L-Rhamnulose 1-Phosphate Aldolase from *Escherichia coli*. Crystallization and Properties*

Teh-Hsing Chiu† and David Sidney Feingold‡

ABSTRACT: L-Rhamnulose 1-phosphate aldolase has been purified and crystallized from L-rhamnose-induced cultures of *Escherichia coli* as well as from a strain of the organism constitutive for L-rhamnose utilization. The enzyme is homogeneous by acrylamide gel disc electrophoresis, by electrophoresis on cellulose acetate, and by immunoelectrophoresis and immunodiffusion in agar gel. It is also homogeneous by sedimentation velocity and density gradient centrifugation. Molecular weight determined by density gradient centrifugation and by Sephadex gel thin-layer chromatography is in the order of 1.3–1.4 × 10^5 daltons. Na^+, Cs^+, NH_4^+, Rb^+, or K^+ is required for activity; K_a for KCl is 6 mM.

The enzyme has a sharp pH optimum of 7.5. K_m is 0.3 mM for L-rhamnulose 1-phosphate, 6.0 mM for L-lactaldehyde, and 3.0 mM for dihydroxyacetone phosphate. K_{equil} for the reaction L-rhamnulose 1-phosphate $\rightleftharpoons$ L-lactaldehyde + dihydroxyacetone phosphate is 8.3 × 10^{-5} M. The enzyme is specific for ketose 1-phosphates which have the configuration D at C-3 and L at C-4 in the Fischer projection formula.

In *Escherichia coli* L-rhamnose is utilized *via* the pathway

$$\text{L-rhamnose} \xrightarrow{\text{isomerase}} \text{L-rhamnulose} \xrightarrow{\text{kinase}} \text{L-rhamnulose-1-P} \xrightarrow{\text{aldolase}} \text{L-lactaldehyde} + \text{dihydroxyacetone phosphate}$$

The last enzyme in the sequence, L-rhamnulose 1-phosphate L-lactaldehyde lyase (L-rhamnulose-1-P aldolase), has been purified partially by Sawada and Takagi (1964) from extracts of L-rhamnose-grown *E. coli* and by Domagk and Heinrich (1965) from suitably induced cells of *Lactobacillus plantarum*. These studies were done with impure enzymes of relatively low specific activity, which were not investigated extensively. In this paper are described the purification and crystallization of L-rhamnulose-1-P aldolase from a strain of *E. coli* as well as studies of its catalytic properties and specificity.

Experimental Procedure

Materials. L-Rhamnulose-1-P was prepared as previously described (Chiu *et al.*, 1966). The procedures used for conversion of ketose 1-phosphates to mixtures of the corresponding epimeric polyol phosphates by reduction with $NaBH_4$ and for isolation and analysis of the products were those of Ginsburg and Mehler (1966). L-Fuculose-1-P and L-lactaldehyde were gifts from Dr. E. C. Heath, Johns Hopkins University. D-Glyceraldehyde-3-P was a gift from Dr. C. E. Ballou, University of California, Berkeley. Dr. H. Lardy, University of Wisconsin, generously provided L-sorbose-1-P, L-sorbose-6-P, and L-sorbose-1,6-P_2. 6-Deoxy-L-sorbose-1-P was synthesized from dihydroxyacetone phosphate and L-lactaldehyde by the action of hexose diphosphate aldolase; the product was isolated and purified as described previously for L-rhamnulose-1-P (Chiu and Feingold, 1964). D-Lactaldehyde was prepared from L-threonine by degradation with ninhydrin (Huff and Rudney, 1959). All other compounds and enzymes either were commercial products or were described previously (Chiu and Feingold, 1964).

Electrophoresis and Chromatography. Electrophoresis of protein was done in 7% polyacrylamide gels, pH 8.9, at 5 mA/tube (Davis, 1964), or on Sepraphore III cellulose polyacetate strips (Gelman Instruments Co., Ann Arbor, Mich.). Thin-layer gel filtration of proteins was carried out on Sephadex G-200 superfine beads by the method of Johansson and Rymo (1962, 1964).

Paper electrophoresis with 0.1 M ammonium acetate (pH 5.8) (Feingold *et al.*, 1958) was performed on either Whatman No. 1 or 3MM filter paper in the GME Model D electrophorator. Paper chromatography was done on Whatman No. 1 paper, using ascending or descending techniques as appropriate. Chromatography on a 0.25-mm thin layer of cellulose on glass plates also was used (Schweiger, 1962).

The following solvent systems were used for paper chromatography: (1) butanone–acetic acid–H_2O (75:25:10, v/v), (2) 1-propanol–NH_4OH (28–30%)–H_2O (60:30:10, v/v), (3) 1-butanol–acetic acid–H_2O (52:13:35, v/v), (4) 80% aqueous phenol, (5) toluene–ethanol–H_2O (270:30:1, v/v), (6) 1-butanol–ethanol–H_2O (10:1:2, v/v), (7) 1-butanol–pyridine–H_2O (3:1:1, v/v), (8) water-sa-

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turated 1-butanol, (9) methanol–NH_4OH (28–30%)–H_2O (6:1:3, v/v), and (10) ethyl acetate–acetic acid–H_2O (3:1:3, v/v).

Spray reagents used to detect compounds on paper electrophoretograms and chromatograms have been described previously (Chiu and Feingold, 1964).

Analytical Methods. Ketohexose and 6-deoxyketohexose were estimated by the method of Dische and Devi (1960), ketopentose by the method of Meijbaum (1939), and ketotetrose by the method of Dische and Dische (1958). L-Rhamnulose-1-P was determined as previously described (Chiu and Feingold, 1964).

The method of Barker and Summerson (1941) was used for determination of lactaldehyde (Ghalambour and Heath, 1962). The Rudolph Model 80 spectropolarimeter was used for determination of optical rotations. Protein was determined by the method of Waddel (1956) with crystalline serum albumin as standard. All other analytical techniques used have been described previously (Chiu and Feingold, 1964).

Enzyme Assays. L-Rhamnulose-1-P aldolase (as well as hexose diphosphate aldolase) activity was measured by determining the rate of dihydroxyacetone phosphate formation from L-rhamnulose-1-P. Dihydroxyacetone phosphate was determined with glycerol phosphate dehydrogenase (EC 1.1.1.8) in the presence of $NADH_2$. Reaction mixtures at 37° contained (micromoles): KCl,[1] 50; $NADH_2$, 0.2; L-rhamnulose-1-P, 2.0; Tris-HCl buffer (pH 7.5), 45; and glycerol phosphate dehydrogenase, 50 μg; in a total volume of 1 ml. Enzyme (10 μl) was added to start the reaction, and the decrease in absorbance was followed at 340 mμ in cells with a 1-cm light path in the Gilford Model 2000 spectrophotometer equipped with a constant-temperature cell chamber. In order to conserve substrate, sometimes the reaction was carried out in a final volume of 0.4 ml, with correspondingly less reagents. A unit of enzyme activity is defined as the amount of enzyme required to release 1 μmole of dihydroxyacetone phosphate/min at 37°.

Ultracentrifugal Studies. Sucrose density gradient ultracentrifugation was carried out according to the method of Martin and Ames (1961). Sedimentation velocity centrifugation was performed in the Beckman Model E ultracentrifuge.

