On the Mechanism of Action of Fructose 1,6-Diphosphatase. Inhibition by Structural Analogs of Fructose 1,6-Diphosphate*

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ABSTRACT: Based on the behavior of the substrate analogs, α-methyl-D-fructofuranoside 1,6-diphosphate (II), 2,5-anhydro-D-glucitol 1,6-diphosphate (III), β-methyl-D-fructofuranoside 1,6-diphosphate (IV), and 2,5-anhydro-D-mannitol 1,6-diphosphate (V), toward fructose 1,6-diphosphatase (FDPase) at pH 9.4 the following conclusions have been reached: (1) that the furanose form of the sugar diphosphate is the active configuration since binding constants determined kinetically and by direct measurement from fluorescence studies are identical with those of fructose 1,6-diphosphate (FDP); (2) that the C-3 and C-4 hydroxyls of the furanose ring are essential since *cis*-2,5-bis(hydroxymethyl)tetrahydrofuran diphosphate did not kinetically assay as a substrate or an inhibitor; (3) that all the analogs, II–V, inclusive, at concentration $<10^{-3}$ M act as competitive inhibitors occupying the active site of FDPase; (4) that a change in kinetic behavior as a function of β configuration (IV, V) at high concentrations ($>10^{-3}$ M) is observed with the enzyme and may be related to inhibition of the enzyme by its own substrate. In general, inhibition of allosteric enzymes by their own substrates may be found in cases where the substrate occurs in two rapidly equilibrating configurations, *i.e.*, the α and the β anomers of FDP. Data from acetylation experiments at pH 7.5 suggest the possibility of interactions between the tyrosyl residues of FDPase and the 2-OH position of FDP.

In an effort to construct a mechanistic hypothesis for the hydrolysis reaction catalyzed by fructose 1,6-diphosphatase (FDPase)[1] we have chosen to explore the active site of this enzyme with FDP analogs. FDPase, isolated from rabbit liver (Pontremoli *et al.*, 1965), in contrast to other phosphatases (Stadtman, 1961) is rather substrate specific in catalyzing the hydrolysis of fructose 1,6- and sedoheptulose 1,7-diphosphates (FDP and SDP) (Pontremoli, 1966), fructose 1-phosphate, and L-sorbose 1,6-diphosphate (McGilvery, 1955) at the second pH optimum (9.4). The order of relative activity of FDPase based on the release of orthophosphate per unit time per unit enzyme with the above substrates is 1:0.7:0.009 and 0.03, respectively. Fructose 1-phosphate occupies a dubious position in this series since it was not observed as a substrate by Pontremoli (1966). In the case of the sugar diphosphates, hydrolysis is restricted to loss of orthophosphate from C-1.

Kinetic investigations have revealed that other D sugar phosphates, including ribulose 1,5-diphosphate, fructose 6-phosphate, ribose 5-phosphate, ribulose 5- phosphate, xylulose 5-phosphate, and sedohepulose 1- and 7-phosphates, are not hydrolyzed by FDPase at pH 9.4 (Pontremoli, 1966). FDPase, however, is markedly inhibited by excess FDP, especially at pH 7.5 (Taketa and Pogell, 1965) and by adenosine 5′-phosphate, although the latter is a unique noncompetitive inhibitor binding at four equivalent allosteric sites (Pontremoli *et al.*, 1966b).

A recent alternate approach to detect enzyme–substrate interactions has utilized a spectrophotometric investigation of the effect of various reagents on the uv absorption spectra of FDPase tyrosyl residues. Effects on the tyrosyl titration curves—shifts to lower pK_a values—caused by addition of FDP are mimicked by fructose 6-, glucose 6-, and fructose 1-phosphates but only at concentrations substantially greater than substrate (Pontremoli *et al.*, 1969).

It is obvious that the design of effective FDPase analogs must minimize permutations of FDP structure. Furthermore, since many of the sugar phosphates already mentioned exist in aqueous solution in a mobile equilibrium involving open-chain (acyclic) and pyranose or furanose forms, the structural analogs tested should have fixed molecular configurations. This study is an initial attempt to delineate the substrate conformations at the active site of FDPase and to test a hypothesis concerning its mechanism of action.

Experimental Section

Rabbit liver FDPase was purified according to published procedures (Pontremoli *et al.*, 1965; Pontremoli, 1966; Sarngadharan *et al.*, 1970). Mean specific activity was 9.5 units/mg of protein. D-Fructose 1,6-diphosphate, D-fructose 1-phosphate, D-fructose 6-phosphate, D-glucose 1,6-diphosphate, hexanediol, and auxiliary assay enzymes were purchased from Sigma. Sucrose, D-glucosamine, D-fructose, D-mannitol, and D-sorbitol (Fisher, reagent grade) were used without further purification.

Unless specifically noted, all studies were carried out at pH 9.2 or 9.4. Reaction rates in the presence of inhibitor were determined by monitoring the release of inorganic phosphate by the procedures of Fiske–Subbarow (1925) or Martin and Doty (1949) as modified by Jencks and Gilchrist (1964) and by the enzymatic procedure of Pontremoli *et al.* (1965). Activity in the absence of inhibitor was assayed by the latter procedure. Protein determinations were by the method of Lowry *et al.* (1951).

