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Carbon-13 Nuclear Magnetic Resonance Studies and Anomeric Composition of Ketohexose Phosphates in Solution†

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ABSTRACT: The Fourier transform ^{13}C NMR spectra of the following ketohexose phosphates were studied: D-fructose 6-phosphate (**1**), D-fructose 1-phosphate (**2**), D-fructose 1,6-bisphosphate (**3**), D-psicose 6-phosphate (**4**), D-tagatose 6-phosphate (**5**), and L-sorbose 6-phosphate (**6**). All ^{13}C resonances were assigned through the use of off-resonance decoupling studies, ^{31}P–^{13}C couplings, and chemical shift comparisons with 2,5-anhydro-D-hexitol phosphates (**7**–**10**). Integration of the signal intensities of the C-2 carbons yielded the following equilibrium percentage compositions for α (**a**) and β (**b**) furanose anomers (±2%): (at 16.5 °C) **1a** 19, **1b** 81, **4a** 76, **4b** 24, **5a** 17, **5b** 83, **6a** 82, **6b** 18; (at 35 °C) **2a** 24, **2b** 76, **3a** 23, **3b** 77. These values can be quantitatively predicted through conformational analysis of the furanose ring of the sugars if it is assumed that (1) ketofuranose 6-phosphates exist in the $^4T_3$, $^OT_2$, and $^OT_5$ twist conformations (or mirror-image conformations for L forms) and (2) a proposed set of twist conformation interaction energies is operative. Due to the presence of the C-2 hydroxymethyl group, a new anomeric interaction ($\Delta 3$ effect) must be proposed for the ketofuranoses that is analogous to Reeves's $\Delta 2$ effect for aldopyranoses. This conformational analysis yields concentrations for the $^4T_3$ conformation of β-D anomers, or the $^3T_4$ conformation of α-L anomers, of ketofuranose 6-phosphates that vary directly with the substrate activity of each sugar for phosphofructokinase (EC 2.7.1.11). This correlation suggests that this enzyme prefers these conformations for the furanose rings of its substrates. The values for $^3J_{POCC}$ allow the rotameric compositions of the phosphate groups of **1**–**10** to be calculated. In all cases, the trans-periplanar rotamer predominates (38–63%).

Ketohexose phosphates play important roles as intermediates and regulators of carbohydrate metabolism. In fact, the isolation of fructose 1,6-bisphosphate (fructose-1,6-P_2)[1] from yeast in 1906 provided the very basis for the modern concept of metabolic "intermediate" (Korman, 1974). Fructose-1,6-P_2 is now known to play a central role in glucose metabolism and to affect the rate of a large variety of physiologically important enzyme reactions (Kirtley & McKay, 1977). Fructose-6-P is the only intermediate common to the glycolytic, gluconeogenic, pentose, and amino sugar pathways. Tagatose phosphates may be important intermediates in galactose metabolism (Bissett & Anderson, 1973; Koerner et al., 1976). The 6-phosphates of fructose, psicose, tagatose, and L-sorbose have all been shown to be substrates of the regulatory enzyme phosphofructokinase (Koerner et al., 1976). The 1,6-bisphosphates of L-sorbose and tagatose as well as fructose are substrates for aldolase (Tung et al., 1954).

An often overlooked aspect of the biochemistry of ketohexose phosphates is that these sugars in solution are mixtures of several constitutional and configurational isomers (tautomers and anomers), each present in different concentrations and capable of displaying different affinities and reactivities (anomeric specificity) for enzyme catalytic and allosteric sites

† From the Department of Biochemistry, Louisiana State University, Baton Rouge, Louisiana 70803. *Received October 11, 1979.* This work was supported by Grant No. AM-21307 from the National Institutes of Health and Grant No. PGM 77-27286 from the National Science Foundation. A preliminary account of this work was presented, in part, at the XIth International Congress of Biochemistry held in Toronto, Canada, July 8–13, 1979. This paper is dedicated to Dr. Richard E. Reeves, whose meritorious contributions to our understanding of the chemistry and metabolism of carbohydrates have been an inspiration to us and to many workers in this field for several decades.

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[1] Abbreviations used: P or PO_3^{2-}, phosphate group; P_2, bisphosphate; δ, chemical shift; $\Delta\delta$, chemical shift difference; $\Delta\Delta\delta$, difference in chemical shift differences; ppm, parts per million; J, coupling constant; FT, Fourier transform; α-1, α-furanose C-1, etc.; β-1, β-furanose C-1, etc.; E and T, envelope and twist conformations of the furanose ring. All sugars are D unless labeled L.

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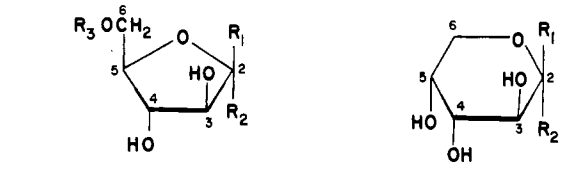
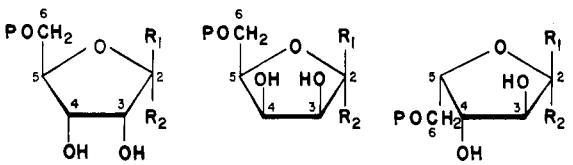


1a: R_1 = CH_2OH, R_2 = OH, R_3 = P
1b: R_1 = OH, R_2 = CH_2OH, R_3 = P
2a: R_1 = CH_2OP, R_2 = OH, R_3 = H
2b: R_1 = OH, R_2 = CH_2OP, R_3 = H
3a: R_1 = CH_2OP, R_2 = OH, R_3 = P
3b: R_1 = OH, R_2 = CH_2OP, R_3 = P
7: R_1 = CH_2OH, R_2 = H, R_3 = P
8: R_1 = CH_2OP, R_2 = H, R_3 = P
9: R_1 = H, R_2 = CH_2OH, R_3 = P
10: R_1 = H, R_2 = CH_2OP, R_3 = P

2c: R_1 = CH_2OP, R_2 = OH
2d: R_1 = OH, R_2 = CH_2OP

FIGURE 1: Structures of fructose phosphates and analogues in solution: fructose-6-P (**1**), -1-P (**2**), and -1,6-P_2 (**3**), 2,5-anhydro-D-glucitol-6-P (**7**) and -1,6-P_2 (**8**), and 2,5-anhydro-D-mannitol-6-P (**9**) and -1,6-P_2 (**10**). Note that the 1-P and 6-P of 2,5-anhydro-D-mannitol are identical.