Antiserum Preparation. Antisera to enzymes of the L-rhamnose pathway in the strain of *E. coli* used in this work were prepared with the partially purified enzyme ($(NH_4)_2SO_4$–I) as antigen. The protein solution was diluted with an equal volume of Freund's adjuvant, and 1 ml of the mixture, containing 20 mg of protein, was injected into the rear footpad of a 2–2.5-kg female rabbit in the first, third, fourth, sixth, and seventh weeks of the immunization period. After 9 weeks the rabbit was exsanguinated by cardiac puncture and the serum was separated from the clotted whole blood by centrifugation. A control serum was obtained from blood drawn prior to immunization.

[1] KCl must be present in assay reaction mixtures since the aldolase requires certain monovalent cations for activity. This point is treated in detail under Results.

Immunochemical Methods. Immunodiffusion in Bactoagar plates was performed as described by Ouchterlony (1958); immunoelectrophoresis was carried out in Bactoagar at pH 8.2 on standard microscope slides at 250 V for 40 min, essentially as described by Scheidegger (1955).

Results

Growth of Cells. E. coli K_{40} (strain derived from K_{12}) was grown in the medium previously described (Chiu and Feingold, 1964) in a New Brunswick 50-l. Model F-50 Fermacell fermenter. Medium (40 l.) was inoculated with 4 l. of a culture in late-log phase (Chiu and Feingold, 1964) (absorbance 0.4 at 420 mμ). Growth was at 37° with 1.5-ft^3/min aeration and 200-rpm agitation. When the culture reached an absorbance of 0.65 at 420 mμ (usually after 12–16 hr), it was cooled quickly to 5–10° and the cells were harvested in a DeLaval Gyro (DeLaval Separator Co., Poughkeepsie, N. Y.). They then were suspended in 1 l. of cold 0.15 M NaCl and packed by centrifugation at 4° for 20 min at 10,000*g*. Approximately 100 g of packed cells was obtained.

Preparation of Cell-Free Extracts. Packed cells were suspended in three times their weight of cold 0.02 M sodium and potassium phosphate buffer (pH 7.0) (buffer I) and 80 ml of the suspension was disrupted in an ice-cooled 150-ml Rosett cell (Rosett, 1965) with the Branson Sonifier Model S-110 (Branson Instrument Inc., Stamford, Conn.) at a 10-A output for 6 min. The broken cell suspension was centrifuged at 4° at 20,000*g* for 20 min and the pellet was discarded.

Purification of L-Rhamnulose-1-P Aldolase. To 400 ml of the supernatant fluid from the previous step (24 mg of protein/ml), 40 ml of 0.5 M $MnCl_2$ was added with stirring and the mixture was left in an ice bath overnight. (This and all subsequent operations were performed at 0–4° unless stated otherwise.) The precipitated nucleoprotein was removed by centrifugation at 30,000*g* for 10 min and the supernatant fluid was retained. $NADH_2$ oxidase, present in the crude extract, was eliminated almost completely in this step. Mercaptoethanol was not added prior to this time because of its protective action on $NADH_2$ oxidase.

$(NH_4)_2SO_4$ Fractionation I. Mercaptoethanol was added to the supernatant fluid to a final concentration of 0.05 M, yielding 405 ml of solution. Solid $(NH_4)_2SO_4$ (94 g) then was added, and the resulting precipitate was spun down and discarded. To the supernatant fluid was added 24 g of $(NH_4)_2SO_4$; the precipitate was collected by centrifugation and dissolved in 130 ml of buffer I, 0.05 M in mercaptoethanol. During the fractionation the pH was kept at 7.0 by addition of 3 M NH_4OH as necessary.

Acetone Fractionation. To one volume of enzyme solution nine-tenths volume of cold acetone (−20°) was added dropwise with stirring. The suspension was centrifuged immediately at 30,000*g* at −6° for 10 min and the precipitate was discarded. An additional six-tenths volume of cold acetone was added to the supernatant fluid and the suspension was centrifuged as above. The supernatant fluid was discarded and the

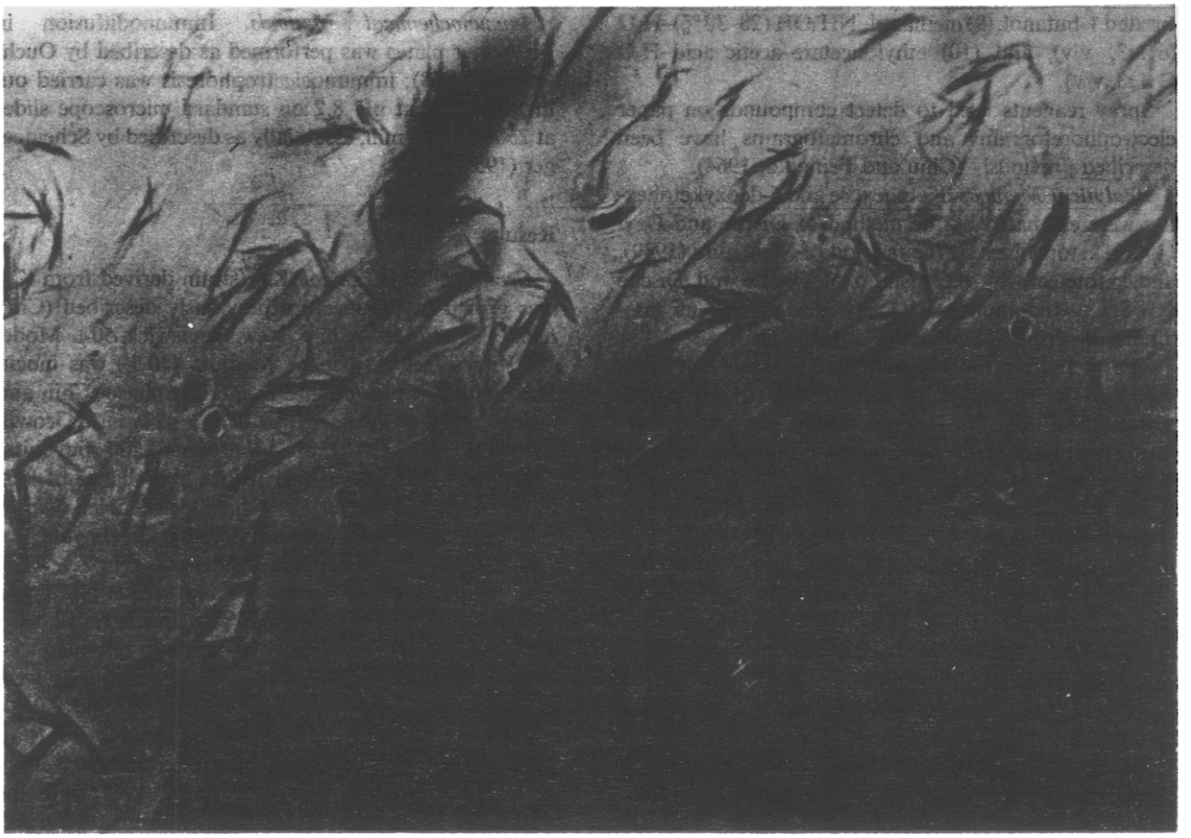


FIGURE 1: Crystalline L-rhamnulose-1-P aldolase (× 100).

precipitate was dissolved in 5 ml of buffer I, 0.01 M in mercaptoethanol; any insoluble precipitate was removed by centrifugation. In order to eliminate residual acetone the enzyme solution was loaded immediately onto a Sephadex G-100 column (3.0 × 85 cm). The column, equilibrated with 0.005 M phosphate buffer (pH 7.0), 0.01 M in mercaptoethanol (buffer II), was eluted with the same buffer. Fractions of 2 ml were collected; active fractions (40–45 ml) were pooled.