The least-squares analysis of the kinetic data for the Lineweaver–Burk plots and the calculation of bound substrate con-

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[1] Abbreviations used are: FDPase, fructose 1,6-diphosphatase; FDP, fructose 1,6-diphosphate; ANS, 1-anilinonaphthalene-8-sulfonate; K_I^C, dissociation constant for competitive inhibitors; K_I^{NC}, dissociation constant for noncompetitive inhibitors.

TABLE I: Solvents for Paper Chromatography.

Systems	Compositions	Reference
Solvent I (sugar phosphates)	Isopropyl alcohol–trichloroacetic acid–NH_4OH (750 ml:5 g of trichloroacetic acid/250 ml of H_2O:2.5 ml)	Karl-Kroupa (1956)
Solvent II (sugars)	*n*-Propyl alcohol–1 N NH_4OH (7:2, v/v)	Horvath and Metzenberg (1963)
Solvent III (sugars)	Ethyl acetate–pyridine–water–benzene (5:3:3:1, v/v, upper phase)	Augestad *et al.* (1953)

centrations from assumed association constants in the fluorescence studies were calculated on a Digital PDP-8/I computer. The Lineweaver–Burk plots, drawn from the computer analyzed data, do not significantly deviate from the double-reciprocal plots of the untreated data.

Acetylimidazole was prepared by the method of Boyer (1952). The acetylation experiments were performed by methods outlined by Pontremoli *et al.* (1966a). Fluorescence emission experiments were carried out at room temperature in a Farrand fluorospectrometer, Mark II, equipped with a xenon light source, in the manner described by Aoe *et al.* (1970). The excitation and emission wavelengths were 378 and 485 nm, respectively. Procedure for a typical run is as follows: FDPase (7.9×10^{-6} M; 0.26 ml), glycine buffer (pH 9.45, 0.014 M; 0.03 ml, and 1-anilinonaphthalene-8-sulfonate (9×10^{-4} M; 0.010 ml) were placed in a micro cell (capacity 0.4 ml). The sugar diphosphates of concentrations varying from 9.9×10^{-7} to 10^{-4} M were titrated directly into the cell. The fluorescence emission was recorded at constant wavelength until stable. All emission intensities are expressed in arbitrary fluorescence units after corrections for the appropriate ANS blanks and dilution.

The purity of the synthesized sugars was determined by melting point (uncorrected), optical rotation (Perkin-Elmer 22 spectrophotometer), vapor-phase chromatography, and paper chromatography. The sugars were phosphorylated as outlined by Hartman and Barker (1965) and isolated as either their cyclohexylammonium or sodium salts. The kinetic behavior noted was not dependent on the nature of the cation. The purity of the esters was determined by paper chromatography, microanalysis, and proton magnetic resonance spectra (Varian A-60). Descending paper chromatograms were obtained at room temperature (25 ± 2°) using Schleicher & Schuell 589c orange ribbon paper employing the solvent systems listed in Table I. Visualization of sugar phosphates was with Hanes–Isherwood spray (1949). Chromatograms of reducing sugars were developed with a solution of benzidine (0.4%) in glacial acetic acid and incubated at 80° for 15 min; nonreducing sugars were detected by spraying with 0.1 N H_2SO_4 in 90% ethanol and incubated at 80° for 15 min.

*2,5-Anhydro-*D*-mannitol 1,6-Diphosphate.* 2,5-Anhydro-D-mannitol was prepared according to the procedure of Bera *et al.* (1956). The complete removal of nickel ions after the reduction of chitose by Raney nickel catalyst (W-2) was accomplished by passing the filtered green aqueous solution through a column of cation exchanger (Amberlite IR-120 H^+). The eluate was freed from acid by shaking it several times with a 5% solution of methyl di-*n*-octylamine in chloroform and was then concentrated. The crystallized product (mp 103–104°, lit. mp 101–102°) was phosphorylated and isolated as the tetracyclohexylammonium salt. The nuclear magnetic resonance (nmr) spectrum (given in δ values) (D_2O) exhibited multiplets at 1.5 (44 H), 3.1, and 4.0 (8 H) relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate. (The area under the multiplet at 3.1 is variable owing to the time-dependent exchange of cyclohexylammonium protons with D_2O.) Paper chromatography in solvent I at 25° revealed two spots (R_F 0.51–0.52 and 0.70–0.71) with the latter identified as inorganic phosphate on comparison with a standard. Maximum mole per cent inorganic phosphate present was <3%.

*2,5-Anhydro-*D*-glucitol 1,6-Diphosphate.* 1,6-Dibenzoyl-2,5-anhydro-D-glucitol was prepared as described by Brigl and Grüner (1933, 1934) and Hockett *et al.* (1946), except that the time of refluxing 1,6-dibenzoylmannitol and *p*-toluenesulfonic acid was reduced from 6 to 1.5 hr (mp 134–137°, $[\alpha]_D^{25°}$ +3.0°; lit. mp 137°, $[\alpha]_D^{25°}$ +3.2°). The benzoyl groups were removed by bubbling ammonia for 45 min into 150 ml of anhydrous methanol containing 2 g of the 1,6-dibenzoyl-2,5-anhydro-D-glucitol. The reaction vessel was stoppered and left standing at 4° for 14 hr before removal of the solvent under vacuum. The semisolid material was treated in a Soxhlet extractor with benzene for 20 hr in order to remove benzamide. The remaining residue was then dried for 2 hr at 60°. The nmr spectrum of the latter material was free of any aromatic absorption. Phosphorylation and isolation of the tetracyclohexylammonium salt yielded a product whose nmr spectrum in D_2O exhibited complex multiplets at 1.5 (44 H), 3.1, and 4.0 (8 H). (The area under the multiplet at 3.1 is variable owing to the time-dependent exchange of cyclohexylammonium protons with D_2O.) Paper chromatography in solvent I revealed the desired product (R_F 0.50, 25°) and a trace of inorganic phosphate contaminant, <1.6 mole %. The sugar phosphate was converted to its Na salt *via* ion-exchange resin (Dowex 50W-X8).

