PATHWAY OF GALACTITOL CATABOLISM IN KLEBSIELLA PNEUMONIAE

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ABSTRACT

PATHWAY OF GALACTITOL CATABOLISM
IN *KLEBSIELLA PNEUMONIAE*

By

John Paul Markwell

Previous investigations in this laboratory established that in *Klebsiella pneumoniae* galactitol is modified by phosphorylation with phosphoenolpyruvate to form L-galactitol 1-phosphate. The data in this dissertation establish that L-galactitol 1-phosphate is further catabolized by a previously unreported pathway, part of which is identical to the D-tagatose 6-phosphate pathway of lactose and D-galactose catabolism first established by this laboratory for *Staphylococcus aureus*. The pathway reactions for L-galactitol 1-phosphate catabolism in *K. pneumoniae* are as follows:

(1) L-Galactitol 1-phosphate dehydrogenase

L-galactitol 1-phosphate + nicotinamide adenine dinucleotide ⟶ D-tagatose 6-phosphate + nicotinamide adenine dinucleotide (reduced) + H^+

(2) D-Tagatose 6-phosphate kinase

D-tagatose 6-phosphate + adenosine 5'-triphosphate ⟶ D-tagatose 1,6-diphosphate + adenosine 5'-diphosphate

(3) D-Tagatose 1,6-diphosphate aldolase

D-tagatose 1,6-diphosphate ⟶ dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate

The enzymes catalyzing these reactions were partially purified and characterized. The products of the individual reactions were identified by enzymatic, chromatographic, and chemical analysis.

L-Galactitol 1-phosphate dehydrogenase was shown to be induced only by growth on galactitol, and was highly specific for L-galactitol 1-phosphate, D-tagatose 6-phosphate, and nicotinamide adenine dinucleotide (oxidized and reduced forms). The K_m values for the substrates were as follows: L-galactitol 1-phosphate, 0.42 mM; oxidized nicotinamide adenine dinucleotide, 0.22 mM; D-tagatose 6-phosphate, 0.10 mM; and reduced nicotinamide adenine dinucleotide, 0.025 mM. The activity was unaffected by monovalent cations, whereas activity and stability showed a strong requirement for divalent cations. The molecular weight of the enzyme was estimated by gel filtration to be 86,000. The equilibrium constant of the reaction, as written above, was determined to be 8.0×10^{-11} moles/liter.

D-Tagatose 6-phosphate kinase was shown to be the same enzyme as the constitutive D-fructose 6-phosphate kinase. This dual specificity was established by coincident chromatography on substituted cellulose, a constant ratio of activity throughout purification, coincident thermal inactivation, competition between the two substrates, and loss of tagatose phosphate activity in a mutant missing D-fructose 6-phosphate kinase. The phosphoryl donor and acceptor specificities were determined, and the apparent K_m values for the

phosphoryl acceptors were shown to be lowered by the addition of adenosine 5'-diphosphate to the assays. The molecular weight of the kinase was estimated by gel filtration to be 92,000.

D-Tagatose 1,6-diphosphate aldolase was induced only by growth on galactitol. The activity was specific for D-tagatose 1,6-diphosphate (K_m = 0.38 mM, relative V_{max} = 1.00) and, to a much lesser extent, D-fructose 1,6-diphosphate (K_m = 0.86 mM, relative V_{max} = 0.04). The enzyme was activated by both monovalent and divalent cations. The molecular weight was estimated by gel filtration to be 157,000.

Three mutants, each missing one of the above enzymes, were isolated. These mutants were unable to grow on galactitol, establishing that this is the sole pathway of physiological significance in this organism. Revertant strains, having regained the ability to grow on galactitol, were shown to contain the formerly missing enzyme activities. The enzymes of this pathway, therefore, constitute the first report of a catabolic route of galactitol utilization in this or any other organism.

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LIST OF ABBREVIATIONS

PEP	phosphoenol pyruvate
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ITP	inosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
UTP	uridine 5'-triphosphate
CTP	cytidine 5'-triphosphate
TTP	thymidine 5'-triphosphate
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
α-methylglucoside	methyl-α-D-glucopyranoside
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetic acid
NEM	N-ethylmaleimide
DTE	dithioerythritol
DTT	dithiothreitol
BME	β-mercaptoethanol; 2-thioethanol
A_{xxx}	absorbance at xxx nanometers

g	acceleration of gravity
g	gram
mg	milligram
Hz	Hertz
min	minutes
ml	milliliter
M	molar
mM	millimolar
cm	centimeter
mm	millimeter
nm	nanometer
Tris	tris (hydroxymethyl) aminomethane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Pyr	pyruvate
P	phosphate

GENERAL INTRODUCTION

The purpose of this work was to elucidate the pathway for the catabolism of galactitol in *Klebsiella pneumoniae*. As will be documented in the Literature Review, galactitol is modified in extracts of *K. pneumoniae* by phosphorylation, to form L-galactitol 1-phosphate. No route for the further catabolism of this compound has yet been reported. It was with this knowledge that I began my studies with this system.

The whole of this research is divided into three parts. The experiments of the first section show that the free hexitol is not modified except by the above-mentioned phosphorylation. A pathway for the catabolism of L-galactitol 1-phosphate is also proposed, along with the reasoning behind it. The activities of the required enzymes (L-galactitol 1-phosphate dehydrogenase, D-tagatose 6-phosphate kinase, and D-tagatose 1,6-diphosphate aldolase) are shown to be present in the extracts of galactitol-grown cells. The second part of this dissertation consists of the partial purification and characterization of these enzymes and their reaction products. These data confirm that the reactions of this pathway are catalyzed *in vitro* by the purified enzymes. The results presented in the third section of this dissertation demonstrate that mutants missing any one of the pathway enzymes cannot grow on

galactitol. This finding confirms the functioning of these enzymes in galactitol catabolism in this organism.

The pathway is as follows: L-galactitol 1-phosphate ⟶ D-tagatose 6-phosphate ⟶ D-tagatose 1,6-diphosphate ⟶ dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate.

LITERATURE REVIEW

While the literature contains many reviews of carbohydrate metabolism in general, only a few devote more than a paragraph to the catabolism of the hexitols. The two extensive reviews of the hexitols (1,2) are now almost 30 years old, and the biochemical data very out-dated. It shall then be the primary purpose of this section to review what is currently known about the bacterial utilization of the hexitols, especially galactitol.

Four of the hexitols have been found in nature (2,3). These naturally occurring hexitols are D-glucitol, D-mannitol, galactitol, and L-iditol. The greatest natural abundance of free galactitol occurs in plants, especially Madagascar manna (2) and the manna of *Gymnosporia diflexa* (4). Large amounts of galactitol have also been found in *Euonymus japonica* (5,6) and in some yeast (7).

The presence of free galactitol is not unknown in mammalian systems. The polyol has been identified in tissues of rats and chicks given large amounts of D-galactose in the diet (8,9). Humans deficient in the galactose kinase or uridyl transferase of the Leloir pathway have also been reported to accumulate galactitol (10,11). Despite these occurrences, there are numerous reports that galactitol is not metabolized in mammals (12,13,14,15). Since galactitol is not a substrate for mammalian polyol dehydrogenases (16,17,18), it seems likely that it is formed when D-galactose

concentrations are high enough to act as a substrate for aldose reductase (19,20) or L-hexonate dehydrogenase (21,22), both of which are known to oxidize galactitol and to have a wide occurrence in mammalian tissues (23,24).

D-Mannitol, D-glucitol and galactitol have long been known to be metabolized by a variety of bacterial species. Growth and fermentation of these hexitols have been used as a basis for the taxonomic classification of the Gram-negative organisms of the colon-typhoid (25) and the colon-aerogenes groups (26,27). Other microbes that have been reported to utilize galactitol include *Salmonella typhimurium* (28), *Streptococcus lactis* (29), *Bacillus subtilis* (30), five species of the yeast genera *Torulopsis* and *Debaryomyces* (31), and four species of the tomato, cabbage, and melon wilt-causing *Fusarium* (32).

Enzyme activities that can modify the hexitols by oxidation have been reported for a number of microbial species. *Pseudomonas fluorescens* cell extract is reported to oxidize D-mannitol to D-fructose, and D-glucitol to a mixture of D-fructose and L-sorbose (33). Two polyol dehydrogenases were reported from *Acetobacter suboxydans* (34). One of these activities, with an acid pH optimum, oxidizes D-mannitol and D-glucitol, but not galactitol, and was cytochrome-linked. The other activity, having an alkaline pH optimum, is NAD-linked, and again is specific for D-mannitol and D-glucitol, but not galactitol. Another particulate dehydrogenase, specific for D-mannitol and D-glucitol, was found in *Gluconobacter oxydans*; this is in addition to soluble NAD- and NADP-linked dehydrogenases catalyzing these same oxidations (35,36). Lin (37) has reported

that D-arabitol dehydrogenase from *Aerobacter aerogenes*, an NAD-specific enzyme, oxidizes D-mannitol to D-fructose. However, since this activity is induced by growth on D-arabitol, and not D-mannitol, it seems unlikely that this enzyme plays a part in mannitol utilization for this organism. An NAD-specific polyol dehydrogenase that oxidizes mannitol and glucitol, but not galactitol, was found in the yeast *Candida utilis* (34,38,39). Polyol dehydrogenases of mammalian tissues have also been studied. These catalyze the oxidation of D-gluticol and L-iditol, but not D-mannitol or galactitol (16,17,18).

None of the microbial enzymes thus far mentioned has been shown to oxidize galactitol. Shaw (40), after futile attempts to detect this activity in *Escherichia coli*, was able to isolate an airborne *Pseudomonas* species that exhibited a galactitol dehydrogenase. The enzyme is NAD specific, and oxidizes galactitol, D-glucitol, and L-iditol, while failing to utilize D-mannitol. Glactitol dehydrogenase activity has also been found in *Staphylococcus* species (41) as well as several species of yeast (31,42).

It was only after the discovery of a D-mannitol 1-phosphate dehydrogenase (43) that researchers considered the initial step for hexitol catabolism to possibly be a phosphorylation. D-Mannitol 1-phosphate dehydrogenase activity has been found in extracts of *Diplococcus pneumoniae* (43), *Lactobacillus arabinosus* (44), *Lactobacillus casei* (45), *Lactobacillus palntarum* (45), *Leuconostoc mesenteroides* (45), *Aerobacter aerogenes* (45,46,47), *Bacillus subtilis* (47), and *Escherichia coli* (45-48). D-Glucitol 6-phosphate dehydrogenase activity has been detected in *Lactobacillus casei* (49), *Aerobacter aerogenes* (46,47), *Klebsiella pneumoniae* (50,51),

and *Escherichia coli* (46,48). L-Galactitol 1-phosphate dehydrogenase has been reported for *Aerobacter aerogenes* (46) and for *Escherichia coli* (46,48,52).

D-Mannitol 1-phosphate dehydrogenase from *A. aerogenes* (47) has been purified and shown to be a 40,000 molecular-weight protein, specific for NAD and D-mannitol 1-phosphate. This enzyme is present in D-glucose grown cells, but is found at much higher specific activities in D-mannitol-grown cells (46,47). D-Glucitol 6-phosphate dehydrognease from the same organism is specifically induced by growth on D-glucitol, utilizes NAD rather than NADP, and oxidizes L-galactitol 1-phosphate at 3% the rate of D-glucitol 6-phosphate (47). Prior to this investigation there had been no report on the characteristics of an L-galactitol 1-phosphate dehydrogenase from any source.

The presence of these hexitol phosphate dehydrogenases led to the search for hexitol kinases (ATP:hexitol phosphotransferase) that would be responsible for the phosphorylation, but none was ever found (47). This lack of a hexitol kinase activity was explained in 1964 when Roseman's group discovered the PEP:carbohydrate phosphotransferase system in *E. coli* (53). The system is composed of at least two soluble and one membrane-bound proteins. It is the membrane-bound protein that is specific for the sugar that is phosphorylated (54). The reader is directed to one of several extensive reviews on the subject (55,56,57). It has been shown that this system is responsible for the phosphorylation of D-mannitol (58) and D-glucitol (50) that initiates the catabolism of these

hexitols. The pathway for D-mannitol and D-glucitol is summarized in Figure 1.

Phosphorylation of galactitol by the PEP-phosphotransferase system was recently shown by Shimamoto (59) in *K. pneumoniae*. It was demonstrated that the formation of the phosphorylated derivative of galactitol was dependent on PEP, and each of the three protein components of the PEP-phosphotransferase system. It was additionally shown that ATP could not substitute for PEP. Furthermore, the product was conclusively identified as L-galactitol 1-phosphate by gas-liquid chromatography, mass spectrometry, periodate oxidation, and enzymatic oxidation. The PEP-dependent phosphorylation of galactitol by *E. coli* was subsequently suggested by Lengler (52,60), but he did not show if the conformation of the product was D-galactitol 1-phosphate or L-galactitol 1-phosphate.

As is true for D-mannitol and D-glucitol catabolism, it might be expected that catabolism of L-galactitol 1-phosphate would involve sequential oxidation to a ketohexose phosphate, phosphorylation to ketohexose diphosphate, and aldolase cleavage to triose phosphates. Oxidation of L-galactitol 1-phosphate would be to D-tagatose 1-phosphate or D-tagatose 6-phosphate, the evidence indicating the latter (48,59). D-Tagatose 6-phosphate is phosphorylated to D-tagatose 1,6-diphosphate in *Staphylococcus aureus* (61) and Group N streptococci (62) in the process of lactose and D-galactose catabolism. The D-tagatose 1,6-diphosphate is cleaved to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (61), which are then assimilated into the glycolytic pathway. Although this pathway is the most likely prospect for further catabolism

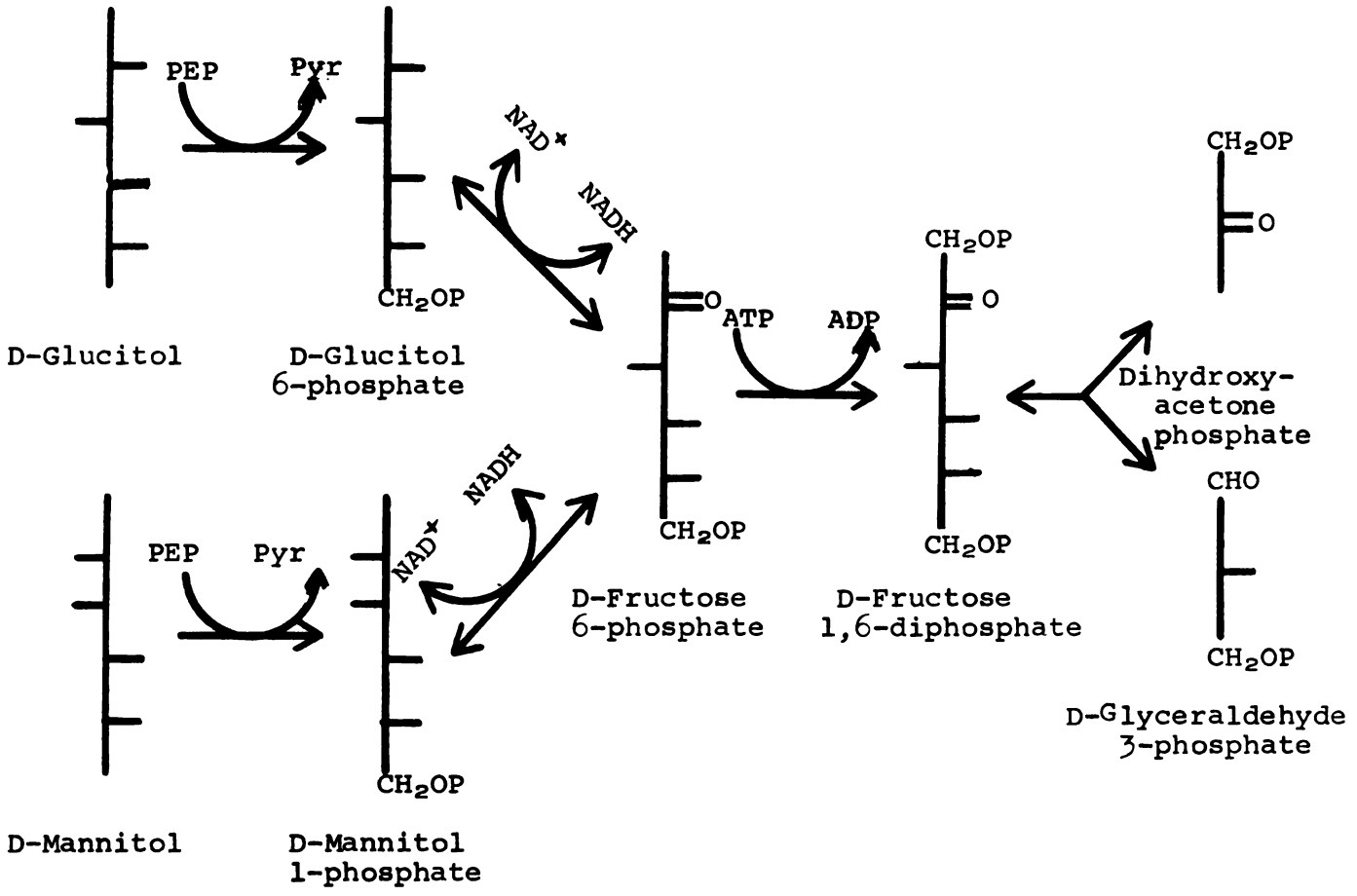


Figure 1. Catabolic pathways for D-mannitol and D-glucitol.

of L-galactitol 1-phosphate, the enzymes of the D-tagatose-6-phosphate pathway have been reported absent from extracts of *K. pneumoniae* cells grown on D-glutoxe, D-galactose, and lactose (62).

In summary, the pathway of galactitol catabolism has never been established for any organism. Cells of *K. pneumoniae* growing on galactitol have been demonstrated to form L-galactitol 1-phosphate (59). The oxidation of this product to D-tagatose 6-phosphate has been suggested (48), while further catablism of D-tagatose 6-phosphate is as yet unknown in this organism. It is the elucidation of this catabolic pathway that my Ph.D. dissertation will address.

SECTION 1

ELUCIDATION OF THE METABOLIC FATE OF GALACTITOL

INTRODUCTION

As described in the Literature Review, the pathway of galactitol catabolism has yet to be established for any organism. Although L-galactitol 1-phosphate has been clearly shown to be a product of the phosphorylation of galactitol by PEP in *Klebsiella pneumoniae* (59), no evidence was presented to show that this is the major, let alone the only, initiation step for the pathway of galactitol catabolism in this organism. The purpose of this section is to show that the formation of L-galactitol 1-phosphate is indeed the prime reaction of the pathway for galactitol utilization. Furthermore, the remaining steps in the catabolic pathway--analogous to the reactions of the D-mannitol and D-glucitol pathways--will be postulated, based on the presence of enzymes found in the cell extract. The proposed pathway is as follows: galactitol ⟶ L-galactitol 1-phosphate ⟶ D-tagatose 6-phosphate ⟶ D-tagatose 1,6-diphosphate ⟶ dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate.

MATERIALS AND METHODS

Bacterial Strain. The organism used was *Klebsiella pneumoniae* PRL-R3 (formerly designated *Aerobacter aerogenes* PRL-R3).

Media. Cells were grown in a mineral medium containing 0.15% KH_2PO_4, 0.71% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, 0.01% $MgSO_4$, and 0.0005% $FeSO_4 \cdot 7H_2O$. Galactitol was used as a carbon source at a concentration of 0.4% (w/v). Nutrient broth was made by mixing 8 g of the dehydrated powder with 1 liter of water.

Sterilization Technique. All media were made sterile by heating for 20 min at 121° in a Wilmot Castle autoclave, model Thermomatic 60. Carbohydrates were sterilized separately from the mineral medium.

Growth of Cells. Cells were grown in 500 ml of mineral medium plus carbohydrate, contained in a 2800 ml Fernbach flask. The flasks were incubated in the dark at 30°, and aerated by constant motion on a New Brunswick Scientific Co. rotary shaker. The inoculum was 7 ml of a culture grown in nutrient broth.

Harvesting of Cells. Continued incubation of cells was halted when a culture reached an A_{600} of approximately 0.6, as measured in 18 x 150 cm tubes with a Coleman Jr. spectrophotometer. Cells were harvested by centrifugation at 6,000 x g for 10 min.

The cells were suspended in 0.85% (w/v) NaCl and centrifuged again at 12,000 x g for 10 min. The cells were used within a hour.

Centrifugation. All centrifugations were done in a Sorvall refrigerated centrifuge, model RC-2B, at 0 to 4°. The rotor radius was either 4.34 or 5.75 inches.

Preparation of Cell Extracts. Cell extracts were prepared by suspending the harvested, washed cells in the appropriate buffer and exposing the solution sonic vibration (10,000 Hz) in a Raytheon sonic oscillator, model DF101, for 20 min. During sonication, the apparatus was cooled with a circulating ice-water mixture. Following disruption of the cells, the solution was centrifuged at 12,000 x g for 10 min to remove whole cells and debris. The resulting supernatant was decanted and termed the cell extract.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (63). Bovine serum albumin was used as a standard.

Removal of Barium from Sugar Phosphates. Barium salts of sugar phosphates were treated with washed, dried Dowex 50W-X8 (H^+ form). The solution was then neutralized with NaOH and diluted to volume.

Preparation of L-Galactitol 1-Phosphate. L-Galactitol 1-phosphate was synthesized by reduction of D-galactose 6-phosphate with $NaBH_4$ as described by Shimamoto (59).

Preparation of D-Tagatose 6-Phosphate. D-Tagatose 6-phosphate was synthesized from D-galacturonic acid as described by Bissett (64).

Gas-Liquid Chromatography. Acetylated derivatives of the hexitols were prepared by incubating 0.1 mg of the polyol with 0.2 ml of acetic anhydride and 1 mg of anhydrous sodium acetate for 2 hr at 100°. Gas-liquid chromatography was performed on a Hewlett Packard 5830A Reporting Gas Chromatograph, employing a 3% XE-60 column, and an oven temperature of 205°. The instrument was programmed to integrate and report the area of each peak.

Preparation of Ion Exchange Resins. Commercial resins were stirred overnight in 2 M NaOH on a magnetic stirrer, allowing the stirring motor to heat the solution. The slurry was washed in water until the pH was neutral, and then washed with 4 M HCl. The resin was then washed again with water, and then washed with the appropriate acid or base to generate the desired ionic form. The resins were then dried at 100° and stored in the dry state.

