reported C_2 and C_3 resonances of 1-deoxy-D-fructose (213.8 and 77.7 ppm, respectively; Angyal et al., 1976), and the hydroxymethyl resonance of dihydroxyacetone phosphate in the keto from (66.2 ppm). No attempt was made to assign the remaining resonances to α-pyranose and furanose forms using natural-abundance ^{13}C NMR because of the limited sensitivity, but these will be discussed in detail elsewhere.

Conformational Free Energies. Values for interaction energies have been proposed to allow the calculation of conformational free energies of pyranose ring forms (Angyal, 1968, 1969; Stoddart, 1971). Using this method, we have calculated conformational free energies for the $^2C_5$ (chair axial) and $^5C_2$ (chair equatorial) conformers of α and β anomers of 5-keto-D-fructopyranose hydrate. For the β anomer, the $^2C_5$ conformer (3.20 kcal/mol) is more stable than the $^5C_2$ conformer (7.05 kcal/mol) and is more stable than either of the α-anomer conformers ($^2C_5$, 4.65 kcal/mol; $^5C_2$, 4.70 kcal/mol). Calculated values of $G°_\beta$ and $G°_\alpha$, corrected for the entropy change on mixing (Stoddart, 1971), are 3.20 and 4.26 kcal/mol, respectively. This method of calculating conformational free energies was derived from experimental observations of the anomeric composition of aldopyranoses and is quite satisfactory for these molecules but overestimates the percentage of α anomers present in ketohexopyranoses. Angyal & Bethell (1976) have discussed reasons for the disagreement between theoretical and experimental ketopyranose anomeric compositions, and in the case of 5KF, which should contain 18% α-pyranose, less than 5% is actually observed. Since we do not observe significant amounts of furanose forms, we have not made similar calculations of conformational free energies for these forms (Koerner et al., 1980).

Relevance to the Specificity of Hexokinase and Fructokinase. 5KF is phosphorylated by rat liver fructokinase to form 5KF-1-P (Englard et al., 1972) with a V_m 44% lower than that for fructose. The homogeneous beef liver enzyme, which also phosphorylates 5KF, appears from substrate specificity studies to require a (2*R*)-2-(hydroxymethyl)-3,4-dihydroxytetrahydrofuran structure for activity (Raushel & Cleland, 1973, 1977). For the beef liver enzyme at 25 °C, the K_m of 1.2 mM for 5KF can be compared to the K_m of 21 μM for D-fructose, as corrected for the amount of the enzymatically active β-furanose form present (21%; Angyal & Bethell, 1976). The 2β,5α anomer of 5KF,[5] which has been proposed as the

Scheme I: Modes of Pyranose and Furanose Binding to Hexokinase[a]

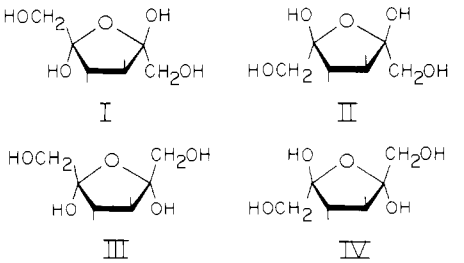


[a] (A) Orientation of α-D-glucopyranose at the hexokinase active site showing hydrogen bonds to acceptor groups. (B) Proposed orientation of β-5-keto-D-fructopyranose hydrate at the active site of hexokinase. (C) Orientation of β-D-fructofuranose at the active site of hexokinase showing hydrogen bonds to acceptor groups. (D) Proposed orientation of 2β-5-keto-D-fructofuranose (5-hydroxy-β-D-fructofuranose) at the active site of hexokinase.

substrate for fructokinase (Raushel & Cleland, 1977), most resembles β-fructofuranose and should bind as tightly to fructokinase, since the enzyme binds and phosphorylates L-sorbose, in which a hydroxymethyl group occupies the position of the C_5 hydroxyl in 5KF. With the assumption that the K_m value for 5KF is the same as that of fructose, then approximately 2% of 5KF should exist as the 2β,5α-furanose anomer at 25 °C. We would not expect to be able to observe this small amount of furanose by natural-abundance ^{13}C NMR, which is consistent with the experimental findings of this study.