Anal. Calcd for $C_{30}H_{66}P_2N_4O_{11}$ (720.84): C, 49.98; H, 9.23; P, 8.59. Found: C, 49.94; H, 9.47; P, 8.43.

*β-Methyl-*D*-fructofuranoside 1,6-Diphosphate.* The β isomer was prepared according to the method of Horvath and Metzenberg (1963). Paper chromatography of the thick syrup ($[\alpha]_D^{25°}$ −44°; lit. $[\alpha]_D^{25°}$ −60°) with solvents II and III revealed the β isomer (R_F 0.54–0.56, 25°; lit. R_F 0.56, 25°) and the previously reported, unidentified fast-moving component (R_F 0.79–0.81, 25°; lit. R_F 0.81, 25°). After several days a very faint spot having an R_F value identical with that of sucrose appeared on the chromatogram. The fast-moving component was apparently eliminated in the further purification of the sugar or by extraction of the aqueous sugar solution with ether. The β-methylfructofuranoside comprised at least 84% of the syrup as determined by the vapor-phase chromatographic analysis of the fully methylated sugar (Walker *et al.*, 1962; Gee and Walker, 1962). The main contaminant by this analysis was the β-methylfructopyranoside (10%). Paper chromatography of the thick syrup incubated with invertase (β-fructofuranosidase) revealed only the expected hydrolysis product, fructose. Paper chromatography of the phosphorylated product in solvent I revealed two spots (R_F 0.57, 0.79, 25°) the latter having an R_F value identical with that of commercial methyl phosphate (R_F 0.79, 25°). The nmr of the cyclohexylammonium salt in D_2O revealed multiplets at 4.0 (7 H), 3.1, and 1.5 (44 H). The area of the multiplet at 3.1 contained both the OCH_3 protons and the ammonium protons

of cyclohexylamine which exchanged with the solvent. The sugar phosphate was converted *via* ion-exchange resin (Dowex 50W-X8) to the Na salt. Maximum mole per cent inorganic phosphate present was <1%.

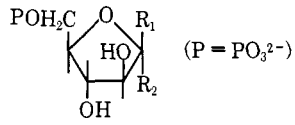
α-Methyl-D-fructofuranoside 1,6-Diphosphate. The α isomer was prepared according to the procedure of Purves and Hudson (1934, 1937) with the final product being obtained as a syrup rather than a crystalline material ($[\alpha]_D^{25°}$ +67°; lit. $[\alpha]_D^{25°}$ +88 to +93°). The composition of the syrup was determined by preparing the fully methylated derivative according to the procedure of Walker *et al.* (1962) and analyzing by gas–liquid chromatography (Gee and Walker, 1962). The identification and relative amounts (%) are listed in order of increasing retention times: (1) methyl 1,3,4,6-tetra-*O*-methyl-α-D-fructofuranoside, 94%; (2) methyl 1,3,4,6-tetra-*O*-methyl-β-D-fructofuranoside, 2%; (3) methyl 1,3,4,5-tetra-*O*-methyl-α-D-fructopyranoside, 0.6%; and (4) methyl 1,3,4,5-tetra-*O*-methyl-β-D-fructopyranoside, 3.4%. The syrup was phosphorylated and isolated as the cyclohexylammonium salt. The sugar phosphate was converted *via* ion-exchange resin (Dowex 50W-X8) to the Na salt. The nmr of the tetrasodium salt in D_2O revealed a multiplet at 4.3 (7 H) and a singlet at 3.45 (3 H). Maximum mole per cent inorganic phosphate present was <2%.

cis-2,5-Bis(hydroxymethyl)tetrahydrofuran 1,6-Diphosphate. cis-2,5-Bis(hydroxymethyl)tetrahydrofuran was prepared according to the procedure of Cope and Baxter (1955) (bp 100° (0.20); lit. bp 105° (2.5)) (ditosylate derivative, mp 129–130°, lit. 128.2–130°). The compound was isolated as its cyclohexylammonium salt upon phosphorylation. Maximum mole per cent inorganic phosphate present ~<0.1%.

1,6-Hexanediol Diphosphate. Hexanediol diphosphate was prepared according to the procedure of Hartman and Barker (1965). The isolated tetrasodium product was essentially free of inorganic phosphate and appeared pure by elemental analysis.