DEAE-Cellulose Chromatography. The DEAE-cellulose was washed with 1 M NaOH, 1 M HCl, and 1 M NaOH as described by Peterson and Sober (65). When not in use, the exchanger was stored in distilled water at 4°. To equilibrate a poured column, the column was washed with buffer until the effluent pH matched that of the buffer. The protein was then passed through the column, and the column was washed with buffer until protein ceased to elute. The adsorbed protein was then eluted with a linear or stepwise gradient of salt. Prior to reuse, the column was washed with 2 M NaCl to remove

tightly bound material. After approximately five runs, the column was unpacked, and the cellulose rewashed in 1 M HCl and water.

Enzymatic Assays. One unit of enzymatic activity is defined as the amount of enzyme that catalyzes the conversion of one µmole of substrate to product per minute at 30°. Assays involving the oxidation or reduction of pyridine nucleotides were monitored in microcuvettes with a 1.0 cm path length at 340 nm, using a thermostated (30°) Gilford spectrophotometer, model 2400. Other assays that involved incubations were temperature controlled in a Precision Scientific Co. water bath. The concentrations of substrate added to an assay were determined on a weight basis of the added substrate.

Galactitol Kinase. Assays (0.15 ml) contained 10 µmoles of glycylglycine buffer (pH 8.0), 0.5 µmole of ATP, 1.0 µmole of $MgCl_2$, 0.05 µmole of NADH, 0.5 µmole of PEP, 7.5 umoles of galactitol, excess pyruvate kinase and lactate dehydrogenase, and rate-limiting amounts of cell extract. Controls were run without galactitol or ATP to correct for ATPase and PEP:galactitol phosphotransferase, respectively.

Galactitol Dehydrogenase. Assays (0.15 ml) contained 10 µmoles of Tris buffer (pH 9.0), 0.01 µmole of $MgCl_2$, 1.0 µmole of NAD^+ or $NADP^+$, and 7.5 µmoles of galactitol. Controls without galactitol were used to correct for NADH oxidase.

Galactitol Epimerase The incubation (5 ml) contained the following: 200 µmoles of Tris buffer (pH 7.5); 1.5 umoles of $NADP^+$; 1.5 µmoles of NAD^+; 0.3 µmole of $MgCl_2$; 50 µmoles of

galactitol; 25 μmoles of α-methylglucoside; and cell extract. The tube was incubated at 30°, and 0.75 ml samples were removed at 30 min intervals. The samples were immediately placed in a 100° water bath for 10 min to stop any enzymatic activity, and the samples were then centrifuged at 5,300 x g for 10 min. One-half milliliter of the supernatant was diluted to 5 ml with water, and treated with dry Amberlite MB-3 to remove all ions. Of the deionized solution, 0.5 ml was placed inside a 2-dram vial and dried using a Buchi Rotovapor. When dry, the samples were acetylated and injected into the gas chromatograph. Alpha-methylglucoside was used as an internal standard for this system. Although the absolute amount of a sugar cannot be accurately determined from its peak area due to variations in sample volume or evaporation during acetylation, its area relative to the area of the α-methylglucoside peak can be accurately determined. The retention times were as follows: α-methylglucoside, 5.12 min; D-mannitol, 8.83 min; galactitol, 9.71 min; and D-glucitol, 11.62 min.

L-Galactitol 1-Phosphate Dehydrogenase. Assays (0.15 ml) contained 10 μmoles of Tris buffer (pH 9.0), 0.05 μmole of NAD^+ or $NADP^+$, 0.2 μmole of L-galactitol 1-phosphate, and limiting amounts of the enzyme solution to be measured.

D-Tagatose 6-Phosphate Kinase. Assays (0.15 ml) contained 10 μmoles of glycylglycine buffer (pH 8.0), 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.05 μmole of NADH, 0.5 μmole of PEP, 0.2 μmole of D-tagatose 6-phosphate; excess of pyruvate kinase and lactate dehydrogenase; and rate-limiting amounts of cell extract. Controls

were run without ATP, D-tagatose 6-phosphate, or both, to correct for L-galactitol 1-phosphate dehydrogenase, ATPase, and NADH oxidase, respectively.

D-Tagatose 1,6-Diphosphate Aldolase. The assay (0.15 ml) contained: 10 μmoles of Tris buffer (pH 7.5); 1.0 μmole of $MgCl_2$; 0.5 μmole of ATP; 0.05 μmole of NADH; 0.05 μmole of D-tagatose 6-phosphate; excess D-fructose 6-phosphate kinase, α-glycerolphosphate dehydrogenase and triose phosphate isomerase; and rate-limiting amounts of cell extract. Controls were run without kinase or D-tagatose 6-phosphate to correct for L-galactitol 1-phosphate dehydrogenase and NADH oxidase, respectively. The cuvettes were incubated at 30° for 5 min before addition of cell extract to allow phosphorylation of the D-tagatose 6-phosphate to D-tagatose 1,6-diphosphate.

Source of Materials. The following were obtained from Sigma Chemical Co.: galactitol; $NaBH_4$; D-galacturonic acid; D-mannitol; D-glucitol; DEAE-cellulose; glycylglycine; ATP; rabbit muscle pyruvate kinase and D-fructose 6-phosphate kinase; Tris (Trizma base); α-methylglucoside; quinacrine; and an α-glycerol phosphate dehydrogenase-triosephosphate isomerase mixture. NADH, PEP, NADP, and rabbit muscle lactic dehydrogenase were obtained from Calbiochem. The Folin and Ciocalteu phenol reagent were from Harleco. NAD^+ was supplied by P-L Biochemicals, Inc. The following reagents were obtained from Mallinckrodt Chemical Works: sodium tartrate; anhydrous sodium acetate; acetic anhydride; and Amberlite MB-3.

Difco Laboratories supplied the nutrient broth, and Sephadex G-25 was purchased from Pharmacia.

RESULTS

To show that the phosphorylation of galactitol with PEP is a metabolically important reaction, it was necessary to preclude other likely modifications of the galactitol molecule. These likely modifications of the free hexitol include phosphorylation with ATP, oxidation, or epimerization.

Absence of Galactitol Kinase, Galactitol Dehydrogenase, and Galactitol Epimerase Activities. To test for the presence of these activities, a culture of *K. pneumoniae* was grown overnight in mineral medium containing galactitol. The harvested, washed cells were sonically disrupted in 0.02 M Tris buffer (pH 7.5) or 0.02 M sodium phosphate buffer (pH 7.5). The cell extract was then passed through a Sephadex G-25 column, equilibrated with the same buffer, to remove low molecular-weight metabolites that would interfere with the assays. No activity could be detected for galactitol kinase (less than 0.003 unit/mg protein) or galactitol dehydrogenase (less than 0.0002 unit/mg protein). During the assay for a galactitol epimerase, large amounts of mannitol were formed in the incubations of up to two hours (87% of the peak area of galactitol at two hours). However, no glucitol was detected, and the amount of galactitol remained unchanged throughout the incubation. Thus, epimerase activity was not detectable (less than 0.0002 unit/mg protein). It seems

reasonable to conclude, therefore, that the kinase, dehydrogenase, and epimerase reactions do not play a part in the initiation of galactitol catabolism, and it seems very likely that PEP-dependent L-galactitol 1-phosphate formation is metabolically important.

Prediction of the Galactitol Pathway. From the presence of an L-galactitol 1-phosphate dehydrogenase, and by analogy to the catabolic pathways for D-mannitol and D-glucitol discussed in the Literature Review, one would expect that L-galactitol 1-phosphate would be oxidized to D-tagatose 6-phosphate. This molecule would be phosphorylated to D-tagatose 1,6-diphosphate, which would in turn be cleaved to yield dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. At this point, the triose phosphates would enter and continue in the normal route of glycolysis. Thus, the proposed enzymes would be L-galactitol 1-phosphate dehydrogenase, D-tagatose 6-phosphate kinase, and D-tagatose 1,6-diphosphate aldolase.

Detection of the Pathway Enzymes. Assay of L-galactitol 1-phosphate dehydrogenase in the cell extracts of cells grown on galactitol proved difficult because of the high levels of NADH oxidase. The results of assays using NAD^+ were inconsistent, while no reduction of $NADP^+$ was observed. Similarly, attempts to use D-tagatose 6-phosphate and NADH as substrates were also hampered by the masking activity of NADH oxidase. The use of the flavine analog, quinacrine, at 0.6 mM, almost totally (98%) inhibited the oxidase, but also quantitatively inhibited the dehydrogenase. It was then found that if the cell extract was passed through a

DEAE-cellulose column, equilibrated in 0.02 M potassium phosphate buffer at pH 7.5, the NADH oxidase would pass through the column, while the dehydrogenase was adsorbed. Elution of the protein with 0.5 M KCl in the same buffer resulted in a preparation of the dehydrogenase free of NADH oxidase. The results of assays with this fraction can be seen in Table 1. It is apparent that the reaction is dependent on L-galactitol 1-phosphate, and is specific for NAD^+. It also appears that the concentrations of NAD^+ used are less than saturating, as judged by its stimulatory effect at higher concentrations.

Table 1. Oxidation of L-galactitol 1-phosphate to D-tagatose 6-phosphate

Reaction mixture	Units/mg protein[b]
Complete[a]	0.00037
Minus L-galactitol 1-phosphate	0.00000
Minus NAD^+	0.00000
Minus NAD^+, plus $NADP^+$	0.00000
Complete (0.1 µmole NAD^+)	0.00080
Complete (0.15 µmole NAD^+)	0.00134

[a]The complete reaction mixture contained 10 µmoles of Tris buffer (pH 9.0), 1.0 µmole of $MgCl_2$, 0.05 µmole of NAD^+, and 0.2 µmole of L-galactitol 1-phosphate in a volume of 0.15 ml.

[b]1 unit = 1 µmole product produced per min at 30°.

D-Tagatose 6-phosphate kinase was found to have a specific activity of 0.013 unit/mg protein. Again, the competing reactions of NADH oxidase, ATPase, and L-galactitol 1-phosphate dehydrogenase require the activity to be measured above a background activity, thereby diminishing the accuracy of the determination.

The specific activity of D-tagatose 1,6-diphosphate aldolase was calculated to be 0.066 unit/mg protein (Table 2). As for the

Table 2. Cleavage of D-tagatose 1,6-diphosphate to triose phosphates

Reaction mixture	Units/mg protein[b]
Complete	0.249[c]
Minus kinase[a]	0.222[d]
Minus kinase,[a] minus D-tagatose 6-phosphate	0.183[e]
Minus D-tagatose 6-phosphate	0.185[e]
Minus cell extract	0.000

[a]Minus D-fructose 6-phosphate kinase.

[b]1 unit = 1 μmole of substrate cleaved per min at 30°.

[c]D-Tagatose 1,6-diphosphate aldolase activity (0.066 units/mg) equals the total activity (0.249 units/mg) minus the activity of NADH oxidase (0.183 units/mg).

[d]Combined NADH oxidase and L-galactitol 1-phosphate dehydrogenase activities. L-Galactitol 1-phosphate dehydrogenase (0.037 units/mg) equals the combined activity (0.222 units/mg) minus the activity of NADH oxidase (0.185 units/mg).

[e]Due to NADH oxidase activity.

other two enzymes, the competing activity was very high in this case also. These data also indicate a value of 0.037 unit/mg protein for the reduction of D-tagatose 6-phosphate with NADH; this value is much higher than that reported above (Table 1) with L-galactitol 1-phosphate and NAD^+ as substrates.

DISCUSSION

The data of this section have suggested that free galactitol is not modified by a kinase, dehydrogenase, or epimerase, as these three activities could not be detected in the cell extract. The threshold for detection of the galactitol kinase is the highest of the three because of the competing activities. The limits of detection for the galactitol dehydrogenase and epimerase were ten-fold more sensitive, making it more certain that these activities are not present in the cell extract. Alpha-methylglucoside was used as an internal standard for the epimerase assay since the only modification that can take place is phosphorylation by PEP-phosphotransferase (55,66), and a Sephadex G-25-filtered extract would not have had this capability. Thus, the amount of α-methylglucoside would remain constant during the incubation, allowing comparison of the amount of galactitol relative to this standard. It is assumed that the formation of large amounts of mannitol in the Sephadex G-25-filtered cell extract was due to the breakdown of some polysaccharide.

Thus, it seems probable that the first step in catabolism is phosphorylation with PEP as reported by Shimamoto (59). The product has been unequivocally identified as L-galactitol 1-phosphate. A pathway for the further metabolism of this compound is now proposed (Figure 2). In this proposal, L-galactitol 1-phosphate is oxidized by a dehydrogenase, with NAD^+ as cofactor, to produce D-tagatose

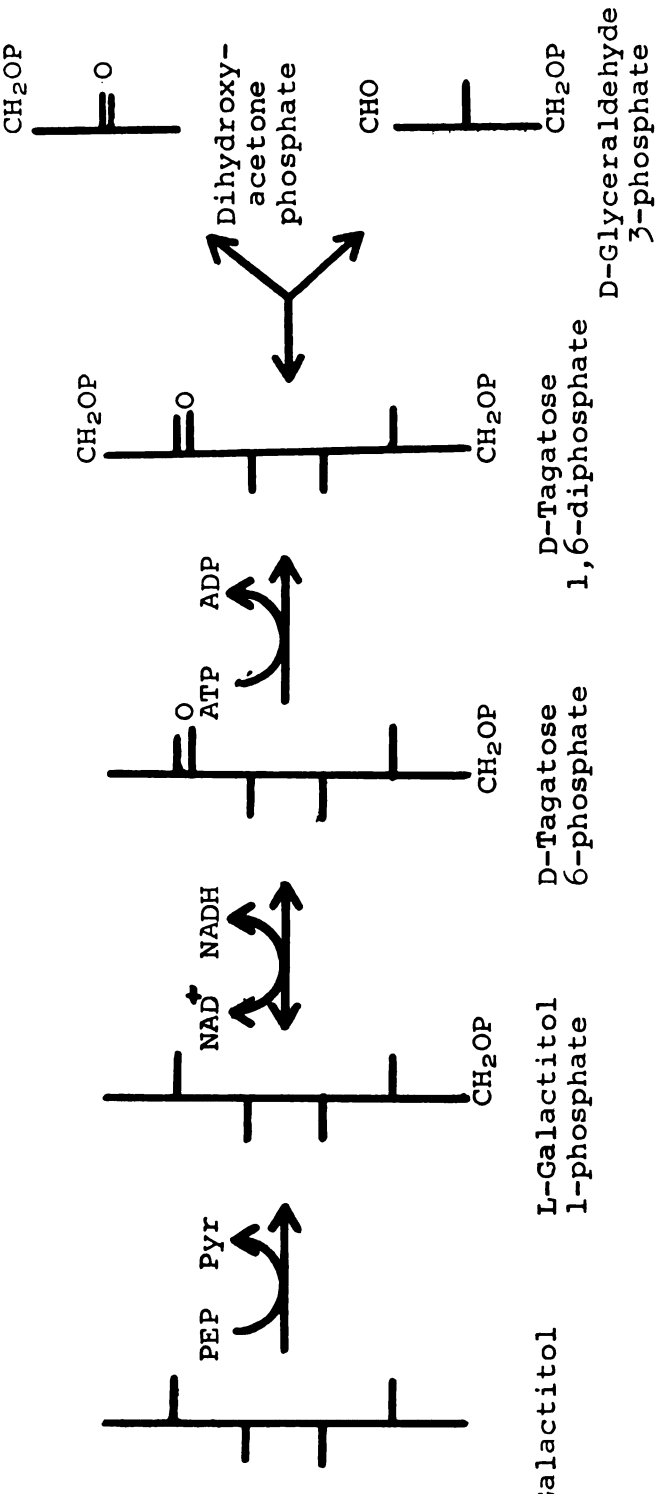


Figure 2. Proposed pathway for galactitol catabolism in *Klebsiella pneumoniae*.

6-phosphate. The D-tagatose 6-phosphate is then phosphorylated by ATP, in a reaction catalyzed by a kinase, to yield D-tagatose 1,6-diphosphate. The diphosphate is then cleaved to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate by an aldolase. This pathway is analogous to the catabolism of D-mannitol and D-glucitol discussed in the Literature Review (Figure 1). It should be noted that the last two steps of this proposed pathway are identical to part of the D-tagatose 6-phosphate pathway of D-galactose metabolism found in other organisms (61,62). Although *K. pneumoniae* does not use the D-tagatose 6-phosphate pathway for the catabolism of lactose and D-galactose (62), cells grown on galactitol have not been previously assayed for the enzymes of this pathway.

The specific activities of the enzymes may be low, especially for the dehydrogenase as measured with L-galactitol 1-phosphate and NAD^+, but it is doubtful that optimal conditions for assaying the enzymes were met by the arbitrary concentrations used in these exploratory experiments. Optimal conditions would best be established with partially purified enzymes. As will be shown in Sections 2 and 3, use of optimal assay conditions greatly increases the specific activity of these activities in cell extracts. The absence of D-tagatose 1,6-diphosphate aldolase in cells of *K. pneumoniae* grown on lactose, D-galactose, or D-glucose (62) could be explained if the enzymes of this pathway are specifically induced by growth on galactitol.

SECTION 2

PURIFICATION AND CHARACTERIZATION OF THE ENZYMES INVOLVED IN THE CATABOLISM OF L-GALACTITOL 1-PHOSPHATE

INTRODUCTION

As was pointed out in the Literature Review, it has been established that in *K. pneumoniae* galactitol is phosphorylated by PEP to form L-galactitol 1-phosphate. Section 1 of this dissertation has shown that cell extracts of this organism grown on galactitol lack kinase, dehydrogenase, or epimerase activities for the free hexitol. These data were interpreted to mean that formation of L-galactitol 1-phosphate was the probable first step in the catabolism of galactitol. Furthermore, preliminary evidence was presented for the presence of L-galactitol 1-phosphate dehydrogenase, D-tagatose 6-phosphate kinase, and D-tagatose 1,6-diphosphate aldolase activities in these extracts. Based on the presence of these activities, a pathway for the conversion of L-galactitol 1-phosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate was proposed.

The purpose of this section is to provide further evidence in support of the proposed pathway. The proteins catalyzing the individual reactions were purified, and their properties examined. The products of each reaction were also identified and shown to be consistent with the proposed pathway. All relevant data are in agreement with these enzymes carrying out such reactions *in vitro*.

MATERIALS AND METHODS

All materials and methods not described here were presented in Section 1.

Growth Conditions. Cells were grown as in Section 1, except that the volume was increased to 1 liter.

Determination of Substrate Concentration. For most assays the concentration of substrate was determined on a weight basis. For assays such as K_m determinations, where the substrate concentration is critical, the concentrations of L-galactitol 1-phosphate, D-tagatose 6-phosphate, D-tagatose 1,6-diphosphate, D-fructose 6-phosphate, D-fructose 1,6-diphosphate, and NAD^+ were determined using an appropriate NAD^+-linked end-point assay. The concentration of NADH was determined by its absorbance at 340 nm.

L-Galactitol 1-Phosphate Dehydrogenase Assays. The assays (0.15 ml) contained: 10 µmoles of Tris buffer (pH 8.5); 1.0 µmole of NAD^+; 0.01 µmole of $MnCl_2$; 0.45 µmole of L-galactitol 1-phosphate; and rate-limiting amounts of L-galactitol 1-phosphate dehydrogenase. The reverse reaction was measured in a volume of 0.15 ml containing 10 µmoles of Tris buffer (pH 7.75), 0.01 µmole of $MnCl_2$; 0.1 µmole of D-tagatose 6-phosphate, 0.05 µmole of NADH, and rate-limiting amounts of L-galactitol 1-phosphate dehydrogenase.

D-Fructose 6-Phosphate Kinase Assays. The standard assays used were developed by Sapico and Anderson (67). Each assay (0.15 ml) contained: 10 μmoles of glycylglycine buffer (pH 8.0); 0.5 μmole of ATP; 1.0 μmole of $MgCl_2$; 0.05 μmole NADH; 1.0 μmole of D-fructose 6-phosphate; excess D-fructose 1,6-diphosphate aldolase, triose phosphate isomerase, and α-glycerolphosphate dehydrogenase; and rate-limiting amounts of D-fructose 6-phosphate kinase. The control contained no ATP.

To show the competition between D-tagatose 6-phosphate and D-fructose 6-phosphate, the following assay (0.15 ml) was used: 10 μmoles of glycylglycine buffer (pH 8.0), 0.05 μmoles of NADH, 1.0 μmole of $MgCl_2$, 0.5 μmole of ATP, 0.5 μmole of PEP, non-limiting amounts of coupling enzymes (D-fructose 1,6-diphosphate aldolase, α-glycerolphosphate dehydrogenase, triose phosphate isomerase, and pyruvate kinase), 0.8 μmole of D-tagatose 6-phosphate, 4.26 μmoles of D-fructose 6-phosphate, and rate-limiting amounts of the kinase.

D-Tagatose 1,6-Diphosphate Aldolase Assays. The assays (0.15 ml) contained: 10 μmoles of glycylglycine buffer (pH 8.0); 0.05 μmole of NADH; 1.0 μmole of $MgCl_2$; 0.5 μmole of ATP; 0.2 μmole of D-tagatose 6-phosphate; non-limiting amounts of D-fructose 6-phosphate kinase from rabbit muscle, triose phosphate isomerase, and α-glycerolphosphate dehydrogenase; and rate-limiting amounts of D-tagatose 1,6-diphosphate aldolase. The cuvettes were incubated at 30° for 5 min before the addition of the aldolase so that the ATP and rabbit muscle D-fructose 6-phosphate kinase could quantitatively convert the D-tagatose 6-phosphate to D-tagatose

1,6-diphosphate (68). When assayed with D-fructose 1,6-diphosphate as the substrate, 0.3 μmole of that compound was added to the assay, and the ATP, $MgCl_2$, and rabbit muscle aldolase were omitted.

D-Tagatose 6-Phosphate Kinase Assays. The standard assays (0.15 ml) contained: 10 μmoles of glycylglycine buffer (pH 8.0); 0.05 μmoles of NADH; 1.0 μmole of $MgCl_2$; 0.5 μmoles of ATP; 0.2 μmole D-tagatose 6-phosphate; 0.075 μmole of ADP; 5 μmoles of KCl; 0.03 μmole of $CoCl_2$; excess triose phosphate isomerase, α-glycerolphosphate dehydrogenase, and D-tagatose 1,6-diphosphate aldolase; and rate-limiting amounts of D-tagatose 6-phosphate kinase.

Alkaline Phosphatase Assays. The assays were as previously described (69), except that they were scaled down to a volume of 0.15 ml.

Glucose 6-Phosphate Dehydrogenase Assays. The assays were as previously reported (70), except that the volumes were scaled down to 0.15 ml.

Lactate Dehydrogenase Assays. The assays were as previously reported (71), except that the volumes were scaled down to 0.15 ml.

