

Bovine Liver Fructokinase: Purification and Kinetic Properties[†]

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ABSTRACT: Fructokinase from beef liver has been purified 2300-fold by acid and heat treatment, ammonium sulfate fractionation, and chromatography on Sephadex G-100, DEAE- and CM-cellulose. The purified enzyme is homogeneous by all criteria examined, has a molecular weight of 56 000, and is a dimer of equal molecular weight subunits. The isoelectric point is 5.7. The Michaelis constant for activation by K⁺ is 15 mM, and the enzyme is also activated by Na⁺, Rb⁺, Cs⁺, NH₄⁺, and Tl⁺. The kinetic mechanism has been determined at pH 7.0, 25 °C. The initial velocity, product, and dead-end inhibition patterns for CrATP, CrADP, and 1-deoxy-D-fructose are consistent with a random kinetic mechanism with the formation of two dead-end complexes. Substrates for fructokinase include: D-fructose, L-sorbose, D-

tagatose, D-psicose, D-xylulose, L-ribulose, D-sedoheptulose, L-galactoheptulose, D-mannoheptulose, 5-keto-D-fructose, D-ribose, 2,5-anhydro-D-mannitol, 2,5-anhydro-D-glucitol, 2,5-anhydro-D-mannose, 2,5-anhydro-D-lyxitol, and D-ribo-γ-lactone. 5-Thio-D-fructose was not a substrate, but was a competitive inhibitor vs. D-fructose. Thus the minimum molecule for substrate activity seems to be (2R)-2-hydroxymethyl-3,4-dihydroxytetrahydrofuran. The configuration of the substituents at carbons 3, 4, and 5 appears not to be critical, but the hydroxymethyl group must have the configuration corresponding to β-D- (or α-L-) keto sugars. The anomeric hydroxyl on carbon 2 is not required (although it contributes to binding), and a wide variety of groups may be present at carbon 5.

Fructokinase (ATP:D-fructose 1-phosphotransferase, EC 2.7.1.3) catalyzes the following reaction:



The enzyme has been purified by a number of workers but has never been prepared in a homogeneous form (Sánchez et al., 1971; Parks et al., 1957; Adelman et al., 1967). Fructokinase will phosphorylate a large number of sugars (Raushel and Cleland, 1973), but only ATP (or 2'-dATP and 3'-dATP) can function as the phosphate donor (Parks et al., 1957; Adelman et al., 1967). It also has an absolute requirement for a monovalent cation for activity (Parks et al., 1957; Sánchez et al., 1971). In this paper we present a method for preparing homogeneous enzyme and report on its physical and chemical properties.

Raushel and Cleland (1973) concluded from the fact that 2,5-anhydro-D-mannitol was a good substrate for fructokinase, while 2,5-anhydro-D-glucitol was phosphorylated on carbon 6, and 2,6-anhydro-D-mannitol and 2,6-anhydro-D-glucitol were not substrates, that fructokinase was specific for the β-furanose anomer of D-fructose and the α-furanose anomer of L-sorbose. We have now extended these studies and report the complete kinetic parameters for 16 substrates of fructokinase. In addition we report product and dead-end inhibition studies which establish the kinetic mechanism to be random.

Materials and Methods

Aldolase B was isolated from beef liver using the procedure of Gracy et al. (1969). D-Xylulose was synthesized by the epimerization of D-xylose with anhydrous pyridine (Touster, 1962) and was used as a mixture of D-xylose and D-xylulose after as much D-xylose as possible was removed by crystalli-

zation. The concentration of D-xylulose was determined by assay with fructokinase. L-Ribulose was prepared by epimerization of L-arabinose by sodium aluminate (Haack et al., 1964). The yield, based on assay with fructokinase, was 9%. L-Arabinose and D-xylose were neither substrates nor inhibitors of fructokinase. CrATP and CrADP were synthesized by the procedure of DePamphilis and Cleland (1973). 1-Deoxy-D-fructose was made according to Ishizu et al. (1967). The procedure of James et al. (1967) was used to prepare D-psicose. 2,5-Anhydro-D-mannitol, 2,5-anhydro-D-lyxitol, 2,5-anhydro-D-glucitol, and 2,5-anhydro-D-mannose were prepared as previously described by Raushel and Cleland (1973). L-Galactoheptulose, D-mannoheptulose, L-glucoheptulose, and D-sedoheptulose hexaacetate were obtained from Dr. E. Zissis. 5-thio-D-fructose was from Dr. R. Whistler, 5-keto-D-fructose was from Dr. S. England, and 1,2-O-isopropylidene-D-psicose was from Dr. A. Perlin. D-Sedoheptulose hexaacetate was deacylated with cold sodium methoxide (Richtmeyer, 1962). Diisopropylidene-D-apiose was obtained from Pfanstiehl Laboratories and converted to D-apiose with 0.5 N H₂SO₄ (Bell, 1962).

Enzyme Assays. Fructokinase activity was measured spectrophotometrically using a pyruvate kinase-lactate dehydrogenase coupling system. A Beckman DU monochromator equipped with a Gilford 200 optical density converter and a 10-mV recorder was used to follow the reaction at 340 nm. Unless otherwise noted, all assays were done at 25 °C.