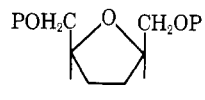
Results

Effects of Diphosphate Analogs on FDPase Activity. Fructose 1,6-diphosphate (I) is the natural substrate of FDPase and is hydrolyzed with the release of inorganic phosphate in the presence of Mn^{2+} or Mg^{2+}. Prolonged incubation (20 hr) of the diphosphates, α-methyl-D-fructofuranoside (II), 2,5-anhydro-D-glucitol (III), β-methyl-D-fructofuranoside (IV), 2,5-anhydro-D-mannitol (V), glucose 1,6-, hexanediol-, and *cis*-2,5-bis(hydroxymethyl)tetrahydrofuran (VI) with FDPase revealed that none of these analogs was hydrolyzed by the enzyme. Importantly only compounds II, III, IV, and V are inhibitors of the enzyme. Results of initial velocity studies of FDPase activity in the presence of various concentrations



(P = PO_3^{2-})

I, R_1 = CH_2OP, R_2 = OH; R_1 = OH, R_2 = CH_2OP
II, R_1 = CH_2OP, R_2 = OCH_3
III, R_1 = CH_2OP, R_2 = H
IV, R_1 = OCH_3, R_2 = CH_2OP
V, R_1 = H, R_2 = CH_2OP



VI

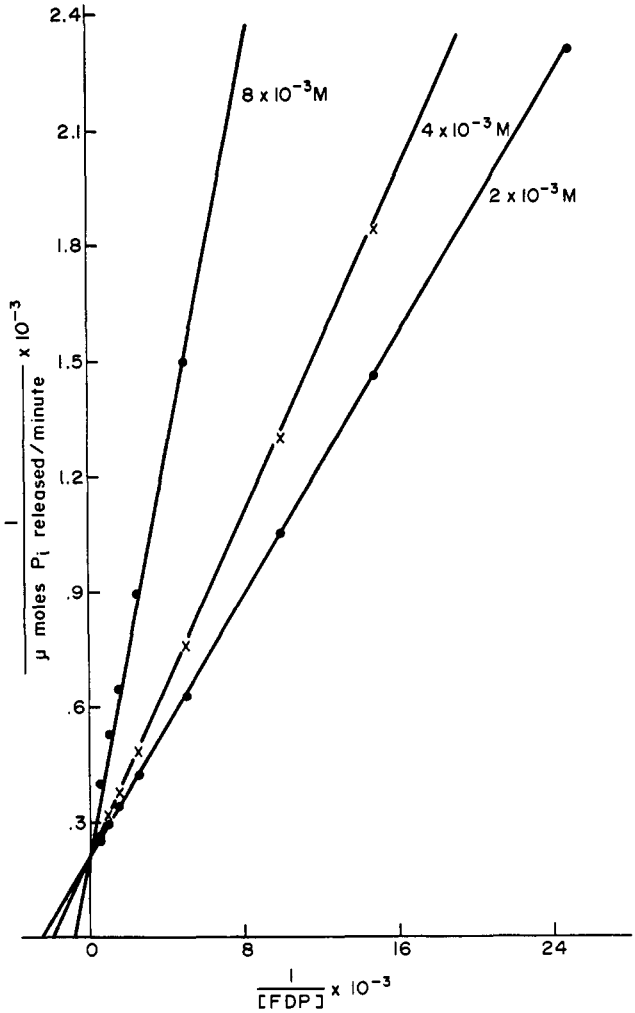


FIGURE 1: Double-reciprocal plot of the rate of enzymic hydrolysis of fructose 1,6-diphosphate against FDP concentration in the presence of α-methyl-D-fructofuranoside 1,6-diphosphate (pH 9.4, 0.04 M glycine, 0.5 mM Mn^{2+}, and 0.03 enzyme unit, 25°). Beside each plot is the inhibitor concentration.

of II, III, IV, and V are presented as double-reciprocal (Lineweaver–Burk) plots of rate *vs.* fructose 1,6-diphosphate (I) concentration in Figures 1, 2, 3, and 4, respectively. None of the analogs whose kinetic behavior was examined competed with AMP for the allosteric site (Sarngadharan *et al.*, 1969) since combined experiments with inhibitors and AMP give additive effects.

Figures 1 and 2 demonstrate that compounds II and III where the phosphoryl moieties are juxtapositioned in a cis relationship, act as competitive inhibitors at all the concentrations employed. Figures 3 and 4 show that IV and V, where the phosphoryl relationship is trans, demonstrate mixed kinetic behavior. At low inhibitor concentrations (~<10^{-3} M), competitive inhibition is observed since the point of intersection on the ordinate, *i.e.*, V_{max}, is identical with the V_{max} in the absence of inhibitor. At higher inhibitor concentrations both slope and intercept effects are observed; hence, by definition, noncompetitive inhibition.

In an earlier paper (Benkovic *et al.*, 1970) a Lineweaver–Burk plot of an anomeric mixture of methyl-D-fructofuranoside 1,6-diphosphates demonstrated competitive kinetic behavior. A graph of the slope of the double-reciprocal plot *vs.* concentration of (α + β)-methyl-D-fructofuranoside 1,6-di-

