Lichtenstein, N., Ross, H. E., and Cohen, P, P. (1953), *J. Biol. Chem. 201*, 117.
Meister, A. (1954), *J. Biol. Chem. 210*, 17.
Meister, A. (1962), *Enzymes 6*, 443.
Meister, A., Bukenberger, M. W., and Strassburger, M. (1963), *Biochem. Z. 338*, 217.
Meister, A., Levintow, L., Greenfield, R. E., and Abendschein, P. A. (1955), *J. Biol. Chem. 215*, 441.
Meister, A., Sober, H. A., and Tice, S. V. (1951), *J. Biol. Chem. 189*, 577, 591.
Pamiljans, V., Krishnaswamy, P. R., Dumville, G., and Meister, A. (1962), *Biochemistry 1*, 153.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Wilson, E. M., and Snell, E. E. (1962), *J. Biol. Chem. 237*, 3171.

An Exploration of the Active Site of Aldolase Using Structural Analogs of Fructose Diphosphate*

Frederick C. Hartman† and Robert Barker‡

ABSTRACT: A number of compounds which are structural analogs of fructose diphosphate were found to be competitive inhibitors of aldolase. It is concluded from comparison of the K_I (enzyme-inhibitor dissociation constant) values of these compounds that the binding of fructose diphosphate is primarily due to the phosphate groups, that hydroxyl groups do not contribute significantly to the binding, that the keto form is not bound preferentially, and that all forms of fructose diphosphate are acted upon by aldolase. The compounds and their K_I values are: D-arabinitol 1,5-diphosphate (1.5×10^{-6}); L-arabinitol 1,5-diphosphate (4.1×10^{-5}); xylitol 1,5-diphosphate (2.8×10^{-6}); ribitol 1,5-diphosphate (2.0×10^{-5}); 1,4-anhydro-DL-ribitol 5-phosphate (4.6×10^{-3}); 1,4-anhydro-DL-xylitol 5-phosphate (3.8×10^{-3}); 1,4-anhydro-D-arabinitol 5-phosphate (1.3×10^{-3}); D-glucitol 1,6-diphosphate (1.2×10^{-5}); 2,5-anhydro-D-mannitol 1,6-diphosphate (3.0×10^{-5}); 2,5-anhydro-D-glucitol 1,6-diphosphate (1.3×10^{-5}); ethylene glycol diphosphate (1.2×10^{-4}); 1,3-propanediol diphosphate (1.1×10^{-4}); 1,4-butanediol diphosphate (5.5×10^{-5}); 1,5-pentanediol diphosphate (2.9×10^{-5}); 1,6-hexanediol diphosphate (2.5×10^{-5}); 1,8-octanediol diphosphate (1.2×10^{-4}); 1,5-pentanediol-2-one diphosphate (3.0×10^{-5}); 3-hydroxytetrahydrofuran (3.6×10^{-2}). The synthesis of these compounds is described.

Ketohexose phosphates which are cleaved by aldolase[1] are fructose 1,6-diphosphate (Meyerhof, 1939), L-sorbose 1,6-diphosphate (Tung *et al.*, 1954; Richards and Rutter, 1961), D-fructose 1-phosphate (Tung *et al.*, 1954; Richards and Rutter, 1961), D-tagatose 1,6-diphosphate (Tung *et al.*, 1954), 5,6-dideoxy-D-fructose 1-phosphate (Lehninger *et al.*, 1955), and L-sorbose 1-phosphate (Tung *et al.*, 1954; Richards and Rutter, 1961). Of these the possibility must be considered that D-tagatose 1,6-diphosphate is cleaved by myogen A which was present in the assay medium and is not, therefore, a substrate for aldolase (Rutter, 1961).

The condensation of a number of aldehydes with dihydroxyacetone phosphate in the presence of aldolases has been observed and the products of such condensations presumably are substrates for the cleavage reaction, although their suitability as substrates relative to fructose 1,6-diphosphate has not been established (Rutter, 1961). Many of the materials which serve as substrates for the cleavage reaction and the normal substrate (fructose 1,6-diphosphate) can exist in carbonyl or hemiketal forms and the question arises, "Which form is the substrate?" The fact that certain compounds which can exist only as the keto form are cleaved by aldolase (Lehninger *et al.*, 1955; Rutter, 1961) does not remove the possibility that one or both of the hemiketal forms is preferentially bound and opened to the keto form prior to cleavage.

The study reported here was carried out in an attempt

* From the Department of Biochemistry, University of Tennessee, Memphis, and the Department of Biochemistry, University of Iowa, Iowa City. *Received January 7, 1965.* Supported in part by a U.S. Public Health Service research grant (GM 09021), and a U.S. Public Health Service fellowship (to F.C.H.) (GM-20585) from National Institute of General Medical Sciences, U.S. Public Health Service.

† Present address: Division of Biochemistry, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Ill.

‡ Department of Biochemistry, University of Iowa, Iowa City, Iowa; to whom inquiries regarding this paper should be addressed.

[1] This communication is concerned only with rabbit muscle aldolase.

to determine which features of a substrate potentiate its binding to aldolase. A number of analogs of fructose 1,6-diphosphate were synthesized; these differed in that they could not undergo aldol cleavage because they do not contain free or potential keto groups. The only keto compound described does not contain a free hydroxyl group and can neither cyclize nor be cleaved.