For activity measurements during the purification of fructokinase, each 3-mL cuvette contained 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 7.0, 100 μg each of salt-free lactate dehydrogenase and pyruvate kinase, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 100 mM KCl, 5.0 mM ATP (pH 7.0), 5.0 mM fructose, and 6.0 mM MgCl₂. A unit of fructokinase is that amount of enzyme that produces 1 μmol of MgADP per min in the above assay system. During the early stages of the purification, activities corresponding to ATPase, myokinase, and sorbitol dehydrogenase had to be subtracted from the observed rate using an appropriate blank. When

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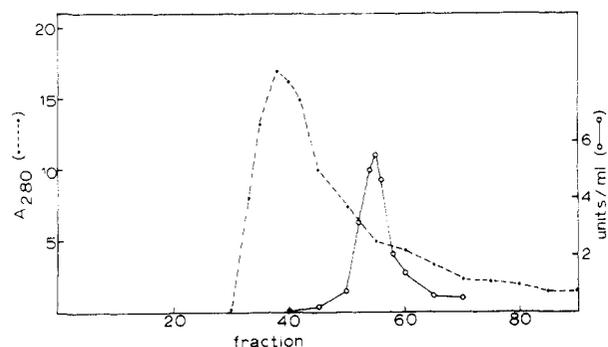


FIGURE 1: Chromatography of bovine liver fructokinase on Sephadex G-100. The fraction volume was 7 mL: (closed circles) protein (A_{280}); (open circles) fructokinase activity.

studying the activation of monovalent cations on fructokinase, activity was measured as above except that all acidic components were neutralized with either Tris¹ or cyclohexylamine, and fructose and MgATP were held at 25 and 3.0 mM.

In the presence of added ADP, fructokinase activity in the forward reaction was measured spectrophotometrically using a liver aldolase B and α -glycerophosphate dehydrogenase coupling system. Each cuvette contained in 3 mL total volume, 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)-KOH (pH 7.0), 1.1 units of aldolase B, 5 units of α -glycerophosphate dehydrogenase, 0.2 mM NADH, 100 mM KCl, 2 mM excess MgCl₂, fructokinase, and various amounts of substrates and inhibitors.

The back reaction was measured using a hexokinase-glucose-6-phosphate dehydrogenase coupling system. Each cuvette contained in 3 mL, 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)-KOH (pH 7.0), 10 units each of hexokinase and glucose-6-P dehydrogenase, 0.3 mM NADP, 2.0 mM glucose, 2 mM excess MgCl₂, 100 mM KCl, fructokinase, and various amounts of D-fructose-1-P and ADP. All assays were at 25 °C and in all cases fructokinase was added last with an adder mixer.

Data Analysis. Reciprocal velocities were plotted graphically against reciprocals of substrate concentration and the data were fitted to eq 2, assuming equal variance for the velocities and using the Fortran programs of Cleland (1967). Data for a sequential initial velocity pattern were fitted to eq 3, for competitive inhibition to eq 4, and for noncompetitive inhibition to eq 5. The nomenclature used in this paper is that of Cleland (1963).

$$v = \frac{VA}{K + A} \quad (2)$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (3)$$

$$v = \frac{VA}{K(1 + (I/K_{is})) + A} \quad (4)$$

$$v = \frac{VA}{K(1 + (I/K_{is})) + A(1 + (I/K_{ii}))} \quad (5)$$

Protein Determination. The protein content of solutions during purification was determined using the method of Warburg and Christian (1941) or Lowry et al. (1951) using bovine serum albumin as a standard. The purified enzyme had

¹ Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl.

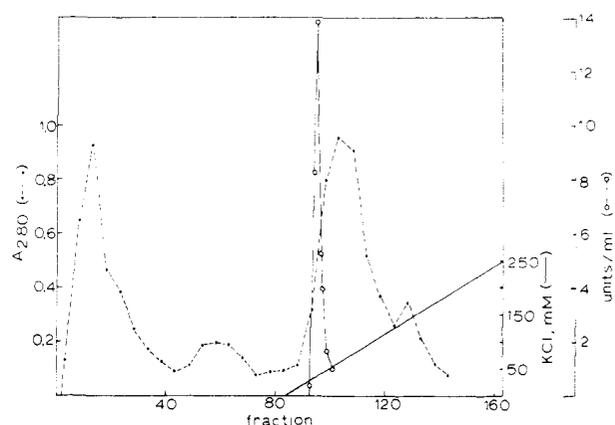


FIGURE 2: Elution of fructokinase from DEAE-cellulose. The fraction volume was 7 mL: (closed circles) protein (A_{280}); (open circles) fructokinase activity.