4a: R_1 = CH_2OH, R_2 = OH **5a**: R_1 = CH_2OH, R_2 = OH **6a**: R_1 = OH, R_2 = CH_2OH
4b: R_1 = OH, R_2 = CH_2OH **5b**: R_1 = OH, R_2 = CH_2OH **6b**: R_1 = CH_2OH, R_2 = OH

FIGURE 2: Structures of the epimers of fructose 6-phosphate in solution: psicose-6-P (**4**), tagatose-6-P (**5**), and L-sorbose-6-P (**6**).

(Wurster & Hess, 1974; Benkovic & Schray, 1976). The importance of the anomeric specificity of these sugars has recently been emphasized by the possibility that such specificity is the basis for a substrate-level regulation of antagonistic enzyme pairs of carbohydrate metabolism, for example, phosphofructokinase and fructose bisphosphatase (Koerner et al., 1977a). It is thus of interest to determine the anomeric and tautomeric composition of ketohexose phosphates and to understand the underlying forces that determine such composition.

Pulsed Fourier transform ^{13}C NMR has emerged as a powerful method for the determination of the anomeric and tautomeric composition of fructose phosphates in solution (Koerner et al., 1973; Gray, 1976), overcoming the problems of continuous-wave ^{13}C NMR (Benkovic et al., 1972) and FT ^{31}P NMR (Gray, 1971). In this communication we extend our earlier ^{13}C NMR studies of fructose-6-P and -1,6-P_2 (Koerner et al., 1973) as well as the limited studies of tagatose-6-P and fructose-1-P (Benkovic et al., 1973; Fishbein et al., 1974) to include all fructose phosphates (Figure 1), their structurally related 2,5-anhydrohexitol phosphates (Figure 1), and all epimers of fructose-6-P (Figure 2). Among the four ketohexose 6-phosphates studied (**1**, **4**, **5**, and **6**) is included one enantiomer of all ketohexose 6-phosphate D,L pairs. Complete ^{13}C resonance assignments, ^{31}P–^{13}C coupling constants, and anomeric compositions are reported. Moreover, we have been able to quantitatively rationalize the anomeric composition data of these sugars through conformational analysis of their furanose ring.

Materials and Methods

The fructose phosphates (**1**–**3**) were obtained from Sigma Chemical Co. as their disodium salts and used directly.

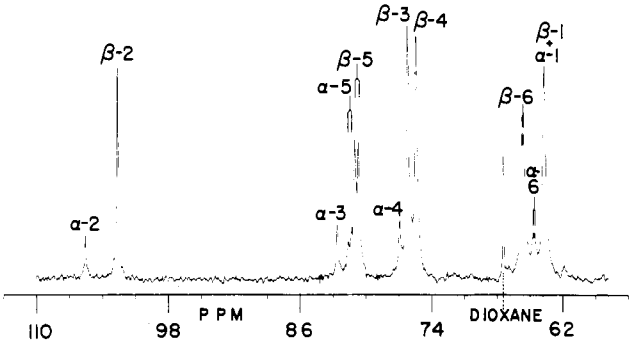


FIGURE 3: Proton-decoupled, natural abundance, FT carbon-13 NMR spectrum of fructose 6-phosphate (**1**) obtained at 25.16 MHz and 16.5 °C. Chemical shifts and ^{31}P coupling constants for the above assigned signals are reported in Tables I and III.

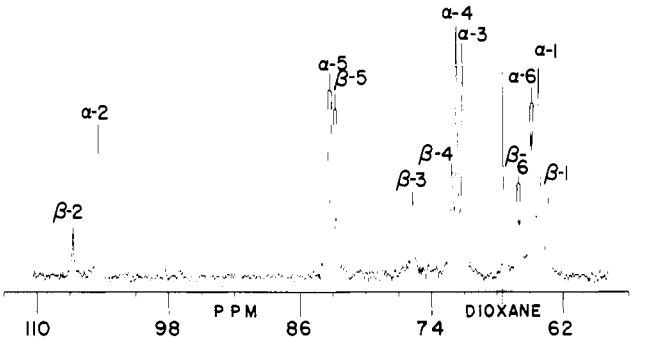


FIGURE 4: Proton-decoupled, natural abundance, FT carbon-13 NMR spectrum of psicose 6-phosphate (**4**) obtained at 25.16 MHz and 16.5 °C. Chemical shifts and ^{31}P coupling constants for the above assigned signals are reported in Tables I and III.

Psicose-6-P (**4**), tagatose-6-P (**5**), and L-sorbose-6-P (**6**) were synthesized as their barium salts according to procedures published earlier (Koerner et al., 1976) and converted to their disodium salts prior to use with an equivalent amount of aqueous sodium sulfate and centrifugation. The 2,5-anhydrohexitol phosphates (**7**–**10**) were prepared according to published procedures (Koerner et al., 1974, 1979; Hartman & Barker, 1965). The 1,6-bisphosphates **8** and **10** were purified from mono- and trisphosphate ester byproducts by DEAE-Sephadex chromatography or by dry column chromatography of their precursors (Voll, Koerner, and Younathan, unpublished results).

^{13}C NMR spectra were obtained on a Varian XL-100A spectrometer equipped for pulsed Fourier transform operation at 25.16 MHz and decoupling. Spectra were recorded at 16.5 °C sample temperature, except for **2** and **3** which were obtained at 35 °C. A sweep width of 2000 Hz with 4000 data points was employed for approximately 4000 transients. Carbon-13 chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane with trace dioxane as internal reference set at δ 67.40. Spectra were obtained both with and without proton and ^{31}P coupling to aid in signal assignment. Each sample was prepared as an approximately 0.6 M solution of its sodium salt in 9:1 (v/v) H_2O/D_2O, pH 8.9 (±0.2), and contained 5 mM Na_2EDTA to prevent line broadening due to possible paramagnetic ion contamination.