Materials and Methods

Rabbit muscle aldolase (Lot 113B-0420), α-glycerophosphate dehydrogenase–triose phosphate isomerase mixture, NADH,[2] and glyceraldehyde 3-phosphate were purchased from the Sigma Chemical Co. and used without purification. The same lots were used throughout this work. Fructose diphosphate tetracyclohexylammonium salt·10 H_2O from the California Corp. for Biochemical Research was used without purification. α,ω-Diols, 3-hydroxytetrahydrofuran, and diphenylphosphorochloridate were obtained from the Aldrich Chemical Co. D-Glucitol was obtained from Pfanstiehl Laboratories, Inc. All other polyols were synthesized in this laboratory by methods to be described elsewhere.

For the evaluation of K_I the aldolase assay used was essentially that described by Blostein and Rutter (1963). Each cuvet contained 0.5 ml of 2.79×10^{-4} M NADH, 0.1 ml of α-glycerophosphate dehydrogenase–triose phosphate isomerase solution (1.0 mg of protein in 4 ml water), and 1.0 ml of 0.178 M glycylglycine buffer, *p*H 7.5, containing 1 mg aldolase per 100 ml. In each experiment, three cuvets were used to evaluate the rate of reaction in the absence of inhibitor; to each was added sufficient water to give a final volume of 3.0 ml when substrate was added. Substrate additions were 0.05 ml of 1.64×10^{-4} M fructose 1,6-diphosphate, 0.08 ml of 1.64×10^{-4} M fructose 1,6-diphosphate, and 0.05 ml of 1.09×10^{-3} M fructose 1,6-diphosphate. Four cuvets were used to measure inhibition; a level of inhibitor which would produce a rate decrease in the presence of 0.05 ml of 1.09×10^{-3} M fructose 1,6-diphosphate to the level obtained in the absence of inhibitor with 0.05 ml of 1.64×10^{-4} M fructose 1,6-diphosphate was usually satisfactory. Inhibitor and water were added to each cuvet to give a volume of 3.0 ml when substrate was added. Substrate additions were 0.08 and 0.15 ml of 1.64×10^{-4} M fructose 1,6-diphosphate, and 0.05 and 0.2 ml of 1.09×10^{-3} M fructose 1,6-diphosphate. Absorbance decreases were measured on a Cary Model 15 spectrophotometer against a standard containing 0.5 ml of 2.79×10^{-4} M NADH and 2.5 ml of water. A decrease of 0.414 in absorbance in this assay is equivalent to 0.1 μmole of fructose 1,6-diphosphate cleaved. Compounds which did not inhibit the reaction were tested at levels up to 0.2 M in the assay medium.

Values of K_m and K_I were estimated according to the method of Lineweaver and Burk (1934). The slopes of the lines which best fitted the experimental points by the criterion of least squares (Wallis and Roberts, 1956) were used in the calculation. To evaluate the range of inhibitor constants the slope of the line for the inhibited reaction plus one standard deviation and the slope of the line for the normal reaction minus one standard deviation were used to recalculate a value of K_I. The difference between this value and that calculated from the lines fitted by the method of least squares is the range reported.

All compounds were tested as inhibitors of α-glycerophosphate triose isomerase mixture using DL-glyceraldehyde 3-phosphate as substrate (1.9×10^{-5} M) and one-half the amount of enzyme mixture used in the aldolase assay. Evaporations were performed at water-aspirator pressure. Carbon and hydrogen analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Paper chromatography of the phosphate esters was carried out by the descending method at room temperature using Whatman No. 1 paper. The phosphate esters were detected with acid molybdate (Hanes and Isherwood, 1949) and, where applicable, periodate-benzidine sprays (Viscontini *et al.*, 1955). All monophosphates had R_F values of approximately 0.5 in solvent 1 (methanol-ammonia-water, 7:2:1) and 0.55 in solvent 2 (ethanol-ammonia-water, 6:2:2). All diphosphates had R_F values of approximately 0.23 in solvent 1 and 0.27 in solvent 2.

Catalytic hydrogenations were performed at room temperature and atmospheric pressure. Periodate oxidations were carried out in unbuffered sodium metaperiodate at room temperature. The consumption of periodate was measured by titration with thiosulfate of the iodine released from a suitable aliquot upon addition of excess sulfuric acid and potassium iodide. The amount of formic acid released during the oxidation was determined by titration with base after destruction of excess periodate with ethylene glycol.

Inorganic phosphate was determined quantitatively by the method of Marsh (1959). Total phosphate was measured as inorganic phosphate released upon digestion of a suitable quantity of compound with sulfuric acid for 2 hours at 150–170°. The pyrophosphates formed during the digestion were hydrolyzed with 2 N sulfuric acid by placing the sample in a boiling-water bath for 30 minutes before analyzing for inorganic phosphate. Acid-labile phosphate was measured as inorganic phosphate released during 10 minutes from a sample in the presence of 2 N sulfuric acid at 100°.

Criteria for Purity of Phosphate Esters. All compounds were examined by paper chromatography using both solvent systems, were analyzed for total phosphate and inorganic phosphate, and, when adjacent hydroxyls were present, were subjected to periodate oxidation. When these procedures indicated that the compound was pure it was subjected to elemental analysis for carbon and hydrogen. The minimum level of purity for each compound is given in the following procedures.

1,4-Di-O-diphenylphosphorylbutanediol. To a solution of 200 mg (2.2 mmoles) of 1,4-butanediol in 5 ml of pyridine was added 2.0 ml (8.9 mmoles) of diphenyl-

[2] Abbreviation used in this work: NADH, reduced diphosphopyridine nucleotide.

phosphorochloridate. The reaction mixture was left at room temperature for 12 hours and then a few drops of water were added to hydrolyze the excess reagent; chloroform (50 ml) was then added. The chloroform solution was washed successively with two 200-ml portions of each of the following: 1 N sulfuric acid, saturated sodium bicarbonate, and water. It was dried over sodium sulfate, filtered, and concentrated to give 1.1 g (92%) of sirup. The sirup was taken up in 10 ml of 2-propanol from which 0.81 g (71%) of crystals was deposited, mp 92–96°. After two recrystallizations from the same solvent, the material melted at 101–103°.