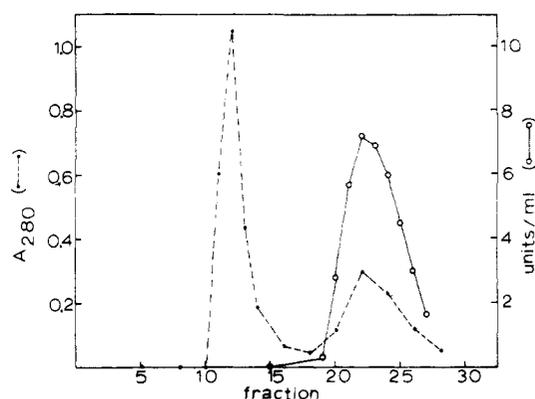


FIGURE 3: Elution of fructokinase from CM-cellulose. The fraction volume was 1.5 mL: (closed circles) protein (A_{280}); (open circles) fructokinase activity.

an extinction coefficient, $E_{1\text{cm}}^{1\%}$, of 8.0 at 280 nm when protein was determined by the method of Lowry et al. (1951) using BSA as a standard.

Purification of Fructokinase. The purification was carried out at 0–4 °C. All centrifugations were for 30 min at 8000g and all buffers contained 1.0 mM dithiothreitol and 0.1 mM EDTA.

Five hundred grams of beef liver were homogenized in 750 mL of 0.25 M mannitol in a Waring blender for 1 min. After centrifugation, the pH was adjusted to 5.0 with cold 1 N HCl. The acid-treated homogenate was centrifuged and the pH of the supernatant solution was adjusted to 7.3 with cold 1 N KOH (fraction I). Fraction I was heated in a stainless steel beaker, with overhead stirring, to 65 °C for 1 min in an 85 °C water bath. The contents were cooled in an ice bath and the precipitated protein was removed by centrifugation and discarded. The supernatant solution (fraction II) was brought to 40% saturation with solid ammonium sulfate (0.23 g/mL). The suspension was stirred for 30 min and centrifuged. The supernatant solution was brought to 60% saturation by the slow addition of solid ammonium sulfate (0.123 g/mL) and centrifuged after 30 min stirring. The precipitate was dissolved in 10 mL of 10 mM triethanolamine hydrochloride buffer, pH 7.3 (fraction III).

The ammonium sulfate fraction was applied to a G-100 column (4.0 × 50 cm) which had been equilibrated with 10 mM triethanolamine hydrochloride, pH 7.3. The column was

TABLE I: Purification of Fructokinase from Bovine Liver.

Fraction	Volume (mL)	Activity (units)	Protein (mg)	Spec. act. (units/mg)	Purification	Yield (%)
(I) pH 5.0 treatment	690	215	29 600	0.007	1	100
(II) Heat treatment	520	185	10 200	0.018	2.5	86
(III) 40–60% (NH ₄) ₂ SO ₄	22	174	2 640	0.066	9.0	81
(IV) Sephadex G-100	51	134	229	0.59	81	62
(V) DEAE-cellulose	29	100	22	4.5	620	47
(VI) CM-cellulose	13	67	4	17	2330	31

eluted with the same buffer at a flow rate of 0.5 mL/min. The fractions containing fructokinase (Figure 1) were pooled (fraction IV) and applied to a column (1.5 × 15 cm) of DEAE-cellulose (adjusted to pH 7.3 in 10 mM triethanolamine hydrochloride, and then washed with more buffer of this pH after the column was poured). The column was washed with 400 mL of 10 mM triethanolamine hydrochloride, pH 7.3, until the *A*₂₈₀ fell below 0.1. Fructokinase was eluted from the column with a 400-mL linear gradient of triethanolamine hydrochloride, pH 7.3, containing 0–250 mM KCl. The active fractions were combined (Figure 2) and adjusted to 10 mM potassium phosphate, pH 5.8, by repeated concentration and dilution in an Amicon ultrafiltration apparatus. The concentrated protein solution (3.0 mL) was applied to a column of CM-cellulose (0.9 × 50 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 5.8. The column was washed with the same buffer and the active fractions were combined (Figure 3).

A summary of a representative purification is found in Table I. The entire purification can be completed in 3 to 4 days. Frozen livers work equally as well. The G-100 and DEAE-cellulose columns have also been operated at room temperature with little loss of activity.

The specific activity of this preparation (17 U/mL) is about ten times greater than the best previously reported specific activity of fructokinase from beef liver (Parks et al., 1957). The enzyme is homogeneous by all criteria examined: disc gel electrophoresis, gel electrofocusing, sedimentation equilibrium centrifugation, and sodium dodecyl sulfate gel electrophoresis. Fructokinase from rat liver has been purified to the same specific activity by Adelman et al. (1967), but no attempt was made to determine the purity of their preparation. Isolation of rat liver fructokinase by a similar procedure resulted in a preparation that appeared homogeneous by sedimentation velocity, but was not totally homogeneous on disc gel electrophoresis and density gradient centrifugation (Sánchez et al., 1971).

Results

Properties of the Purified Enzyme. The molecular weight of bovine liver fructokinase was determined by sedimentation equilibrium centrifugation in a Spinco Model E analytical ultracentrifuge according to the method of Yphantis (1964). The protein (0.4 mg/mL) was dissolved in 10 mM triethanolamine hydrochloride (pH 7.3)–100 mM KCl–1 mM dithiothreitol and centrifuged for 20 h at 23 150 rpm at 20 °C. Analysis of the data supported the homogeneity of the purified enzyme. Assuming a partial specific volume of 0.73 mL/g, the molecular weight is 56 000.