DEAE-Sephadex A-50 Fractionation. The pooled active fractions were fractionated further on a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with buffer II, 0.01 M in mercaptoethanol. The enzyme solution was placed on the column, which then was washed with 75 ml of the same buffer. The column was eluted with an increasing gradient of NaCl obtained with 80 ml of buffer II, 0.01 M in mercaptoethanol, in the mixing chamber and 500 ml of the same buffer, 0.5 M in NaCl and 0.01 M in mercaptoethanol, in the reservoir; 2-ml fractions were collected. The enzyme emerged after approximately 120 ml had been eluted from the column. Active fractions were pooled, yielding 88 ml of solution.

$(NH_4)_2SO_4$ Fractionation II. The protein in the active fractions was precipitated by addition of 32.6 g of solid $(NH_4)_2SO_4$ to 60% saturation. The pH was kept at 7.0 as mentioned previously. The precipitate was extracted in order with 1.0 ml each of 35, 30, and 28% saturated $(NH_4)_2SO_4$ in buffer I. The extracts were discarded and the remaining precipitate was dissolved in 2 ml of buffer I, 0.05 M in mercaptoethanol; insoluble material was eliminated by centrifugation.

At this point the protein concentration in the solution was approximately 8 mg/ml. Solid $(NH_4)_2SO_4$ was added slowly to the solution until it was faintly cloudy; usually this required 0.28 g. This cloudy suspension then was placed in a bath at 25° whereupon rapid crystallization occurred. After 10 min an additional 0.3 g of $(NH_4)_2SO_4$ was added over a period of 5 min. The suspension was cooled in an ice bath for 5 min; the crystals were removed by centrifugation in the cold. The crystals were

TABLE I: Purification of L-Rhamnulose-1-P Aldolase.

Fraction	Sp Act.[a]	Purifcn (-fold)	% Recov
$MnCl_2$	0.26	1	100
$(NH_4)_2SO_4$-I	1.1	4.2	82
Acetone	2.6	10.0	66
Sephadex G-100	3.7	14.2	65
DEAE-Sephadex	5.4	20.8	62
$(NH_4)_2SO_4$-II	16.9	65.0	38
First crystallization	17.2	66.0	33
Second crystallization	17.2	66.0	32

[a] Units per milligram of protein at 37°.

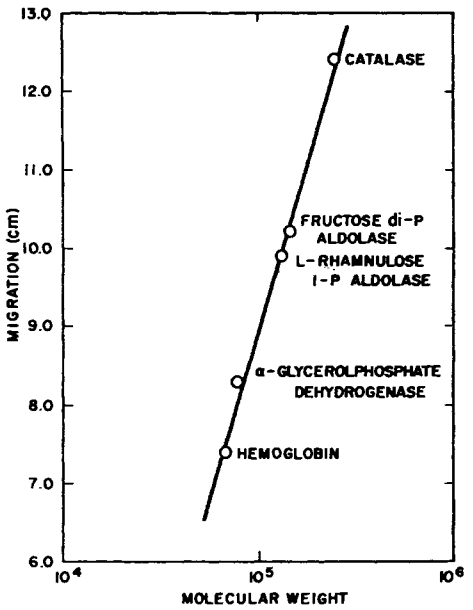


FIGURE 2: Sephadex G-200 gel chromatography of L-rhamnulose-1-P aldolase. Approximately 10 μg of each of the proteins listed in the figure was chromatographed on a 0.25-mm layer of Sephadex G-200 in 0.05 M sodium and potassium phosphate (pH 7.0). Staining was with Amido Black. The following were used as standards: hemoglobin, mol wt 64,000 (Benhamou and Weill, 1957); glycerolphosphate dehydrogenase from rabbit muscle (EC 1.1.1.8), mol wt, 78,000 (van Eys *et al.*, 1959); fructose diphosphate aldolase from rabbit muscle (EC 4.1.2.13), mol wt 147,000 (Taylor and Lowry, 1956); and beef liver catalase (EC 1.11.1.6), mol wt 244,000 (Samejima *et al.*, 1962). Migration distance is plotted against the logarithm of the molecular weight of each of the reference substances.

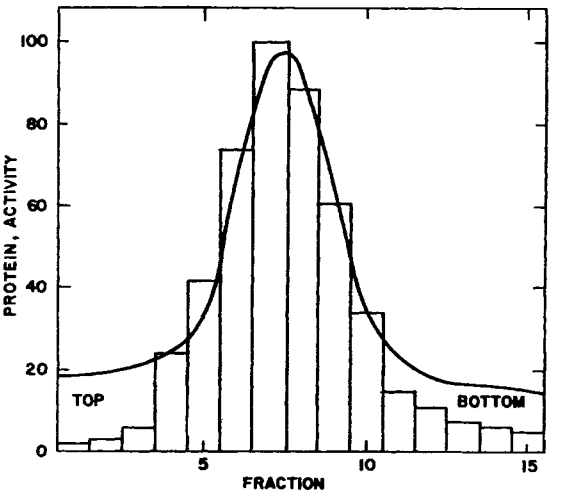


FIGURE 3: Sucrose density gradient centrifugation of L-rhamnulose-1-P aldolase. L-Rhamnulose-1-P aldolase (0.1 ml of a solution containing 25–250 μg of protein) was layered onto 4.5 ml of a 5–40% sucrose gradient. Centrifugation was performed with a SW-39 rotor in the Spinco Model L2 preparative ultracentrifuge at 38,000 rpm for 16 hr at 4°. Egg-white lysozyme (EC 3.2.1.17), mol wt 14,400 (Kaminski, 1955); crystalline yeast alcohol dehydrogenase (EC 1.1.1.1), mol wt 151,000 (Kagi and Vallee, 1961); and crystalline beef liver catalase (EC 1.11.1.6), mol wt 244,000 (Samejima *et al.*, 1962), were used as reference standards. After centrifugation, the tubes were eluted in the ISCO Model D density gradient fractionator (Instrument Specialties Co., Inc., Lincoln, Neb.); ultraviolet absorption of the fractions was recorded either at 254 mμ with the ISCO Model UA recording ultraviolet analyzer or at 215 mμ with the Gilford Model 2000 automatic recording spectrophotometer equipped with Model 209 flow cells. Approximately 18 0.25-ml fractions were collected per tube. The distribution of the reference enzyme in the fractions was established as described by Martin and Ames (1961) and of L-rhamnulose-1-P aldolase by activity determination using the assay method described in this paper. Absorption at 315 mμ, solid curve; activity (absorbance change at 340 mμ/min in the standard assay), blocks. The scale is arbitrary.

redissolved in 2 ml of buffer I, 0.05 M in mercaptoethanol, and recrystallized as before. The crystals were dissolved in 2 ml of buffer I, 0.01 M in mercaptoethanol, and the solution was stored at 0°. The enzyme crystallizes in slender needles, 15–20 μ long (Figure 1). The purification is summarized in Table I.

The identical procedure was used to prepare crystalline enzyme from a strain of *E. coli* (P72 Met$^+$Rha$^-$201) constitutive for L-rhamnose utilization, obtained from Dr. J. Power, University of California, Santa Barbara. The organism was grown as described, with 0.5% glycerol in place of L-rhamnose. The pure crystalline enzyme so obtained had the same specific activity and immunochemical behavior as that from the inducible strain.