Results and Discussion

Chemical Shift Assignments. The spectra of the four ketohexose 6-phosphates are shown in Figures 3–6. Chemical shift assignments for the ^{13}C resonances of all ketohexose phosphates are reported in Table I, and those for their structurally related 2,5-anhydrohexitol phosphates are reported

Table I: ^{13}C Chemical Shifts of the Anomers of Ketohexose Phosphates at 25.16 MHz[a]

compound	C-1	C-2	C-3	C-4	C-5	C-6
α-D-fructofuranose-6-P (**1a**)	63.8 (+0.0)	105.3 (−0.2)	82.6 (−0.3)	76.9 (−0.1)	81.4 (−0.8)	64.5 (+2.6)
β-D-fructofuranose-6-P (**1b**)	63.8 (+0.2)	102.4 (−0.2)	76.2 (−0.2)	75.4 (+0.0)	80.8 (−0.8)	65.4 (+2.2)
α-D-fructofuranose-1-P (**2a**)	*b*	*c*	83.0 (+0.1)	77.0 (+0.0)	83.0 (+0.8)	62.6 (+0.7)
β-D-fructofuranose-1-P (**2b**)	66.0 (+2.4)	*c*	77.4 (+1.0)	75.2 (−0.2)	81.3 (−0.3)	63.3 (+0.1)
α-D-fructopyranose-1-P (**2c**)	*b*	*c*	*b*	*b*	*b*	61.9
β-D-fructopyranose-1-P (**2d**)	67.4 (+2.7)	99.0 (−0.1)	69.0 (+0.6)	70.4 (−0.1)	70.1 (+0.1)	64.4 (+0.3)
α-D-fructofuranose-1,6-P_2 (**3a**)	65.4 (+1.6)	105.7 (+0.2)	82.4 (−0.5)	77.5 (+0.5)	82.7 (+0.5)	65.4 (+3.5)
β-D-fructofuranose-1,6-P_2 (**3b**)	66.9 (+3.3)	102.0 (−0.6)	76.9 (+0.5)	75.1 (−0.3)	80.4 (−1.2)	65.4 (+2.2)
α-D-psicofuranose-6-P (**4a**)	64.2 (+0.0)	104.4 (+0.4)	71.1 (−0.1)	71.7 (+0.5)	83.2 (−0.4)	64.9 (+2.7)
β-D-psicofuranose-6-P (**4b**)	63.4 (+0.1)	106.7 (+0.3)	75.7 (+0.2)	72.1 (+0.3)	82.9 (−0.7)	66.1 (+2.4)
α-D-tagatofuranose-6-P (**5a**)	*b*	106.1 (+0.4)	78.2 (+0.6)	72.3 (+0.4)	79.4 (−0.6)	*b*
β-D-tagatofuranose-6-P (**5b**)	63.6 (+0.1)	103.6 (+0.3)	71.4 (−0.3)	71.8 (+0.0)	80.3 (−0.6)	63.7 (+1.8)
α-L-sorbofuranose-6-P (**6a**)	64.3 (+0.0)	102.8 (+0.3)	76.6 (−0.4)	76.2 (+0.0)	77.9 (−0.7)	63.8 (+2.2)
β-L-sorbofuranose-6-P (**6b**)	*b*	106.4	80.9	76.6	81.2	*b*

[a] Measured at 16.5 °C (except **2** and **3** at 35 °C) in 9:1 (v/v) H_2O/D_2O for ~0.6 M solutions, pH 9. Estimated error ±0.2 ppm. Numbers in parentheses refer to difference in chemical shift (Δδ) with the corresponding carbon of the parent ketohexose as assigned by Angyal & Bethell (1976) by using the relationship $\Delta\delta = \delta_{\text{ketose-P}} - \delta_{\text{ketose}}$. [b] Not observed, obscured by other resonance. [c] Not detected.

Table II: ^{13}C Chemical Shifts of 2,5-Anhydrohexitols and Phosphorylated Derivatives at 25.16 Mhz[a]

compound	C-1	C-2	C-3	C-4	C-5	C-6
2,5-anhydro-D-glucitol	61.0 (+2.8)	81.8 (+23.7)	77.8 (+5.1)	78.9 (−1.9)	85.6 (−3.4)	62.5 (−0.6)
2,5-anhydro-D-glucitol-6-P (**7**)	60.9 (+2.9)	81.8 (+23.5)	77.7 (+4.9)	78.8 (−1.9)	84.6 (−3.2)	64.9 (−0.4)
2,5-anhydro-D-glucitol-1,6-P_2 (**8**)	62.5 (+2.9)	81.6 (+24.1)	77.9 (+4.5)	79.1 (−1.6)	85.5 (−2.8)	65.1 (−0.3)
2,5-anhydro-D-mannitol	61.8 (+1.8)	83.1 (+19.5)	77.2 (−0.8)	77.2 (−1.8)	83.1 (−1.5)	61.8 (+1.4)
2,5-anhydro-D-mannitol-6-P[b] (**9**)	62.0 (+1.8)	83.4 (+19.0)	77.2 (−1.0)	77.7 (−2.3)	82.6 (−1.8)	64.7 (+0.7)
2,5-anhydro-D-mannitol-1,6-P_2 (**10**)	64.9 (+2.0)	82.4 (+19.6)	77.2 (−0.3)	77.2 (−2.1)	82.4 (−2.0)	64.9 (+0.5)

[a] Determined at 16.5 °C in 9:1 (v/v) H_2O/D_2O for ~0.6 M solutions, pH 9. Estimated error ±0.2 ppm. Numbers in parentheses refer to the difference in chemical shift (Δδ) with the corresponding carbons of fructofuranose-6-P and -1,6-P_2 by using the relationship $\Delta\delta = \delta_{\text{ketose-P}} - \delta_{\text{analogue}}$. α anomers are compared with **7** and **8** and β anomers with **9** and **10**. 2,5-Anhydro-D-glucitol and 2,5-anhydro-D-mannitol are compared with α- and β-fructofuranose, respectively. [b] Properly named 2,5-anhydro-D-mannitol-1-P.