The molecular weight of fructokinase was also estimated by gel filtration on Sephadex G-100 according to the method of Andrews (1964). When bovine serum albumin, ovalbumin, and myoglobin were used as standards, a molecular weight of

TABLE II: Activation by Monovalent Cations, pH 7.0, 25 °C.

Cation	<i>K_m</i> (mM)	Relative <i>V_{max}</i>
K ⁺	14.7 ± 0.9	100
Na ⁺	72 ± 8	44 ± 2
Rb ⁺	53 ± 3	102 ± 3
Cs ⁺	55 ± 7	12 ± 1
Tl ⁺	1.2 ± 0.07	70 ± 1
NH ₄ ⁺	32.4 ± 0.6	40 ± 1

56 000 was obtained.

The subunit molecular weight of fructokinase was determined using sodium dodecyl sulfate gel electrophoresis in 10% gels according to the method of Weber and Osborn (1969). The purified enzyme was incubated with 0.1% sodium dodecyl sulfate in 10 mM phosphate buffer, pH 7.2, containing 10 mM dithiothreitol for 10 min at 65 °C. The enzyme migrated as a single band as detected by staining with Coomassie blue. The molecular weight of the sodium dodecyl sulfate treated enzyme yielded a value of 29 000 when BSA, ovalbumin, lactate dehydrogenase, and cytochrome *c* were used as standards, and thus fructokinase appears to be a dimer of equal size subunits.

The enzyme from rat liver has a reported native molecular weight of 28 000 as determined by density gradient centrifugation using lysozyme as a standard (Sánchez et al., 1971). Apparently the beef liver enzyme is a dimer while the rat liver enzyme is a monomer. When compared with the list compiled by Morrison and Heyde (1972), fructokinase is one of the smallest phosphotransferases known.

Gel electrofocusing was used to determine the isoelectric point of fructokinase according to the method described by Wrigley (1971). LKB (pH 3–10) ampholytes were used with 7.5% polyacrylamide gels. Only one band was detected when the gels were treated with 5% trichloroacetic acid and it corresponded to an isoelectric point of 5.7.

Activation by Monovalent Cations. Fructokinase has an absolute requirement for a monovalent cation (Parks et al., 1957). Potassium and other monovalent cations were found to activate hyperbolically and the data were fitted to eq 2. The Michaelis constants and relative *V_{max}* values for various monovalent cations appear in Table II.

Substrate Specificity. The kinetic constants from fits of the data to eq 2 or 3 for those compounds found to be substrates for fructokinase are shown in Table III. The relative *V_{max}* values were determined using the same enzyme solution by varying each substrate over a tenfold concentration range at a level of 10 mM MgATP and fitting the data to eq 2. Since fructokinase is known to be specific for the β-D- (or α-L-) furanose anomer (Raushel and Cleland, 1973), the Michaelis constants and *V/K* values were corrected for the percentage of active isomer for these ketoses whose anomeric equilibria

TABLE III: Kinetic Constants from Initial Velocity Experiments.^a

Substrate	$K_{(MgATP)}$ (mM)	$K_{i(MgATP)}$ (mM)	$K_{(sub)}$ (mM)	$K_{i(sub)}$ (mM)	Rel V_{max}	Rel $V_{max}/K_{(sub)}$
D-Fructose ^c (I)	0.17 ± 0.02	0.51 ± 0.08	0.10 ± 0.01 (0.021)	0.29 ± 0.04 (0.061)	100 ^b	100 (480)
L-Sorbose ^c (II)	0.05 ± 0.02	0.73 ± 0.23	0.43 ± 0.1 (0.022) ^d	6.7 ± 2 (0.34) ^d	44 ± 5	10 ± 3 (200) ^d
D-Tagatose ^c (XIII)	0.17 ± 0.02	1.0 ± 0.2	0.9 ± 0.1 (0.036)	5.7 ± 0.9 (0.23)	103 ± 3	11 ± 2 (275)
D-Psicose ^c (XIV)	0.51 ± 0.06	1.8 ± 0.2	11 ± 1 (1.6)	38 ± 6 (5.7)	59 ± 3	0.53 ± 0.05 (3.7)
D-Xylulose ^c (V)	0.38 ± 0.03	0.8 ± 0.2	1.4 ± 0.2 (0.87)	2.9 ± 0.6 (1.8)	78 ± 2	5.6 ± 0.8 (9.0)
L-Ribulose (VI)	0.14 ± 0.02	0.5 ± 0.1	0.37 ± 0.05	1.3 ± 0.3	49 ± 3	13 ± 2
D-Sedoheptulose (VII)	0.89 ± 0.07	2.2 ± 0.2	2.2 ± 0.2	5.3 ± 0.5	137 ± 3	6.0 ± 0.6
L-Galactoheptulose (VIII)	0.33 ± 0.02	1.1 ± 0.2	1.8 ± 0.2	5.7 ± 0.4	134 ± 4	7.4 ± 1
D-Mannoheptulose (IX)			120 ± 10		72 ± 4	0.06 ± 0.01
5-Keto-D-fructose (XVI)	0.35 ± 0.03	1.5 ± 0.2	1.2 ± 0.1	4.9 ± 0.5	47 ± 1	3.9 ± 0.3
D-Ribose (XII)	1.0 ± 0.1	1.8 ± 0.3	142 ± 16	252 ± 39	63 ± 5	0.044 ± 0.006
2,5-Anhydro-D-mannitol (XIX)	0.32 ± 0.03	2.4 ± 0.2	1.6 ± 0.2	12 ± 1	222 ± 6	14 ± 2
2,5-Anhydro-D-glucitol (III)	0.38 ± 0.04	1.4 ± 0.2	2.9 ± 0.3	11 ± 1	109 ± 5	3.8 ± 0.4
2,5-Anhydro-D-mannose (XX)	0.30 ± 0.02	1.8 ± 0.2	1.0 ± 0.1	5.8 ± 0.6	59 ± 2	5.9 ± 0.09
2,5-Anhydro-D-lyxitol (IV)	0.7 ± 0.3	1.3 ± 0.6	10 ± 3	19 ± 13	25 ± 2	0.25 ± 0.07
D-Ribono- γ -lactone (XI)	3.9 ± 0.3	1.5 ± 0.2	58 ± 5	22 ± 2	64 ± 4	0.11 ± 0.01