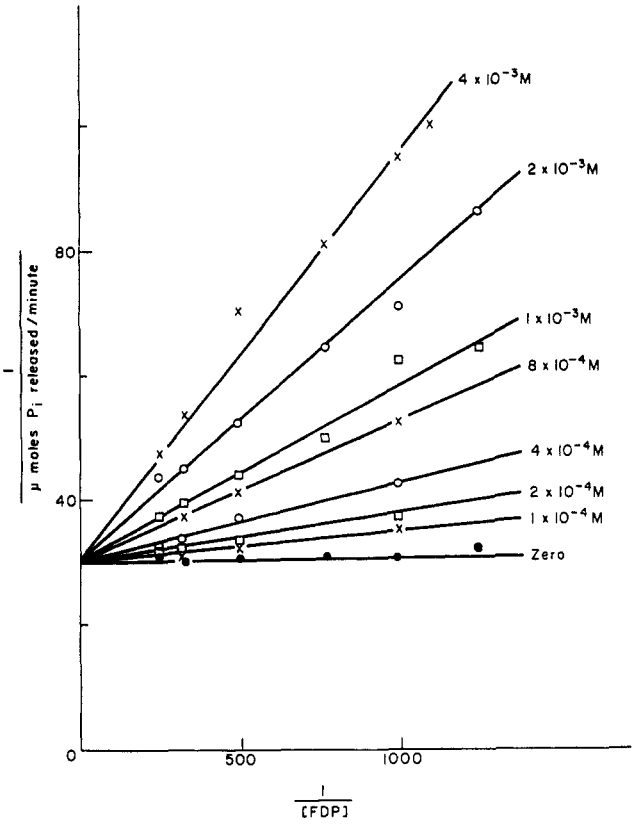


FIGURE 2: Double-reciprocal plot of the rate of enzymic hydrolysis of fructose 1,6-diphosphate against FDP concentration in the presence of 2,5-anhydro-D-glucitol 1,6-diphosphate (pH 9.4, 0.04 M glycine, 0.5 mM Mn^{2+}, and 0.03 enzyme unit, 25°). Beside each plot is the anhydro-D-glucitol concentration.

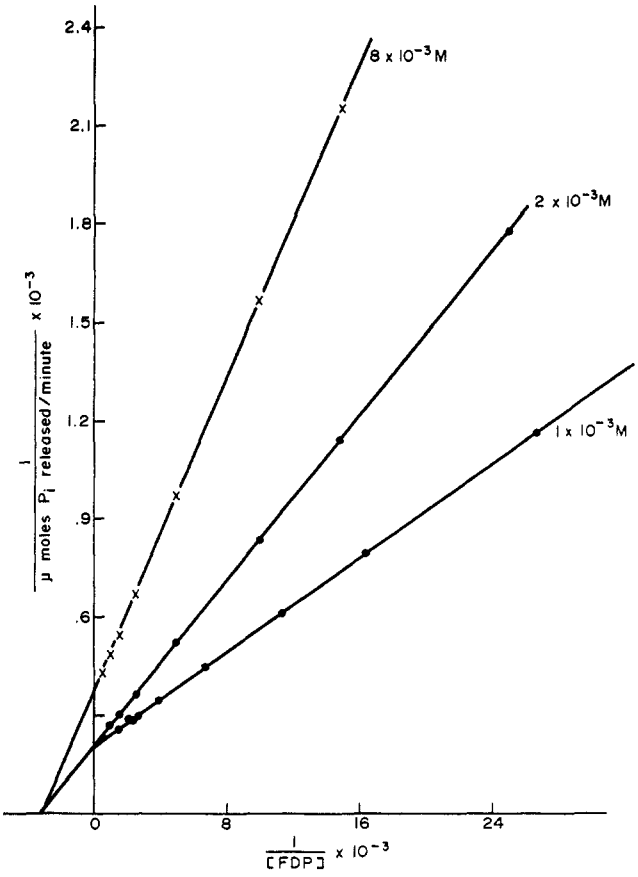


FIGURE 3: Double-reciprocal plot of the rate of enzymic hydrolysis of fructose 1,6-diphosphate against FDP concentration in the presence of β-methyl-D-fructofuranoside 1,6-diphosphate (pH 9.4, 0.04 M glycine, 0.5 mM Mn^{2+}, and 0.03 enzyme unit, 25°). Beside each plot is the inhibitor concentration.

phosphate appeared hyperbolic rather than linear. However, the plot may indeed be linear as values at higher inhibitor concentrations are lowered by increased ionic strength which effects a decrease in enyzme activity. If this is the case, noncompetitive behavior would be expected at high inhibitor and substrate concentrations. The influence of ionic strength on the activity of FDPase is shown in Table II.

Dissociation Constants. The dissociation constants of several of the analogs were determined independently from both the kinetic and fluorescence data and are compiled in Table III.

TABLE II: Influence of Ionic Strength on FDPase Activity.

		% FDPase[a]
	Concn (M)	Act. Retained
$NaNO_3$	0.1	77
	0.5	50
	1.0	30
KCl	0.1	110
	0.5	80
	1.0	50
Glycine buffer (pH 9.3)	0.067	87.3
	0.133	45.4
	0.20	16.4
	0.40	2.9

[a] 100% activity is for enzyme in 0.04 M glycine (pH 9.3) and 0.5 mM Mn^{2+}.