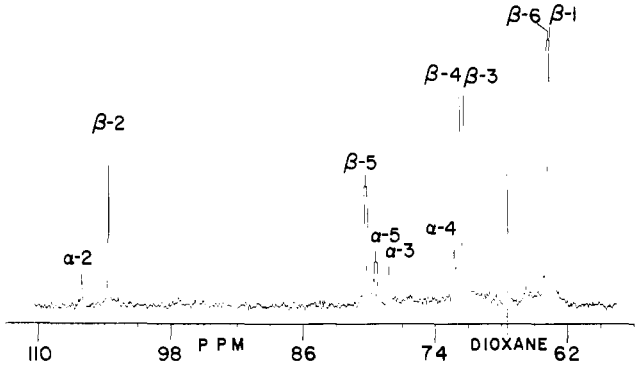


FIGURE 5: Proton-decoupled, natural abundance, FT carbon-13 NMR spectrum of tagatose 6-phosphate (**5**) obtained at 25.16 MHz and 16.5 °C. Chemical shifts and ^{31}P coupling constants for the above assigned signals are reported in Tables I and III.

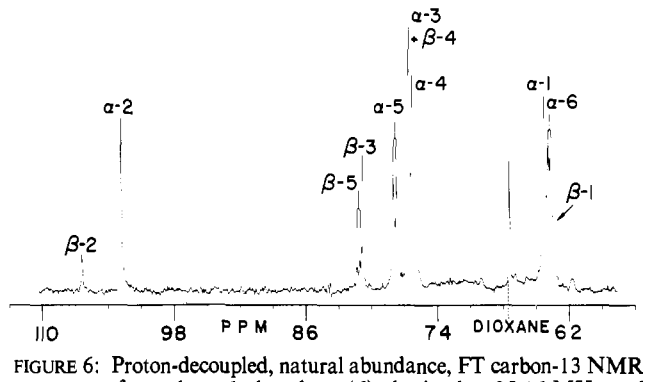


FIGURE 6: Proton-decoupled, natural abundance, FT carbon-13 NMR spectrum of L-sorbose 6-phosphate (**6**) obtained at 25.16 MHz and 16.5 °C. Chemical shifts and ^{31}P coupling constants for the above assigned signals are reported in Tables I and III.

in Table II. The ^{13}C resonances of the latter were assigned by comparison of their chemical shifts with those of their parent anhydrohexitols (Que & Gray, 1974) and with their acetylated and methylated derivatives (Voll, Koerner, and Younathan, unpublished results), as well as with their ^{31}P–^{13}C couplings (Table III).

The ^{13}C resonances of the ketohexose 6-phosphates were assigned through a combination of approaches. Inspection of Figures 3–6 reveals a major and a minor set of six signals based on peak height. Each set of signals must correspond to one anomeric form since keto and hydrated keto forms of the sugars are undetectable in the ^{13}C NMR spectra of samples lacking ^{13}C isotopic enrichment (Midelfort et al., 1976). Observation of $^1J_{CH}$ couplings through off-resonance decoupling studies shows that each set of signals is composed of two methylene (C-1, C-6), three methine (C-3, C-4, C-5), and one quaternary (C-2) carbon. The anomeric (C-2) carbons are thus unambiguously identified. Since Que & Gray (1974) have shown that a cisoid substitution of diols on the tetrahydrofuran ring leads to shielding of the hydroxylated carbons relative to transoid substitution, the downfield (δ 105–107) anomeric signals are assigned to **1a**, **4b**, **5a**, and **6b** and the upfield (δ 102–104) anomeric signals to **1b**, **4a**, **5b**, and **6a**. These assignments also allow each set of major and minor signals to be allocated to a specific anomeric form.

The methylene carbons of each anomer are readily assigned since C-6 has a ^{31}P coupling and C-1 does not. Of the methine carbons, C-5 is also unambiguously assigned due to its ^{31}P coupling (Koerner et al., 1973). However, the assignments of C-3 and C-4 are equivocal.

In the case of fructofuranose-6-P (**1**), the C-3 and C-4 methine carbons were assigned through a study of C-3 and C-4 of 2,5-anhydroglucitol-6-P (**7**) and 2,5-anhydromannitol-6-P (**9**), which are structural analogues of α- and β-fructofuranose-6-P, respectively (Table II). The shielding of cisoid-substituted diols described by Que & Gray (1974), as noted previously, is 5.4 ppm and should be present for C-3

Table III: ^{31}P–^{13}C Coupling Constants of Ketohexose Phosphates and 2,5-Anhydrohexitol Phosphates and Rotameric Compositions of Their Phosphate Groups[a]

compound	spin-coupled nuclei	$^2J_{POC}$ (Hz)	$^3J_{POCC}$ (Hz)	rotameric compn (%)[b] i + ii	rotameric compn (%)[b] iii
α-D-fructofuranose-6-P (**1a**)	P,C-6,C-5	4.4[c]	8.1	49	51
β-D-fructofuranose-6-P (**1b**)	P,C-6,C-5	2.9[c]	7.4	55	45
α-D-fructofuranose-1-P (**2a**)	P,C-1,C-2	*d*	*d*	*e*	*e*
β-D-fructofuranose-1-P (**2b**)	P,C-1,C-2	4.5	*d*	*e*	*e*
α-D-fructopyranose-1-P (**2c**)	P,C-1,C-2	*d*	*d*	*e*	*e*
β-D-fructopyranose-1-P (**2d**)	P,C-1,C-2	4.6	6.6	62	38
α-D-fructofuranose-1,6-P_2 (**3a**)	P-1,C-1,C-2	*d*	*d*	*e*	*e*
	P-6,C-6,C-5	*d*	*d*	*e*	*e*
β-D-fructofuranose-1,6-P_2 (**3b**)	P-1,C-1,C-2	4.0	*d*	*e*	*e*
	P-6,C-6,C-5	4.5	*d*	*e*	*e*
α-D-psicofuranose-6-P (**4a**)	P,C-6,C-5	4.4	7.4	55	45
β-D-psicofuranose-6-P (**4b**)	P,C-6,C-5	4.4	9.5	37	63
α-D-tagatofuranose-6-P (**5a**)	P,C-6,C-5	*d*	8.0	50	50
β-D-tagatofuranose-6-P (**5b**)	P,C-6,C-5	5.2	7.3	56	44
α-L-sorbofuranose-6-P (**6a**)	P,C-6,C-5	4.4	6.6	62	38
β-L-sorbofuranose-6-P (**6b**)	P,C-6,C-5	*d*	7.3	56	44
2,5-anhydro-D-glucitol-6-P (**7**)	P,C-6,C-5	3.8	8.1	49	51
2,5-anhydro-D-glucitol-1,6-P_2 (**8**)	P-1,C-1,C-2	4.3	7.2	57	43
	P-6,C-6,C-5	4.4	7.6	53	47
2,5-anhydro-D-mannitol-6-P[f] (**9**)	P,C-6,C-5	5.0	8.1	49	51
2,5-anhydro-D-mannitol-1,6-P_2 (**10**)	P-1,C-1,C-2	4.9	7.9	51	49
	P-6,C-6,C-5	4.9	7.9	51	49