Substrate	$K_{(MgADP)}$ (mM)	$K_{i(MgADP)}$ (mM)	$K_{(sub)}$ (mM)	$K_{i(sub)}$ (mM)	Rel V_{max}	Rel $V_{max}/K_{(sub)}$
D-Fructose-1-P ^c	0.10 ± 0.02	0.19 ± 0.06	1.8 ± 0.3 (0.27)	3 ± 1 (0.5)	0.42 ± 0.04	0.023 ± 0.005 (0.15)

^a From fits to eq 2 and 3 of data at pH 7.0, 25 °C, 100 mM KCl. Roman numerals refer to structures in the text. ^b Corresponds to a turnover number of 480 min⁻¹ assuming a dimer of molecular weight 56 000 and a specific activity of 17 μ mol min⁻¹ (mg of enzyme)⁻¹. ^c The values in parentheses have been corrected assuming that only the β -D- (or α -L-) furanose anomer is the active species. The correction factors are: D-fructose (21%), D-tagatose (4%), D-psicose (15%), (Angyal and Bethell, 1976), D-fructose-1-P (15%) (Benkovic et al., 1973), D-xylulose (62%), (Angyal et al., 1976), and L-sorbose (2 or 5%). ^d Assuming 5% α -L-furanose (Que and Gray, 1974). ^e Assuming 2% α -L-furanose (Angyal and Bethell, 1976).

are known, and the corrected constants are shown in Table III.

At concentrations of at least 50 mM, L-glucoheptulose, D-glucitol, D-mannitol, D-apiose, tetrahydrofurfuryl alcohol, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D- and L-arabinose, D- and L-xylose, and D-lyxose were not substrates for fructokinase. No activity could be detected with 5-thio-D-fructose at a concentration of 0.75 mM, but it was a competitive inhibitor vs. D-fructose with a K_i of 2.4 mM.²

Appropriate tests were performed to ensure that the observed activity with the various compounds in Table III was not due to small amounts of impurities. Since both D-psicose and D-ribose have been reported not to be substrates for rat liver fructokinase (Adelman et al., 1967), the activities of these compounds relative to fructose were determined through an entire purification and found to remain constant in fractions II-VI of Table I. In addition, all three activities coincided exactly on elution from Sephadex G-100.

An attempt was made to determine if fructokinase would also phosphorylate fructose at C-6 by having fructose inverted from its normal position at the active site. Formation of any fructose-6-P was determined by coupling with glucose-6-P isomerase and glucose-6-P dehydrogenase. At a concentration

of 2.0 mM D-fructose, the rate of formation of fructose-6-P was less than 0.1% that of fructose-1-P.

Anomeric Specificity. Shown in Figure 4 are the time courses of phosphorylation by fructokinase of a newly prepared solution of D-fructose and a solution that has been allowed to equilibrate overnight. This experiment was run at pH 6 and at 15 °C so that the mutarotation rate of fructose would be slow ($t_{1/2}$ about 4 min).

Back Reaction. The kinetic constants of the back reaction from a fit to eq 3 appear in Table III. The ratio of maximal velocities for the forward and reverse reactions at pH 7.0 is 240.

Product and Dead End Inhibition. MgADP is a competitive inhibitor of the forward reaction with MgATP as the varied substrate. With D-fructose as the varied substrate, MgADP gives noncompetitive inhibition. The product inhibitor, D-fructose-1-P, is competitive vs. D-fructose and noncompetitive vs. MgATP. The kinetic constants from fits of the data to eq 4 or 5 appear in Table IV. Also shown in Table IV are the data from dead end inhibition studies with 1-deoxyfructose, CrATP, and CrADP.

Discussion

Anomeric Specificity. Both Kuyper (1959) and Sánchez et al. (1971) claimed there was no difference in the rate of phosphorylation of a newly prepared solution of fructose (β -pyranose) and an equilibrated solution of fructose. However, calculations show that the mutarotation rate under the con-

² 5-Thio-D-fructose is a substrate for hexokinase, $V = 2.6\%$ of D-glucose, $K_m = 1.7$ mM, and 5-thio-D-fructose-6-P is a substrate for phosphofructokinase, $V = 5.9\%$ of F6P, $K_m = 0.09$ mM (unpublished observations).