The kinetic dissociation constants (K_I's) for the competitive inhibitors (K_I^C) were calculated from either the intercept on the base line of the Lineweaver–Burk plot $[K_M(1 + [I]/K_I)]^{-1}$, where $K_M = 6.7 \times 10^{-6}$, the Michaelis constant of the free enzyme, [I] = the competitive inhibitor concentration; K_I, the dissociation constant of the inhibitor from the free enzyme; or by plotting the slope term, $K_M/V_{max}(1 + [I]/K_I)$ (typical graph in Figure 5) *vs.* concentration of inhibitor, where V_{max} is the velocity of the reaction in the absence of inhibitor. The K_I^C for IV and V were determined at concentrations $\leq 2 \times 10^{-3}$ M. The dissociation constants for the noncompetitive inhibitors were calculated from the magnitude of the intercept on the vertical axis of the Lineweaver–Burk plot, $(1/V_{max})(1 + [I]/K_I^{NC})$. K_I^{NC} could not be calculated from a plot of reciprocal velocity *vs.* inhibitor concentration (Dixon plot) since the kinetic data were collected at substrate concentrations greater than 100 K_M.

To complement the kinetic data, the K_I's for II and IV were determined *via* a fluorescence technique using the procedure developed for this particular enzyme by Aoe *et al.* (1970) at pH 9.2. The enzyme was labeled with the fluorescent dye, 1-anilinonaphthalene-8-sulfonate (ANS), and titrated with the inhibitors directly into the cell of the spectrophotofluorometer. The intensity and emission wavelength of the substrate analogs paralled FDP in the absence of metal. Taketa *et al.* (1971) at neutral pH also have found that the addi-

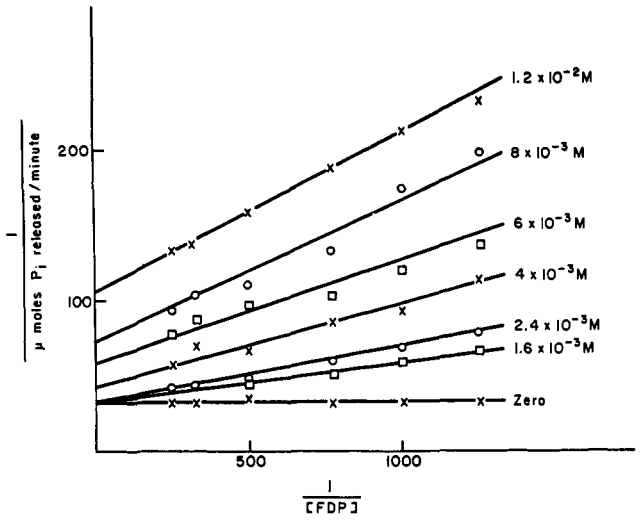


FIGURE 4: Double-reciprocal plot of the rate of enzymic hydrolysis of fructose 1,6-diphosphate against FDP concentration in the presence of 2,5-anhydro-D-mannitol 1,6-diphosphate (pH 9.4, 0.04 M glycine, 0.5 mM Mn^{2+}, and 0.03 enzyme unit, 25°). Beside each plot is the anhydro-D-mannitol concentration.

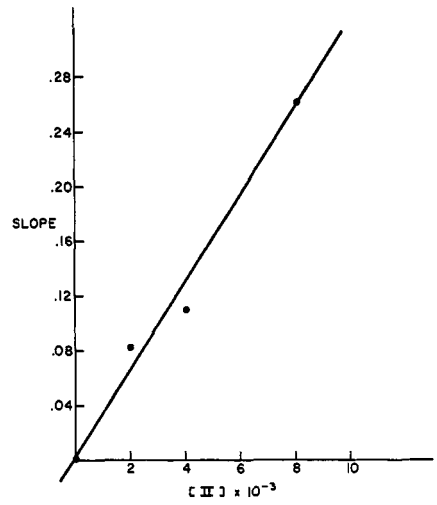


FIGURE 5: Slope of double-reciprocal plot (see Figure 1) *vs.* concentration of inhibitor, α-methyl-D-fructofuranoside 1,6-diphosphate (II).

tion of either (α + β)-methyl-D-fructoside 1,6-diphosphate or FDP to the FDPase–ANS complex in the absence of metal ion decreased equally the fluorescence yield, which is very specific for substrate. The fluorescence spectra of the analogs in the presence of metal ions are not significantly altered. The dissociation constants were determined by plotting the changes in fluorescence intensity as a function of the bound inhibitor calculated from assumed association constants. Figure 6 shows the results of such an experiment in which the concentration of the enzyme and ANS were held constant and the decrease in intensity, ($[I_0] - [I]$), was measured at varying bound α-methyl-D-fructofuranoside 1,6-diphosphate concentrations per mole of enzyme at pH 9.2. The dissociation constant giving the best straight line intersecting at zero was chosen. The large error limits are due mainly to the small, but real changes in the fluorescence intensity at pH 9.2.

TABLE III: Dissociation Constants at pH 9.4.

Analogs	Kinetic	Fluorescence
Fructose 1,6-diphosphate[a]	6.7×10^{-6}	
(α + β)-Methyl-D-fructofuranoside 1,6-diphosphate[b]	$7.1 \pm 3.0 \times 10^{-6}$	
α-Methyl-D-fructofuranoside 1,6-diphosphate (II)	$3.8 \pm 3.0 \times 10^{-5}$	$3.2 \pm 3.0 \times 10^{-5}$
β-Methyl-D-fructofuranoside 1,6-diphosphate (IV)	$6.4 \pm 2.5 \times 10^{-3}$ $3.8 \pm 3.0 \times 10^{-5}$	$2.8 \pm 2.0 \times 10^{-5}$
2,5-Anhydro-D-mannitol 1,6-diphosphate (V)	$6.4 \pm 3.5 \times 10^{-3}$ $2.7 \pm 2.0 \times 10^{-5}$	
2,5-Anhydro-D-glucitol 1,6-diphosphate (III)	$7.5 \pm 3.0 \times 10^{-6}$	

[a] Dissociation constant of 2×10^{-7} obtained by Pontremoli *et al.* (1968) *via* gel filtration technique. [b] Benkovic *et al.* (1970).