[a] Measured at 16.5 °C (except **2** and **3** at 35 °C) in 9:1 (v/v) H_2O/D_2O for 0.6 M solutions, pH 9. Estimated error ±0.1 Hz. Calculated according to method described under Results and Discussion; i and ii are gauche rotamers and iii is the trans-periplanar rotamer. [b] Error estimated to ±6%. [c] To be noted is the correction of a typographical error (Koerner et al., 1973). [d] Unresolved. [e] No data available for calculation. [f] Properly named 2,5-anhydro-D-mannitol-1-P.

but not C-4 of the furanose ring when compared to the tetrahydrofuran ring. Thus, calculation of $\Delta\Delta\delta$, the difference in chemical shift differences ($\Delta\delta$) from Table II, should give $\Delta\Delta\delta$(C-3) = 5.4 and $\Delta\Delta\delta$(C-4) = 0 ppm for fructofuranose-6-P [calculated (**1a**–**7**) – (**1b**–**9**)]. Of the four possible sets of assignments of the ^{13}C resonances of C-3 and C-4 of α- and β-fructofuranose-6-P, only one set gives $\Delta\Delta\delta$ values within experimental error of those expected, namely, $\Delta\Delta\delta$(C-3) = 5.9 and $\Delta\Delta\delta$(C-4) = 0.4 ppm, and the following assignments (δ) are made: α-3 82.6, α-4 76.9, β-3 76.2, and β-4 75.4.

The C-3 and C-4 resonances of the other ketohexose 6-phosphates (**4**, **5**, and **6**) are assigned by comparison with α- and β-fructofuranose-6-P (**1**) and using the rule that the α-3 and β-3 signals should differ by approximately 5 ppm whereas the α-4 and β-4 signals should have approximately the same chemical shift. Thus, all carbons of the ketohexose 6-phosphates are assigned as shown in Table I. These assignments are in agreement with those of Angyal & Bethell (1976) when allowances are made for the changes in C-6 and C-5 after phosphorylation but differ in several instances with the assignments of Que & Gray (1974) and Doddrell & Allerhand (1971). Included in Table I are the chemical shift differences ($\Delta\delta$) between the carbons of **1**–**6** and the corresponding carbons of the parent ketohexose furanose forms, as assigned by Angyal & Bethell (1976). It should be noted that the assignment for the carbons of β-L-sorbofuranose-6-P (**6b**) is the first reported for the β-furanose form of this sugar.

The ^{13}C resonances of fructofuranose-1-P (**2**) and -1,6-P_2 (**3**) are assigned by comparison of chemical shifts with fructofuranose-6-P (**1**) and the parent sugar (Angyal & Bethell, 1976) and knowledge that phosphorylation of a hydroxymethyl group leads to a 2–3-ppm carbon deshielding (Koerner et al., 1973). Resonances of fructose-1-P tautomers are assigned by comparison with the ^{13}C chemical shifts of 3-*O*-methyl-D-fructose (Koerner et al., 1978).

The assignments of C-3 and C-4 of **3a** and **3b** were confirmed by comparison with the chemical shifts of the 2,5-anhydrohexitol bisphosphates **8** and **10** and calculation of $\Delta\Delta\delta$(C-3) and $\Delta\Delta\delta$(C-4), as demonstrated above for **1a** and **1b**.

^{31}P–^{13}C Coupling Constants. Included in Table III are the ^{31}P–^{13}C coupling constants of each anomer of **1**–**6** as well as their analogues (**7**–**10**). As noted previously (Koerner et al., 1973), the vicinal couplings ($^3J_{POCC}$) are greater than the two-bond couplings ($^2J_{POC}$). As recently noted (O'Connor et al., 1979), the maximum trans-periplanar coupling of $^3J_{POCC}$ is larger than the 8 Hz which was previously reported (Lapper et al., 1973; Lapper & Smith, 1973).

Anomeric and Tautomeric Compositions. Integration of the signal intensities of the anomeric (C-2) resonances yields the equilibrium anomeric compositions of **1**–**6**, which are reported in the last column of Table IV. It is noted that the experimentally found predominant anomers of **1**, **4**, **5**, and **6** are as previously predicted (Koerner et al., 1976). The tautomeric and anomeric compositions of fructose-1-P (**2**) are α-furanose (**2a**) 5%, β-furanose (**2b**) 16%, α-pyranose (**2c**) 5%, and β-pyranose (**2d**) 73%. No keto or hydrated keto forms were detected for any sugar phosphate within the limits of sensitivity (approximately 2%). However, as pointed out by Midelfort et al. (1976), the very rapid exchange of the C-2 carbon between the keto and the anomeric forms of isotopically unenriched sugars may have produced sufficient line broadening to render the carbonyl carbon of the keto form or its hydrate undetectable, even if present at concentrations greater than 2%.