Pyruvate Kinase Assays. The assays were as reported (72), except that the volumes were scaled down to 0.15 ml.

D-Fructose 1-Phosphate Kinase Assays. The assays were as described previously (67) with the addition of 5.0 μmoles of KCl (73).

D-Fructose 1,6-Diphosphate Aldolase Assays. The assays were the same as for D-tagatose 1,6-diphosphate aldolase when assayed with D-fructose 1,6-diphosphate.

Ketohexose Determination. Ketohexoses were determined by the method of Roe (74) as described by Ashwell (75). D-Fructose, D-fructose 6-phosphate, or D-fructose 1,6-diphosphate was used as a standard, depending on whether the compound being measured was a free ketohexose, or its mono- or diphosphate derivative.

Phosphate Determination. Inorganic phosphate was determined by a modification of the Fiske-SubbaRow procedure (76) as described by Clark (77). For total phosphate analysis, the samples were hydrolyzed overnight in 1 M HCl at 100° and assayed as above.

Bentonite Treatment. The enzyme solution was stirred in an ice-water bath as the proper amount of bentonite was added slowly. After 10 min, the solution was centrifuged at 12,000 x g, the supernatant decanted and the pelleted material discarded.

Ammonium Sulfate Precipitation. The enzyme solution was stirred at 0° as the desired amount of enzyme-grade ammonium sulfate was slowly added to bring the concentration to the desired percent of saturation (78). After the ammonium sulfate had dissolved, the solution was stirred for an additional 15 min, and then centrifuged at 12,000 x g for 15 min. If the supernatant was to be treated with additional ammonium sulfate, the above procedure was repeated. Precipitated protein containing the desired enzyme activity was dissolved in buffer following centrifugation.

Sephadex G-25 Chromatography. The gel was swollen by soaking in water for 24 hours. When not in use, the gel was stored in 0.02% (w/v) sodium azide at 4°. After the gel had been poured, the column was washed with 4 volumes of the proper buffer. After use, the column was washed with 0.02% sodium azide for storage stability. Sample volumes were usually 5-15% of the bed volume.

Sephadex G-150 Chromatography. The gel was swollen in water for 2 days, and then heated on a steam bath for 12 hours. The gel was stored, and columns washed, as with Sephadex G-25. Sample volumes did not exceed 5% of the bed volume for preparative work or 2% of the bed volume for molecular weight determinations.

Hydroxyapatite Adsorption. The powdered form of hydroxyapatite was suspended in 0.25 M sodium phosphate buffer (pH 7.5) and left at room temperature overnight. The hydroxyapatite was then washed exhaustively with water by alternate centrifugation and resuspension. Finally the pelleted mineral was suspended in the enzyme solution containing not more than 5 mM phosphate buffer (pH 8.0). After 10 min, the hydroxyapatite was removed by centrifugation. Phosphate buffer (0.1 M, pH 8.0) was used in an attempt to elute enzyme activity from the hydroxyapatite.

Alumina Cγ Adsorption. Aged alumina gel Cγ was washed extensively with water by alternate suspension and centrifugation. It was then used to treat an enzyme solution as described for hydroxyapatite.

Hexokinase Assays. The assays were as described previously (79), except that all amounts were scaled down to a volume of 0.15 ml.

Protein Assays. Protein concentrations of greater than 0.5 mg/ml were determined by the method of Lowry et al. (63). Protein concentrations of less than 0.5 mg/ml were determined by absorbance at 220 nm (80). Bovine serum albumin was used as a standard with both methods.

Preparation of NAD^+- or AMP-Sepharose. Sepharose 4-B was activated with cyanogen bromide as described by Cuatrecasas and Anfinsen (81). The activated Sepharose was then reacted with adipic dihydrazide (82). The ribosyl moiety of the nucleotide was oxidatively cleaved, and attached to the adipic dihydrazide-Sepharose (82). Coupling was confirmed with the 2,4,6-trinitrobenzene sulfonate test (81).

Dialysis. Dialysis tubing was prepared by boiling in a 0.2% (w/v) solution of EDTA for 15 min. The tubing was then thoroughly washed with distilled water. Dialysis was performed overnight at 4°, against at least 100 volumes of the appropriate buffer.

Polyacrylamide Gel Electrophoresis. Electrophoresis on 7.5% polyacrylamide gels at pH 8.9 was performed as reported by Gabriel (83). Following electrophoresis, the protein bands were stained with Coomassie blue (84).

Paper Chromatography. Descending paper chromatography was done in a water-saturated phenol solvent (85) with Whatman No. 1 paper (23 x 56 cm). The paper was soaked in 0.1 M citric acid and allowed to dry before use. Glycerol was used as a marker for this system because it appears as a white spot, and migrates faster than the ketohexoses. After the run, the paper was dried at room temperature, and the chromatograms developed for ketohexoses using an orcinol spray (86) or for total carbohydrates with a silver nitrate spray (87).

Ketohexose Differentiation. The four different 2-ketohexoses were distinguished from one another by the cysteine-H_2SO_4 reaction. Sorbose was identified by its spectrum after reacting for 20 hours (88), while fructose, tagatose, and psicose were distinguished by the rate of color development (89).

Effect of $NaBH_4$. Solutions of aldolase, in the presence and absence of the ketohexose diphosphate substrates, were maintained at pH 6.0 by adding 2 M acetic acid during the addition of $NaBH_4$ at 0°. Samples were periodically removed for assay. The levels of $NaBH_4$ in the enzyme solution did not affect the assays.

Effect of NEM. Thiol protectants were removed from the solution by chromatography on a Sephadex G-25 column equilibrated with the proper buffer. The enzyme was treated with NEM for 30 min at 20°. The small amount of NEM in the enzyme solution did not affect the assay.

Dephosphorylation of Sugar Phosphates. Sugar phosphates were dephosphorylated by the method of Bissett (64). The sugar phosphate was combined with Sephadex G-25 filtered wheat germ acid phosphatase in 80 mM sodium acetate buffer (pH 4.6) at 25°. The sample was then desalted by passage through a mixed-resin column of Dowex 50W-X8 (H^+) and Amberlite IR 45 (OH^-); the resins were present in a roughly 1:1 proportion of basic to acidic exchange capacities.

Monovalent Cation Activation. Monovalent cations were removed from substrates and cofactors by treatment with Dowex 50W-X8 (H^+) and neutralization with cyclohexylamine (this must be done very rapidly with NADH because of its acid lability). Enzyme solutions were chromatographed on Sephadex G-25 columns equilibrated with Tris or cyclohexylammonium-neutralized phosphate buffers.

Divalent Cation Activation. To remove divalent cations, enzyme solutions were treated with 10 mM EDTA for 15 min at room temperature, and passed over a column of Sephadex G-25 which had been equilibrated with the proper divalent cation-free buffer. A similar amount of untreated enzyme solution was then filtered through Sephadex G-25 to act as a control for specific activity determination.

Determination of Dehydrogenase Equilibrium Constant. The dehydrogenase equilibrium constant was determined by placing known amounts of L-galactitol 1-phosphate and NAD^+ into cuvettes with the standard assay components and 0.01 unit of L-galactitol 1-phosphate dehydrogenase at 30°. The progress of the reaction was continually monitored at 340 nm. When the reaction had reached completion,

the amount of NADH formed was calculated from the increase in absorbance. As will be seen later in Section 2, the oxidation of L-galactitol 1-phosphate is coupled to the reduction of NAD^+ with a reaction stoichiometry of 1:1. Since the amount of D-tagatose 6-phosphate formed equaled the NADH, these values could be subtracted from the initial concentrations of the substrates, giving the final concentrations of NAD^+ and L-galactitol 1-phosphate. Determinations were made with three different initial amounts of substrates at each of two pH values. The pH of the assay mixtures was checked after each of the determinations to make sure that it had not changed.

Source of Materials. The following were purchased from Sigma Chemical Co.: D-fructose 6-phosphate; D-fructose 1,6-diphosphate; D-galactose 6-phosphate; *E. coli* alkaline phosphatase; D-glucose 6-phosphate; D-fructose 1-phosphate; yeast hexokinase; alumina Cγ gel; 2,4,6-trinitrobenzene sulfonate; Coomassie brilliant blue; orcinol; cysteine; DTT; DTE; BME; AMP; HEPES; ITP; GTP; and diethylacetyl-D-glyceraldehyde 3-phosphate. Resorcinol and sodium meta periodate were purchased from Mallinckrodt Chemical Works. Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann, while NADPH and Sephadex G-150 were purchased from P-L Biochemicals and Pharmacia, respectively. The acrylamide, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine used for electrophoresis were purchased from Canalco, Inc. Paper used for chromatography was Whatman grade 1. Adipic dihydrazide and cyclohexylamine were purchased from Eastman Organic Chemicals. The following were

purchased from the indicated sources: cyanogen bromide, Matheson, Coleman, and Bell; D-mannose 6-phosphate, Nutritional Biochemicals Corp.; rabbit muscle D-fructose 1,6-diphosphate aldolase, Calbiochem; wheat germ acid phosphatase, Worthington Biochemical Corp.; bentonite, Fischer Scientific Co.; hydroxyapatite HTP, Bio Rad Laboratories. The following compounds were donated by the indicated person: D-tagatose, Dr. H. Lardy; L-sorbose 1-phosphate, Dr. Ronald Simkins; and D-mannitol 1-phosphate and D-glucitol 6-phosphate, G. T. Shimamoto. D-Fructose 1-phosphate kinase was purified as described by Sapico and Anderson (67).

RESULTS

A. L-Galactitol 1-Phosphate Dehydrogenase (L-Galactitol 1-Phosphate:NAD^+ Oxidoreductase)

Purification. During the course of purification of L-galactitol 1-phosphate dehydrogenase, some techniques, methods, or conditions were found to be ineffective or counterproductive. Substantial losses of activity were found when the enzyme was stored overnight in buffers with a pH less than 8.0. Acid precipitation of the activity at pH values less than 6.0 resulted in irrecoverable loss of activity. The activity was not adsorbed to hydroxyapatite and alumina gel in 5 mM sodium phosphate buffer (pH 8.0) or Tris buffer (pH 8.5). Addition of bentonite caused loss of activity at high concentrations, and only slight purification at low concentrations. The activity failed to be adsorbed to, or be retarded on, columns of NAD^+-Sepharose or AMP-Sepharose in 20 mM Tris buffer (pH 8.0 or 9.0) in the presence or absence of 15 mM L-galactitol 1-phosphate.

Cell extract. The washed cells from 1 liter of galactitol-grown *K. pneumoniae* were suspended in 0.025 M Tris buffer (pH 8.5) containing 10% (v/v) glycerol and 0.5 mM DTE. The cell extract was prepared by sonication. All purification steps were carried out at 0-4°. A summary of the purification is found in Table 3.

Table 3. Purification of L-galactitol 1-phosphate dehydrogenase

Fraction	Volume[a]	Total protein[b]	Total units of activity[c]	Units / mg protein	Recovery[d]
Cell extract	21.8	296	15.9	0.053	100
DEAE-cellulose	28	35	11.8	0.338	74
Ammonium sulfate	2.2	16.3	9.85	0.605	62
Sephadex G-150	7.7	2.4	3.26	1.37	20

[a]Volume is in ml.

[b]Protein is in mg.

[c]1 unit = 1 μmole of product formed per min at 30°.

[d]Recovery is in %.

DEAE-cellulose chromatography. The cell extract was loaded onto a 1.2 x 6 cm DEAE-cellulose column that had been equilibrated with 0.025 M Tris buffer (pH 8.5) containing 10% (v/v) glycerol and 0.5 mM DTE. A 200 ml linear gradient of 0-0.35 M KCl in the above buffer was used to elute the protein from the column (Figure 3). Fractions of 2 ml were collected. Fractions 28 to 43, containing the peak of the activity, were pooled for further purification.

Ammonium sulfate precipitation. To the 28 ml of the pooled activity was slowly added 5.38 g of ammonium sulfate to bring the concentration to 35% of saturation. The solution was stirred for 15 min, and then centrifuged at 12,000 x g for 10 min. The 30 ml of supernatant was decanted, and 6.6 g more of ammonium sulfate added to bring the concentration to 70% saturation. After 15 min, the solution was centrifuged at 12,000 x g for 10 min. The supernatant was discarded, and the pelleted material dissolved in 2 ml of 0.025 M Tris buffer containing 10% (v/v) glycerol and 0.5 mM DTE.

Sephadex G-150 chromatography. The 35-70% ammonium-sulfate-precipitated protein was layered onto a 1.4 x 82 cm column of Sephadex G-150 equilibrated with 0.025 M Tris (pH 8.5) containing 10% (v/v) glycerol and 0.5 mM DTE. The column was eluted with the same buffer. Fractions of 1 ml were collected. Fractions 55-60 (Figure 4) containing the peak of the activity were pooled. This solution of L-galactitol 1-phosphate dehydrogenase was 25-fold purified, and contained 20% of the initial activity. D-Tagatose

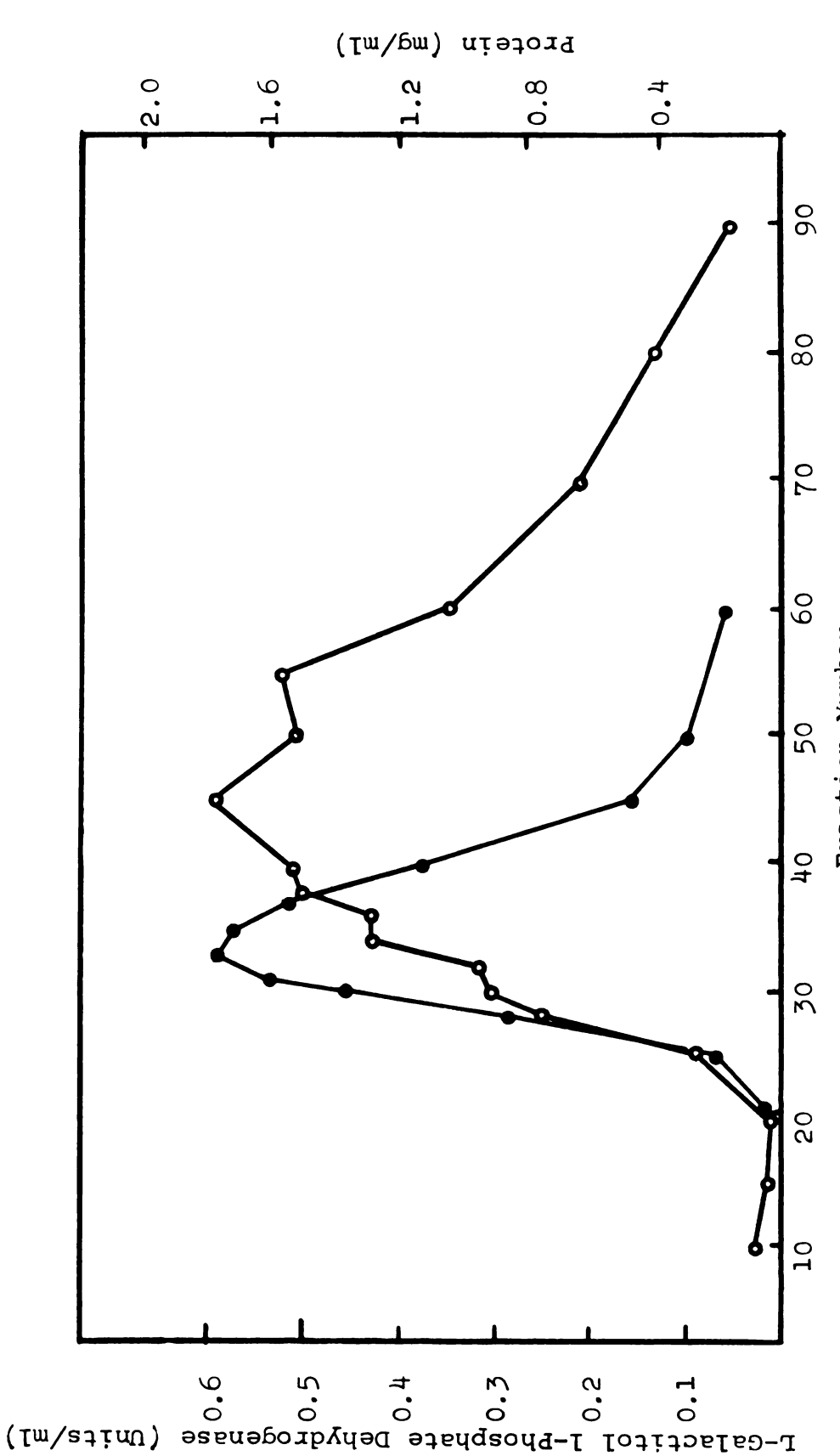


Figure 3. Chromatography of L-galactitol 1-phosphate dehydrogenase on DEAE-Cellulose. Symbols: (●) L-galactitol 1-phosphate dehydrogenase, (○) protein. Fraction volume is 2 ml.

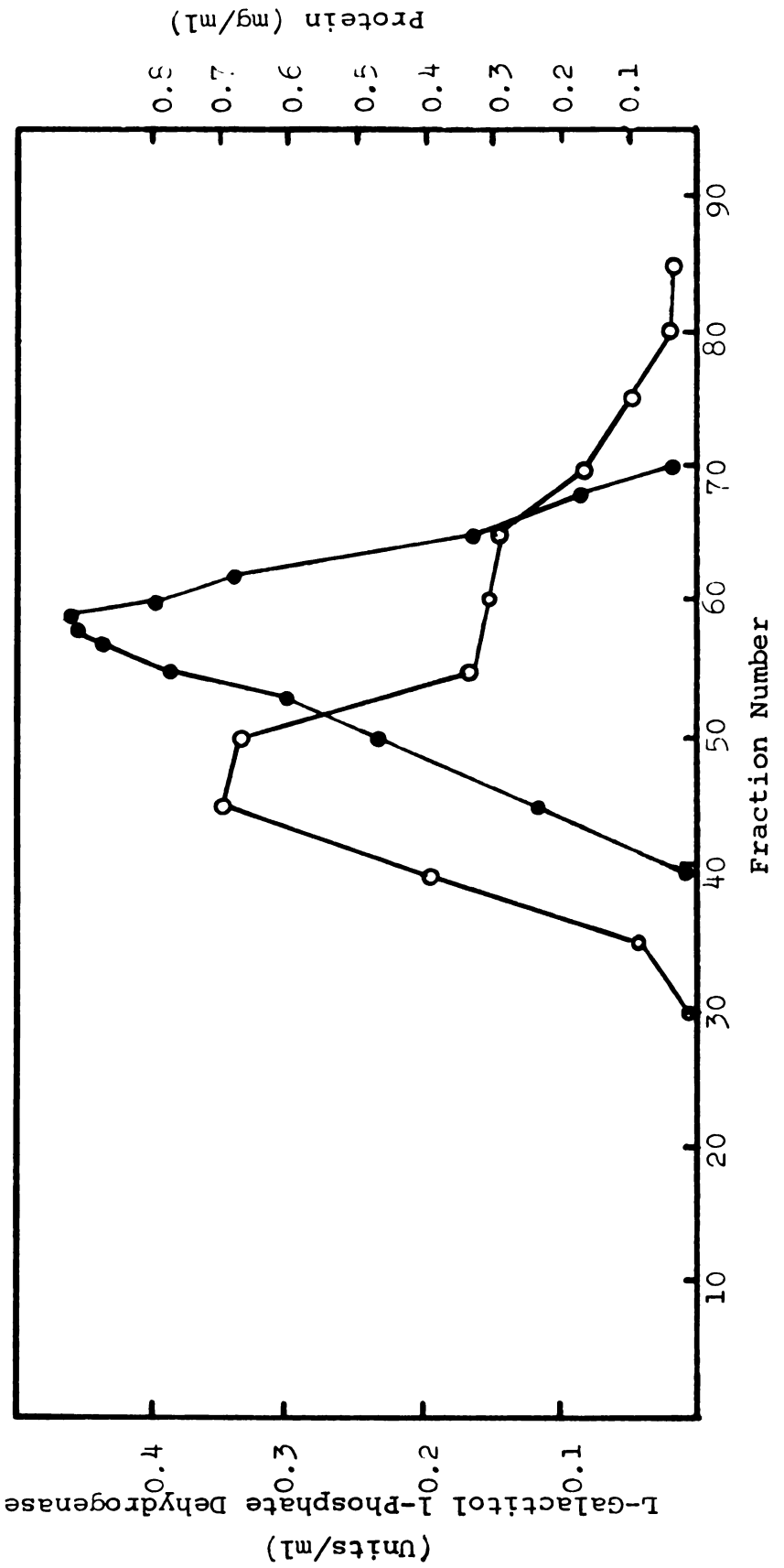


Figure 4. Chromatography of L-galactitol 1-phosphate dehydrogenase on Sephadex G-150. Symbols: (●) L-galactitol 1-phosphate dehydrogenase, (○) protein. Fraction volume is 1 ml.

6-phosphate kinase or D-tagatose 1,6-diphosphate aldolase could not be detected (less than 0.005 unit/ml).

Properties

Stability. The dehydrogenase was found to retain most of its activity when stored at 0° or at -20° for at least two weeks. The presence of glycerol and DTE were found to be essential for good recovery (greater than 85%) of activity for periods of time as short as 12 hours.

pH optima. The pH optimum for the dehydrogenase was measured for both the oxidation and reduction of sugar phosphate (Figure 5). The optimum for the reaction using L-galactitol 1-phosphate and NAD^+ as substrates was pH 8.75, while the optimum with D-tagatose 6-phosphate and NADH as substrates was pH 7.75. In both cases, Tris buffer gave higher activity than glycylglycine buffer at all pH values tested.