Binding Sites. In Figure 7 are shown data for FDP (I) in the absence and presence of the inhibitors II and IV, according to the Hill eq 1, where $V = v$ maximum. The Hill

$$\log \frac{v}{V - v} = n_H \log [FDP] - \log K \qquad (1)$$

coefficient, n_H, is approximately 1.0 for FDP and the substrate analogs. This is indicative of noncooperativity among the sites.

The number of binding sites at pH 9.2 has not been determined in the presence of the substrate analogs. However, at pH 7.5, by measurement of the proton relaxation rate of water in the presence of the ternary complex (FDPase–Mn^{2+}–analog) 4 moles of (α + β)-methyl-D-fructofuranoside 1,6-diphosphate was bound per mole of enzyme (S. J. Benkovic and

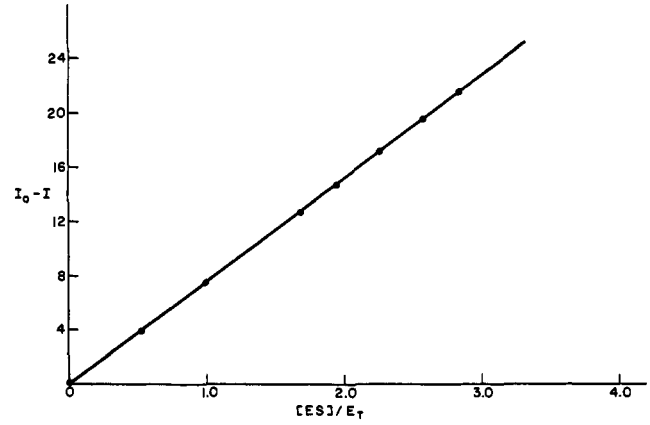


FIGURE 6: Plot of decrease in relative fluorescence intensity, $[I]_0 - [I]$, *vs.* moles of substrate bound per mole of enzyme, $[ES]/[E_T]$. The dissociation constant was selected from the straight line bisecting zero as determined by the digital PDP-8/I computer.

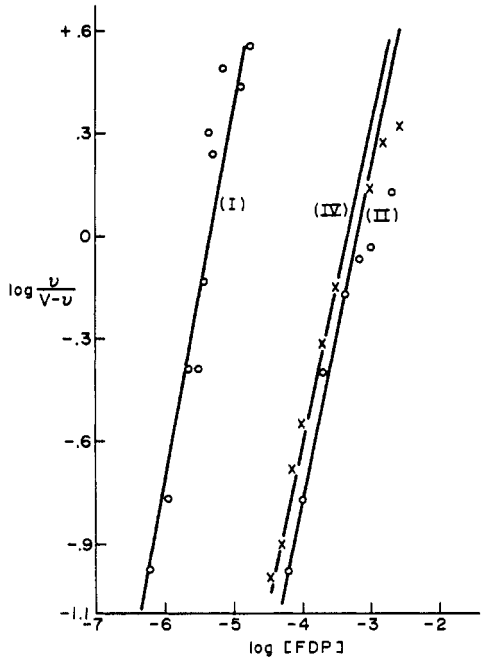


FIGURE 7: Hill plots for fructose 1,6-diphosphate (I), α-methyl-D-fructofuranoside 1,6-diphosphate (II), and β-methyl-D-fructofuranoside 1,6-diphosphate (IV) at pH 9.4. The concentrations of II and IV are 0.004 M.

K. J. Schray, to be published, 1971). It thus appears reasonable to assume that 4 moles of the inhibitors also is bound per mole of FDPase at pH 9.4. This is consistent with the findings of Pontremoli *et al.* (1968) of the equivalence of four binding sites of FDPase with FDP at pH 9.2 using a gel filtration technique in the absence of metal ion.

Acetylation. Treatment of the reactive enzyme in the absence of substrate with acetylimidazole results, after an initial 20-min lag phase, in the progressive decrease of almost 90% of the catalytic activity after a period of 60 min (Pontremoli *et al.*, 1966a). The decrease in enzymic activity has been attributed to the acetylation of essential tyrosyl residues; those residues nonessential for catalytic activity being acetylated in the initial twenty minutes followed by acetylation of additional critical residues over the remaining forty minutes (Pontremoli *et al.*, 1966b). Acetylation of FDPase, attempted in the presence of I, showed that the natural substrate protects the tyrosyl residues so that catalytic activity is retained. The data in Table IV summarize the results of acetylation experiments conducted in the presence of substrate analogs. None of the inhibitors examined protects the active site from acetylation and, moreover, the acetylation of essential tyrosyl residues apparently is immediate.