Anal. Calcd for $C_{28}H_{28}O_8P_2$ (554.46): C, 60.65; H, 5.09. Found: C, 60.4; H, 5.26.

1,4-Butanediol Diphosphate Tetracyclohexylammonium Salt. A solution of 0.70 g of 1,4-di-*O*-diphenylphosphorylbutanediol in 50 ml of methanol was hydrogenated over platinum oxide (0.2 g). The hydrogen consumption was 510 ml (103%) after 18 hours. The catalyst was removed by filtration, and the filtrate was neutralized with cyclohexylamine and concentrated to a residue which was dissolved in 10 ml of water. The *p*H of the resulting solution was adjusted to 10 with cyclohexylamine. Dropwise addition of approximately 50 ml of acetone caused the deposition of 450 mg (50%) of crystals which were chromatographically pure. Before the compound was used in enzymatic studies, it was twice recrystallized in the manner described. The material was free of inorganic phosphate and acid-labile phosphate and contained 2.03 ± 0.06 molar equivalents of total phosphate based on a molecular weight of 718.9, minimum purity 97%.

1,3-Propanediol Diphosphate, 1,5-Pentanediol Diphosphate, 1,6-Hexanediol Diphosphate, and 1,8-Octanediol Diphosphate. These compounds were synthesized from the appropriate diol and isolated as tetracyclohexylammonium salts by the procedure employed for the synthesis of 1,4-butanediol diphosphate. None of the di-*O*-diphenylphosphoryldiols was crystallized. The yields obtained of the tetracyclohexylammonium salts of propanediol, pentanediol, hexanediol, and octanediol diphosphate were 56, 43, 40, and 60%, respectively. The products were free of inorganic and acid-labile phosphate and appeared pure by paper chromatography and elemental analysis.

Ethylene Glycol Diphosphate. To a solution of 100 mg (1.6 mmoles) of ethylene glycol in 10 ml pyridine was added 2 ml (8.9 mmoles) of diphenylphosphorochloridate. After the initial reaction had subsided, the reaction mixture was heated to boiling and then left at room temperature for 2 hours. The remainder of the synthesis was performed as for 1,4-butanediol diphosphate. A 53% yield (575 mg) of apparently pure tetracyclohexylammonium salt was obtained.

2,5-Anhydro-D-glucitol 1,6-Diphosphate. To a solution of 358 mg (292 mmoles) of 2,5-anhydro-D-glucitol in 10 ml of pyridine at −20° was added 0.93 ml (4.2 mmoles) of diphenylphosphorochloridate. After the reaction mixture had remained at −20° for 30 minutes, it was warmed to 0° and was left for 12 hours, at which time the product was isolated by the method used to isolate 1,4-di-*O*-diphenylphosphorylbutanediol. A methanol solution of the sirup obtained (1.1 g, 80%) was hydrogenated for 12 hours using 0.2 g of platinum oxide as catalyst; the hydrogen consumption was 680 ml (102%). After removal of the catalyst by filtration, the filtrate was neutralized with cyclohexylamine and concentrated. Dissolution of the residue in 10 ml of water, adjustment of the *p*H to 10.0 with cyclohexylamine, and addition of approximately 60 ml of acetone caused the deposition of 560 mg (33%) of crystals of 95% minimum purity.

2,5-Anhydro-D-mannitol 1,6-Diphosphate. This compound was prepared as described for 2,5-anhydro-D-glucitol 1,6-diphosphate. A 50% yield of the tetracyclohexylammonium salt was obtained which was at least 98% pure.

1,5-Pentanediol-2-one Dimethyl Ketal. A solution of 5 g of α-ketoglutaric acid in a mixture of 50 ml of methanol and 20 ml of trimethyl orthoformate containing 0.5 ml of concentrated sulfuric acid was refluxed for 12 hours. The reaction mixture was cooled to 0° and rapidly neutralized with 1 N sodium hydroxide. After the addition of 50 ml of water, the solution was extracted with five 50-ml portions of chloroform. The chloroform extracts were dried over sodium sulfate, filtered, and concentrated to give 7.1 g (94%) of dimethyl α-ketoglutarate dimethyl ketal as a mobile, yellow sirup.

To a well-stirred, refluxing suspension of 2 g of powdered lithium aluminum hydride in 75 ml of dioxane was added dropwise a solution of 4 g of dimethyl α-ketoglutarate dimethyl ketal in 25 ml of dioxane. After addition was complete, the mixture was refluxed for 15 minutes and cooled to room temperature. Excess lithium aluminum hydride was destroyed by the dropwise addition of ethyl acetate. The product was isolated by a modification of the method used by Woodward *et al.* (1952). Hydrolysis of the alcoholate was effected by the addition of 10 ml of saturated aqueous sodium sulfate solution, followed by boiling for 15 minutes. After removal of the water with anhydrous magnesium sulfate (20 g) the mixture was filtered, and the residue was washed thoroughly with dioxane. Concentration of the combined filtrate and washings gave 2.6 g (90%) of 1,5-pentanediol-2-one dimethyl ketal as a viscous, slightly yellow sirup. A 200-mg portion of this compound was converted to its di-*p*-nitrobenzoyl ester in the usual fashion. Crystallization of the derivative from 25 ml of ethanol gave 485 mg (81%) of crystals, mp 85–87°. After two recrystallizations from the same solvent, the material melted at 86–87°.