Substrate specificity. L-Galactitol 1-phosphate and D-tagatose 6-phosphate were the only compounds found to serve as substrates for the dehydrogenase. The following compounds were tested as substrates for the dehydrogenase at concentrations of 10 mM, with NAD^+ as the cofactor, with no activity (<0.0006 unit/mg) detectable: D-glucose 6-phosphate, D-mannitol 1-phosphate, D-glucitol 6-phosphate, D-fructose 1-phosphate, and D-galactose 6-phosphate. Similarly, the following compounds were tested, using NADH as cofactor, without success: D-galactose 6-phosphate,

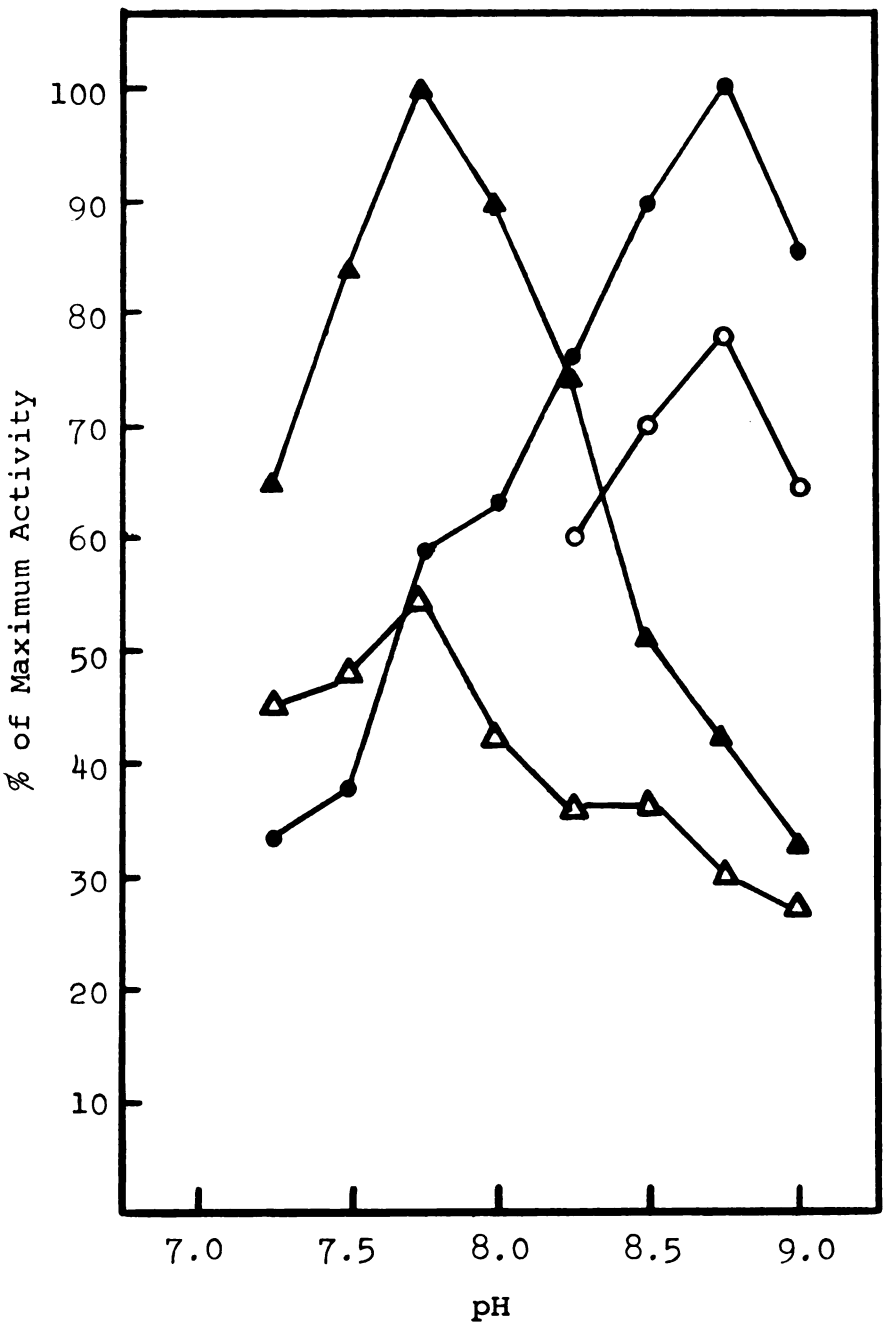


Figure 5. pH optimum of L-galactitol 1-phosphate dehydrogenase. Symbols: (●) Tris and (○) glycylglycine with L-galactitol 1-phosphate and NAD as substrates; and (▲) Tris and (△) glycylglycine with D-tagatose 6-phosphate and NADH as substrates. The maximum specific activities obtained were 1.66 units/mg protein with L-galactitol 1-phosphate as substrate, and 0.41 units/mg protein with D-tagatose 6-phosphate as substrate.

D-glucose 6-phosphate, D-fructose 1-phosphate, D-fructose 1,6-diphosphate, D-fructose 6-phosphate, and L-sorbose 1-phosphate.

All attempts to detect activity using $NADP^+$ (6.7 mM) or NADPH (0.33 mM) as the pyridine nucleotide cofactor failed.

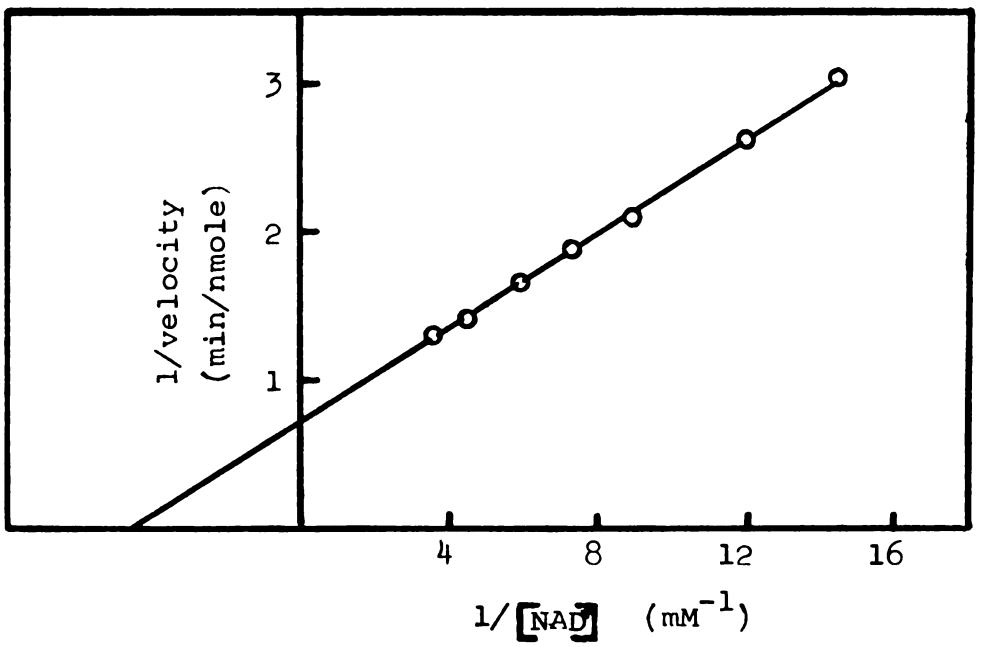
Cation activation. The dehydrogenase was assayed in the presence of the following monovalent cations at a final concentration of 33 mM: NaCl, KCl, NH_4Cl, LiCl, RbCl, and CsCl. None of the cations caused a significant increase or decrease in the enzyme activity. On the other hand, divalent cations were shown to have a great effect on the activity of EDTA-treated dehydrogenase (Table 4). Without added divalent cations, the enzyme had no detectable activity. The cations causing the greatest restoration of activity were Cd^{++}, Mn^{++}, and Co^{++}. The restoration of 36% of the control activity by 6.7×10^{-5} M $MnCl_2$ took place 15 min after the Sephadex G-25 filtration, but if the same concentrations of $MnCl_2$ were added after 2.5 hours, only 4% of the control activity was restored. The enzyme is apparently destabilized by the EDTA treatment and subsequent storage in divalent cation-free buffer.

Kinetic constants. The concentrations of L-galactitol 1-phosphate and NAD^+ were varied, each in the presence of saturating amounts of the other. The K_m values obtained were 0.22 mM for NAD^+ and 0.42 mM for L-galactitol 1-phosphate (Figure 6). When the experiment was carried out with D-tagatose 6-phosphate and NADH, the K_m values were 0.10 mM and 0.025 mM, respectively (Figure 7).

Table 4. Divalent cation activation of EDTA-treated L-galactitol 1-phosphate dehydrogenase

Divalent cation	Specific activity (% of control)		
	6.7×10^{-6} M	6.7×10^{-5} M	6.7×10^{-4} M
$CdSO_4$	3	42	53
$MnCl_2$	1	36	41
$CoCl_2$	ND	30	41
$ZnCl_2$	ND	15	14
$NiCl_2$	ND	ND	2
$MgCl_2$	ND	ND	4
$BaCl_2$	ND	ND	2
$CaCl_2$	ND	2	14
$CuSO_4$	ND	ND	ND
$FeSO_4$	ND	ND	ND

N.D.: The activity was not detectable (less than 1% of the control).



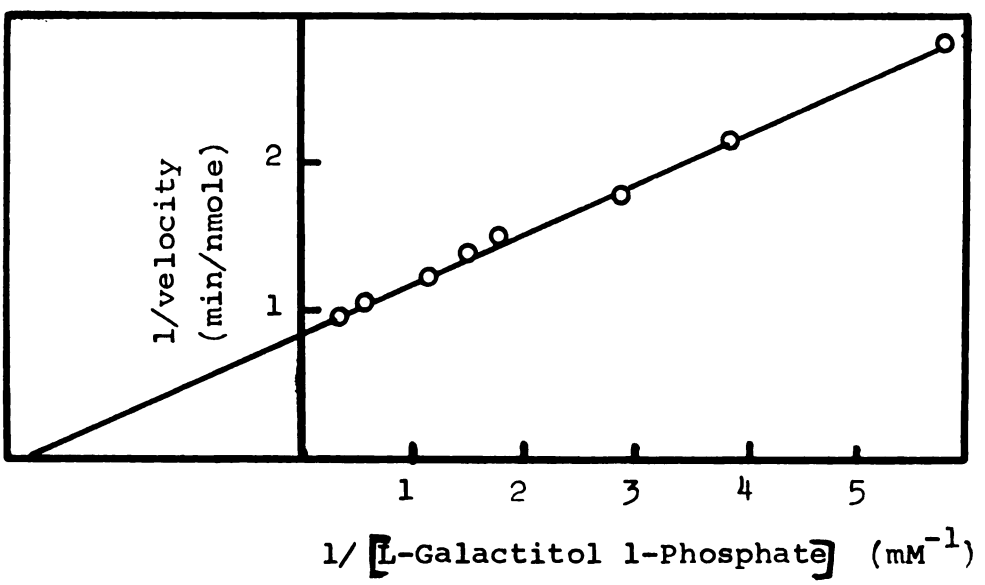


Figure 6. Lineweaver-Burk plots of L-galactitol 1-phosphate dehydrogenase with L-galactitol 1-phosphate and NAD^+ as substrates.

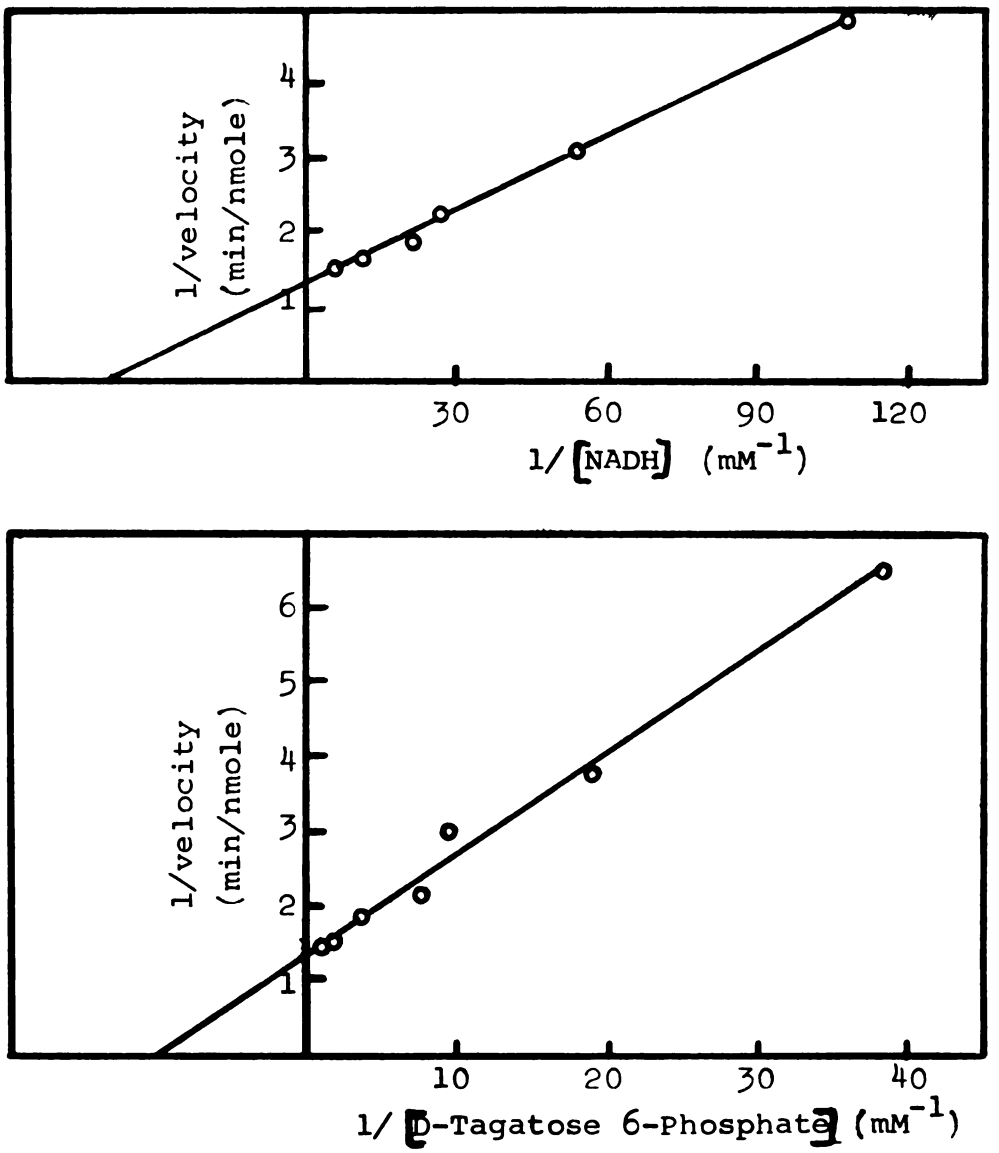


Figure 7. Lineweaver-Burk plots of L-galactitol 1-phosphate dehydrogenase with D-tagatose 6-phosphate and NADH as substrates.

Molecular weight determination. The molecular weight of the dehydrogenase was determined by chromatography on a 1.45 x 80 cm column of Sephadex G-150, with standard proteins of known molecular weight. Fractions of 1 ml were collected. The standard proteins and their molecular weights are: rabbit muscle D-fructose 1,6-diphosphate aldolase, 150,000 (90); yeast D-glucose 6-phosphate dehydrogenase, 128,000 (90); *E. coli* alkaline phosphatase, 86,000 (91); and yeast hexokinase, 51,000 (92). When the fraction containing the peak of activity for each protein was plotted against the log of the molecular weight, a linear relationship was observed (Figure 8). The peak of the dehydrogenase eluted concidently with the peak of alkaline phosphatase, indicating a molecular weight of 86,000. This method of molecular weight estimation assumes a spherical shape for the protein molecule.

Reaction Characteristics

Product identification. The product of the oxidation of L-galactitol 1-phosphate was identified as D-tagatose 6-phosphate by Shimamoto (59), using L-galactitol 1-phosphate dehydrogenase supplied by this investigator. The product was identified by means of gas-liquid chromatography of the trimethylsilyl derivative before and after dephosphorylation, acid lability, ketohexose to phosphate ratio, periodate oxidation, and enantiomeric configuration.

Stoichiometry. For each mole of L-galactitol 1-phosphate, as measured by the organic phosphate assay, 0.94 moles of NAD^+ was reduced to NADH when the reaction was allowed to run to completion

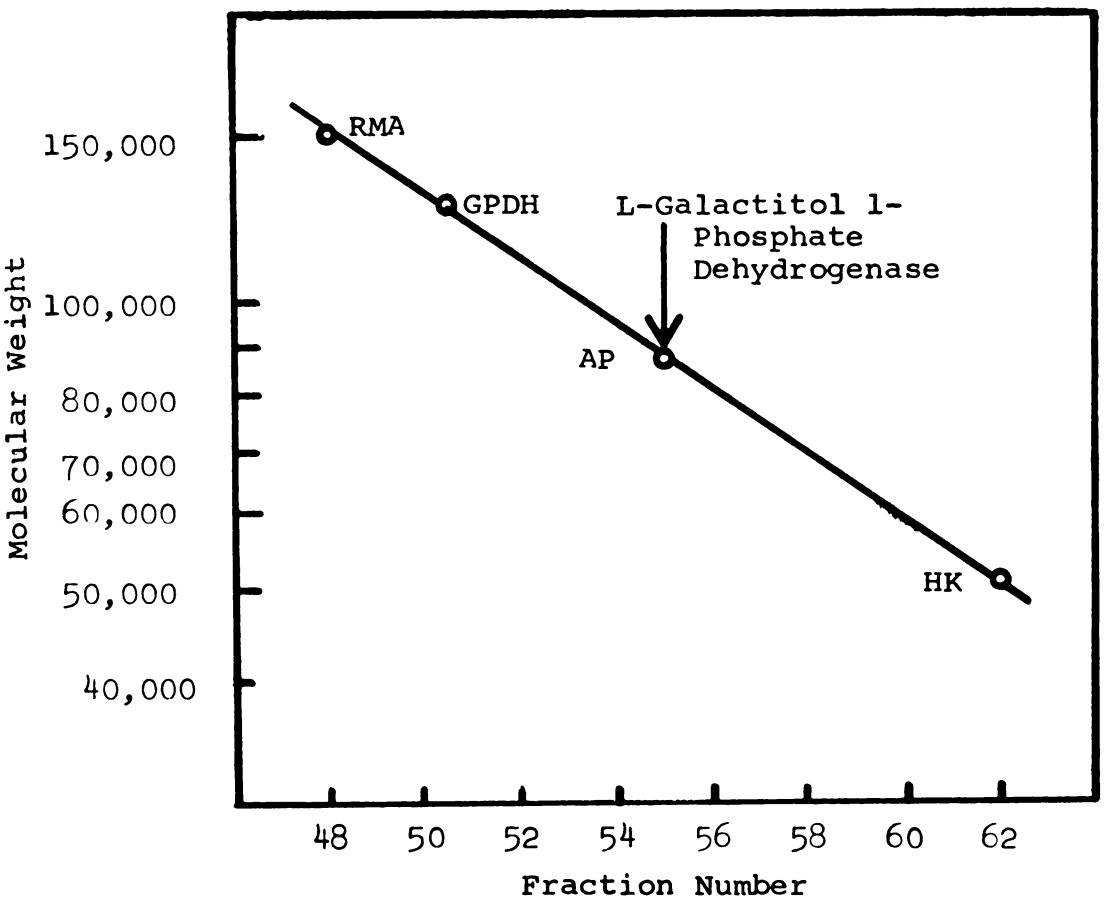


Figure 8. Molecular weight determination of L-galactitol 1-phosphate dehydrogenase. The molecular weight markers are: RMA, rabbit muscle D-fructose 1,6-diphosphate aldolase; GPDH, D-glucose 6-phosphate dehydrogenase; AP, *E. coli* alkaline phosphatase; and HK, hexokinase.

at pH 9.0. Thus, the reaction stoichiometry is one mole of NAD^+ reduced for each mole of L-galactitol 1-phosphate oxidized.

Determination of equilibrium constant. The equilibrium constant was determined by placing known amounts of NAD^+ and L-galactitol 1-phosphate into cuvettes with 0.01 units of the dehydrogenase, buffered at one of two different pH values. The absorbance at 340 nm was monitored and the reaction allowed to go to completion at 30°. From the increase in absorbance, the amount of NADH formed could be calculated from its molar absorbtivity. As shown by the reaction stoichiometry, the amount of D-tagatose 6-phosphate formed was equal to the amount of NADH, and both could be subtracted from the initial concentrations of NAD^+ and L-galactitol 1-phosphate, to give the final concentrations of these two substrates. Determinations were made at both pH 8.5 and pH 9.0 (Table 5). The equation used for equilibrium constant is

$$K_{eq} = \frac{[NADH]\,[D\text{-tagatose-6-P}]\,[H^+]}{[NAD^+]\,[L\text{-galactitol-1-P}]}$$

The mean value for these determinations was 8.0×10^{-11} M with a standard deviation of 1.1×10^{-11} M.

Discussion. The data presented for the partially purified L-galactitol 1-phosphate dehydrogenase establish that this enzyme is indeed capable of functioning in the proposed pathway, oxidizing L-galactitol 1-phosphate to D-tagatose 6-phosphate. D-Tagatose 6-phosphate has conclusively been shown to be the product of this oxidation by Shimamoto (59). My data have also shown that the

Table 5. Determination of the equilibrium constant for the L-galactitol 1-phosphate dehydrogenase reaction

pH	Initial [NAD^+]	Initial [L-galactitol 1-phosphate]	Final [NADH]	K_{eq}
8.5	69.5×10^{-6} M	37.6×10^{-6} M	6.50×10^{-6} M	6.8×10^{-11} M
8.5	69.5×10^{-6} M	75.2×10^{-6} M	9.73×10^{-6} M	7.7×10^{-11} M
8.5	139.0×10^{-6} M	75.2×10^{-6} M	14.60×10^{-6} M	9.5×10^{-11} M
9.0	69.5×10^{-6} M	37.6×10^{-6} M	10.40×10^{-6} M	6.7×10^{-11} M
9.0	69.5×10^{-6} M	75.2×10^{-6} M	16.30×10^{-6} M	8.5×10^{-11} M
9.0	139.0×10^{-6} M	75.2×10^{-6} M	22.90×10^{-6} M	8.6×10^{-11} M
			Mean value =	8.0×10^{-11} M
			Standard deviation =	1.1×10^{-11} M

dehydrogenase was specific for NAD^+ as its pyridine nucleotide co-factor, and the reaction had a stoichiometry of one NAD^+ reduced for each molecule of L-galactitol 1-phosphate oxidized to D-tagatose 6-phosphate. The K_m value for L-galactitol 1-phosphate (0.42 mM) seems reasonable for a reaction that physiologically follows a phosphorylative step, with its resultant large decrease in free energy. The K_m value for NAD^+ (0.22 mM) is higher than for most dehydrogenases, but is not without precedence [e.g., D-mannitol 1-phosphate dehydrogenase (0.38 mM) and D-glucitol 6-phosphate dehydrogenase (0.42 mM) (47)]. The specificity of the dehydrogenase appears to be rather high, with only L-galactitol 1-phosphate and D-tagatose 6-phosphate acting as substrates, out of all the compounds tested.

The activity was unaffected by monovalent cations, whereas divalent cations were necessary for activity and stability. Although Cd^{++} gave the highest amount of activity, Mn^{++} was used in routine assays of activity because its greater natural abundance led me to assume it would more likely be the physiologically important cation.