TABLE IV: Residual Specific Activity of FDPase (%).[a]

Acetylation Time[b] (min)	Methyl-D-fructofuranoside 1,6-Diphosphate (α + β)	2,5-Anhydro-D-glucitol 1,6-Diphosphate	2,5-Anhydro-D-mannitol 1,6-Diphosphate
0	100	100	100
10	83	85	88
20[c]	66	71	76
30	50	56	65
60[d]	0	14	29

[a] Error ±10%. [b] Reaction solution: substrate analog, 1 mM; FDPase, 3.4 mg; acetylimidazole, 4 mg/ml; pH 7.4 (0.05 M borate) 22°, total volume 5 ml. [c] In the absence of reagent, 90–95% activity remains after 20 min, 0% after 60 min. [d] With fructose 1,6-diphosphate 94–98% activity remains after 60 min.

Discussion

Replacement of the 2-OH by OCH_3 (II and IV) or H (III and V) converts the sugar diphosphate from a substrate into an inhibitor. Other diphosphates, glucose 1,6, *cis*-2,5-bis-(hydroxymethyl)tetrahydrofuran, the unresolved mixture of D-mannitol and D-sorbitol 1,6- (Benkovic *et al.*, 1970), and 1,6-hexanediol, act neither as substrates nor inhibitors. It now appears that the most reactive substrate for FDPase should contain two phosphoryl moieties (1–6) and a furanose configuration bearing hydroxyl groups at C-3 and C-4.

A comparison of the dissociation constants (K_I) of Table III obtained from either kinetic or fluorescence studies reveals the near identity of the inhibitor dissociation constants for II–V, inclusive. The similarity between K calculated from gel filtration experiments and K_M, the Michaelis constant determined kinetically at pH 7.3 for FDP, suggests that the breakdown of the enzyme–substrate complex is rate determining (Sarngadharan *et al.*, 1969). Consequently, it will be assumed that constants calculated from either the kinetic or fluorescence data approximate the dissociation constants of the enzyme–inhibitor complexes at pH 9.4. The possibility that K_I is controlled by substituents at C-2 is eliminated by the equality in K_I for the various sugar diphosphate inhibitors. Likewise, the equivalence between K_I and K_M and the fluorescence spectra of the enzyme–dye–analog systems with and without Mn^{2+} indicates that the primary role of the metal ion is not one of binding substrate to enzyme.

Substantial evidence has accumulated to support the postulate that the inhibitors II–V at concentrations of $<10^{-3}$ M bind to the active site of FDPase. The data include (a) the competitive kinetic behavior, (b) the similarity in K_I's for the analogs, II–IV, and K_M for I and (c) the specific and equivalent decrease in the fluorescence yield of the enzyme–dye complex for FDP and inhibitors tested at identical excitation and emission wavelengths. Moreover the enzyme–dye complex saturated with FDP does not show any significant changes in the fluorescence intensity when titrated with the analogs at concentrations less than 10^{-3} M. In accord with the Hill coefficient of nearly unity (Figure 7) the inhibitors and FDP show no cooperative interactions at pH 9.2 at the previously reported four binding sites (Pontremoli *et al.*, 1968; Sarngadharan *et al.*, 1969).

Compounds II and III (α anomers), with the C-2 position bearing OCH_3 and H, respectively, and with the geometry of the phosphoryl moieties cis, are competitive inhibitors at concentrations up to 8×10^{-3} M. Compounds IV and V (β anomers), where the phosphoryl groups are trans, exhibit competitive inhibition at concentrations of $\lesssim 10^{-3}$ M and noncompetitive inhibition at higher concentrations. Their diverse kinetic behavior is interpreted as the binding at higher concentrations to some additional site on the enzyme other than the allosteric site. Pontremoli *et al.* (1969) also have detected addi-

tional binding sites on FDPase at saturating concentrations of FDP with a binding constant of the order of magnitude of 10^{-3} M. This value is in agreement with K_I^{NC} of Table III, calculated from Figures 3 and 4 at high concentrations ($>10^{-3}$ M) of IV and V, respectively, where noncompetitive behavior is manifest. If such inhibition is observed at neutral pH, it may be related to the inhibition of the enzyme by its own substrate (Taketa and Pogell, 1965).

In general, inhibition of allosteric enzymes by their own substrates may be found in cases where the substrate occurs in two rapidly equilibrating configurations, *i.e.*, α and β forms. Recently, Gray and Barker (1970) and Swenson and Barker (1971) have reported that FDP (I) in aqueous solution at neutral pH exists almost exclusively in the furanose configuration as one anomer, the β. This finding, in view of our data, tends to implicate substrate configuration with FDPase regulation.

The difference between FDP and the inhibitors toward FDPase in the absence of metal ions is manifest in the acetylation data of Table IV at pH 7.4. This pH was adopted since it is optimal for the stability of acetylimidazole. FDP protects the tyrosyl residues essential for catalytic activity (Pontremoli *et al.*, 1966b). None of the inhibitors examined however prevents acetylation; moreover, the acetylation apparently is immediate. Comparison of the dissociation constants of Table III for III and V with I does not predict the loss of FDPase activity. Presumably, the 2-OH of the substrate may interact with tyrosyl residues, and thereby prevent their acetylation, perhaps through hydrogen-bond formation. Replacement of OH at C-2 of FDP with OCH_3 or H renders this type of interaction impossible.

Compounds II, III, IV, and V are very sensitive probes of enzyme subunit interactions and implicate the 2-OH in the catalytic mechanism. We plan to pursue these aspects further and examine the intact system, enzyme–substrate analog–AMP–Mn^{2+}, with nmr techniques.

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