TABLE IV: Product and Dead-End Inhibition Constants.^a

Inhibitor	Variable Substrate	Fixed Substrate	K_{is} (mM)	K_{ii} (mM)	Inhibition
Fructose-1-P	Fructose	MgATP, 0.25 mM	12 ± 1		C
Fructose-1-P	MgATP	Fructose, 0.10 mM	21 ± 3	42 ± 8	NC
MgADP	Fructose	MgATP, 0.20 mM	0.15 ± 0.02	0.20 ± 0.01	NC
MgADP	MgATP	Fructose, 0.60 mM	0.08 ± 0.01		C
1-Deoxyfructose	Fructose	MgATP, 0.25 mM	6.1 ± 0.7		C
1-Deoxyfructose	MgATP	Fructose, 0.10 mM	7.6 ± 0.9	34 ± 9	NC
CrATP	Fructose	MgATP, 0.10 mM	1.8 ± 0.3	2.7 ± 0.3	NC
CrATP	MgATP	Fructose, 1.0 mM	1.2 ± 0.2		C
CrADP	Fructose	MgATP, 0.25 mM	1.1 ± 0.2	1.3 ± 0.1	NC
CrADP	MgATP	Fructose, 0.20 mM	0.78 ± 0.05		C

^a pH 7.0, 100 mM KCl. K_{is} , slope inhibition constant; K_{ii} , intercept inhibition constant; C, competitive inhibition; NC, noncompetitive inhibition.

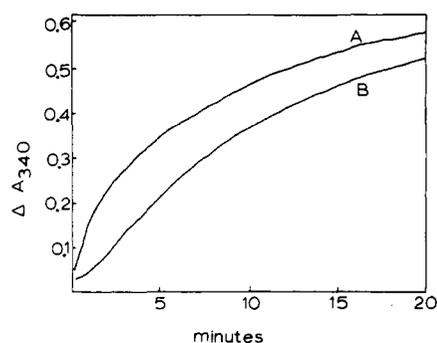


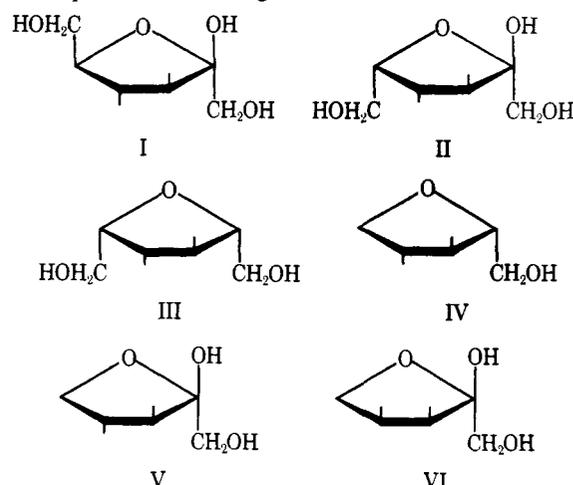
FIGURE 4: Phosphorylation of an equilibrated solution of D-fructose (A) and a solution of freshly dissolved fructose (β -pyranose) (B). Conditions: 100 mM KCl, 5.0 mM MgATP, 50 mM each in acetate and cacodylate, pH 6.0, 0.09 mM D-fructose, 0.67 unit/mL fructokinase, 15 °C.

ditions used by these workers was probably faster than the rate of phosphorylation (Isbell and Pigman, 1969; Pigman and Isbell, 1968). As shown in Figure 4 when the mutarotation rate has been decreased by lowering the pH and temperature of the reaction, the time course for the newly prepared solution of D-fructose clearly shows a lag. These results are consistent with the conclusions of Raushel and Cleland (1973) from substrate analogue studies that only the β -furanose anomer of D-fructose is a substrate for fructokinase.

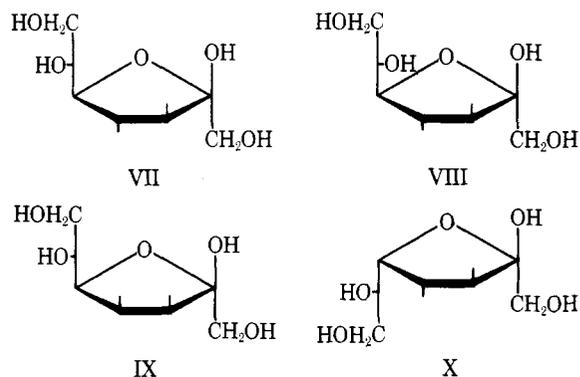
Substrate Specificity. Although fructokinase from bovine liver is specific for the β -furanose anomer of D-fructose (I), a number of modifications in the D-fructose structure can be made and still retain activity. The $-\text{CH}_2\text{OH}$ group at C-5 can also be in the L configuration as shown by activity with L-sorbose (II) and 2,5-anhydro-D-glucitol (III) (which is phosphorylated at the 6 position (Raushel and Cleland, 1973)), or it can be missing altogether as shown by activity with 2,5-anhydro-D-lyxitol (IV), D-xylulose (V), and L-ribulose (VI).