Properties of L-Rhamnulose-1-P Aldolase. ENZYMIC PURITY. The crystalline enzyme had a specific activity of 17.2 at 37°. It yielded one spot in Sephadex G-200 thin-layer gel filtration; molecular weight calculated from its chromatographic mobility by the method of Andrews (1964) was 135,000 (Figure 2). Centrifugation in a sucrose gradient gave a single symmetrical peak with correspondence between protein and activity (Figure 3). The density gradient centrifugation data yielded a molecular weight of 130,000–140,000. Sedimentation velocity experiments in the Spinco Model E ultracentrifuge yielded a single sedimenting component. Further sedimentation behavior will be described in a subsequent paper. One band was obtained upon polyacrylamide gel electrophoresis at pH 8.9 (Figure 4), as well as upon cellulose polyacetate electrophoresis over the pH range 4.5–8.3. The apparent isoelectric point, determined from the relative mobilities at different pH values, is 5.05 (Figure 5). Only one band of enzyme–antibody precipitate was detectable by the Ouchterlony (1958) double-diffusion technique and by immunoelectrophoresis (Figure 6). On the basis of these criteria the enzyme is homogeneous. The turnover number calculated for a molecular weight of 135,000 is 2300 moles of L-rhamnulose-1-P split per min per mole of enzyme.

ABSORPTION SPECTRUM AND AMINO ACID ANALYSIS. Pure enzyme gave a typical protein absorption spectrum with a single peak at 280 mμ. A solution containing 1 mg of enzyme/ml (calculated from amino acid analysis) in buffer I gave an absorbance of 1.73 at this wavelength.

The composition of L-rhamnulose-1-P aldolase as determined on a Beckman amino acid analyzer is given in Table II.

STABILITY. Solutions of the crystalline enzyme containing from 0.2 to 1.0% protein in buffer I, 0.01 M in

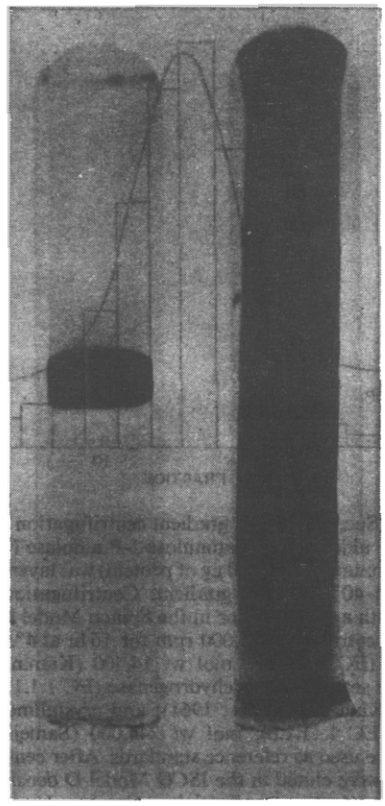


FIGURE 4: Polyacrylamide gel electrophoresis of L-rhamnulose-1-P aldolase: left: $MnCl_2$ step; right: crystalline enzyme. The samples contained 50 μg of protein.

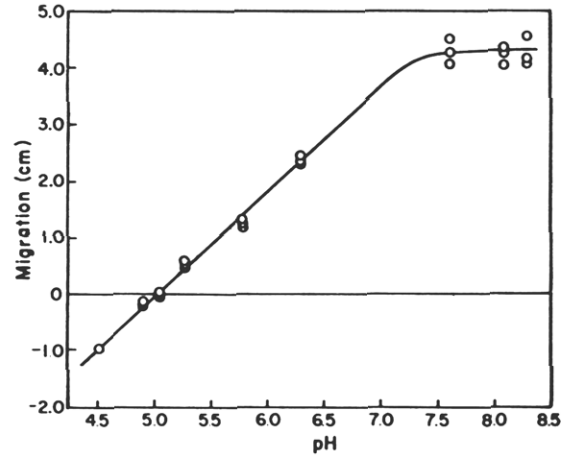


FIGURE 5: Determination of the isoelectric point by electrophoresis of L-rhamnulose-1-P aldolase on cellulose polyacetate strips. Electrophoresis was carried out on 2.5 × 17 cm strips of Sepraphore III in 0.05 ionic strength sodium acetate–acetic acid between pH 4.5 and 5.8, and collidine–HCl between pH 6.3 and 8.3. Approximately 10 μg of protein was applied per strip. After electrophoresis at 10 V/cm for 105 min, protein was stained with Ponceau S. Apalon yellow was used as a nonmobile marker. Anodal migration is plotted as a positive value.

mercaptoethanol, retained full activity for at least 3 months when stored at 0–4°.

OPTIMUM pH. The optimum is 7.5, with activity declining rather steeply on both sides (Figure 7).

IDENTIFICATION OF REACTION PRODUCTS. Dihydroxyacetone phosphate was characterized by its reactivity with glycerol phosphate dehydrogenase in the presence of $NADH_2$ and by its paper electrophoretic mobility. Only one electrophoretically mobile compound was released from L-rhamnulose-1-P by the aldolase. Upon paper electrophoresis at pH 5.8 this compound had the mobility and spray reactions with $AgNO_3$ and molybdic acid of authentic dihydroxyacetone phosphate. L-Lactaldehyde was characterized by (1) its positive color reaction in the Barker and Summerson (1941) test, (2) reduction of Fehling's solution in the cold (Prager and Jacobson, 1918), (3) paper chromatography in solvents 2, 9, and 10, (4) immobility upon paper electrophoresis, and (5) bis-2,4-dinitrophenylhydrazone formation. The 2,4-dinitrophenylhydrazone derivative was prepared as follows. The reaction mixture consisted of (in millimoles): L-rhamnulose-1-P, 0.2; hydrazine sulfate (to trap L-lactaldehyde produced), 1.0; glycylglycine buffer (pH 7.5), 1.5; KCl, 2; and L-rhamnulose-1-P aldolase, 2 mg, in a total volume of 40 ml. After incubation for 1 hr at 37° 10.5 ml of 50% trichloroacetic acid was added and the precipitated protein was removed by centrifugation. Then 50 ml of 2 N HCl containing 200 mg of 2,4-dinitrophenylhydrazine was added to the supernatant fluid. The solution was kept at room temperature for 5 hr, heated on a steam bath for 30 min, and stored in the refrigerator overnight. The precipitate which formed was washed with ethyl acetate and dissolved in 20 ml of pyridine, and insoluble material was removed by centrifugation. The solution was concentrated to about 2 ml by evaporation under vacuum and kept at 4° for 4 hr. The red crystals which formed were isolated by centrifugation, recrystallized twice from pyridine, and dried under vacuum. A decomposition point of 298–301° uncor (which corresponds to that reported by Neuberg and Kobel (1928)) was observed, with no melting point depression upon addition of the authentic dinitrophenylhydrazone. Since this derivative was optically inactive, it was not possible to determine the configuration at this point.