Anal. Calcd for $C_{21}H_{22}N_2O_{10}$ (462.42): C, 54.55; H, 4.79. Found: C, 54.4; H, 4.68.

1,5-Pentanediol-2-one Diphosphate. To a solution of 0.2 g of 1,5-pentanediol-2-one dimethyl ketal in 10 ml of pyridine was added 1.0 ml of diphenylphosphorochloridate. After the reaction had remained at room temperature for 8 hours, a few drops of water were

added to hydrolyze the excess reagent. The product (0.81 g, 97%), isolated by the procedure described for the isolation of 1,4-di-*O*-diphenylphosphorylbutanediol, was obtained as a sirup. A methanol solution of the sirup was hydrogenated in the presence of 0.2 g of platinum oxide. The product (0.42 g, 48%) was obtained as the tetracyclohexylammonium salt by crystallization from water-acetone. Chromatography in solvent 2 revealed the presence of two components, the major component having R_F 0.40 and the minor component having R_F 0.30. The compound present in largest quantities was probably 1,5-pentanediol-2-one dimethyl ketal; the other compound was 1,5-pentanediol-2-one diphosphate, since acid treatment of the mixture resulted in all of the material being converted to the compound with the lower R_F value.

A 200-mg portion of the crystalline mixture was added to an aqueous slurry of 5 g of Dowex 50 (H^+), and after 8 hours the resin was removed by filtration. The *p*H of the filtrate was adjusted to 10 with cyclohexylamine. Addition of acetone to the solution caused deposition of 145 mg (76%) of crystalline 1,5-pentanediol-2-one diphosphate tetracyclohexylammonium salt. This compound gave a single spot in both solvents and had a carbonyl absorption at 1722 cm^{-1}, estimated minimum purity 94%.

D-*Arabinitol 1,5-Diphosphate.* A solution of 0.55 g (1.3 mmoles) of 2,3,4-tri-*O*-benzyl-D-arabinitol (Gray *et al.*, 1965) in 15 ml of pyridine was cooled in a dry-ice bath. When the solution began to solidify on the sides of the vessel, 4.3 ml (8.1 mmoles) of dibenzylphosphorochloridate (Kenner *et al.*, 1952) was added. The reaction mixture was left in the dry-ice bath for 30 minutes, transferred to an ice bath at 0° for 4 hours, and then 3 ml of saturated sodium carbonate solution and 50 ml of chloroform were added. The chloroform solution was washed successively with two 300-ml portions of 1 N sulfuric acid saturated with sodium chloride, 5% sodium bicarbonate saturated with sodium chloride, and saturated sodium chloride. The chloroform solution was dried over sodium sulfate, filtered, and concentrated to give 1.8 g (150%) of sirupy 2,3,4-tri-*O*-benzyl-1,5-di-*O*-dibenzylphosphoryl-D-arabinitol, which was contaminated with benzyl chloride from the dibenzylphosphorochloridate.

The impure product was applied to a column containing 50 g of Florisil prepared from a slurry in cyclohexane. Elution with 200 ml of benzene gave 120 mg of benzyl chloride. Elution with 200 ml of ether gave 140 mg of unidentified material. Elution with 200 ml of acetone gave 0.95 g (77%) of presumed 2,3,4-tri-*O*-benzyl-1,5-di-*O*-dibenzylphosphoryl-D-arabinitol as a viscous, yellow sirup which could not be induced to crystallize.

One g of 10% palladium chloride on charcoal slurried in 50 ml of methanol was hydrogenated for 1 hour, at which time it was collected by filtration and thoroughly washed with methanol to remove hydrogen chloride. The reduced palladium was added to a solution of 0.95 g of 2,3,4-tri-*O*-benzyl-1,5-di-*O*-dibenzylphosphoryl-D-arabinitol in 50 ml of methanol, and the resulting mixture was hydrogenated. During 2 hours, 153 ml (99%) of hydrogen was consumed. The palladium was removed by filtration, and the filtrate was neutralized with cyclohexylamine and concentrated. Crystallization of the residue from water-acetone (*p*H 10) gave 550 mg (79%) of the tetracyclohexylammonium salt of D-arabinitol 1,5-diphosphate, which was revealed by paper chromatography to be contaminated with a substance having the same R_F value as inorganic phosphate. No periodate-oxidizable impurities were detected. Repeated recrystallization from water-acetone did not result in any appreciable purification. The material was estimated to be 85% pure.

L-*Arabinitol 1,5-Diphosphate.* The crystalline 2,3,4-tri-*O*-benzyl derivative of L-arabinitol was prepared by the method previously described for the preparation of the corresponding D- isomer (Gray *et al.*, 1965). L-Arabinitol 1,5-diphosphate was prepared by the procedure described for the preparation of D-arabinitol 1,5-diphosphate. The product, isolated as the tetracyclohexylammonium salt, was approximately 90% pure. Chromatography of the impure product demonstrated that there were no impurities present which consumed periodate. The impurity appeared identical to the impurity present in D-arabinitol 1,5-diphosphate.

Xylitol 1,5-Diphosphate and Ribitol 1,5-Diphosphate. These compounds were synthesized from the appropriate tri-*O*-benzylpentitol (Gray *et al.*, 1965) by the procedure described for the preparation of D-arabinitol diphosphate and isolated as their tetracyclohexylammonium salts. The purities of these compounds were comparable to the purities of D- and L-arabinitol 1,5-diphosphate.