Conformational Analysis of Anomeric Compositions. Angyal (1969) has shown that the anomeric equilibrium compositions of pyranose sugars can be predicted quantitatively through conformational analysis. Such an analysis has never been reported for furanose sugars. This is partly due to the complication that most furanose sugars preferentially undergo tautomeric equilibria to form more stable pyranose or other ring forms and partly due to the fact that accurate determination of the anomeric compositions of ketofuranoses had to await the availability of FT ^{13}C NMR. More importantly, the required interaction energies for the conformations of the

Table IV: Relative Standard Free Energies (G°) of Ketofuranose Phosphate Anomers and Their Constituent Conformations and the Calculated and Found Anomeric Compositions in Aqueous Solution

ketofuranose	conformational free energies[a] (kcal/mol)			anomeric free energies[b] (G°_{anomer}) (kcal/mol)	anomeric compn[c] (%)	
	$^4T_3$ (D)	T_2 (D)	T_5 (D)		theory	found (±2%)
α-D-fructofuranose-6-P (**1a**)	3.05	3.80	3.45	2.72	19	18
β-D-fructofuranose-6-P (**1b**)	2.05	4.10	2.80	1.90	81	82
α-D-fructofuranose-1-P (**2a**)	3.05	3.80	3.45	2.69	21	24[d]
β-D-fructofuranose-1-P (**2b**)	2.05	4.10	2.80	1.88	79	76[d]
α-D-fructofuranose-1,6-P_2 (**3a**)	3.05	3.80	3.45	2.69	21	23
β-D-fructofuranose-1,6-P_2 (**3b**)	2.05	4.10	2.80	1.88	79	77
α-D-psicofuranose-6-P (**4a**)	3.50	2.90	4.20	2.68	79	76
β-D-psicofuranose-6-P (**4b**)	4.15	4.70	3.75	3.45	21	24
α-D-tagatofuranose-6-P (**5a**)	4.65	4.30	3.90	3.57	18	17
β-D-tagatofuranose-6-P (**5b**)	3.10	3.95	3.25	2.70	82	83
α-L-sorbofuranose-6-P (**6a**)	4.60	2.20	3.45	2.13	78	82
β-L-sorbofuranose-6-P (**6b**)	4.55	3.50	3.15	2.87	22	18

[a] Calculated by summation of interaction energies in Table V. The conformations listed are for D sugars; for L-sorbofuranose the free energies are for the mirror-image $^3T_4$ (L), ^2T_O (L), and ^5T_O (L) conformations, respectively. [b] Calculated at 16.5 °C, except **2** and **3** at 35 °C. [c] Measured at 16.5 °C, except **2** and **3** at 35 °C, in 9:1 (v/v) H_2O/D_2O, pH 9.0, by integration of ^{13}C NMR signals of anomeric carbons. [d] Percentage is that of total furanose tautomer only and ignores pyranose tautomer.

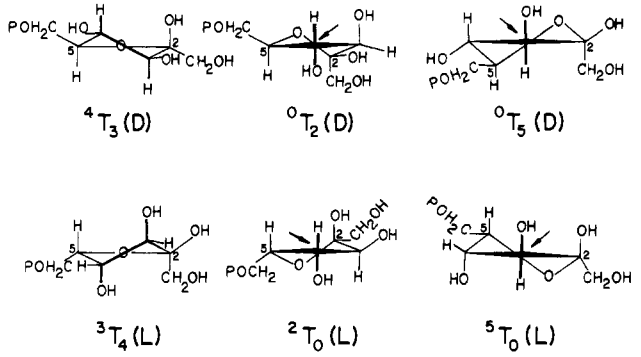


FIGURE 7: Probable predominant conformations for the furanose ring of ketohexose 6-phosphates. (above) $^4T_3$, $^0T_2$, and $^0T_5$ twist conformations of β-D-fructofuranose-6-P (**1b**); (below) $^3T_4$, ^2T_O, and ^5T_O twist conformations of α-L-sorbofuranose-6-P (**6a**). An arrow indicates carbons through which C_2 axis of symmetry passes (C_2 carbons). Conventional carbon numbering is used.

furanose ring have not been available. The determination of the anomeric compositions of the complete series of ketohexose 6-phosphates (Table IV) (all of which are confined to the furanose ring) allows a quantitative prediction of furanose anomeric compositions, based on a conformational analysis.

There are a priori 20 conformations possible for the furanose ring. These include 10 envelope (E) and 10 twist (T) conformations. If one assumes that the bulky phosphoryl hydroxymethyl group must be quasi-equatorial, then all but seven of these conformations can be eliminated. Moreover, assuming that the anomeric effect is operative for furanoses (Angyal, 1969; Stevens & Fletcher, 1968), then the anomeric (C-2) carbon must be puckered or adjacent to a puckered atom to allow for quasi-axial and quasi-equatorial substituents. This constraint leaves only four conformations for further consideration, namely, $^4T_3$, ^0E, $^0T_2$, and $^0T_5$. Finally, it is assumed that conformations containing eclipsed 1,2 interactions do not occur when similar conformations are available that avoid such interactions. Since the ^0E conformation contains eclipsed substituents at C-3 and C-4, it can be dismissed. Thus, the D-ketohexose 6-phosphates in solution can be assumed to be confined to the three twist conformations $^4T_3$, $^0T_2$, and $^0T_5$, which are shown in Figure 7. Likewise, the same reasoning indicates that L-ketohexose 6-phosphates in solution should exist predominantly in the mirror-image $^3T_4$, ^2T_O, and ^5T_O twist conformations, which are also shown in Figure 7. These conformations are favorably interconvertible on the pseudorotation itinerary of Stoddart (1971).