STOICHIOMETRY. The reaction mixture (in micromoles) consisted of: L-rhamnulose-1-P, 0.4; $NADH_2$, 0.5; KCl, 25; glycylglycine buffer (pH 7.5), 200; glycerol-P dehydrogenase, 0.5 mg; and 4 μg of L-rhamnulose-1-P aldolase. After 30 min at 25°, the reaction was stopped by heating at 100° for 1 min; analysis showed that the following quantities (micromoles) of products were present: dihydroxyacetone phosphate, 0.080; L-lactic aldehyde, 0.070; L-rhamnulose-1-P, 0.30. Thus 1 mole each of dihydroxyacetone phosphate and L-lactic aldehyde is formed per mole of L-rhamnulose-1-P cleaved.

REVERSIBILITY AND EQUILIBRIUM. Many aldolase reactions are known to be reversible. The reversibility of the reaction catalyzed by L-rhamnulose-1-P aldolase was shown as follows. L-Lactaldehyde and dihydroxyacetone phosphate (2 μmoles of each) were incubated under standard conditions for 1 hr with purified L-rhamnulose-1-P aldolase. A spot corresponding in mobility to L-rhamnulose-1-P was detected upon chromatog-

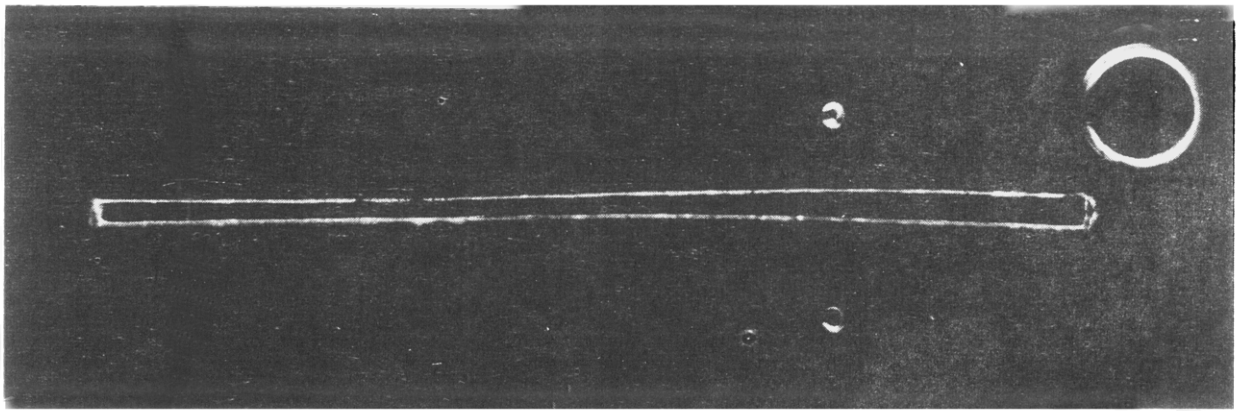


FIGURE 6: Immunoelectrophoresis of L-rhamnulose-1-P aldolase. Top: crystalline enzyme. Bottom: $MnCl_2$ step. Center slot: antiserum.

TABLE II: Amino Acid Composition of L-Rhamnulose-1-P Aldolase.

	Hydrolysis Time (hr)				
	20	40	70	100	
Amino Acid	Moles/Mole of Enzyme	Moles/Mole of Enzyme	Moles/Mole of Enzyme	Moles/Mole of Enzyme	
Lysine	42.6	48.4	51.9	53.3	
Histidine	31.7	33.1	35.4	34.5	34.9[a]
NH_4^+	47.2	53.3	90.7	96.0	28.2[a]
Arginine	34.0	37.1	39.4	38.5	
Half-cysteine				13.3	
Aspartic	128	133	123	127.3	
Threonine	94.8	97.2	88.2	86.5	99.2[a]
Serine	61.8	57.8	50.8	48.5	64.5[a]
Glutamic acid	128	134	129	126	
Proline	68.3	80.5	60.3	76	
Glycine	115	118	115.5	111.5	
Alanine	107.5	108	101	101.7	
Valine	79.3	88.2	88.8	89.3	
Methionine	28	28.7	29.4	32.4	
Isoleucine	54	60.8	63.6	62	
Leucine	117.5	123.5	126	126.3	
Tyrosine	18.4	19.8	22.2	23.3	
Phenylalanine	43.4	47.3	52.7	53.5	
Tryptophan					14[b]

[a] Extrapolated to time zero. [b] From spectroscopic measurement in 0.1 N NaOH.

raphy of the reaction mixture in solvent 2. Acid phosphatase hydrolysis of the product yielded a single compound with the mobility of authentic L-rhamnulose upon paper chromatography (solvents 1, 3, and 8) which gave a positive reaction with silver nitrate (Partridge, 1948), *p*-anisidine phosphate (Feingold *et al.*, 1958), and urea phosphate (Wise *et al.*, 1955) spray reagents.

A quantitative study of the equilibrium, starting with either L-rhamnulose-1-P, or dihydroxyacetone phosphate and L-lactaldehyde, was carried out at a substrate concentration of 0.001 M. As shown in Figure 8 the reaction mixture contained approximately 75% L-rhamnulose-1-P and 25% dihydroxyacetone phosphate and L-lactaldehyde after equilibrium was reached. The equilibrium constant, K_{equil}, for the reaction

$$\text{L-rhamnulose-1-P} \rightleftharpoons \text{DHAP} + \text{L-lactaldehyde}$$

is 8.3×10^{-5} M.

SUBSTRATE SPECIFICITY. The substrate specificity of L-rhamnulose-1-P aldolase for cleavage of different ketose phosphates was determined by substituting the com-

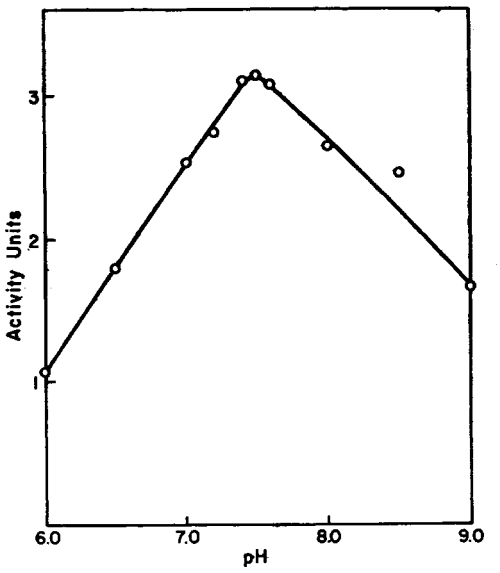


FIGURE 7: The effect of pH on enzyme activity. Assay conditions are as described in the text except that the buffer and pH were varied. Potassium phosphate buffer was used between pH 6.0 and 7.6 and Tris-HCl above pH 7.5.

pounds for L-rhamnulose-1-P in the standard assay. Table III lists the relative activity of L-rhamnulose-1-P aldolase ($(NH_4)_2SO_4$–II fraction) with a variety of substrates. These results indicate that L-rhamnulose-1-P aldolase is highly specific for its substrate and also show that there is minimal contamination with fructose diphosphate aldolase, which might be expected to be present in *E. coli* extracts.