As discussed in the Literature Review, it was reported that D-glucitol 6-phosphate dehydrogenase from *A. aerogenes* is capable of oxidizing L-galactitol 1-phosphate (47), presumably to D-tagatose 6-phosphate. It can be clearly shown that the L-galactitol 1-phosphate dehydrogenase reported here is not in fact the result of the non-specificity of D-glucitol 6-phosphate dehydrogenase. First, the L-galactitol 1-phosphate dehydrogenase does not use D-glucitol 6-phosphate as a substrate. Second, D-fructose

6-phosphate does not serve as a substrate, as would be expected for D-glucitol 6-phosphate dehydrogenase. And third, as will be seen in Section 3, L-galactitol 1-phosphate dehydrogenase is specifically induced by growth on galactitol, and was not detected in extracts of cells grown on D-glucitol, which induces the D-glucitol 6-phosphate dehydrogenase (50).

B. D-Tagatose 6-Phosphate Kinase [ATP:D-Fructose 6-Phosphate (D-Tagatose 6-Phosphate) 1-Phosphotransferase] (EC 2.7.1.11)

Purification. During early attempts at purifications of the D-tagatose 6-phosphate kinase activity, some methods were found to be ineffective. These procedures are as follows. No binding or retardation was found when the activity was passed through a column of AMP-Sepharose in either the presence or absence of D-fructose 6-phosphate (1 or 10 mM), with and without 1 mM $MgCl_2$. Treatment with hydroxyapatite resulted in a loss of most of the activity, which could not be recovered by elution with 0.5 M phosphate buffer. The enzyme did not adsorb to alumina gel Cγ in 5 mM phosphate buffer. Adjustment of the pH to 5.0 with 2 M acetic acid resulted in high recovery only when the protein concentrations were kept fairly high (about 10 mg/ml or greater), but gave little purification beyond that of the bentonite treatment. High protein concentration was also necessary for the high recoveries reported previously with Sephadex chromatography and ammonium sulfate precipitation (67). The use of a heat step (5 min at 60°) gave excellent recovery of activity, but little purification was achieved beyond that of the bentonite treatment.

Cell extract. The washed cells from 1 liter of a nutrient-broth-grown culture were suspended in 20 ml of 0.02 M sodium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol. The cells were sonically disrupted. Whole cells were removed by centrifugation at 12,000 x g for 10 min. The extract was then diluted to 40 ml with the above buffer. All the following steps took place at 0-4°. A summary of the purification is presented in Table 6.

Bentonite treatment. To the stirred cell extract was slowly added 1.2 g of bentonite. The mixture was stirred for 10 min, and then centrifuged at 12,000 x g for 10 min. The supernatant was decanted and reserved.

DEAE-cellulose chromatography. The bentonite supernatant was passed through a 1 x 8 cm DEAE-cellulose column that had been previously equilibrated with 20 mM sodium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol. The column was washed in the same buffer. A linear gradient of 0 to 0.2 M ammonium sulfate in 160 ml of the above buffer was used to elute the adsorbed protein. Eluted fractions of approximately 2 ml were collected. The elution profile can be seen in Figure 9. Fractions 43-62 were pooled for further studies, and dialyzed against 50 mM sodium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol and 0.1 M ammonium sulfate.

The enzyme was 15.6-fold purified following the DEAE-cellulose step, with a recovery of 66%. The cells were grown on nutrient broth, rather than a medium containing galactitol, so there was no detectable L-galactitol 1-phosphate dehydrogenase or D-tagatose

Table 6. Purification of D-tagatose 6-phosphate kinase

Fraction	Volume[a]	Total protein[b]	Total activity[c]	Units / mg protein	Recovery[d]	F6PK/T6PK[e]
Cell extract	40	248	13.1	0.053	100	1.5
Bentonite	34.5	60	13.4	0.226	109	1.6
DEAE-cellulose	40	11.2	8.9	0.795	66	1.6

[a]Volume is in ml.

[b]Protein is in mg.

[c]1 unit = 1 μmole of product formed per min at 30°.

[d]Recovery is in %.

[e]Ratio of the specific activity of D-fructose 6-phosphate kinase to D-tagatose 6-phosphate kinase.

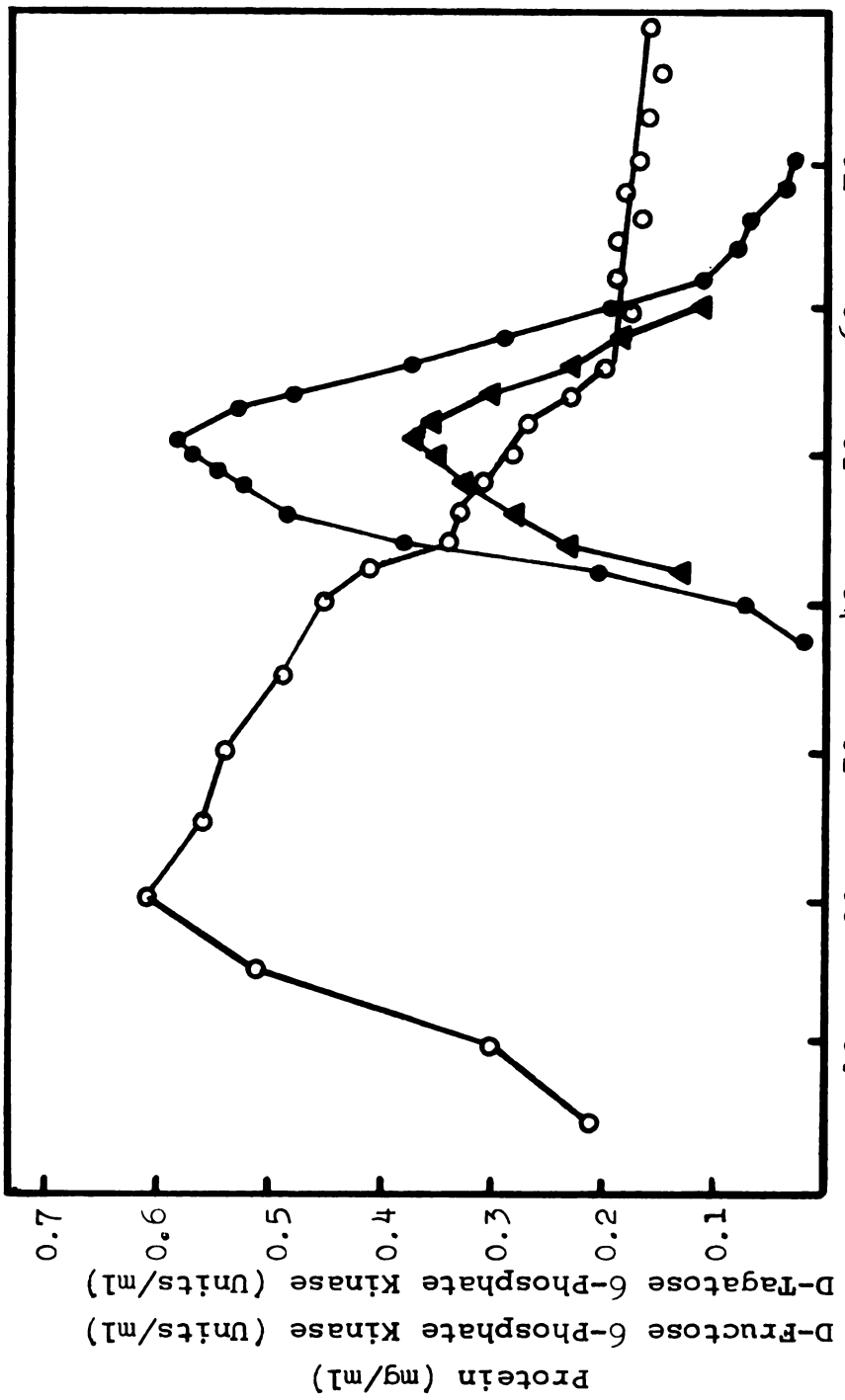


Figure 9. Chromatography of D-fructose 6-phosphate kinase and D-tagatose 6-phosphate kinase on DEAE-cellulose. Symbols: (●) D-fructose 6-phosphate kinase; (▲) D-tagatose 6-phosphate kinase; and (○) protein.

1,6-diphosphate aldolase in the cell extract. Because of the absence of these two other enzymes from the galactitol catabolic pathway, as well as the competing NADH oxidase activity, it was not deemed necessary to further purify the kinase. The constant specific activity ratios for D-fructose 6-phosphate kinase and D-tagatose 6-phosphate kinase during purification, as well as their coincident elution from DEAE-cellulose, suggest that these two activities are catalyzed by the same enzyme.

Properties

Stability. The DEAE-cellulose fractions were stable when kept on ice, having a half-life of approximately two weeks. Samples stored frozen at -20° for a month showed little loss of activity. Storage of the enzyme in 20 mM sodium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol for 24 hours at 0° or 4° caused 79% and 100% loss of activity, respectively.

Thermal inactivation. Samples of the kinase treated in a water bath at 64° for various times were assayed for activity with both D-fructose 6-phosphate and D-tagatose 6-phosphate (Figure 10). These data show that the loss of both activities is concident, supporting the contention that both activities reside in one protein.

pH optimum. For the D-fructose 6-phosphate kinase, as well as the D-tagatose 6-phosphate kinase activities, the highest activity occurred in Tris buffer, with the optimum at pH 8.25 (Figure 11). Use of glycylglycine gave an optimum of pH 8.0, while potassium phosphate buffer shifted the optimum to pH 7.75.

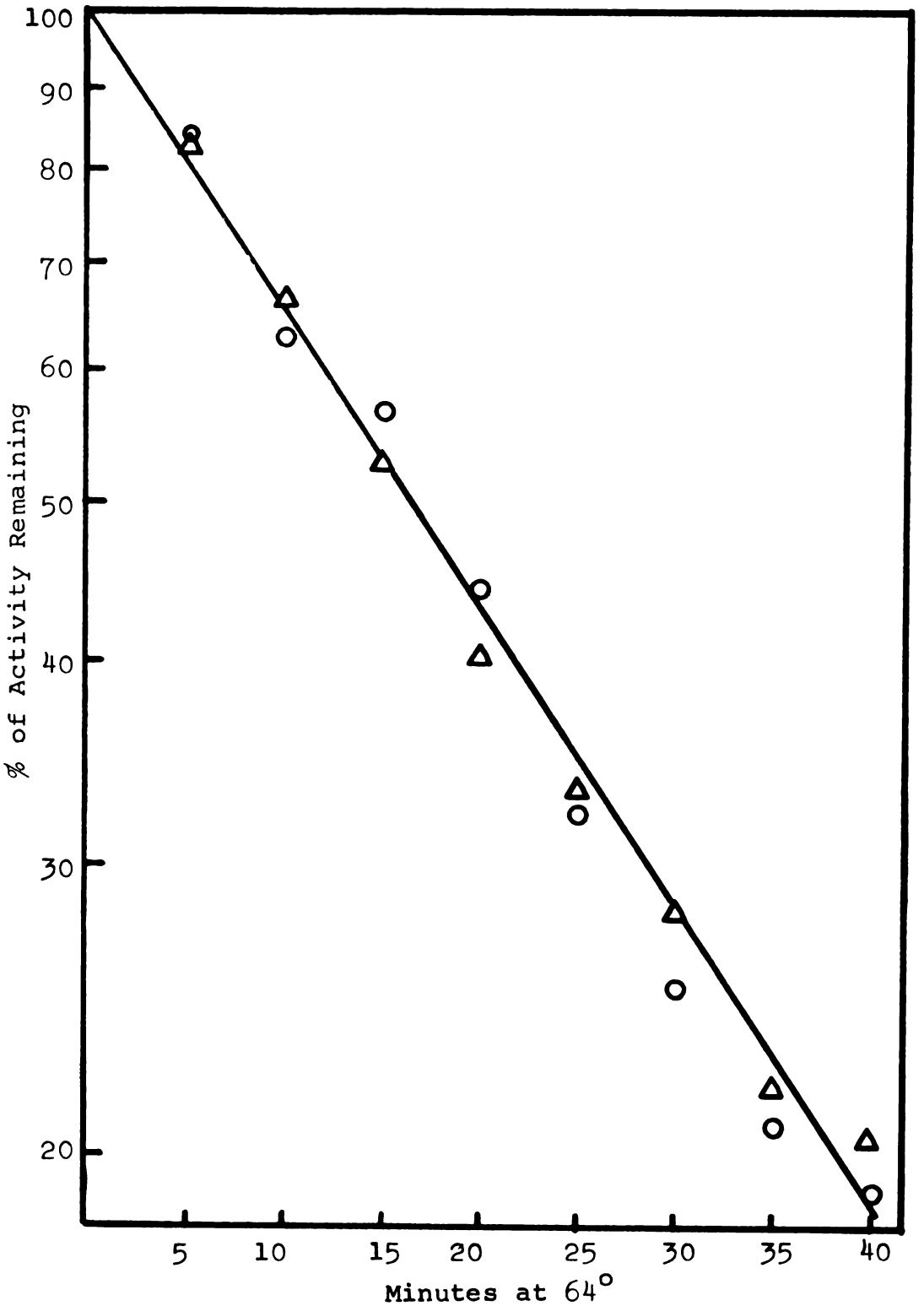


Figure 10. Thermal inactivation of D-tagatose 6-phosphate kinase and D-fructose 6-phosphate kinase. Symbols: (Δ) D-tagatose 6-phosphate kinase; (O) D-fructose 6-phosphate kinase.

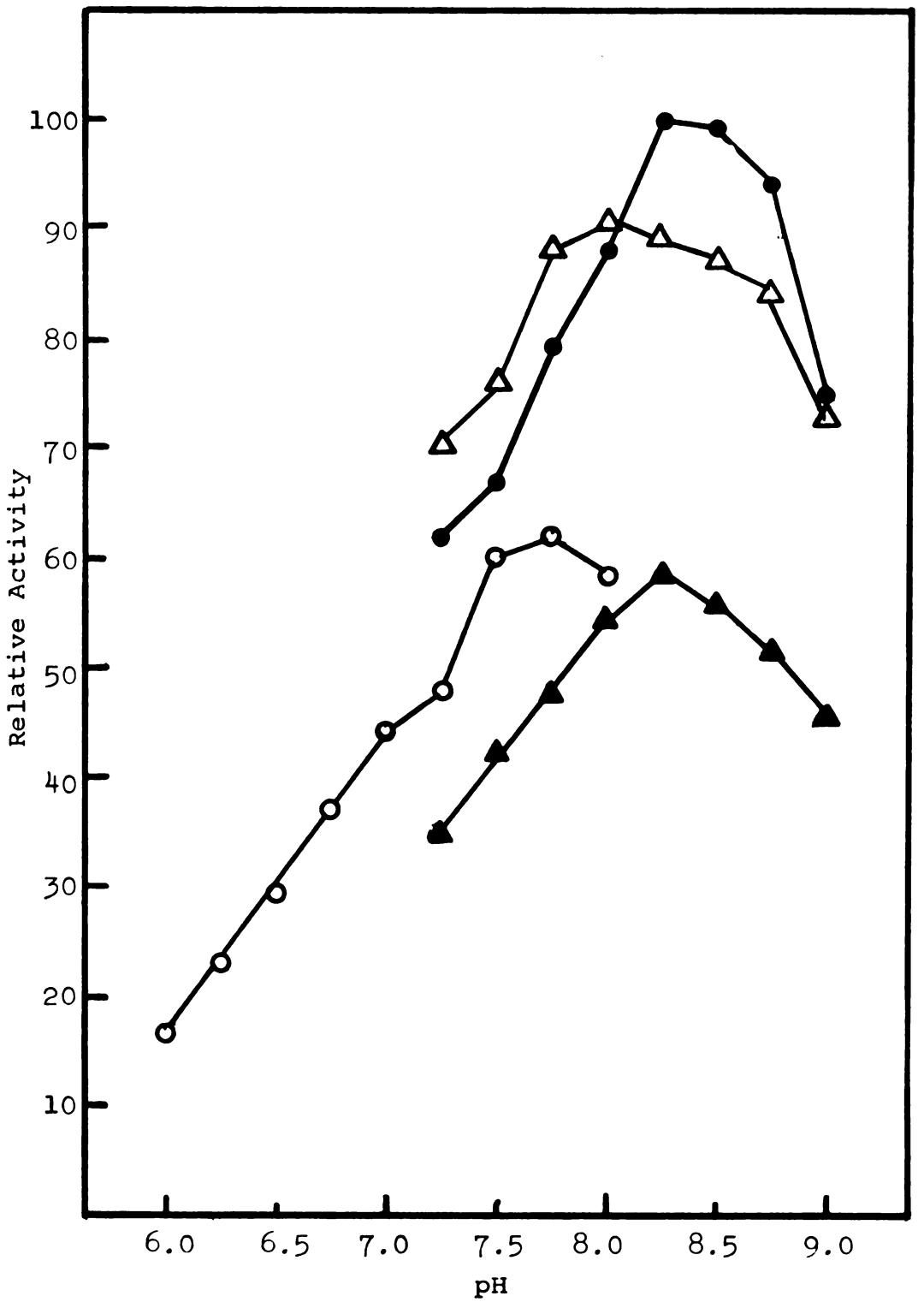


Figure 11. pH optimum of D-fructose 6-phosphate kinase and D-tagatose 6-phosphate kinase. Symbols: D-fructose 6-phosphate kinase in Tris (●), glycylglycine (△), and KPO_4 (○); and D-tagatose 6-phosphate kinase in Tris (▲).

Phosphoryl acceptor specificity and kinetic constants. As has already been shown, both D-fructose 6-phosphate and D-tagatose 6-phosphate act as substrates for the kinase. Plots of substrate concentration versus activity (Figures 12 and 13) show a sigmoidicity which indicates allosteric behavior toward these substrates (93). Substrate concentrations that gave half-maximal velocity were 0.25 mM and 1.1 mM for D-fructose 6-phosphate and D-tagatose 6-phosphate, respectively, in the absence of ADP, and 0.4 mM for D-tagatose 6-phosphate in the presence of ADP. The value for D-fructose 6-phosphate is in good agreement with the published value of 0.3 mM (67). It can also be seen that the V_{max} for D-tagatose 6-phosphate is 62% of that for D-fructose 6-phosphate. This is in good agreement with the data for pH optimum where in Tris buffer the D-tagatose 6-phosphate kinase activity was 58% of the D-fructose 6-phosphate kinase activity at the optimal pH (Figure 11).

To establish that these two substrates are competing for the same activity, the following experiment was devised. The standard D-fructose 6-phosphate kinase assay using non-limiting amounts of rabbit muscle aldolase was employed, except that the D-fructose 6-phosphate concentration was made 1.04 mM (four times its K_m value), and pyruvate kinase and 3.3 mM PEP were added to remove all ADP. When D-tagatose 6-phosphate was added to a final concentration of 5.33 mM (approximately five times its K_m value), the rate of D-fructose 1,6-diphosphate formation was inhibited 46%. It was possible to measure formation of D-fructose 1,6-diphosphate independently from formation of D-tagatose 1,6-diphosphate because of the rabbit muscle D-fructose 1,6-diphosphate aldolase specificity

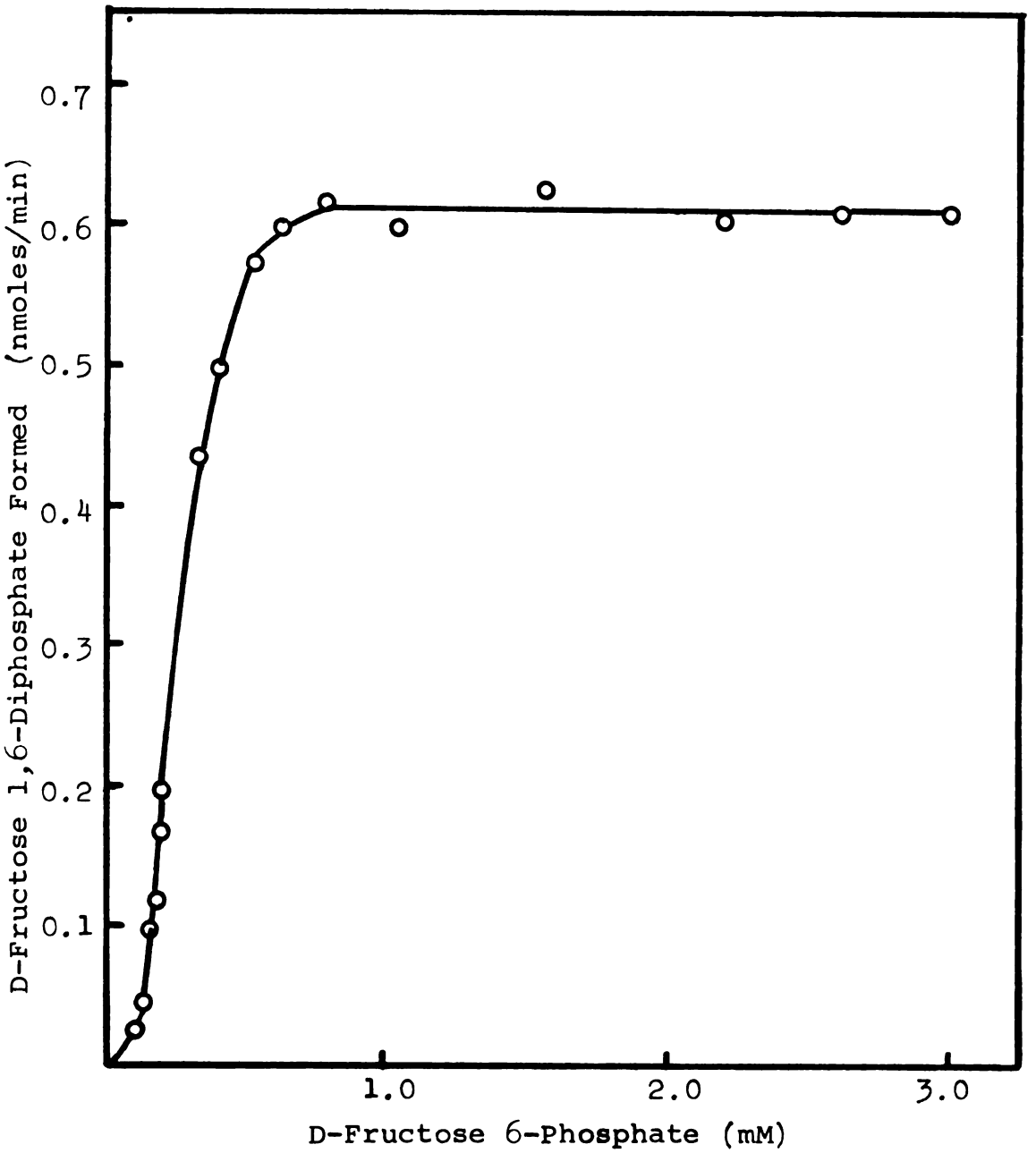


Figure 12. The effect of D-fructose 6-phosphate concentration on the kinase activity. The assay contained 57.5 µg of the purified kinase.