Table V: Estimated Relative Interaction Energies for the Twist Conformation of Ketofuranose Rings[a]

type of interaction	interaction energies (kcal/mol) involving non-C_2 carbons	involving C_2 carbons
(1) Nonbonded Group Interaction		
O_1–O_2	0.35 (1,2 a/e or e/e)	0.45 (1,2 a/i or e/i)
C_1–O_2	0.45 (1,2 a/e or e/e)	0.55 (1,2 a/i or e/i)
O_a–H_a	0.45 (1,3 a/a)	0.40 (1,2 a/i)
C_a–H_a	0.90 (1,3 a/a)	0.70 (1,3 a/i)
O_a–O_a	1.5 (1,3 a/a)	1.2 (1,3 a/i)
C_a–O_a	2.5 (1,3 a/a)	2.0 (1,3 a/i)
(2) Anomeric Interactions (O–X)		
O–OH (0–3 e)	0.55	
O–OH (0–3 a or i) Δ2 effect	1.0	1.0
O–OH (0–3 and 0–4 a or i) Δ2 effect	0.85	0.85
O–CH_2OH (0–3 e)	0.45	
O–CH_2OH (0–3 a or i) Δ3 effect	2.0	2.0
O–CH_2OH (0–3 and 0–4 a or i) Δ3 effect	1.7	1.7

[a] The C_2 carbon is that carbon of a particular twist conformation through which the C_2 axis of symmetry passes (see Figure 7). In parentheses are shown the interaction distances and orientations of substituents using the abbreviations a, axial; e, equatorial; and i, isoclinal. There are two interactions possible with isoclinal substitution, one at 19° and the other at 101°. Only the 19° interaction is considered destabilizing.

In the case of fructose-6-P (**1**) and fructose-1,6-P_2 (**3**), the above assumptions are supported by recent 200- and 300-MHz proton NMR studies (Koerner, Voll, and Younathan, unpublished results) that show approximately 6–8-Hz values for the vicinal coupling constants between H-3 and H-4 and between H-4 and H-5 of the β-furanose rings of these sugars. Such coupling constants are large when compared with the coupling constants of the closely related tetrahydrofuran ring (Koerner et al., 1977b), indicating that the dihedral angles betwen H-3 and H-4 and between H-4 and H-5 must be

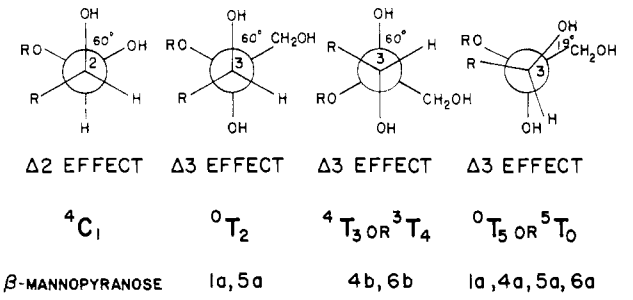


FIGURE 8: Newman projections illustrating the relationship of substituents in each conformation for which the Δ3 effect of ketofuranoses is operative. The Δ2 effect of the $^4C_1$ conformation of aldopyranoses is shown for comparison. Under each conformation are listed examples.

greater than 160°. Such dihedral angles are consistent with the three assumed twist conformations, especially the $^4T_3$ conformation.

In order to calculate the relative free energy of each twist conformation, a set of values for the nonbonded and the anomeric interaction energies of their substituents is needed. We wish to report an estimate of these values (Table V), which we arrived at through the following rationale. For substituents of ring carbons other than the C_2 carbon (the carbon through which the C_2 axis of symmetry of the twist conformation passes; Figure 7), the nonbonded interactions were assumed to be the same as for the substituents of the pyranose ring (Stoddart, 1971; Angyal, 1968). This is reasonable since such substituents of the twist conformation have the same dihedral angle as those of the pyranose ring within 10° and nonbonded interactions are essentially equatorial and axial interactions. However, for interactions involving the C_2 carbon substituents (axial/isoclinal and equatorial/isoclinal interactions), estimates had to be made that differ from those based on the pyranose ring (Table V). For the two 1,2 interactions (O_1–O_2 and C_1–O_2) of the twist conformation for which the dihedral angle is 19° instead of 60°, the interaction energies were assumed to be approximately 1.25-fold those of the chair conformation. For the four 1,3-diaxial interactions of the twist conformation, for which neither the angle nor the distance of the interaction is as unfavorable as that of the chair conformation, the interaction energies were assumed to be 0.8-fold those of the chair conformation.

The anomeric effect (O–OH), the unfavorable presence of an equatorial hydroxyl group at the anomeric carbon, was assumed to have the same value for the twist conformation of the furanose ring as for the chair conformation of the pyranose ring (Stoddart, 1971). Likewise, the same values were assumed for the anomeric effect in which an axial hydroxyl group is present at the adjacent carbon or at two adjacent carbons [Δ2 effect of Reeves (1949, 1950, 1951)]. However, the unique structural feature of the ketose, the presence of a hydroxymethyl group at the anomeric (C-2) carbon, necessitated the estimation of the destabilization energy of a new anomeric interaction. It was assumed that for the twist conformation of the furanose ring, an equatorial hydroxymethyl group at C-2 in the presence of a C-3 axial hydroxyl group causes a 2.0 kcal/mol destabilization and in the presence of both C-3 and C-4 axial hydroxyl groups a 1.7 kcal/mol destabilization. By its analogy with the Δ2 effect of aldopyranoses (Reeves, 1951), we propose to name this new anomeric interaction (O–CH_2OH) the Δ3 effect (Figure 8). The change in numbers reflects the fact that the axial hydroxyl group that is the basis of the effect is at C-2 of an aldose and C-3 of a ketose. Whereas the Δ2 effect is thought to be a special case of the anomeric effect which is predominantly electronic in origin, the Δ3 effect is probably dominantly steric in origin but may also be partially electronic. The estimated value for the Δ3 effect is based on an analogy with the energy difference between the O_a–O_a and C_a–O_a 1,3 interactions (1.0 kcal/mol). Thus, the Δ3 effect is assumed to cost 1.0 kcal/mol more than the Δ2 effect, or 2.0 kcal/mol. In analogy with the 0.85 reduction for the Δ2 effect, the Δ3 effect is reduced to 1.7 kcal/mol when an axial hydroxyl group is present at C-4.