Investigation of the specificity of L-rhamnulose-1-P

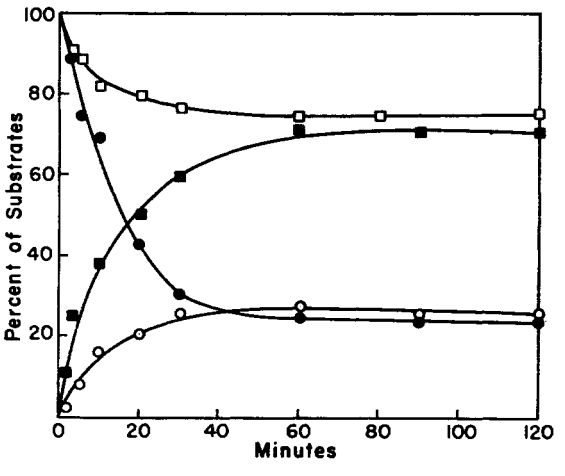


FIGURE 8: Reversibility of L-rhamnulose 1-phosphate aldolase action. Reaction mixtures at 37° contained (micromoles) either L-rhamnulose-1-P, 3; or dihydroxyacetone phosphate, 3, and L-lactaldehyde, 3; glycylglycine buffer (pH 7.5), 135; and L-rhamnulose-1-P aldolase, 20 μg; KCl, 150; in a total volume of 3.0 ml. At the times indicated 0.25-ml samples were removed and inactivated for 1 min at 100°; denatured protein was removed by centrifugation. The ratio of dihydroxyacetone phosphate to L-rhamnulose-1-P in the supernatant fluid was determined (Chiu and Feingold, 1964). (□—□ and ■—■) L-Rhamnulose-1-P; (○—○ and ●—●) dihydroxyacetone phosphate.

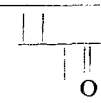
TABLE III: Substrate Specificity of L-Rhamnulose-1-P Aldolase.

Substrate	C-3,4 Configuration	Rel Act.
L-Rhamnulose-1-P	*trans*	100
D-Fructose-1,6-P_2	*trans*	1.8
L-Fuculose-1-P	*cis*	1.7
D-Ribulose-1,5-P_2	*cis*	1.3
6-Deoxy-L-sorbose-1-P	*trans*	0.8
L-Sorbose-1-P	*trans*	0.3
L-Sorbose-1,6-P_2	*trans*	0.0
D-Fructose-1-P	*trans*	0.0
L-Sorbose-6-P	*trans*	0.0
D-Fructose-6-P	*trans*	0.0

aldolase for aldehydes in the direction of condensation was carried out to determine the relative activity of the enzyme with various aldehydes (Table IV). It is evident that dihydroxyacetone phosphate is able to condense with D-glyceraldehyde, glycolaldehyde, acetaldehyde, formaldehyde, and D-lactaldehyde, in addition to L-lactaldehyde, in the presence of L-rhamnulose-1-P aldolase. In order to determine the configuration of the hydroxyl groups at C-3 and C-4, large-scale experiments were conducted to prepare ketose phosphate from dihydroxyacetone phosphate and L-lactaldehyde, D-glyceraldehyde, and glycolaldehyde, respectively. Reaction mixtures consisted of (millimoles) dihydroxyacetone phosphate, 0.2; aldehyde, 0.25; KCl, 3.0; glycylglycine buffer (pH 7.5), 2.5; and enzyme, 2 mg; in a total volume of 60 ml. After 2 hr at 30°, the mixtures were held at 100° for 5 min, and then concentrated to 5 ml by vacuum evaporation. The products were isolated by chromatography on Dowex 1-×8 formate columns and shown to be ketose 1-phosphates by periodate oxidation followed by isolation and characterization of phosphoglycolic acid as described previously (Chiu and Feingold, 1964). The compounds obtained from L-lactaldehyde and D-glyceraldehyde had the mobilities of authentic L-rhamnulose-1-P and L-sorbose-1-P, respectively, upon paper chromatography in solvent 2 and upon paper electrophoresis at pH 5.8. The ketose released from each of the ketose phosphates by acid phosphatase migrated as a single spot and had the chromatographic properties of L-rhamnulose, L-sorbose, and D-xylulose, respectively, in solvents 1, 6, and 7.

Phenylosazones prepared from each of the ketoses according to Vogel (1948) had the same crystalline shape and cellulose thin-layer chromatographic mobilities (solvent 5) as the authentic compounds; in addition, the melting points of the phenylosazones prepared from the ketoses isolated from reaction mixtures containing L-lactaldehyde and glycolaldehyde were 182–184 and 161–163°, respectively, in agreement with the literature value for the melting points of the phenylosazones of L-rhamnose and D-xylose (Vogel, 1948). The optical rotations

TABLE IV: Aldehyde Specificity of L-Rhamnulose-1-P Aldolase.[a]

Aldehyde	Rel Rate[b]	Product	Configuration
L-Lactaldehyde	100	L-Rhamnulose-1-P	
D-Glyceraldehyde	50	D-Sorbose-1-P	
Glycolaldehyde	40	L-Xylulose-1-P	
Acetaldehyde	15	5-Deoxy-L-xylulose-1-P[c]	
Formaldehyde	10	D-Erythrulose-1-P[c]	
D-Lactaldehyde	10	6-Deoxy-D-sorbose-1-P[c]	
L-Glyceraldehyde	0		
D-Glyceraldehyde-3-P	0		
Propionaldehyde	0		

[a] The reaction mixture at 37° consisted of (micromoles): dihydroxyacetone phosphate, 2.0; aldehyde, 2.5; glycylglycine buffer (pH 7.0), 40; in a total volume of 1.0 ml. The reaction was started by addition of 2.5 μg of enzyme in 10 μl of solution. At 0, 1, 3, 5, 10, and 20 min, 0.15-ml samples were removed and inactivated at 100° for 1 min. Residual dihydroxyacetone phosphate in the samples was determined with glycerol phosphate dehydrogenase and $NADH_2$. Disappearance of dihydroxyacetone phosphate equalled 6-deoxyketose appearance in mixtures containing L-lactaldehyde. Formation of ketose was qualitatively demonstrated by the appropriate color reactions for 6-deoxyketohexose and ketohexose by the method of Dische and Devi (1960), ketopentose by the method of Meijbaum (1939), and ketotetrose by the method of Dische and Dische (1958). In addition, each of these reaction mixtures contained an organic phosphate with the expected paper chromatographic (solvent 2) and paper electrophoretic (pH 5.8) behavior. Treatment of the reaction products with acid phosphatase released carbohydrates with the relative paper chromatographic (solvents 1, 6, and 7) mobilities expected for the free ketoses. [b] The reaction rate with L-lactaldehyde is taken as 100. [c] In assigning structures to these compounds it is assumed that the enzyme catalyzes formation of only the isomer with the configuration D at C-3 and L at C-4.

of the ketoses (Table V), determined polarimetrically, are in reasonable agreement with the recorded values (Merck and Co., 1960; Chiang and Knight, 1961). These data establish the identity of the first three compounds in Table IV. The structures assigned to the ketose phosphates formed from dihydroxyacetone phosphate and acetaldehyde, formaldehyde, and D-lactaldehyde, while not rigorously established, are consistent with the demonstrated specificity of the enzyme.

EFFECT OF MONOVALENT CATIONS. In the absence of monovalent cations the specific activity of L-rhamnulose-1-P aldolase is in the order of 0.6. In Figure 9 it can be noted that NaCl, CsCl, NH_4Cl, RbCl, and KCl, in order of increasing effectiveness, markedly enhance enzyme activity. LiCl is an inhibitor strictly competitive with KCl; K_i = 8 mM. K_m for L-rhamnulose-1-P remains essentially unchanged at KCl concentrations of 0.02, 0.05, and 0.2 M, and K_a for K^+ does not vary at L-rhamnulose-1-P concentrations of 0.9, 1.8, and 3.5 mM.