5KF is also phosphorylated by yeast hexokinase to form 5KF-1-P (Avigad & Englard, 1968), with a V_m 1.5 times greater than that for glucose and a 10-fold higher K_m. Hexokinase will utilize either α or β anomers of D-glucose (Salas et al., 1965), and although suggestions have been made that the thermodynamically preferred $^4C_1$ conformer of D-glucose binds to hexokinase and is then forced to assume $^1C_4$ geometry prior to phosphorylation (Crane, 1962), an alternative explanation has been proposed in which the $^4C_1$ conformer is bound and phosphorylated (Purich et al., 1973). Crystallographic studies of the glucose–hexokinase complex (Anderson et al., 1978) have shown that glucose exists in the $^4C_1$ con-

[5] The anomeric composition of furanose forms of ketohexoses has been shown to depend principally on the configuration of the C_2 hydroxymethyl and C_3 hydroxyl groups (Que & Gray, 1974). In all cases, the predominant furanose anomer in solution had these substituents trans. Due to the presence of two anomeric centers in furanose forms of 5KF, four furanose anomers are possible (I, 2β,5α; II, 2β,5β; III, 2α,5α; IV, 2α,5β). Of these four forms, two are identical (II and III), while both



I and IV possess C_2 symmetry. The predominant anomer will be structure I in which both the C_2 and C_5 hydroxymethyl groups are trans to the C_3 and C_4 hydroxyl groups. Structures II and III possess one such unfavorable interaction, while in structure IV, both the C_2 and C_5 hydroxymethyl groups are cis to the C_3 and C_4 hydroxyl groups, respectively.

formation with the C_3 and C_4 hydroxyls engaged in an extensive hydrogen-bonding network with active-site residues, and with the C_6 hydroxyl bonded to a carboxyl group (Scheme I, A).

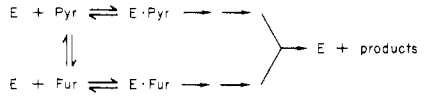
The predominant form of 5KF in aqueous solution is the β-pyranose form, and we have considered this structure as a substrate for hexokinase. The binding orientation of this ring form of 5KF, relative to β-D-glucopyranose, to hexokinase is shown in Scheme I, structure B. In this proposed binding orientation, the β-pyranose ring of 5KF is superimposable on the $^4C_1$ conformation of D-glucose. The C_1 hydroxymethyl group of 5KF occupies the position of the C_6 hydroxymethyl group of glucose and would undergo phosphorylation to give 5KF-1-P. The C_4 and C_3 hydroxyls of 5KF have the required orientation at positions analogous to the critical C_3 and C_4 hydroxyls of glucose, respectively. The C_5 *gem*-diol of 5KF occupies the same site as C_2 of glucose, and since both mannose and glucose are good substrates for hexokinase, there should be no loss in binding affinity for 5KF at this position. The C_2 hydroxyl group of 5KF binds at the same site as C_5 of glucose; however, no conclusive data regarding the steric tolerance at this position in a pyranose ring have been reported. Finally, the C_6 of 5KF binds at the same site as the anomeric carbon position of D-glucose. 1-Deoxy sugars such as 1,5-anhydroglucitol and 1,5-anhydromannitol are substrates for hexokinase, although their K_m's are relatively high (20 mM for 1,5-anhydroglucitol; DeDomenech & Sols, 1980) and their V_m's are low (6% of that for glucose for 1,5-anhydromannitol; Viola & Cleland, 1978). These data suggest that 5KF can fulfill the substrate requirements of a 1-deoxy sugar. However, the V_m for 5KF is 1.5-fold higher than that for glucose, and the K_m is 600 μM, which in inconsistent with the reported values for 1-deoxy analogues. These inconsistencies suggest that the β-pyranose form of 5KF in this proposed orientation may not account for the entire activity of this sugar with hexokinase. Furthermore, β-fructopyranose, which is the major species of fructose in solution, appears not to undergo phosphorylation at C_1, although its structure resembles the β-pyranose form of 5KF, lacking only the equatorial C_5 OH group, and would be expected to bind in an orientation identical with that of 5KF. We therefore considered the possibility that the furanose form of 5KF is a substrate for hexokinase.

Fructose must be phosphorylated by hexokinase in the furanose form, since the observed product is fructose 6-phosphate. The orientations of β-fructofuranose and the 2β,5α-furanose anomer of 5KF are shown in Scheme I, structures C and D. This orientation of the fructofuranose ring fulfills the hydrogen-bonding requirements observed for pyranose rings. The β anomer of such furanoses is preferred, and the C_2 hydroxyl is important for furanose binding to hexokinase, as 2,5-anhydro-D-mannitol has a K_m of 6.3 mM (Raushel & Cleland, 1973). The furanose form of 5KF shown in Scheme I is identical with β-fructofuranose, with the exception of an additional hydroxyl at C_5, and 5KF-6-P would be the expected product of phosphorylation of this anomeric form. However, the symmetric nature of this furanose anomer of 5KF precludes the distinction between 5KF-1-P and 5KF-6-P and is compatible with the identification of the product as 5KF-1-P based on the formation of fructose-1-P after reduction of the open-chain form at C_5 with NADPH and the *G. cerinus* dehydrogenase (Avigad & Englard, 1968). If approximately 2% of 5KF exists in the 2β,5α-furanose form, which is the amount we calculate from the kinetic data for fructokinase and is consistent with the ^{13}C NMR data, then we calculate a corrected K_m of 12 μM for this form, or 15 times lower than the correspondingly corrected value of 180 μM for β-fructofuranose. This is a large decrease in the magnitude of K_m as a result of the presence of a single additional hydroxyl group (and its presumptive hydrogen bond) at C_5 of 5KF.