D-*Glucitol 1,6-Diphosphate.* The 2,3,4,5-tetra-*O*-benzyl derivative of D-glucitol was prepared in the same manner as the 2,3,4-tri-*O*-benzylpentitols and obtained as a thick sirup. None of the intermediates involved in this synthesis was crystallized. D-Glucitol 1,6-diphosphate was synthesized from 2,3,4,5-tetra-*O*-benzyl-D-glucitol in the same manner as the pentitol 1,5-diphosphates were synthesized from the 2,3,4-tri-*O*-benzylpentitols and obtained as the tetralithium salt. The compound was estimated to be 90% pure.

1,4-Anhydro-DL-*ribitol 5-Phosphate, 1,4-Anhydro*-DL-*xylitol 5-Phosphate, and 1,4-Anhydro*-D-*arabinitol 5-Phosphate.* These compounds were synthesized from the appropriate 2,3-di-*O*-benzyl-1,4-anhydropentitol (Gray *et al.*, 1965) by the same procedure described for the synthesis of D-arabinitol 1,5-diphosphate and isolated as analytically pure dicyclohexylammonium salts. The yields obtained were 76% for 1,4-anhydro-DL-ribitol 5-phosphate, 81% for 1,4-anhydro-DL-xylitol 5-phosphate, and 95% for 1,4-anhydro-D-arabinitol 5-phosphate.

Results

Aldolase Activity. The average K_m for the aldolase used was determined to be 2.8×10^{-6} M. Twenty separate determinations were made using five concen-

TABLE I: Compounds Tested as Inhibitors of Aldolase.

Compound	Concentration in Assay Medium (M)	K_I (M)
3-Hydroxytetrahydrofuran	0.1	$3.6 \pm 0.6 \times 10^{-2}$
D-Arabinitol 1,5-diphosphate	8.9×10^{-6}	$1.5 \pm 0.1 \times 10^{-6}$
L-Arabinitol 1,5-diphosphate	3.1×10^{-4}	$4.1 \pm 0.3 \times 10^{-5}$
Xylitol 1,5-diphosphate	1.8×10^{-5}	$2.8 \pm 0.2 \times 10^{-6}$
Ribitol 1,5-diphosphate	2.1×10^{-4}	$2.0 \pm 0.2 \times 10^{-5}$
1,4-Anhydro-DL-ribitol 5-phosphate	1.24×10^{-2}	$4.6 \pm 0.6 \times 10^{-3}$
1,4-Anhydro-DL-xylitol 5-phosphate	7.6×10^{-3}	$3.8 \pm 0.5 \times 10^{-3}$
1,4-Anhydro-D-arabinitol 5-phosphate	1.24×10^{-2}	$1.3 \pm 0.3 \times 10^{-3}$
D-Glucitol 1,6-diphosphate	4.5×10^{-5}	$1.2 \pm 0.1 \times 10^{-5}$
2,5-Anhydro-D-mannitol 1,6-diphosphate	1.8×10^{-4}	$3.0 \pm 0.6 \times 10^{-5}$
2,5-Anhydro-D-glucitol 1,6-diphosphate	9.0×10^{-5}	$1.3 \pm 0.3 \times 10^{-5}$
Ethylene glycol diphosphate	7.44×10^{-4}	$1.2 \pm 0.3 \times 10^{-4}$
1,3-Propanediol diphosphate	2.5×10^{-4}	$1.2 \pm 0.1 \times 10^{-4}$
1,4-Butanediol diphosphate	3.66×10^{-4}	$5.5 \pm 0.8 \times 10^{-5}$
1,5-Pentanediol diphosphate	9.4×10^{-5}	$2.9 \pm 0.7 \times 10^{-5}$
1,6-Hexanediol diphosphate	9.1×10^{-5}	$2.5 \pm 0.5 \times 10^{-5}$
1,8-Octanediol diphosphate	2.7×10^{-4}	$1.2 \pm 0.2 \times 10^{-4}$
2-Pentanone 1,5-diphosphate	9.1×10^{-5}	$3.0 \pm 0.7 \times 10^{-5}$

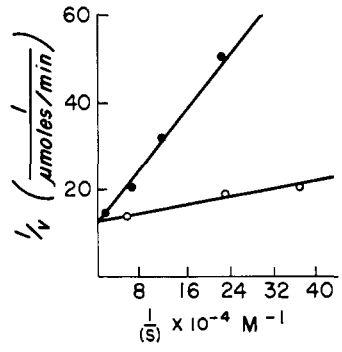


FIGURE 1: A Lineweaver-Burk plot of typical data obtained in a single experiment to evaluate K_I for D-arabinitol 1,5-diphosphate. (○), no inhibitor; (●), D-arabinitol 1,5-diphosphate, 8.9×10^{-6} M. All other conditions as given in the text.

trations of substrate in each. The range of values was $2.5\text{–}3.1 \times 10^{-6}$ M. The average value of V_{max} was 1150 moles of fructose 1,6-diphosphate cleaved per min per mole of aldolase (assuming that the molecular weight of aldolase is 150,000).