EFFECT OF SUBSTRATE CONCENTRATION. The effects of L-lactaldehyde, dihydroxyacetone phosphate, and L-rhamnulose-1-P concentration on enzyme activity are shown in Figure 10; K_m values determined by the method of Lineweaver and Burk (1934) are 6.0, 3.0, and 0.3 mM, respectively.

The latter value, which is significantly lower than was reported previously (Chiu and Feingold, 1965), was obtained with pure crystalline substrate (Chiu *et al.*, 1966); the L-rhamnulose-1-P previously used probably contained inhibitory impurities. K_m values for D-sorbose-1-P and L-xylulose-1-P, determined in the same way, are 1.8 and 0.2 mM, respectively.

INHIBITION BY SUBSTRATE ANALOGS. Since L-rhamnulose-1-P has a number of distinct structural features

TABLE V: Optical Rotation of Ketoses Isolated from Reaction Mixtures.

Ketose	$[\alpha]_D^{30}$ (deg)
Authentic L-rhamnulose	+36
From condensation of L-lactaldehyde and dihydroxyacetone phosphate	+30
Authentic L-sorbose	−42
From condensation of D-glyceraldehyde and dihydroxyacetone phosphate	+56
Authentic D-xylulose	−34
From condensation of glycolaldehyde and dihydroxyacetone phosphate	+33

which could be involved in binding, inhibition of cleavage of L-rhamnulose-1-P by a number of substrate analogs was tested. The following were not inhibitory when incorporated into standard reaction mixtures at concentrations of 1.5 mM or greater: D-fructose-1-P, D-fructose-6-P, D-fructose-1,6-P_2, L-fuculose-1-P, D-glucitol-6-P, a mixture of D-mannitol-1,6-P_2 and D-glucitol-1,6-P_2, D-glucose-6-P, α-D-glucopyranosyl-P, β-glycerol phosphate, D-mannitol-6-P, D-mannose-6-P, L-rhamnose, P_i, L-rhamnulose, L-sorbose-6-P, and D-xylose-1-P.

Inhibition by the product obtained by reduction of L-rhamnulose-1-P with $NaBH_4$ (a mixture of 6-deoxy-L-mannitol-1-P and 6-deoxy-L-glucitol-1-P) was strictly competitive with substrate and K_i = 0.1 mM. However, splitting of L-rhamnulose-1-P was not inhibited appreciably by the reduction products of D-sorbose-1-P (5 mM) or of L-xylulose-1-P (1 mM).

Discussion

Sawada and Takagi (1964) have reported that partially purified L-rhamnulose-1-P aldolase yields L-rhamnulose-1-P and another unidentified ketose phosphate when incubated with L-lactaldehyde and dihydroxyacetone phosphate. The evidence presented in this paper shows that L-rhamnulose-1-P is the only product of the reaction; possibly the compound found by Sawada and Tagaki (1964) was due to the presence of an epimerase in their enzyme preparation which converted the L-rhamnulose-1-P to L-fuculose-1-P. We have occasionally observed such activity in partially purified enzyme preparations.[3]

L-Rhamnulose-1-P aldolase displays a high degree of specificity for cleavage of ketose phosphates. Only those compounds which have the D configuration at C-3 and L at C-4 in the Fischer projection formula are split (Table III), and only such isomers are formed from aldehydes and dihydroxyacetone phosphate (Table IV), as shown by characterization of the products of condensation of dihydroxyacetone phosphate with L-lactaldehyde and two alternative aldehydes, D-glyceraldehyde and glycolaldehyde, each of which reacts at approximately one-half the rate of L-lactaldehyde.

Chiu and Feingold (1967b) have shown that the stereochemistry of tritium labeling of dihydroxyacetone phosphate by L-rhamnulose-1-P aldolase is the opposite of that observed with hexose disphosphate aldolase (Rose, 1958). Since L-rhamnulose-1-P is the diastereoisomer of D-fructose-1,6-P_2 at C-3 and C-4, the enediols formed from dihydroxyacetone phosphate by the two enzymes must be stereoisomers of each other. It would be of interest to examine the stereospecificity of tritium labeling of dihydroxyacetone phosphate by L-fuculose-1-P aldolase, which employs the same substrates as L-rhamnuclose-1-P aldolase but forms a product with the D configuration at C-3 as well as C-4 (Ghalambour and Heath, 1962).

Rose (1966), in an analysis based on the data of Ghalambour and Heath (1962) for L-fuculose-1-P aldolase and on our preliminary results with L-rhamnulose-1-P aldolase (Chiu and Feingold, 1965), has pointed out that the stereochemistry of the new bond is determined not only by which C-3 hydrogen of dihydroxyacetone phosphate is activated but also by stereospecific attack on the aldehyde. He also pointed out that were the condensation of L-lactaldehyde and dihydroxyacetone phosphate nonspecific and determined only by thermodynamic considerations, one would expect to find both isomers among the products, since a comparison of the K_{equil} of formation of L-fuculose-1-P (Ghalambour and Heath, 1962) and L-rhamnulose-1-P shows only a 5.5-fold difference in favor of the latter compound. The data presented in this paper fully substantiate these views.

The results of inhibition studies of L-rhamnulose-1-P aldolase make it possible to delineate partially the substrate binding sites on the enzyme. Neither L-rhamnulose nor P_i are inhibitors, showing that the structure $-C(O)CH_2OP$ present in L-rhamnulose-1-P plays a major role in binding of substrate to enzyme. The OH groups at C-3 and C-4 also must be involved in binding as shown by failure of compounds like L-fuculose-1-P and D-fructose-1-P to inhibit.

While the presence of the phosphate moiety at C-1 and of the proper configuration at C-3 and C-4 are necessary for binding of (and inhibition by) polyol phosphates, they are not sufficient. Of polyol phosphates tested, only the mixture of epimers resulting from the $NaBH_4$ reduction of L-rhamnulose-1-P was inhibitory. Since the inhibition is strictly competitive with substrate and K_i is comparable with K_m, it can be concluded that the inhibitor binds to the active site of the aldolase with approximately the same affinity as substrate. That this binding requires the presence of the terminal methyl group is shown by failure of the reduction products of D-sorbose-1-P and L-xylulose-1-P to inhibit splitting of L-rhamnulose-1-P. The parent ketose phosphates themselves, however, are good substrates for the aldolase, and therefore do bind, doubtless due to specificity for the structural features at carbons 1 to 4. Conversion of the carbonyl into a hydroxyl group probably weakens the binding to the extent that only a compound which contains an additional group remote from the phosphate

[3] T. H. Chiu and D. S. Feingold, unpublished experiments.