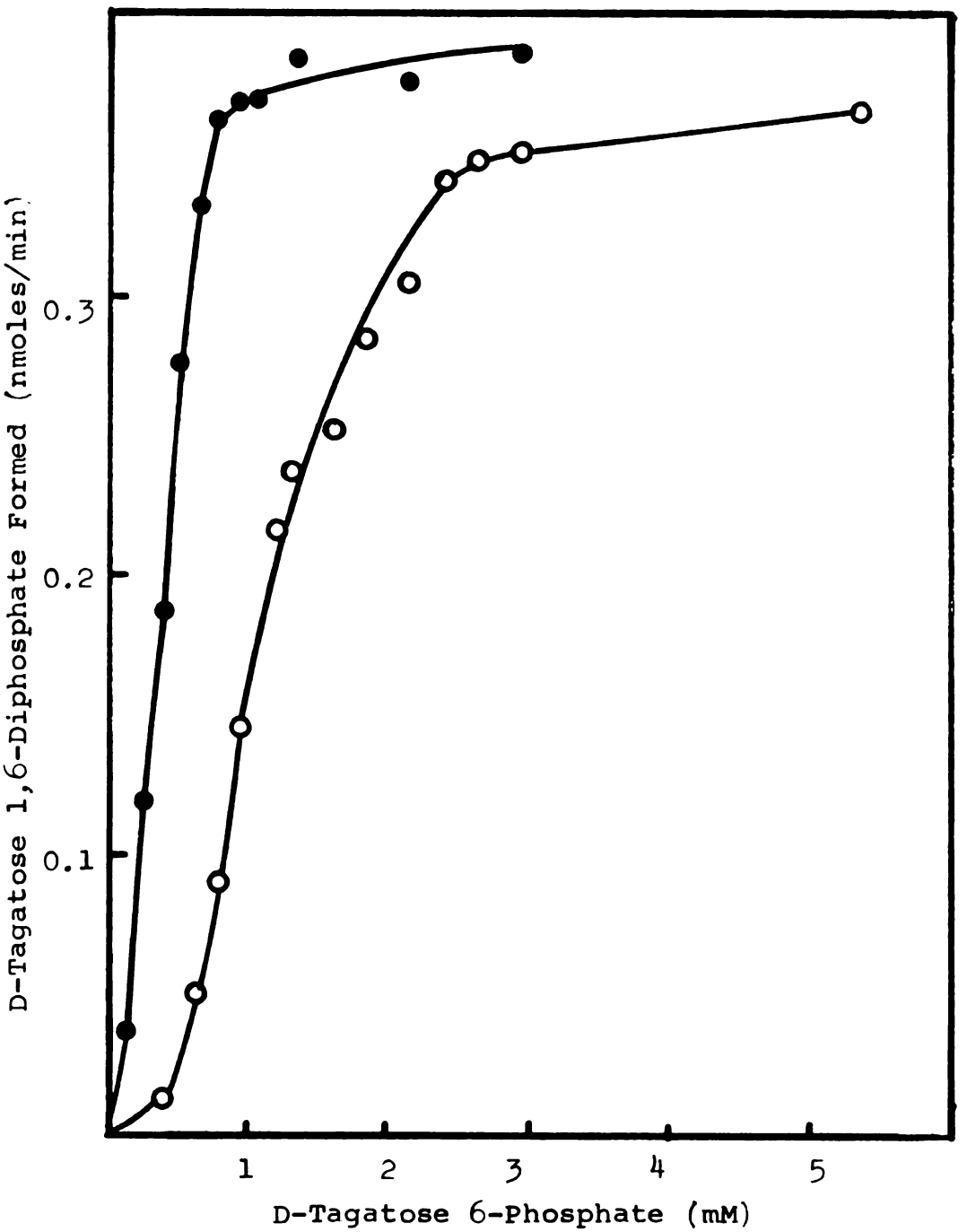


Figure 13. The effect of D-tagatose 6-phosphate concentration on the kinase activity. Symbols: (○) minus ADP, (●) plus 1 mM ADP. The assay contained 57.5 µg of the purified kinase.

for the former (61,94). Because the presence of both substrates in the cuvette would change the kinetic constants from those obtained with either substrate present alone, there are not sufficient data with which to calculate the expected percent inhibition. However, the fact that a considerable inhibition does occur suggests that the two sugar phosphates are substrates for the same enzyme.

Other substrates of the kinase would also be expected to cause an inhibition of the phosphorylation of D-fructose 6-phosphate. The following compounds were added to the standard D-fructose 6-phosphate kinase assay at a concentration of 10 mM to observe if any would cause inhibition as did D-tagatose 6-phosphate: L-sorbose 1-phosphate, L-galactitol 1-phosphate, D-galactose 6-phosphate, D-glucose 6-phosphate, D-mannose 6-phosphate, D-fructose 1-phosphate, and D-fructose. None of these compounds affected the rate of D-fructose 1,6-diphosphate formation, indicating that they are not substrates for the kinase. In addition, the formation of D-fructose 1-phosphate from D-fructose (measured with D-fructose 1-phosphate kinase) could not be detected.

Phosphoryl donor specificity. The relative rates of phosphorylation of D-fructose 6-phosphate by the six common nucleoside triphosphates has been reported previously (67). Their efficiency in the standard assay at a concentration of 3.3 mM was (%): ATP, 100; ITP, 93; GTP, 86; UTP, 57; CTP and TTP, 50. Three of these were tested in the standard D-tagatose 6-phosphate kinase assay with the following results (%): ATP, 100; ITP, 94; and GTP, 84. Thus, it is seen that the results for the phosphorylation of

D-tagatose 6-phosphate parallel the results obtained with D-fructose 6-phosphate.

Sulfhydryl requirement. The thiol reagent NEM was incubated with the enzyme at a final concentration of 0.2 mM for 30 min. No loss of activity was noted. This is in contrast to the D-tagatose 6-phosphate kinase of *S. aureus*, which was 85% inhibited under identical conditions. The addition of BME or DTT to the buffer during my attempts at purification did not enhance the recovery over attempts without these compounds. Thus, there is no apparent sulfhydryl requirement for the kinase.

Molecular weight. The kinase was applied to a Sephadex G-150 column along with several proteins of known molecular weight (92). The standards used, and their molecular weights, were as follows: rabbit muscle lactate dehydrogenase, 140,000 (90,95); yeast D-glucose 6-phosphate dehydrogenase, 128,000 (90); *E. coli* alkaline phosphatase, 86,000 (91); and *K. pneumoniae* D-fructose 1-phosphate kinase, 75,000 (67). When the log of the molecular weights of the standards is plotted against the fraction of each with the greatest activity (Figure 14), a linear function is evident. This plot indicates the kinase has a molecular weight of 92,000. This determination assumes a spherical shape for the protein molecule.

Product Identification

Production of product. To produce the product of the kinase reaction for identification, a reaction mixture (15 ml) was

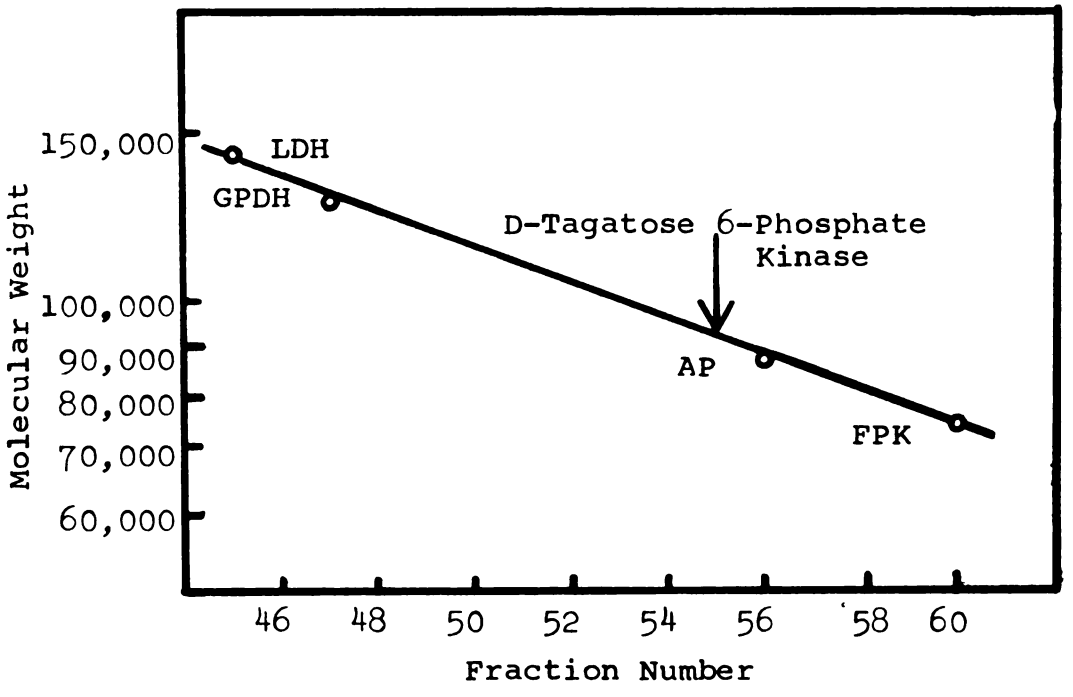


Figure 14. Molecular weight determination of D-tagatose 6-phosphate kinase. The standard proteins are lactate dehydrogenase (LDH), D-glucose 6-phosphate dehydrogenase (GPDH), *E. coli* alkaline phosphatase (AP), and D-fructose 1-phosphate kinase (FPK).

prepared containing 1.0 mmole of glycylglycine buffer (pH 7.5), 100 μmoles of $MgCl_2$, 50 μmoles of ATP, 50 μmoles of D-tagatose 6-phosphate, and D-tagatose 6-phosphate kinase. The reaction was monitored by removing samples at timed intervals, and assaying for the amount of ADP present. When the formation of ADP had stopped, the reaction mixture was adjusted to pH 8.5 with 6 M NH_4OH.

Isolation of the product. The reaction mixture was immediately applied to a Dowex 1-X8 chloride column (1.1 x 18 cm). The column was washed successively with the following solutions as described by Khym and Cohn (96): 0.001 M HN_4OH; 0.025 M HN_4Cl, 0.01 M $K_2B_4O_7$; 0.025 M NH_4Cl, 0.0025 M NH_4OH, 0.00001 M $K_2B_4O_7$; 0.01 M HCl, 0.02 M KCl; and 0.02 M HCl, 0.2 M KCl. Fractions of the 0.02 M HCl, 0.02 M KCl eluate that contained ketohexose were combined, lyophilized, dissolved in 10 ml of water, and the pH adjusted to neutrality with NaOH.

Identification of the product. The isolated ketohexose product was identified as D-tagatose 1,6-diphosphate by the following criteria: (i) it eluted from the Dowex 1-X8 column in the fractions where sugar diphosphates normally elute, (ii) the assayed phosphate to ketohexose ratio was 1.93:1, (iii) the product was cleaved by D-tagatose 1,6-diphosphate aldolase, as was authentic D-tagatose 1,6-diphosphate produced in the aldolase assay mixture from D-tagatose 6-phosphate as described in Materials and Methods, (iv) cleavage of the product with D-tagatose 1,6-diphosphate aldolase in the presence of α-glycerolphosphate dehydrogenase resulted in 0.48 moles of NADH oxidized for each mole of organic phosphate in the

product, indicating it reacted through the pathway: kinase product ⟶ dihydroxyacetone phosphate ⟶ α-glycerol phosphate, (v) cleavage of the product with D-tagatose 1,6-diphosphate aldolase in the presence of triose phosphate isomerase and α-glycerolphosphate dehydrogenase resulted in 1.01 moles of NADH oxidized for each mole of organic phosphate in the product, indicating it reacted through the pathway: kinase product ⟶ dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate ⟶ 2 dihydroxyacetone phosphate ⟶ 2α-glycerol phosphate, and (vi) the product was not cleaved by rabbit muscle aldolase, as is known not to happen with D-tagatose 1,6-diphosphate (61,94).

The product was enzymatically dephosphorylated for further analysis. When subjected to paper chromatography, the free keto-hexose migrated identically with tagatose, but separated from fructose ($R_{tagatose}$ = 1.14), sorbose ($R_{tagatose}$ = 0.96), and psicose ($R_{tagatose}$ = 1.22). When the dephosphorylated product was subjected to the cysteine-H_2SO_4 reaction (89), the half-time required for color development was 6.7 min, as it was for tagatose, while psicose took 14.5 min and fructose 45.7 min. If this reaction were allowed to proceed for 20 hours (88), the A_{412} to A_{605} ratio was 0.52 for sorbose, 3.48-3.61 for the other 2-ketohexoses, and 3.58 for the dephosphorylated product. All the evidence therefore indicates that the dephosphorylated product was tagatose.

Reaction stoichiometry. The stoichiometry of the kinase reaction was determined by the simultaneous assay for ADP and D-tagatose 1,6-diphosphate formed in separate cuvettes, as shown

in Table 7. These data demonstrate that for each mole of ATP and D-tagatose 6-phosphate, one mole each of ADP and D-tagatose 1,6-diphosphate were formed. This, combined with the product identification data, substantiates that D-fructose 6-phosphate kinase phosphorylates D-tagatose 6-phosphate with ATP, generating D-tagatose 1,6-diphosphate and ADP.

Discussion. The data presented in this section clearly establish that the product of the L-galactitol 1-phosphate dehydrogenase reaction (D-tagatose 6-phosphate) is enzymatically phosphorylated with ATP to yield D-tagatose 1,6-diphosphate. Furthermore, determination of the reaction stoichiometry has revealed that for each mole of D-tagatose 1,6-diphosphate formed, one mole of ATP is converted to ADP.

The isolated product of the enzymatic reaction was identified as D-tagatose 1,6-diphosphate on the basis of the following criteria: it eluted from Dowex 1-X8 as expected for a ketohexose diphosphate; the phosphate to ketohexose molar ratio was 2:1; the product was cleaved quantitatively by *K. pneumoniae* D-tagatose 1,6-diphosphate aldolase, but was not cleaved by rabbit muscle D-fructose 1,6-diphosphate aldolase; and finally, when cleaved by the appropriate aldolase, the two triose phosphates formed were those expected for D-tagatose 1,6-diphosphate. Dephosphorylation of the reaction product released a ketohexose that was proven to be tagatose. Paper chromatography showed identical migration of the dephosphorylated product and tagatose. In addition, when subjected to the cysteine-H_2SO_4 reaction, the dephosphorylated product developed its chromophore

Table 7. Determination of the product stoichiometry of D-tagatose 6-phosphate kinase

Both cuvettes contained 9.4 nmoles of ATP and 9.4 nmoles of D-tagatose 6-phosphate. Reaction A, which measured ADP formed, also contained 0.05 µmole of PEP, non-limiting amounts of pyruvate kinase and lactate dehydrogenase, and D-tagatose 6-phosphate kinase. Reaction B, which measured D-tagatose 1,6-diphosphate, also contained the standard components of the D-tagatose 1,6-diphosphate aldolase assay.

Time[a]	ADP formed in reaction A[b]	D-Tagatose 1,6-diphosphate formed in reaction B[b]
10	1.95	2.34
20	3.90	4.39
30	5.65	6.09
40	6.92	7.31
50	8.00	8.29
60	8.68	8.87
70	9.07	9.17
80	9.36	9.26
90	9.46	9.36
100	9.46	9.36

[a] Time is in minutes.

[b] nmoles.

at the same rate as authentic D-tagatose, and after 20 hours had the same A_{412}/A_{605} ratio as the tagatose.

That the D-tagatose 6-phosphate kinase is the same protein as the D-fructose 6-phosphate kinase is demonstrated by the following data: (i) the activities have a constant ratio of specific activity during the purification and elute coincidentally from DEAE-cellulose; (ii) both activities have the same rate of inactivation at 64°; (iii) the pH optima in Tris buffer are identical; (iv) the relative rates of phosphorylation of the two ketohexose phosphates by ATP, ITP, and GTP were essentially identical; and (v) D-tagatose 6-phosphate inhibits the phosphorylation of D-fructose 6-phosphate. Thus, all the evidence is consistent with the two kinase activities being the result of the dual specificity of a single protein. This will be confirmed by the mutant analysis in Section 3. There is also precedence in the literature for mammalian D-fructose 6-phosphate kinases being able to phosphorylate D-tagatose 6-phosphate (68).

Regulation of the enzyme activity *in vivo* may be due, at least in part, to the effects of ADP. ADP lowered the K_m of the enzyme for D-tagatose 6-phosphate, while apparently not changing the V_{max}. This effect has already been reported to occur with the D-fructose 6-phosphate substrate (67). The net result would be an activation of kinase activity at ketohexose diphosphate concentrations which are less than saturating in the absence of ADP.

C. D-Tagatose 1,6-Diphosphate Aldolase (D-Tagatose 1,6-Bisphosphate D-Glyceraldehyde 3-Phosphate-Lyase)

Purification. During preliminary purification attempts, a few techniques were not successful either because of low purification or low recovery. Precipitation by the addition of solid ammonium sulfate to the cell extract resulted in poor recovery of the activity. Batchwise treatment of the cell extract of DEAE-cellulose-chromatographed fractions with hydroxyapatite in 0.005 M sodium phosphate buffer (pH 7.5) failed to cause adsorption of the aldolase activity. The addition of small amounts of bentonite to the cell extract provided up to a 1.7-fold purification, but if enough were added to remove the contaminating NADH oxidase, the loss of significant amounts of the aldolase activity occurred. Precipitation of the activity at pH values less than 6 resulted in irrecoverable loss of the activity, while retaining almost all of the D-fructose 1,6-diphosphate aldolase activity.

Preparation of cell extract. Cell extracts from galactitol-grown cultures of *K. pneumoniae* were prepared in 0.02 M sodium phosphate buffer (pH 7.5), containing 10% (v/v) glycerol. All steps took place at 0-4°. A summary of the purification scheme appears in Table 8.

DEAE-cellulose chromatography I. A DEAE-cellulose column (1.2 x 10 cm) was equilibrated with the cell-extract buffer. The extract was applied to the column, and the column washed with 100 ml of the same buffer. The adsorbed protein was eluted with a 150-ml linear gradient of 0 to 0.45 M KCl in 0.02 M sodium phosphate buffer

Table 8. Purification of D-tagatose 1,6-diphosphate aldolase

Fraction	Volume[a]	Total protein[b]	Total activity[c]	Units / mg protein	Recovery[d]
Cell extract	43	653	41.6	0.0637	100
DEAE-cellulose I	44	60.3	46.5	0.770	112
Sephadex G-150	67	28.8	40.8	1.40	98
DEAE-cellulose II	35	2.35	22.6	3.07	54

[a]Volume is in ml.

[b]Protein is in mg.

[c]1 unit = 1 μmole of substrate cleaved per min at 30°.

[d]Recovery is in %.

(pH 7.5), containing 10% (v/v) glycerol. Fractions of 2 ml were collected (Figure 15). The fractions containing the bulk of the activity (numbers 35-66) were pooled.

Sephadex G-150 chromatography. The combined DEAE-cellulose fractions were concentrated to approximately 15 ml by pressure filtration in an Amicon Diaflo apparatus. This concentrate was applied to a Sephadex G-150 column (3.2 x 81 cm) equilibrated with 0.02 M sodium phosphate buffer (pH 7.5) containing 10% glycerol. The column was eluted with the same buffer, and fractions of approximately 4 ml were collected (Figure 16). Fractions 50-67 were pooled for further purification.

DEAE-cellulose chromatography II. The combined activity from the Sephadex G-150 column was applied to a DEAE-cellulose column (1.2 x 5 cm) previously equilibrated with 0.02 M sodium phosphate buffer (pH 7.5), containing 10% (v/v) glycerol. The column was then washed with 100 ml of the same buffer. The adsorbed protein was then eluted with a 200-ml linear gradient of 0 to 0.4 M KCl in 0.02 M sodium phosphate buffer (pH 7.0), containing 10% (v/v) glycerol. Fractions of approximately 2 ml were collected (Figure 17). DEAE-cellulose II fractions 57-75, containing the majority of the activity, were combined. The aldolase was 48-fold purified with a 54% yield. The activities of L-galactitol 1-phosphate dehydrogenase and D-tagatose 6-phosphate kinase could not be detected (less than 0.0001 unit/ml). The preparation was tested for purity by electrophoresis in 7.5% polyacrylamide gels at pH 8.9.