A number of compounds were tested which did not inhibit aldolase when present in the assay medium at a concentration of 0.2 M. They are 1,4-anhydroribitol, 1,4-anhydro-D-arabinitol, 1,4-anhydroxylitol, 1,4-anhydro-D-mannitol, 2,5-anhydro-D-glucitol, 2,5-anhydro-D-mannitol, 2,5-anhydro-L-iditol, xylitol, ribitol, D-arabinitol, D-glucitol, D-mannitol, and tetrahydrofurfuryl alcohol. The compounds which were found to be competitive inhibitors of aldolase are listed in Table I. A Lineweaver-Burk plot of a typical set of experimental data is shown in Figure 1. Each value for K_I given in Table I represents the average of three experiments, and the three determinations used to evaluate K_m in each case fell well within the range for K_m as described.

Discussion

It is assumed throughout this discussion that competitive inhibitors act at the active site of the enzyme and that K_I calculated from kinetic data is equivalent to the dissociation constant (K_{dis}) of the enzyme-inhibitor complex (EI).

Several conclusions can be drawn from the results of the experiments described: (1) Aldolase has two binding sites for phosphate. All of the diphosphate esters were approximately 100 times more effective as inhibitors then the monophosphates (Table I), indicating much tighter binding. This finding agrees with the conclusions drawn from the K_m values reported for substrates which contain one phosphate ester group as compared

to two (Richards and Rutter 1961; Tung *et al.*, 1954).

(2) The binding sites for phosphate are such that diphosphates are most tightly bound when the maximum distance which can be achieved between P atoms is between 10 and 12 A (Table II). Decreased stability

TABLE II: Distance between Phosphorus Atoms in Various Diphosphates as Measured on Dreiding Models.

Compound	Maximum Distance (A)	Minimum Distance (A)
Ethylene glycol diphosphate	7.0	5.9
1,3-Propanediol diphosphate	8.2	5.9
1,4-Butanediol diphosphate	9.4	5.9
1,5-Pentanediol diphosphate	10.6	5.9
1,6-Hexanediol diphosphate	11.8	5.9
1,8-Octanediol diphosphate	14.2	5.9
2,5-Anhydro-D-glucitol 1,6-diphosphate	10.0	5.9
2,5-Anhydro-D-mannitol 1,6-diphosphate	10.24	5.9

of the complex might be owing to the strain induced in the inhibitor portion of the complex, in the enzyme portion, or both.

It is probable that, because of strong repulsions between similarly charged phosphate groups, the linear diphosphates exist in solution with a majority of the molecules fully extended so that the carbon chain has a zigzag conformation. Consider 1,8-octanediol diphosphate in which the maximum phosphate separation is 14.2 A; the number of molecules having a P-P distance in the range of 10–12 A would be expected to be approximately 15% of the total.[3] If this proportion of the total inhibitor is the true inhibitor, then the value of K_I becomes approximately 2.0×10^{-5} M, in reasonable agreement with the value 2.5×10^{-5} M for the compounds possessing a phosphate separation nearer to the distance expected in fructose 1,6-diphosphate. If the difference in the apparent K_I values between octanediol diphosphate and hexanediol diphosphate resides in the proportion of the molecules having a P-P distance conducive to maximum complex stability, then, relative to these compounds, the enzyme serves as a fairly rigid template on which the complex is formed.

For those inhibitors with maximum P-P distances less than that required for firmest binding the strain in the complex may be owing to strain in the enzyme portion or simply to lack of fit. It is our conclusion that the phosphate-binding sites of aldolase are constrained within a limited tolerance of 10–12 A.

(3) Hydroxyl groups contribute little to the binding of inhibitors or the substrate. This conclusion is based on the finding that K_I for hydroxyl-containing inhibitors with P-P distances of 10–12 A is not greatly increased over that for inhibitors without hydroxyl groups which have similar P-P distances. It is apparent that hydroxyl groups play some role in the binding since D-arabinitol 1,5-diphosphate and xylitol 1,5-diphosphate bind somewhat more tightly than any of the other inhibitors tested and at least as tightly as the substrate fructose 1,6-diphosphate (Westhead *et al.*, 1963).

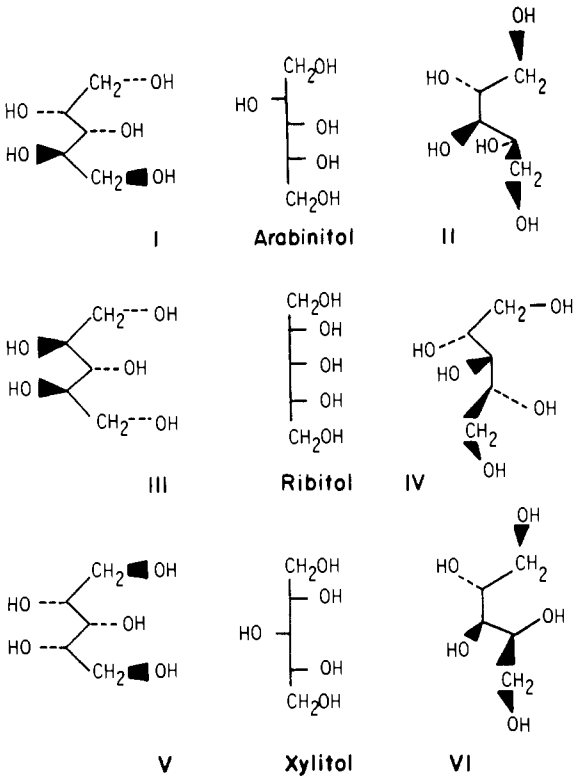
It is possible that the hydroxyl groups function not as binding sites for complex formation but to modify the conformation of the carbon chain owing to dipole interactions. The most probable conformation of an alditol is one in which adjacent hydroxyl groups are gauche when viewed in Newman projection. In this arrangement, as opposed to that obtained when the carbon chain is most fully extended (zigzag), the distances between adjacent hydroxyl groups are less, but those between hydroxyl groups β to each other are greater (compare structures I and II in which D-arabinitol is represented with all hydroxyls gauche [II], and with an extended carbon chain [I]). Conformer II would be more stable because it results in a greater average separation of the repelling groups.