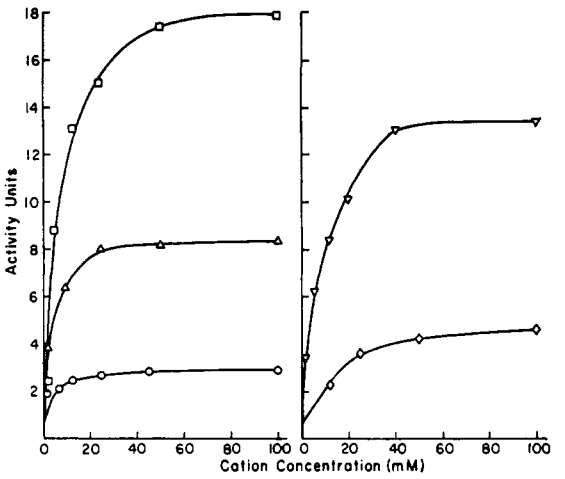
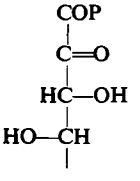


FIGURE 9: Effect of increasing monovalent cation concentration on activity of L-rhamnulose-1-P aldolase. The aldolase and the indicator enzyme were freed from monovalent cations by dialysis overnight against 2 × 10^4 volumes of 0.05 M Tris-HCl buffer (pH 7.2) with one change of buffer. Reaction mixtures at 37° contained (micromoles) $NADH_2$, 0.05 (disodium salt); Tris-HCl buffer (pH 7.5), 17.0; dicyclohexylammonium salt of L-rhamnulose-1-P, 1.4; L-rhamnulose-1-P aldolase, 0.8 μg; and glycerol-P dehydrogenase, 25 μg; in a total volume of 0.4 ml. The reaction rate was noted over a period of 3 min, and then 5 μl of a solution containing the desired concentration of salt was added and the effect on reaction rate was noted. Salts used were NaCl, O—O; CsCl, ◇—◇; NH_4Cl, △—△; RbCl, ▽—▽; and KCl, □—□.

(such as the methyl group in reduced L-rhamnulose-1-P) which can bind strongly and specifically to the enzyme can act as an inhibitor. This binding is so specific that conversion of the methyl group of L-rhamnulose-1-P to the hydroxymethyl group as in D-sorbose-1-P interferes with binding at the methyl group site, causing a sevenfold increase in the K_m, and making reduced D-sorbose-1-P noninhibitory. On the other hand, elimination of the methyl group, as in L-xylulose-1-P, has little effect on K_m.

These observations lead to the conclusion that L-rhamnulose-1-P aldolase has sites which specifically bind compounds with the structure



at C-1 to C-4, and that, in addition, there is a binding site on the enzyme which is specific for the methyl group of the L-rhamnulose-1-P.

L-Rhamnulose-1-P aldolase probably has an absolute requirement for one of the monovalent cations shown in Figure 9. In the absence of added K^+ (the most effective activator) activity did not exceed 3.4% of the maximum attainable. This small residual activity probably was due to Na^+ introduced as the disodium salt of $NADH_2$ used in the assay reaction mixture. The effectiveness of a monovalent cation as an activator does not seem to

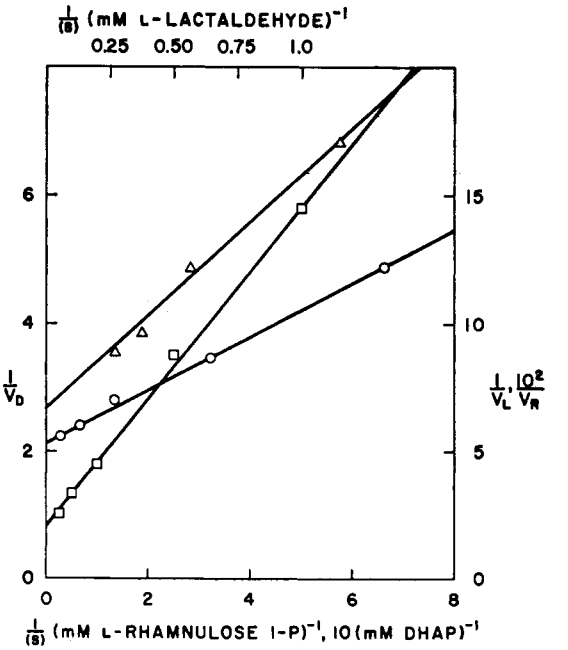


FIGURE 10: Effect of L-rhamnulose-1-P, dihydroxyacetone phosphate, and L-lactaldehyde concentrations on reaction velocity. L-Rhamnulose-1-P, O—O; reaction velocity, V_R. Reaction conditions were as described for the assay except that L-rhamnulose-1-P concentration was varied as indicated. Decrease in absorbance at 340 mμ was followed. Dihydroxyacetone phosphate: △—△, reaction velocity, V_D. Reaction mixtures contained (micromoles): L-lactaldehyde, 3.2; glycylglycine buffer (pH 7.5), 10; $(NH_4)_2SO_4$, 40; dihydroxyacetone phosphate as indicated; and L-rhamnulose-1-P aldolase, 8 μg; in a total volume of 0.5 ml. All reagents were adjusted to pH 7.5 before use. At appropriate time intervals samples were removed and their L-rhamnulose-1-P content was determined (Chiu and Feingold, 1964). L-Lactaldehyde, ▽—▽; reaction velocity, V_L. Reaction mixtures contained (μmoles): DHAP, 4.3; glycylglycine buffer (pH 7.5), 10; $(NH_4)_2SO_4$, 40; L-lactaldehyde as indicated; and L-rhamnulose-1-P aldolase, 8 μg; in a total volume of 0.5 ml. All reagents were adjusted to pH 7.5 before use. At appropriate time intervals samples were removed and their L-rhamnulose-1-P content was determined (Chiu and Feingold, 1964).

be correlated in any obvious way with its affinity for the enzyme. K_a values calculated from the data of Figure 9 by the method of Lineweaver and Burk (1934) are (millimolar): NaCl, 3; CsCl, 16; NH_4Cl, 2; RbCl, 9; and KCl, 6. Also Li^+ binds to the same site as K^+ with essentially the same affinity, yet it is an inhibitor rather than an activator. The obvious single characteristic which all the activating ions have in common is the size of their hydrated radii; the radii of the active ions are approximately similar and markedly different from that of Li^+, which inhibits. Comparable observations of the effect of monovalent cations have been made by Kachmar and Boyer (1952) with ATP–pyruvate phosphotransferase, by Vasquez (1964) in a study of binding of [^{14}C]chloramphenicol to ribosomes, and by Levine *et al.* (1966) in an investigation of the aminoacyl-transfer reaction in protein synthesis.

Kachmar and Boyer (1952) in their study point out that if for each reactive site for substrate more than one

activator atom must be bound for full activation of enzyme, velocity should be related to a higher power of activator concentration. Plots linear in respect to slope for each activator are obtained by plotting the data of Figure 9 as A/v *vs.* A, indicating that only 1 mole of activator/mole of L-rhamnulose-1-P need be bound to completely activate L-rhamnulose-1-P aldolase. It can reasonably be assumed that the activating ions (as well as Li^+) combine with a specific site on the enzyme, thereby effecting a conformational change which markedly affects V but has little or no effect on substrate affinity. This aspect is at present under investigation.

There are two distinct classes of aldolases known: class I aldolase, the prototype of which is fructose-P_2 aldolase of mammalian muscle, and class II aldolase, the prototype of which is fructose-P_2 aldolase from yeast. Class I aldolases are not activated by K^+ and are unaffected by chelating agents, while class II enzymes are activated by K^+ and other monovalent cations and are inactivated by chelating agents (Rutter, 1964). L-Rhamnulose-1-P aldolase is clearly a class II aldolase, since in addition to being activated by monovalent cations as described in this paper, it is completely inhibited by the zinc chelator, 1,10-phenanthroline (Chiu *et al.*, 1968). It is of interest that L-rhamnulose-1-P aldolase of *Lactobacillus plantarum* is activated by NH_4^+ but not by K^+ (Domagk and Heinrich, 1965), which represents a much higher specificity for monovalent cations than that of the *E. coli* enzyme.

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