Since both the β-pyranose and 2β,5α-furanose forms of 5KF appears to fulfill the substrate requirements of hexokinase as deduced from both substrate specificity studies and analysis of crystallographic data, the possibility that both forms are bound and phosphorylated by hexokinase was considered. Suitable equations have been derived which allow estimation of the kinetic parameters for the β-pyranose and 2β,5α-furanose structures of 5KF, assuming that both ring forms are substrates.[6] If the two forms are in rapid equilibrium, the observed K_m will be an intermediate value between the true K_m's for the pyranose and the furanose forms. Similarly, the observed V_m will reflect contributions from each sugar form. Although there are numerous values which can be assigned to the variables in these equations to fit the experimental data, we have chosen values which are consistent with the kinetic parameters observed previously for similar sugars which are phosphorylated in either the pyranose or the furanose form. Thus, the V_m value of 3 which we assign to the furanose form (relative to 1 for glucose) is identical with the value for fructose, while the V_m value of 0.05 assigned to the pyranose form is comparable to the value for 1,5-anhydro-D-mannitol (0.06; Viola & Cleland, 1978). Calculated K_m's for the furanose and pyranose forms of 5KF are 25 μM and 1.15 mM, respectively. These values are approximately 7 and 17 times lower than the K_m's of fructose and 1,5-anhydroglucitol, respectively. Since other sugars which have such an additional hydroxyl group at the C_5 position have not been tested as substrates for yeast hexokinase, the validity of the above analysis must await further experimental verification. However, it is clear that both the β-pyranose and 2β,5α-furanose forms of 5KF are possible substrates, and the furanose form of 5KF is possibly the best hexokinase substrate yet examined.

Conclusions. 5-Keto-D-fructose is an unusual, symmetric hexose. It exists as a pyranose–furanose dimer both in the crystal and in anhydrous Me_2SO, in which the C_5 keto group of the β-pyranose ring forms a cyclic hemiketal with the C_2 and C_3 hydroxyl groups of the furanose ring. In aqueous solution, it exists predominantly (>95%) as a β-pyranose with a *gem*-diol ketone hydrate at C_5. Monoketohexoses do not exist appreciably in hydrated forms, preferring instead cyclic or open-chain keto structures (Angyal et al., 1976). However, diketohexoses studies to date, in particular the 2,5-diketo-

[6] For a sugar in which there is a rapid equilibration of pyranose and furanose ring forms, both of which are substrates for an enzyme, the following scheme can be written:



If [Fur]= 0.02[Pyr], then the following expressions can be derived:

$$\text{app } V_m = V_m^{Pyr}K_m^{Fur} + 0.02V_m^{Fur}K_m^{Pyr}/(K_m^{Fur} + 0.02K_m^{Pyr}) \quad (1)$$

and

$$\text{app } K_m = K_m^{Pyr}K_m^{Fur}/(K_m^{Fur} + 0.02K_m^{Pyr}) \quad (2)$$

Dividing eq 1 by eq 2 yields

$$\text{app } V/K = (V/K)_{Pyr} + 0.02(V/K)_{Fur}$$

For app V_m = 1.46 and app K_m = 0.6 mM, then the following are a possible set of values which fulfill eq 1 and 2: V_m^{Fur} = 3; V_m^{Pyr} = 0.05; K_m^{Fur} = 0.025 mM; K_m^{Pyr} = 1.15 mM.

hexoses (Andrews et al., 1979; Dizdaroglu et al., 1976), exist predominantly or exclusively as *gem*-diol hydrates in both furanose and pyranose structures.

The proportion of pyranose and furanose forms present in aqueous solutions of 5KF, as measured by ^{13}C NMR, provides insight into the specificity of the hexose kinases for which 5KF is a substrate. Liver fructokinase phosphorylates the minor (2%) 2β,5α-furanose anomer of 5KF, while yeast hexokinase appears to be able to phosphorylate both the predominant β-pyranose and minor 2β,5α-furanose forms of 5KF. Due to experimental difficulties in determining the proportion of furanose and free carbonyl forms of 5KF in solution at 25 °C by natural-abundance ^{13}C NMR, we are presently preparing C_2,C_5 ^{13}C-enriched 5KF to extend these studies, as well as to study the solution structures and anomeric composition of the monophosphate ester, a substrate for phosphofructokinase (Avigad & Englard, 1974), and the diphosphate ester, a slow substrate for rabbit muscle aldolase (Healy & Christen, 1972).

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