The effect of phosphate groups on the conformation of the polyol is difficult to evaluate but it is apparent that, since the phosphates repel each other, they would cause some elongation of the molecule compared to the nonphosphorylated form. However, the energy differences between conformers I and II owing to the closer approach of phosphate groups in the 1 and 5 positions in conformer II is 0.43 kcal/mole, whereas the increased interaction of 1,3-hydroxyl groups in conformer I as compared to II constitutes an opposing energy barrier of approximately the same size. In the case of arabinitol, the energy differences between the gauche and zigzag arrangements are small as compared to those which would be expected between conformers in ribitol (conformers III and IV) and xylitol (conformers V and VI), in which the zigzag conformers (III and V) have the 2 and 4 hydroxyl groups eclipsed leading to interaction free energies of at least 1.0 kcal/mole opposing assumption of this form (Lemieux, 1964).

The dipole interactions of the hydroxyl groups have a pronounced effect on the conformation that the various alditol phosphates will assume. As compared to ribitol, the xylitol and arabinitol isomers will exist predominantly as conformers having the carbon chain disposed as it would be in the tetrahydrofuran ring system and the substituent hydroxyls *trans* as would be found in fructose 1,6-diphosphate. These two compounds would be expected to fit very well on the active site since they bear a close resemblance to the substrate (see below under [5]).

(4) *keto*-D-Fructose diphosphate is not the true or preferred substrate for aldolase. If the *keto* form of fructose 1,6-diphosphate were the only form which could act as

[3] Calculated from the energy required (approximately 1 kcal/mole) to decrease the distance between diionic phosphates from 14 to 10 A at 25° in water.

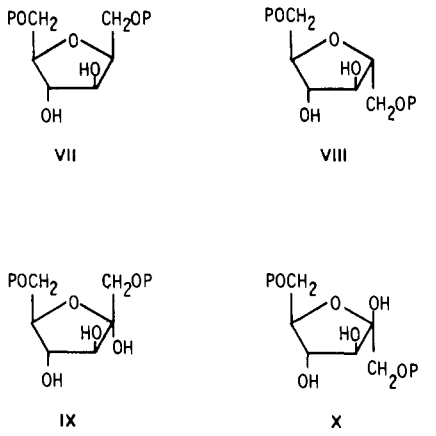


a substrate, the observed K_m for the reaction would be in error to the extent that the hemiketal forms of the substrate exist in aqueous solution. The E_M^{280} of the fructose diphosphate tetracyclohexylammonium salt used in this study is 1.06. If it is assumed that the *keto* form has the same extinction coefficient as does acetone in aqueous solution (E_M^{285} 17.5, Nagakura *et al.*, 1957), the concentration of *keto* form is 6% of the total concentration of fructose 1,6-diphosphate. Correction of K_m by a factor of 17 gives a value of 1.6×10^{-7} M for the *keto* form as the only substrate. However, unless a remarkable mechanism exists whereby the enzyme is not inhibited by the α- and β-furanose forms of fructose 1,6-diphosphate, even this value of K_m would be much too large. The *keto* group would have to confer a large increase in binding if the *keto* form is the only substrate and gives the observed rates of reaction. On this basis a compound containing two phosphates and a suitably situated ketone function would be expected to be an exceedingly potent inhibitor. 1,5-Pentanediol-2-one diphosphate was found to inhibit only to the same extent as 1,5-pentanediol diphosphate. The *keto* group did not increase the stability of the enzyme-inhibitor complex. This finding is in accord with the dissociation constant for enzyme-dihydroxyacetone phosphate complex (1.6×10^{-3} M) reported by Westhead *et al.* (1963); the stability of this enzyme-substrate complex is approximately the same as that of the monophosphate ester–enzyme complexes reported here and elsewhere (Tung *et al.*, 1954; Richards and Rutter, 1961), indicating that the *keto* group does not increase the binding energy. The comparison of dissociation constants for species involved in the forward and back reactions is valid since the same kind of intermediate is involved.

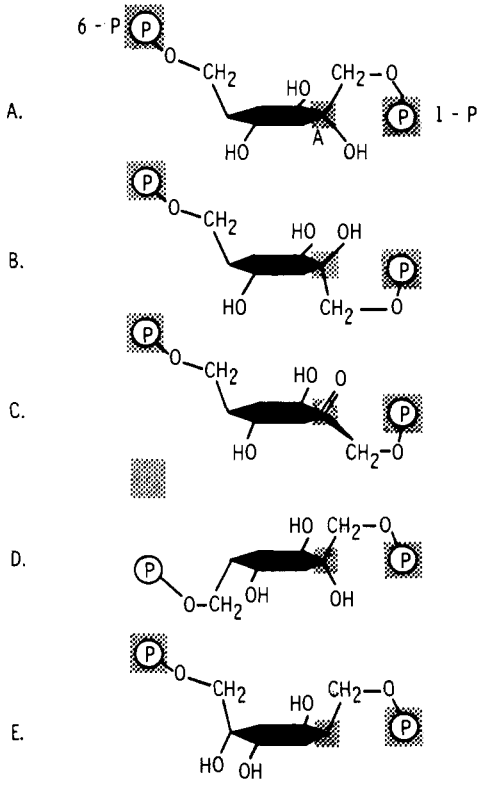
(5) Aldolase *binds* all forms of fructose 1,6-diphosphate present in solution.