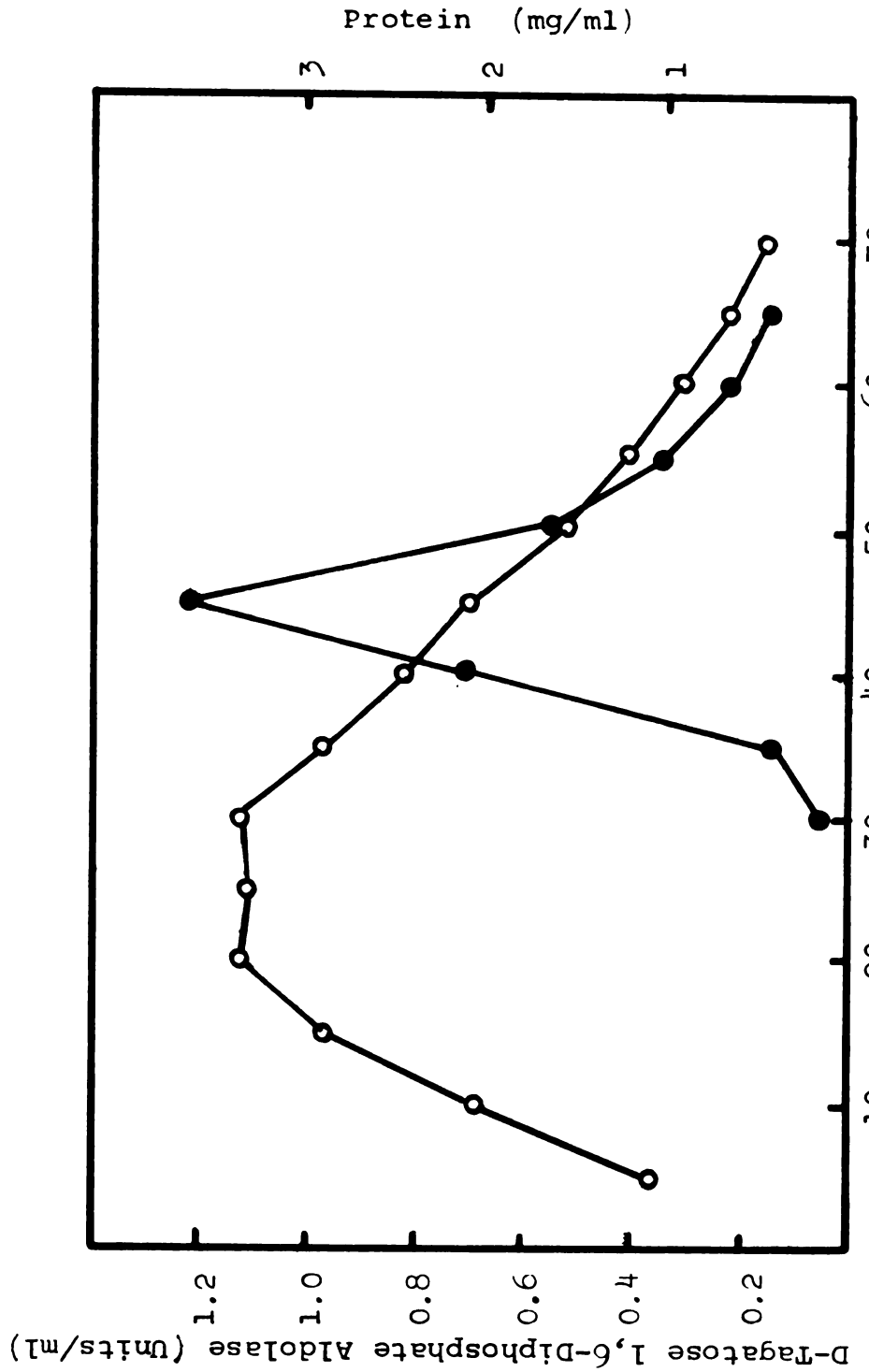


Figure 15. DEAE-Cellulose chromatography I of D-tagatose 1,6-diphosphate aldolase. Symbols: (●) D-tagatose 1,6-diphosphate aldolase, and (○) protein.

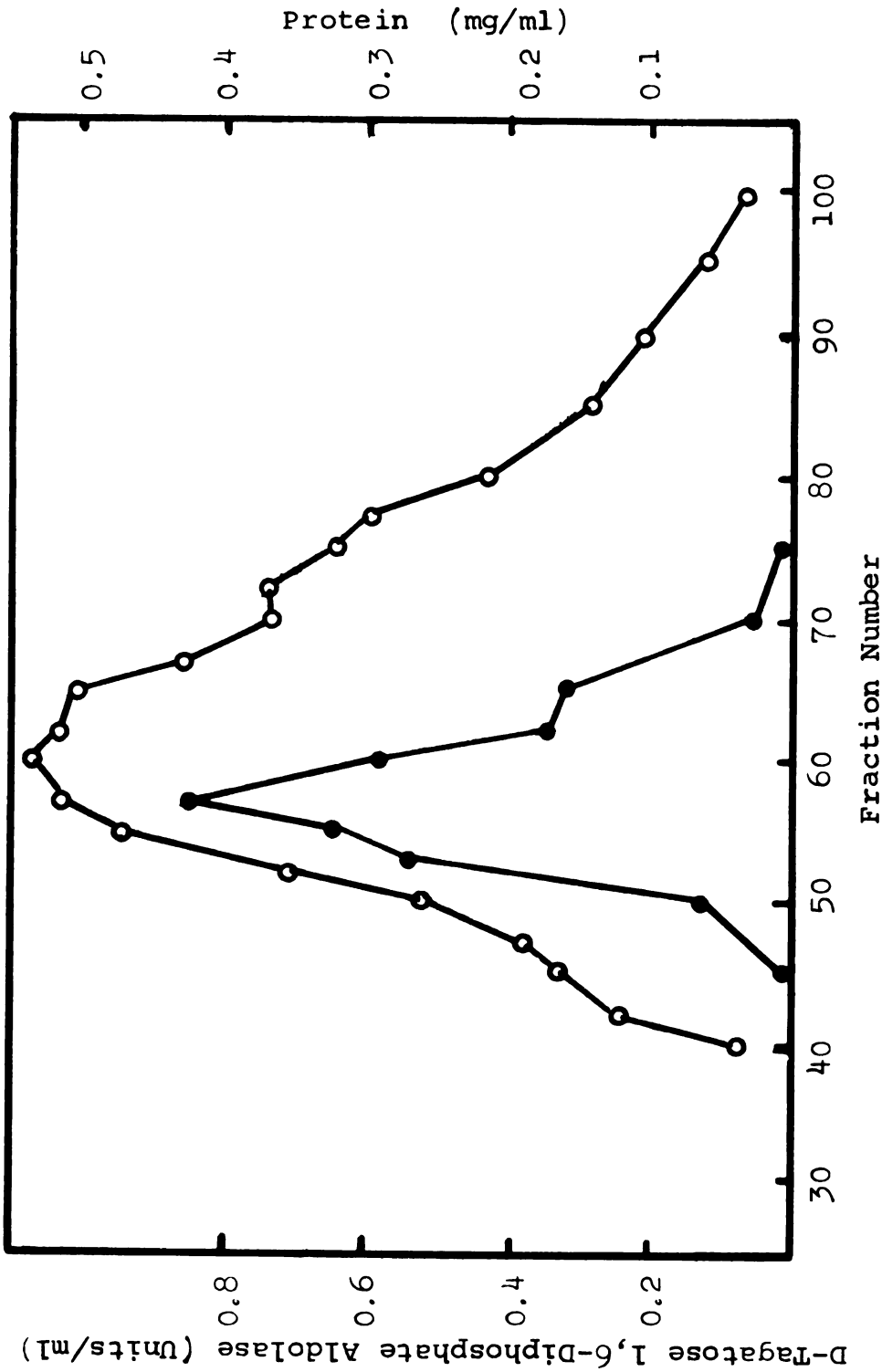
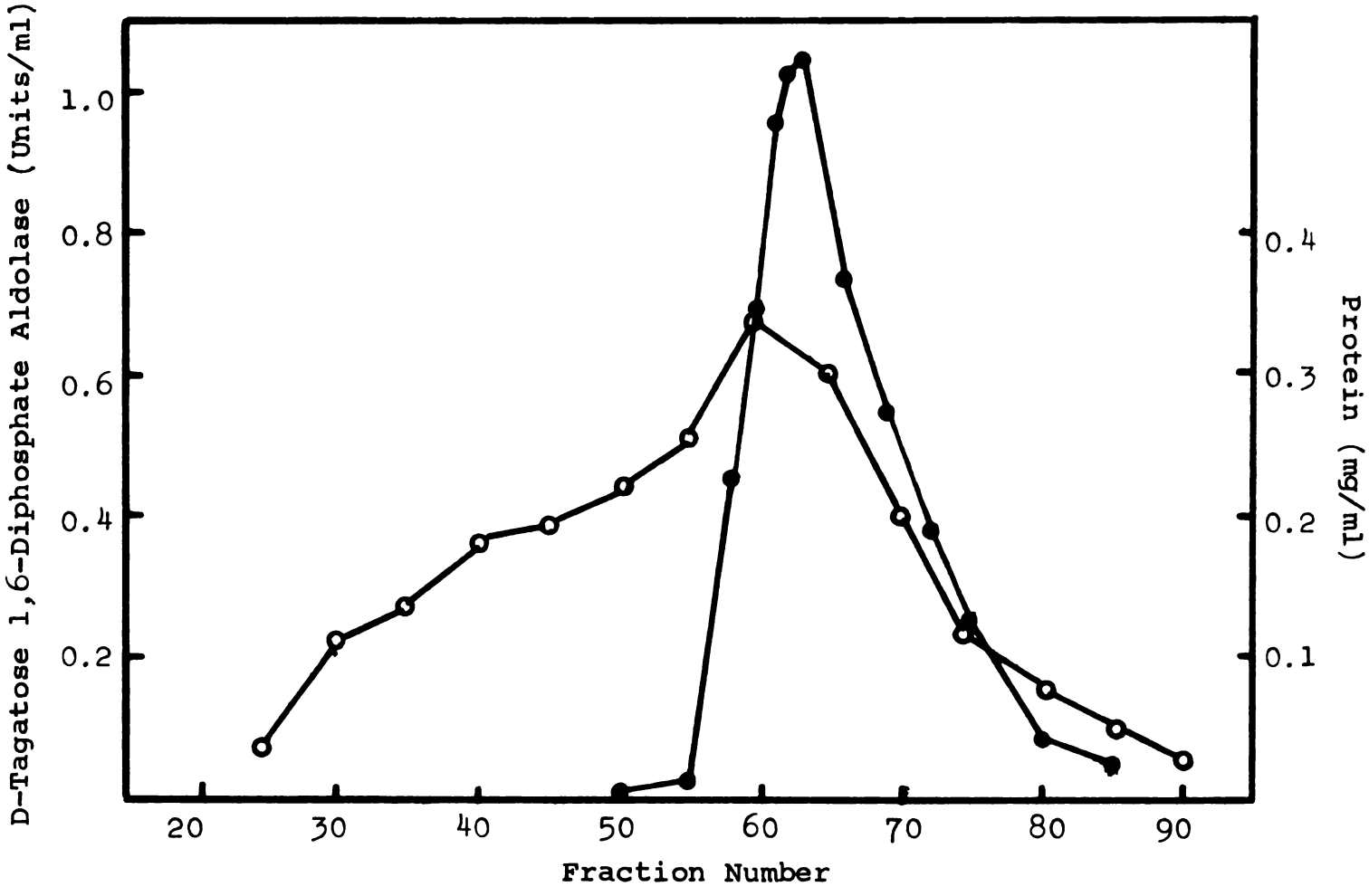


Figure 16. Chromatography of D-tagatose 1,6-diphosphate aldolase on Sephadex G-150. Symbols: (●) D-tagatose 1,6-diphosphate aldolase, and (○) protein. Fraction volume was 4 ml.



Fraction 17. DEAE-Cellulose chromatography II of D-tagatose 1,6-diphosphate aldolase. Symbols: (●) D-tagatose 1,6-diphosphate aldolase, and (○) protein. Fraction volume is 2 ml.

When stained for protein, the gels showed one major, and at least four minor, bands, indicating that the aldolase is not homogeneous.

Properties

Stability. The activity-containing fractions from the second DEAE-cellulose treatment were kept on ice for five weeks, with loss of approximately one-third of the activity. Identical fractions were kept frozen at -20° for three months with little loss of activity.

pH optimum. The pH optimum of the aldolase activity was determined in two different buffer systems. The highest activity occurred at pH 8.0 in HEPES buffer, and at pH 8.25 in glycylglycine buffer (Figure 18).

Substrate specificity and kinetic constants. D-Tagatose 1,6-diphosphate and D-fructose 1,6-diphosphate are substrates for the aldolase. Lineweaver-Burk plots show that the K_m values for D-tagatose 1,6-diphosphate and D-fructose 1,6-diphosphate (Figure 19) were 0.38 mM and 0.86 mM, respectively, while the V_{max} is 25-fold higher for cleavage of the former. The following sugar phosphates were tested as substrates for the aldolase at concentrations of 10 mM: D-fructose 6-phosphate, D-tagatose 6-phosphate, L-sorbose 1-phosphate, D-fructose 1-phosphate, D-glucose 6-phosphate, D-galactose 6-phosphate, D-mannose 6-phosphate, and L-galactitol 1-phosphate. D-Fructose 1-phosphate was also tested at a concentration of 50 mM. With a limit of detection of 0.0005 unit/mg protein, none of these compounds was cleaved by the aldolase.

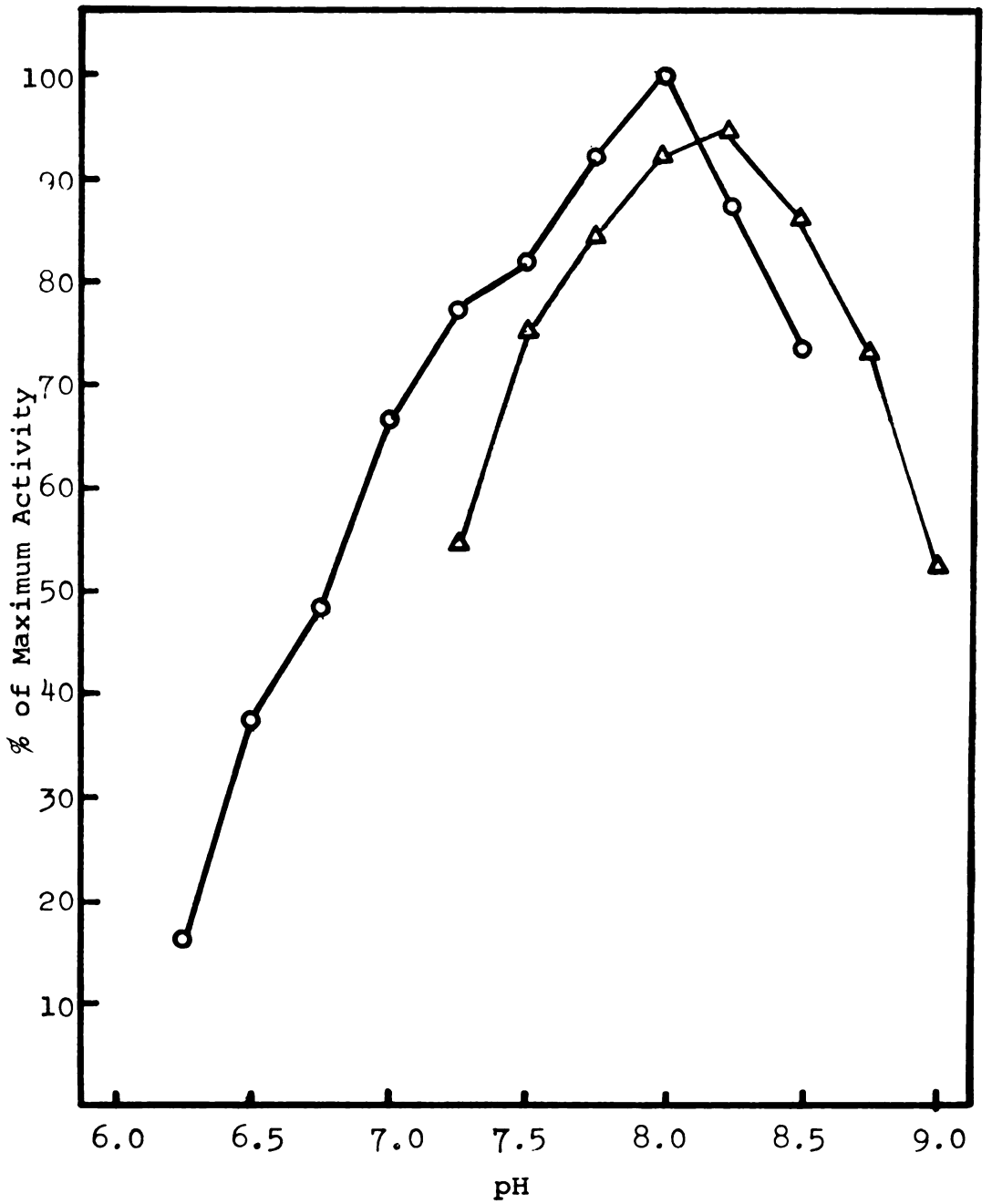


Figure 18. pH optimum of D-tagatose 1,6-diphosphate aldolase. The buffers used were HEPES (●) or glycyl-glycine (▲).

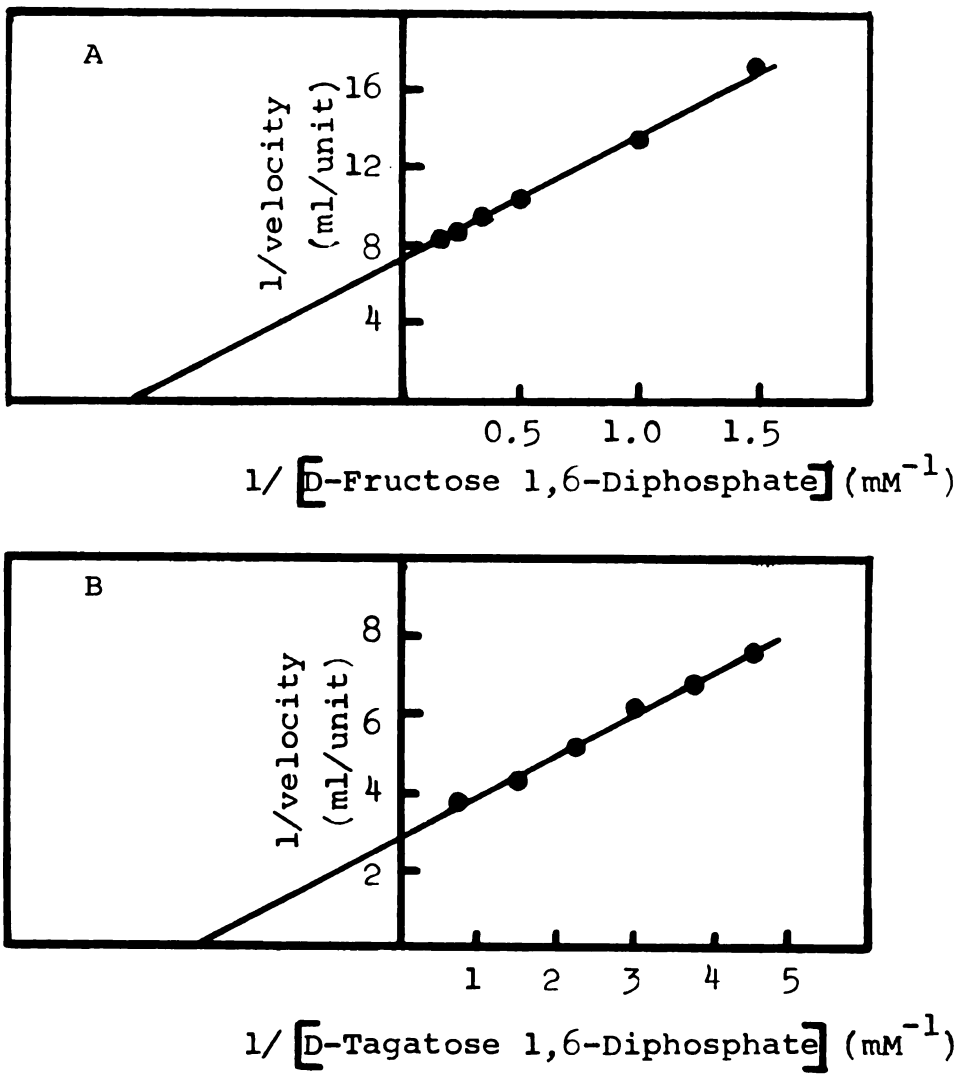


Figure 19. Lineweaver-Burk plots for D-tagatose 1,6-diphosphate aldolase. Determination A was done with the assay utilizing D-fructose 1,6-diphosphate. The data in B were obtained with the standard assay utilizing D-tagatose 1,6-diphosphate generation. In addition, both assays contained 0.5 μmoles of KCl and 0.03 μmoles of $CoCl_2$. The specifi V_{max} values are 0.65 units/mg protein and 16.8 units/mg protein for A and B respectively.

That the cleavage of D-fructose 1,6-diphosphate is due to the D-tagatose 1,6-diphosphate aldolase can be shown in several ways. First, 145-fold purified D-fructose 1,6-diphosphate aldolase from *K. pneumoniae*, which does not utilize D-tagatose 1,6-diphosphate as a substrate, has a K_m of 0.14 mM for D-fructose 1,6-diphosphate (unpublished observations), as compared to the value of 0.86 mM obtained in the above study. And second, when the D-tagatose 1,6-diphosphate aldolase was heated in a water bath at 64°, there was a coincident decay in the two activities (Figure 20). These data are consistent with the cleavage of both substrates by D-tagatose 1,6-diphosphate aldolase.

Unlike the D-tagatose 1,6-diphosphate aldolase from *S. aureus* (64), the enzyme from *K. pneumoniae* does not cleave D-psicose 1,6-diphosphate or D-sorbose 1,6-diphosphate. To demonstrate this, a solution containing 100 μmoles of DL-glyceraldehyde 3-phosphate was adjusted to pH 8.0 with NaOH. Triose phosphate isomerase and D-tagatose 1,6-diphosphate aldolase were added to make the final volume 5.0 ml. The solution was incubated at 25°. Samples were withdrawn at timed intervals to monitor the progress of the reaction. When the formation of ketohexose had halted, the solution was adjusted to pH 8.5 with NaOH and applied to a 1.2 x 16 cm Dowex 1-X8 bicarbonate column. The column was washed with 300 ml of water and then 150 ml each of 0.15 M, 0.3 M, and 0.45 M $KHCO_3$ (51). The 0.15 M wash contained monophosphates, which were discarded. The diphosphates were eluted in the 0.3 M wash. The ketohexose-containing fractions were combined and acidified by addition of Dowex 50W-X8 (H^+ form) until the pH was 2.0. The sample was then

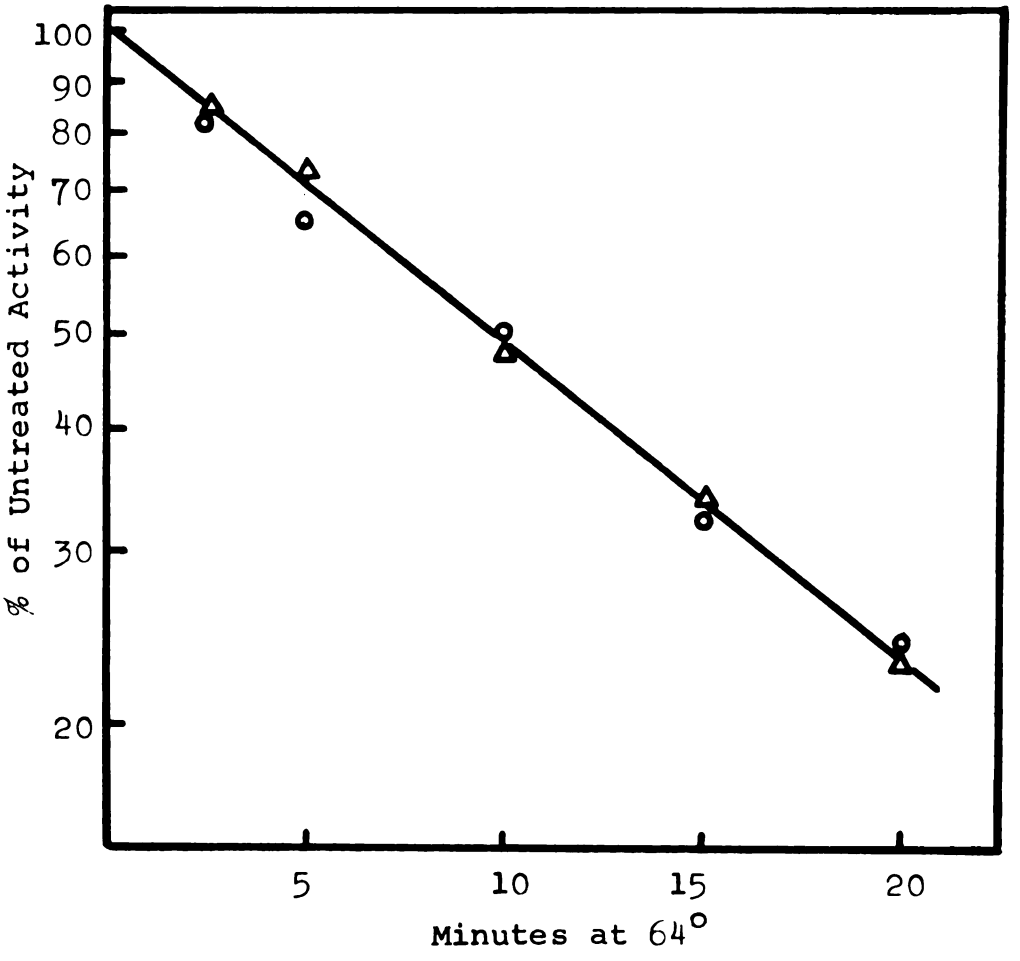


Figure 20. Concomitant thermal inactivation of D-tagatose 1,6-diphosphate aldolase and D-fructose 1,6-diphosphate aldolase activities. Symbols: D-tagatose 1,6-diphosphate aldolase activity (O), and D-fructose 1,6-diphosphate aldolase activity (Δ).

lyophilized and dissolved in 5 ml of water. The pH was adjusted to 7.0 with NaOH.