The effect of the above anomeric interaction energies is to alter the anomeric composition of ketofuranoses in a way that is consistent with qualitative observations reported in the literature. Thus, a cis arrangement of hydroxyl and hydroxymethyl groups in a furanose ring is very unfavorable (Angyal, 1969; Angyal & Bethell, 1976). Moreover, in furanose forms of ketohexoses, the C-2 hydroxymethyl group and the C-3 hydroxyl group are trans in the predominant anomer (Gray, 1976).

The relative standard free energy ($G°$) of each twist conformation (numbered 1, 2, and 3) can be calculated with the interaction energies given in Table V by summing all nonbonded and anomeric interactions of the substituents (Table IV). The mole fraction (N) of each conformation is then calculated from the difference in relative standard free energies of the conformations ($\Delta G°$) by using

$$N_2/N_1 = e^{-\Delta G°/RT}$$

The $G°$ and N of each conformation are used to calculate the relative standard free energy of each anomer ($G°_{\text{anomer}}$) by using

$$G°_{\text{anomer}} = \sum_{i=1}^{3} G°_i N_i + \sum_{i=1}^{3} RTN_i \ln N_i$$

in which the weighted average of the conformation free energies is corrected for the entropy of mixing (Eliel et al., 1965). From the relative anomeric standard free energies, the theoretical anomeric composition (N_α and N_β) of each ketohexose-6-P is calculated from

$$G°_\alpha - G°_\beta = -RT \ln (N_\beta/N_\alpha)$$

and

$$N_\alpha + N_\beta = 1$$

In Table IV, the relative standard free energies and theoretical compositions are listed for each of the anomers of the ketohexose 6-phosphates and fructose-1,6-P_2. Good agreement is observed between the calculated anomeric compositions and those experimentally obtained by ^{13}C NMR.

Thus, the anomeric compositions of the ketohexose 6-phosphates are seen to be quantitatively predicted through conformational analysis of the furanose ring. This result strongly supports the validity of the proposed nonbonded and the anomeric interaction energies of the twist conformation of the furanose ring (Table V). The few percent disparity between calculated and found anomeric compositions is less than that reported by Angyal (1969) for aldopyranose anomeric compositions. Such a small disparity may be due to the difference in temperature between our experiments (16.5 °C) and the experiments on which the values for the interaction energies of the chair/pyranose ring are based (22, 25, and 40 °C) that are in turn the basis for our proposed twist/furanose ring interaction energies. Further studies may also allow for refinement of the values of the interaction energies that will lead to more precise predictions of anomeric compositions. Finally, our assumption that furanose rings exist predominantly in the $^4T_3$, $^0T_2$, and $^0T_5$ twist conformations may be more

general and applicable to furanose rings not possessing 6-phosphorylated hydroxymethyl groups since our conformational analysis is applicable also to the anomeric composition of fructofuranose-1-P (**2a**, **2b**, Table IV).

Relevance to the Specificity of Phosphofructokinase. It is of interest to see if the same conformational analysis that predicts the anomeric compositions of the ketohexose 6-phosphates also sheds any light on the nature of the substrate specificity of the enzyme phosphofructokinase (EC 2.7.1.11), for which these sugars are all substrates (Koerner et al., 1976). Inspection of Table IV reveals that the lowest relative free energy of any anomer of these sugars is that of β-fructofuranose-6-P (1.90 kcal/mol), the natural substrate of the enzyme. The $^4T_3$ conformation has not only the lowest relative free energy of all conformations of β-fructofuranose-6-P (2.05 kcal/mol) but also the lowest relative free energy of all conformations of the anomers of the ketohexose-6-P substrates of phosphofructokinase. This suggests that the $^4T_3$ conformation of β-fructofuranose-6-P may be an energy minimum for the structure of ketohexose 6-phosphates in solution and as such was selected by the enzyme during evolution. In fact, a plot of the natural logarithm of V_{max}/K_m (a measure of the efficacy of a compound as substrate) vs. the natural logarithm of the amount of $^4T_3$ (D)-β- or $^3T_4$ (L)-α-furanose form of each ketohexose-6-P substrate yields a straight line. This observation suggests that such a relationship is due to an ideal angle and distance between the C-1 hydroxyl and C-6 phosphate that the $^4T_3$ conformation or its enantiomer imparts to a ketohexose-6-P substrate. Studies to expand and verify such a structure–activity relationship for phosphofructokinase and its phosphoryl acceptor substrates are under way in our laboratory.

Rotamers of Phosphate Groups. In solution, the phosphate groups of **1**–**10** will rotate about the C-6/O-6 or C-1/O-1 bond to populate one of three low-energy conformations (rotamers). In two of these rotamers (i and ii), the dihedral angle of the POCC fragment is gauche (60°), and in one of them (iii), the dihedral angle is trans-periplaner (180°). Since studies with cyclic nucleotides (Lapper et al., 1973; Lapper & Smith, 1973) and glycosyl phosphates (O'Connor et al., 1979) have established a Karplus relationship between vicinal ^{31}P–^{13}C coupling constants and the POCC dihedral angle, $^3J_{POCC}$ from Table III can be used to calculate the rotameric compositions of the phosphate groups of **1**–**10** with

$$^3J_{POCC} = (N_i + N_{ii})^3J_g + (N_{iii})^3J_t$$

and

$$N_i + N_{ii} = 1 - N_{iii}$$

where N_i, N_{ii}, and N_{iii} are the mole fractions of three rotamers, and ^3J_g and ^3J_t are the coupling constants for the gauche and trans-periplanar rotamers, respectively. Assuming these two coupling constants have values similar to those previously found (O'Connor et al., 1979), ^3J_g = 2 Hz and ^3J_t = 14 Hz, we calculated the rotameric compositions (Table III). In every case, we found that the trans-periplanar rotamer is predominantly populated (38–63%). Further elucidation of the orientation of the phosphate group requires knowledge of the vicinal proton couplings of the $POCH_2$ group with its adjacent methine proton, as obtained by high-resolution proton NMR. These studies will be reported elsewhere.

Acknowledgments

We wish to thank Dr. Clinton E. Ballou, University of California at Berkeley, for inspiring us to undertake this study and Dr. Jack F. Kirsch for having one of us (E.S.Y.) as his laboratory guest during a sabbatical leave.

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