This statement is based in part on the argument under (4) that the *keto* form of fructose 1,6-diphosphate would need to have an actual K_m of much less than 1×10^{-7} M to produce the apparent K_m of 2.8×10^{-6} M in the presence of a large excess of furanose forms which should be excellent "inhibitors" (*vide infra*). If the *keto* form is the true substrate *keto* analogs would be expected to be the best inhibitors, but they are not.

This conclusion is also supported by the finding that 2,5-anhydro-D-glucitol 1,6,diphosphate (compound VII) and 2,5-anhydro-D-mannitol 1,6-diphosphate (compound VIII) inhibit to approximately the same extent as the open-chain compounds having the same degree of phosphate separation. These two inhibitors are excellent analogs of the α (compound IX) and β (compound X) forms, respectively, of fructofuranose diphosphate. They should possess all of the conformational characteristics of the furanose forms except for the absence of the anomeric hydroxyl. This difference should not result in any steric inhibition of binding. It was expected that if aldolase acted preferentially on the α- or β-furanose form of fructose 1,6-diphosphate, either compound VII or VIII would be a much better inhibitor of fructose 1,6-diphosphate cleavage.



The simplest conclusion that can be drawn from the foregoing data is that aldolase acts on all forms of fructose 1,6-diphosphate and converts them all to the same intermediate in the enzymatic process. A model of the active site of aldolase can be constructed, embodying the conclusions which have been drawn. In it there are two phosphate-binding sites and a functional site which engages the carbonyl group or anomeric carbon to form the intermediate. The position of the latter is such that either anomer of fructose 1,6-diphosphate can be acted on with equal facility. One possible organization of these three sites is illustrated in Scheme 1, in which the furanose forms of fructose 1,6-diphosphate are shown bound to a surface with the ring oxygen toward the surface. The site for 6-phosphate binding is above the plane of the ring, that for 1-phosphate in the plane of the ring, and the "anomeric" site is below the anomeric carbon.



SCHEME 1: The α,β-furanose and *keto* forms of fructose 1,6-diphosphate fitted to the hypothetical active site of aldolase (A, B, and C, respectively). Molecules are oriented so that the ring oxygen (or 5-OH) is away from the observer toward the surface of the enzyme; 6-P = binding site for the 6-phosphate, 1-P = binding site for the 1-phosphate, and A = reaction site for the anomeric center. (A, B, C) All bind tightly and are cleaved rapidly; (D) α-L-sorbofuranose 1,6-diphosphate fitted to the same site is weakly bound ($K_m \cong 1 \times 10^{-3}$ M) and cleaved slowly; (E) β-L-sorbofuranose 1,6-diphosphate fitted to the site is bound tightly ($K_m \cong 1 \times 10^{-5}$ M), cannot cleave, and would act as a potent competitive inhibitor.

This arrangement of groups allows an explanation of the finding by Richards and Rutter (1961) that L-sorbose 1,6-diphosphate is bound tightly by aldolase but cleaved slowly (Scheme 1). The α-furanose form could fit on the 1-phosphate site and the "anomeric" site and be cleaved. However it would probably be weakly bound ($K_{dis} \cong 10^{-3}$ M) and cleaved slowly since the 6-phosphate is not properly placed to bind. On the other hand, the β-form could fit on both phosphate sites and be tightly bound ($K_{dis} = 10^{-5}$ M), but could not be cleaved because it is inverted, C-5 occupying the anomeric site.

The *keto* form of fructose 1,6-diphosphate could readily occupy the site as described since the only change would involve C-2 becoming trigonal and planar. There is also the attractive possibility of a concerted reaction of the anomeric carbon with the "anomeric site" at the time of formation of the complex which is being formed principally as a result of binding of the phosphate groups.

References

Blostein, R., and Rutter, W. J. (1963), *J. Biol. Chem. 238*, 3280.

Gray, G. R., Hartman, F. C., and Barker, R. (1965), *J. Org. Chem. 30*, 2020.

Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.

Kenner, G. W., Todd, A. R., and Weymouth, F. J. (1952), *J. Chem. Soc.*, 3675.

Lehninger, A. L., Sice, J., and Jensen, E. V. (1955), *Biochim. Biophys. Acta 17*, 285.

Lemieux, R. U. (1964), *Mol. Rearrangements* (Part 2), 737.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Marsh, B. B. (1959), *Biochim. Biophys. Acta 32*, 357.

Meyerhof, M. O. (1939), *Bull. Soc. Chim. Biol. 21*, 965.

Nagakura, S., Minegishi, A., and Stanfield, K. (1957), *J. Am. Chem. Soc. 79*, 1033.

Richards, O. C., and Rutter, W. J. (1961), *J. Biol. Chem. 236*, 3185.

Rutter, W. J. (1961), *Enzymes 5*, 341.

Tung, T., Ling, K. H., Byrne, W. L., and Lardy, H. A. (1954), *Biochim. Biophys. Acta 14*, 488.

Viscontini, M., Hoch, D., and Karrer, P. (1955), *Helv. Chim. Acta 38*, 642.

Wallis, W. A., and Roberts, H. V. (1956), Statistics: A New Approach, Glencoe, Ill., Free Press, pp. 534–538.

Westhead, E. W., Butler, L., and Boyer, P. D. (1963), *Biochemistry 2*, 927.

Woodward, R. B., Sondheimer, F., Taub, D., Heusler, K., and McLamore, W. M. (1952), *J. Am. Chem. Soc. 74*, 4223.