Although the corrected K_m value for L-sorbose is the same or lower than that for D-fructose (depending on what value is assumed for the proportion of α -furanose present), V is reduced by a factor of 2 when the CH_2OH group is L as opposed to D. The absence of the CH_2OH group causes higher K_m values, although V values are still good as long as the anomeric hydroxyl is present to promote binding in the proper mode. With 2,5-anhydro-D-lyxitol, however, nonproductive binding in which C-1 occupies the normal position of C-6 of fructose cannot be prevented by the binding of an anomeric hydroxyl,

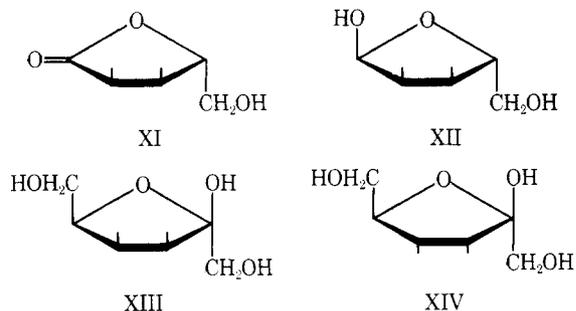
and the V value that is only 25% that of fructose suggests that such nonproductive binding does occur.



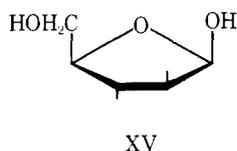
Heptuloses with a D configuration at C-5 (D-sedoheptulose (VII), L-galactoheptulose (VIII), D-mannoheptulose (IX)) are also good substrates with high V values, but no activity was detected with L-glucoheptulose (X) which has an L configuration at this position. The most likely explanation is steric hindrance with the enzyme when there is a large group in the L configuration at C-5, but L glucoheptulose (X) is also expected to exist in the furanose conformation to an extent less than L-sorbose (Perlin et al., 1973). A very small amount of furanose in solution is the most likely explanation for the high K_m of D-mannoheptulose (IX), and the lower proportion of the correct furanose than with fructose probably explains the higher K_m 's of VII and VIII, whose anomeric composition has not been reported.



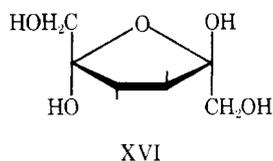
Since D-ribo- γ -lactone (XI) is a substrate, it is evident that a carbonyl can also occupy the position at C-5, although it does not seem to contribute much to binding. It was surprising to find that D-ribose (XII) was such a good substrate, although its Michaelis constant is quite high. D-Ribose (XII) must be phosphorylated at C-5 with the C-3 and C-2 hydroxyls oriented like the C-3 and C-4 hydroxyls of D-tagatose (XIII) and L-ribulose (VI). Binding in this orientation is clearly preferred over the one in which the C-3 and C-2 hydroxyls are oriented like the C-3 and C-4 hydroxyls of D-psicose (XIV) because the corrected Michaelis constant of D-tagatose is a factor of 45 lower than that of D-psicose.



No activity is seen with D-arabinose (XV), which may be due in part to the small amount of furanose in solution (~3%) (Angyal, 1969). Also, since the C-2 and C-3 hydroxyls are trans to each other, there is no preference for the orientation of these groups as there is with D-ribose (XII), and specific binding of the anomeric hydroxyl would lead to predominantly nonproductive binding as shown in XV.



Englard et al. (1972) have shown that 5-keto-D-fructose is phosphorylated by fructokinase. Since a five-membered ring is required for activity, the most likely anomer phosphorylated is the dihemiacetal XVI. This assignment is supported by the

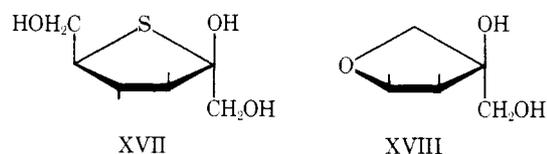


fact that the crystalline form of 5-keto-D-fructose (Hansen et al., 1976) is a dimer in which the 5-keto group of one molecule in the β -pyranose form forms a cyclic acetal with the 2- and 3-hydroxyls of a second molecule in a furanose form similar to XVI, except that carbon 6 is exo to the acetal ring, rather than to carbon 1.

The orientation of the hydroxyl at C-4 is relatively unimportant since D-tagatose (XIII) and L-ribulose (VI) are good substrates. The hydroxyl at C-3 can also be in both the D and L configurations since D-psicose (XIV) is a substrate. There is, however, a 76-fold increase in the corrected Michaelis constant of D-psicose relative to D-fructose, compared with a 1.7-fold increase for D-tagatose, suggesting that the L configuration is greatly preferred at C-3.

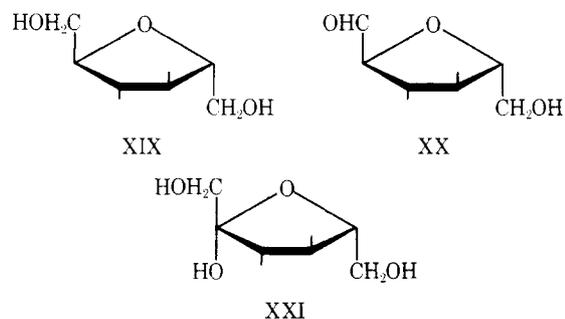
The hydroxyls at C-3 and C-4 are needed for substrate activity as neither tetrahydrofurfuryl alcohol nor *cis*-2,5-bis(hydroxymethyl)tetrahydrofuran (Raushel and Cleland, 1973) are substrates.

The replacement of the ring oxygen by sulfur in 5-thio-D-fructose (XVII) results in no detectable activity, although this compound is a competitive inhibitor ($K_i = 2.4$ mM) vs. D-fructose and thus binds well.³ No activity of D-apiose (XVIII)



with fructokinase confirms the requirement for an oxygen in the proper position in the ring.

The anomeric hydroxyl is not essential since a number of anhydro sugar alcohols are substrates. However, the Michaelis constants of 2,5-anhydro-D-mannitol (XIX) and 2,5-anhydro-D-mannose (XX) are larger than the corrected value for



D-fructose by factors of 76 and 48. Similarly the K_m values for 2,5-anhydro-D-glucitol and 2,5-anhydro-D-lyxitol are larger than those for the α -furanose anomer of L-sorbose and the β -furanose anomer of D-xylulose by factors of 130 to 320 (depending on the proportion of α -furanose assumed) and 12. Thus this hydroxyl is very important in binding. It has a more important role, however, in preventing binding of fructose in the configuration XXI, which would result in phosphorylation at position 6, rather than 1. With sensitive assays, we failed to find any fructose-6-P as the product of phosphorylation. Since the anomeric hydroxyl will fit in the position shown in XXI, as shown by activity with the dihemiacetal of 5-ketofructose (XVI), the lack of phosphorylation at the 6 position must result from the specific binding of the anomeric hydroxyl to some group on the enzyme. When the anomeric hydroxyl is absent and the anhydro sugar alcohol will fit in the nonproductive mode, such binding appears to occur, as indicated by the lower than expected V values for 2,5-anhydro-D-lyxitol and 2,5-anhydro-D-mannose (this lowers their apparent K_m values as well).

Thus, the substrate specificity of fructokinase requires a (2*R*)-2-hydroxymethyl-3,4-dihydroxytetrahydrofuran molecule, with the L configuration for the hydroxyl strongly preferred at C-3, and the D configuration preferred at C-4. However, only the configuration of the hydroxymethyl group that is phosphorylated shows absolute stereospecificity. An

³ However, 6-thio-D-fructose, which exists almost entirely as the thiapyranose, is a substrate (Chmielewski et al., 1976). Recent experiments in this laboratory by C. E. Grimshaw using material supplied by Dr. R. L. Whistler show that the β -furanose is the true substrate, and the ring opening of the thiapyranose is base catalyzed and rapid enough at pH 7 (0.1 min^{-1}) or above not to limit the enzymatic rate. The result is little change in V , but a drastic increase in K_m , relative to the values for fructose. If the β -furanoses of 6-thiofructose and fructose have equal affinity for the enzyme, the calculated level of furanose present for the thio sugar is 0.4%. This work will be reported in detail at a later date.

anomeric hydroxyl at C-2 strongly increases binding and prevents nonproductive binding, while a wide variety of groups may be attached to C-5.

Kinetic Mechanism. The intersecting initial velocity patterns for both the forward and reverse reactions indicate that the mechanism is sequential. The inhibition patterns of the forward reaction by the products, MgADP and D-fructose-1-P, are characteristic of a random kinetic mechanism with two dead-end complexes (E·MgADP-fructose and E·MgATP-fructose-1-P) rather than an ordered one. A Theorell-Chance mechanism, which gives the same product inhibition patterns, is ruled out by the observation that the initial velocity patterns for the forward and reverse reactions both intersect above the horizontal axis. In a Theorell-Chance mechanism the vertical coordinate of the crossover point of the initial velocity pattern for the forward reaction is equal to $1/V_1 - 1/V_2$, and since $V_1 \gg V_2$, the pattern would have to intersect far below the horizontal axis and look nearly parallel.

The inhibition patterns for the dead-end inhibitors CrATP, CrADP, and 1-deoxy-D-fructose confirm that the kinetic mechanism is random; if the mechanism were ordered, at least one of these inhibition patterns would have been uncompetitive.

If the rapid equilibrium assumption is valid for this enzyme, then the kinetic constants from the initial velocity studies will be actual dissociation constants. In particular, the dissociation constant of MgATP from the E·MgATP complex, $K_{i(\text{MgATP})}$, should be constant for all of the substrates listed in Table III. However, the observed values range from 0.5 to 2.4 mM, which suggests that the rapid equilibrium assumption does not hold, at least for some of the substrates. The fact that $K_{i(\text{MgATP})}$ is not constant also eliminates an ordered mechanism in which MgATP is the first substrate to add to the enzyme. The rapid equilibrium assumption will be examined more closely in the following paper, which presents equilibrium isotope exchange and isotope partitioning experiments that determine the stickiness of the substrates.

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