All of the ketohexose eluted from the Dowex column as would be expected for a diphosphate, and had a phosphate to ketohexose ratio of 1.92:1. For each mole of organic phosphate in the product, 1.04 moles of NADH were oxidized when incubated with D-tagatose 1,6-diphosphate aldolase, triose phosphate isomerase, and α-glycerolphosphate dehydrogenase. The reaction sequence was as follows: ketohexose diphosphate ⟶ dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate ⟶ 2 dihydroxyacetone phosphate ⟶ 2 α-glycerol phosphate. This implies that only the D-isomer of the commercial DL-glyceraldehyde 3-phosphate was utilized in the condensation reaction, and that dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate are the products of the cleavage reaction.

For further identification, the ketohexose diphosphate was enzymatically dephosphorylated, and subjected to analysis by paper chromatography. The amounts used were 2 μmoles for the dephosphorylated product, and 100 nmoles for the 2-ketohexose standard, whereas the threshold of detection for the visualizing reagents was less than 10 nmoles. The carbohydrates were visualized with an orcinol spray for ketohexose, and a duplicate chromatogram was developed with a silver nitrate spray for total carbohydrates. The only spots visible from the dephosphorylated product corresponded to tagatose and fructose ($R_{tagatose}$ = 1.12). Spots corresponding to sorbose ($R_{tagatose}$ = 0.96) and psicose ($R_{tagatose}$ = 1.22) were not visible. Thus, neither sorbose nor psicose could constitute as much

as one percent of the dephosphorylated product. The absence of sorbose, which migrates closely to tagatose, can be confirmed by the cysteine-H_2SO_4 reaction (88). After 20 hours, the A_{412} to A_{605} ratio for sorbose was 0.45, whereas the ratio for the other three 2-ketohexoses was 3.46-3.6. Analysis of the dephosphorylated product gave a ratio of 3.54, indicating the absence of sorbose in the solution.

Effect of cations. Because the assay for D-tagatose 1,6-diphosphate aldolase contains D-fructose 6-phosphate kinase, which requires magnesium ions, this assay could not be used to assess the effect of divalent cations. Instead, D-fructose 1,6-diphosphate was used for these assays, thereby obviating the use of D-fructose 6-phosphate kinase. The effect of nine divalent cations on the activity of EDTA-treated aldolase was determined (Table 9). Without the addition of divalent cations, the EDTA-treated enzyme had no detectable activity. The divalent cations that restored the activity to levels much higher than the untreated enzyme were Co^{++} and Cd^{++}. The activity was restored to approximately the untreated levels by Mn^{++} and Zn^{++}, whereas Fe^{++} and Ca^{++} only partially restore the activity. The activity was not restored by Mg^{++}, Ni^{++}, or Cu^{++}.

The addition of monovalent cations to D-tagatose 1,6-diphosphate aldolase which has not been treated with EDTA (Table 10) shows that the cations which stimulate the most are K^+ and NH_4^+. The effect of potassium on the kinetic constants of non-EDTA-treated, but Co^{++} supplemented, aldolase is shown by comparing Lineweaver-Burk plots

Table 9. Effect of divalent cations on EDTA-treated D-tagatose 1,6-diphosphate aldolase

The standard assay using D-fructose 1,6-diphosphate was used to obtain these results.

Metal used[a]	Units/mg protein	
	2×10^{-5} M	10^{-3} M
None	<0.002	<0.002
$MgCl_2$	<0.002	<0.002
$NiCl_2$	<0.002	<0.002
$CuSO_4$	<0.002	<0.002
$FeSO_4$	0.025	<0.002
$ZnCl_2$	0.077	<0.002
$CaCl_2$	0.009	0.077
$MnCl_2$	0.090	0.201
$CoCl_2$	0.201	0.188
$CdSO_4$	0.225	0.233

[a]Untreated enzyme = 0.074 units/mg protein.

Table 10. Stimulation of D-tagatose 1,6-diphosphate aldolase by monovalent cations

5 μmoles of monovalent cation were added to the standard assay utilizing D-fructose 1,6-diphosphate as substrate.

Addition	Relative velocity
None	100
KCl	1440
NH_4Cl	1540
NaCl	620
RbCl	179
LiCl	116
CsCl	100

in the presence (Figure 19B) and absence (Figure 21A) of KCl. The presence of KCl lowers the K_m for D-fructose 1,6-diphosphate from 1.27 mM to 0.86 mM, and stimulates the V_{max} approximately 2.5-fold. Varying the potassium concentration showed that it has a K_a of approximately 6 mM (Figure 21B).

Thus, it can be seen that both monovalent and divalent cations have significant effects on the enzyme activity.

Effect of $NaBH_4$. Inhibition of the aldolase activity by $NaBH_4$, in the presence and absence of substrate (0.5 mM D-tagatose 1,6-diphosphate or 25 mM D-fructose 1,6-diphosphate), was assessed using 10 mM or 100 mM of the reducing agent. Significant loss of activity (greater than 2%) 15 min after addition of the ketohexose

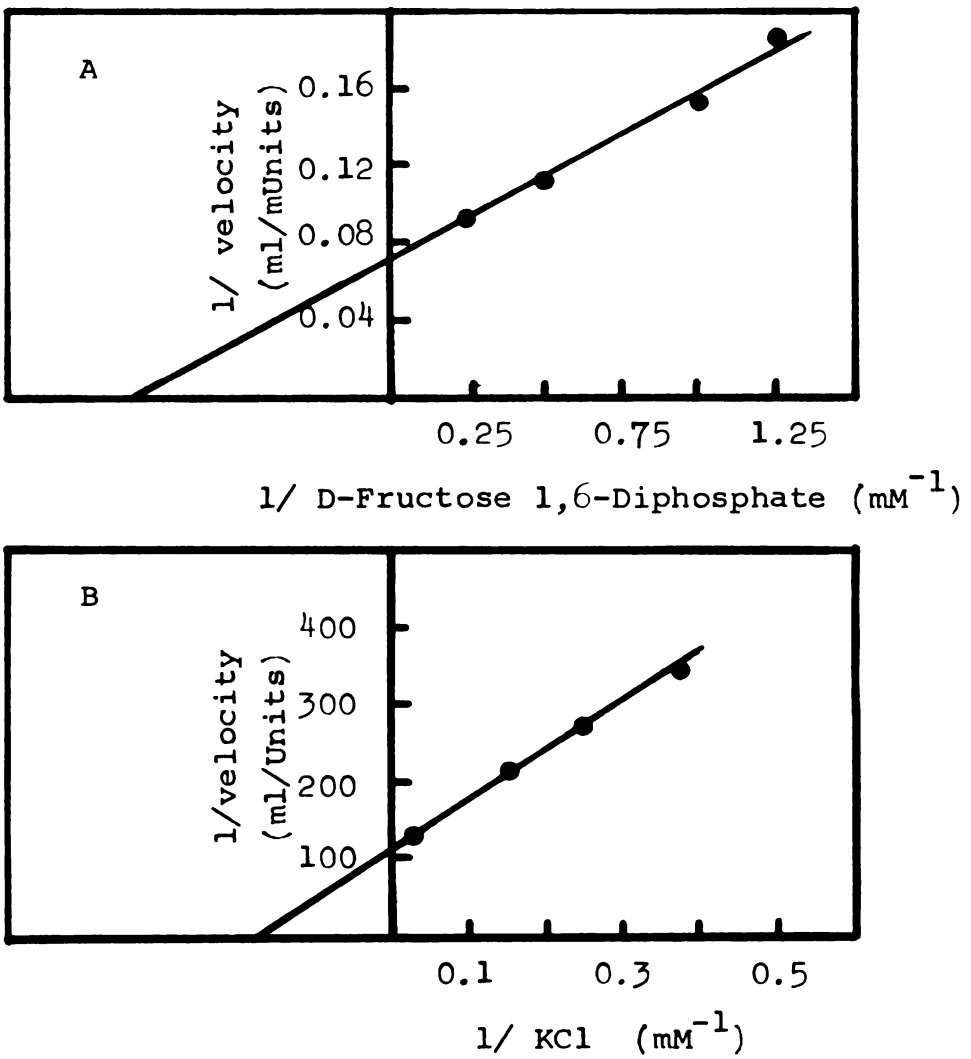


Figure 21. Effect of potassium ion on D-tagatose 1,6-diphosphate aldolase. Both A and B were obtained using the aldolase assay utilizing D-fructose 1,6-diphosphate as substrate with 0.03 µmole of $CoCl_2$. The D-fructose 1,6-diphosphate concentration in B is 4 mM. The specific V_{max} for A is 0.24 units/mg protein.

diphosphate was not found. A control incubation containing rabbit muscle aldolase was 96% inhibited by 10 mM $NaBH_4$ in the presence of 1.5 mM D-fructose 1,6-diphosphate, after 6 min. These data indicate that D-tagatose 1,6-diphosphate aldolase does not form a Schiff's base intermediate as part of the reaction mechanism (97,98). The aldolase can therefore be classified as class II type (97,98).

Molecular weight. The elution of the aldolase from a 1.45 x 88 cm Sephadex G-150 column, with standards of known molecular weight, was used to determine the molecular weight of the aldolase. The standards and their molecular weights are as follows: rabbit muscle pyruvate kinase, 237,000 (99); beef heart lactic dehydrogenase, 140,000 (90,95); yeast glucose 6-phosphate dehydrogenase, 128,000 (90); and *E. coli* alkaline phosphatase, 86,000 (91). Plotting the elution versus the log of the molecular weight of the aldolase results in a linear relationship (Figure 22). Assuming a spherical shape for the aldolase, the molecular weight can be estimated as 157,000.

Discussion. As demonstrated by the preceding data, an enzyme can be purified from cell extracts of galactitol-grown cells that is capable of catalyzing the proposed cleavage of D-tagatose 1,6-diphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The aldolase was found to be very specific, cleaving only D-tagatose 1,6-diphosphate and, to a lesser extent, D-fructose 1,6-diphosphate. Evidence was presented showing that neither D-psicose 1,6-diphosphate, D-sorbose 1,6-diphosphate, D-fructose 1-phosphate, nor any of a variety of sugar phosphates was cleaved

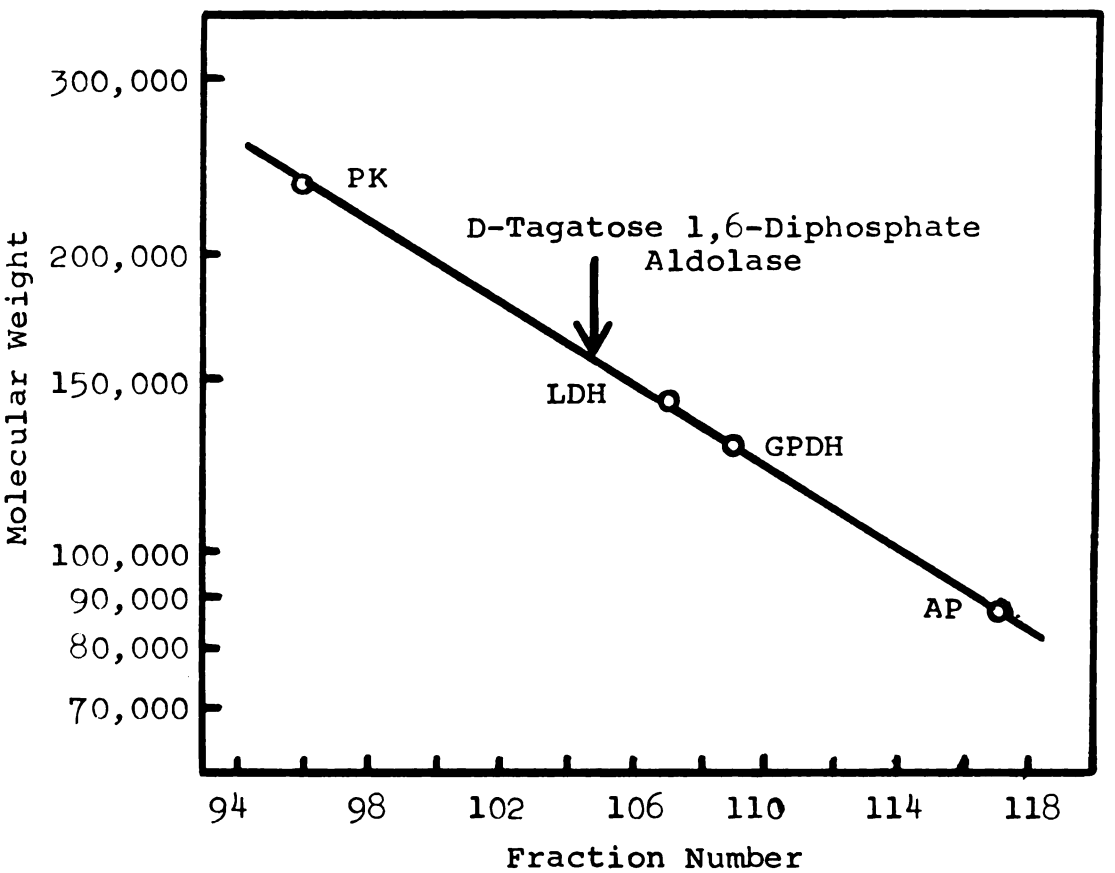


Figure 22. Molecular weight determination of D-tagatose 1,6-diphosphate aldolase. The protein standards used were pyruvate kinase (PK), D-glucose 6-phosphate dehydrogenase (GPDH), lactate dehydrogenase (LDH), and alkaline phosphatase (AP).

by the aldolase. The relatively low V_{max} for the cleavage of D-fructose 1,6-diphosphate probably implies that it could not play a significant role in catabolism of compounds that are converted to D-fructose 1,6-diphosphate.

The effect of cations on the aldolase was shown to be considerable. Removal of divalent cations with EDTA resulted in loss of the activity, which could be restored by a number of cations with varying degrees of success, Co^{++} and Cd^{++} being the best. In the presence of an activating divalent cation, monovalent cations provided further activation, the effect of K^{+} being to lower the K_m and raise the V_{max}.

The lack of inhibition of activity in the presence of $NaBH_4$ and ketohexose diphosphate substrates allows designating the aldolase as a class II type. This class of aldolase has been assumed to be typical for bacterial species. Typically they require divalent cations, and are activated by monovalent cations. One characteristic of the aldolase that is more characteristic of class I (mammalian) aldolases is the molecular weight of 157,000. Most class I aldolases have molecular weights of 150,000-160,000, whereas class II aldolases generally have molecular weights of 70,000-80,000 (97). That this aldolase is of bacterial origin cannot alone be used as a criterion for classification, as a few bacterial class I aldolases have now been identified (100-105), including the D-tagatose 1,6-diphosphate aldolase from *S. aureus* (64). Except for the discrepancy in molecular weight, the data fit the criteria for a class II aldolase.

It is interesting to note the differences in the D-tagatose 1,6-diphosphate aldolase from *K. pneumoniae* and *S. aureus*, the only two examples of this enzyme ever studied. The aldolase from *S. aureus* (64) is a 40,000 molecular weight protein, utilizes a Schiff's base intermediate, is not inhibited by EDTA or activated by monovalent cations, and can cleave all four D-2-ketohexose diphosphates (D-fructose 1,6-diphosphate at 47% of the rate for D-tagatose 1,6-diphosphate). The *K. pneumoniae* enzyme, on the other hand, is a 157,000 molecular weight protein, utilizing a divalent cation in its reaction mechanism, rather than a Schiff's base intermediate, is activated by monovalent cations, and is specific for D-tagatose 1,6-diphosphate and D-fructose 1,6-diphosphate (cleaving the latter at 4% the rate of the former). Except for catalyzing the same physiological reaction, the two enzymes seem to have little in common. Perhaps as more organisms are studied with respect to this enzyme, an evolutionary or genetic basis for these differences will become apparent.

SECTION 3

GENETIC EVIDENCE FOR THE PHYSIOLOGICAL SIGNIFICANCE OF THE PROPOSED PATHWAY FOR GALACTITOL CATABOLISM

INTRODUCTION

A pathway for the catabolism of galactitol through L-galactitol 1-phosphate, a known metabolite in *Klebsiella pneumoniae* (59), has been established. The data of the first two sections of this dissertation indicate potential participation of a dehydrogenase, kinase, and aldolase, involved in oxidation of L-galactitol 1-phosphate to D-tagatose 6-phosphate, phosphorylation to D-tagatose 1,6-diphosphate, and cleavage to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. These three enzymes have been demonstrated in cell extracts of galactitol-grown cells. In addition, these enzymes have been partially purified, and their K_m values and substrate specificities found to be consistent with their role in this pathway.

The studies of this section show the inducibility of the dehydrogenase, kinase, and aldolase, as well as genetic evidence for their physiological role in galactitol catabolism. It is demonstrated that although the kinase activity is constitutive, the dehydrogenase and aldolase activity are specicifically induced by growth on the cells on galactitol. Analysis of mutants missing either the dehydrogenase, kinase, or aldolase shows that each of these enzymes is essential to the ability of *K. pneumoniae* to utilize galactitol as a carbon source for growth.

MATERIALS AND METHODS

All materials and methods not presented here were described in the Materials and Methods of Sections 1 and 2.

Organisms. The wild type organism was *Klebsiella pneumoniae* PRL-R3 (formerly designated *Aerobacter aerogenes* PRL-R3). All the mutants described were derived from a deletion mutant of PRL-R3 that is auxotrophic with respect to growth on uracil (U^-).

Media. Routine growth of cells was done in nutrient broth. For mineral agar plates, agar and mineral medium were made up and sterilized separately at such concentrations that the final mixture was 1.5% (w/v) agar. Growth of cells on specific sugars was at a concentration of 0.5% (w/v). Growth or induction of uracil auxotrophs was in media supplemented with 0.005% (w/v) uracil.

Enzyme Induction. Induction of the pathway enzymes in different strains was initiated by adding 7 ml of nutrient broth containing 1% galactitol to a 7 ml nutrient broth culture of cells. The tubes were then incubated on a reciprocal shaker at 30°. Hourly sampling of wild-type cells under these conditions, and subsequent assays of the extracts for the dehydrogenase and aldolase, established that these enzymes were maximally induced after six hours. Following this time, the specific activity of the activities decreased,

dropping to about half of the maximal values after 10 hours. All other strains were also assayed after six hours of incubation.

Monitoring of Cell Growth. Cell growth was monitored with a Coleman Junior Spectrophotometer, model 6A, at 600 nm. These cells were grown in 18 x 150 mm tubes containing 7 ml of mineral medium supplemented with 0.5% (w/v) of carbohydrate. The tubes were incubated at 30° on a reciprocal shaker. A plot of absorbance versus growth was prepared using actual A_{600} values from undiluted samples, and corrected absorbance values from diluted samples. This plot was used to correct for light scattering at higher absorbance values.

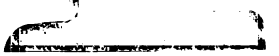
Enzymatic Assays. All assays were as described in Section 2, except that the D-tagatose 1,6-diphosphate aldolase assay additionally contained 5 μmoles of KCl and 0.15 μmoles $CoCl_2$ for optimal activity.

Mutant A9-1 (D-Fructose 6-Phosphate Kinase Negative). Mutant A9-1 was the strain isolated as reported previously (106) and found to be missing D-fructose 6-phosphate kinase.

Isolation of Mutants Missing L-Galactitol 1-Phosphate Dehydrogenase (VP-14) or D-Tagatose 1,6-Diphosphate Aldolase (VP-8). To isolate mutants selectively impaired for growth on galactitol, an overnight culture of *K. pneumoniae* PRL-R3 (U^-) in nutrient broth (7 ml) was harvested by centrifugation in a sterile centrifuge tube, and suspended in 7 ml of mineral medium containing 0.2 M ethyl methanesulfonate. After incubation for two hours on a shaker at 30°, the cells were harvested and washed three times with 7 ml of

mineral medium. The cells were grown in nutrient broth until there was a 10-fold increase in cell number. The cells were suspended in mineral medium containing 0.5% (w/v) galactitol and 2000 units/ml of Penicillin G. After four hours, the cells were harvested, washed in mineral medium, and grown for 10 generations in mineral medium plus 0.5% (w/v) D-fructose. The penicillin-treated bacteria were placed on uracil-supplemented mineral agar containing 0.5% galactitol plus 0.005% D-glucose. After 48 hours at 30°, the smallest colonies were selected and grown in nutrient broth. The cells were screened for growth in mineral agar plates containing 0.5% (w/v) of galactitol, mannitol, D-glucose, or D-fructose. Mutants VP-8 and VP-14 were selected as strains which grew well on D-mannitol, D-glucose, and D-fructose, but failed to grow on galactitol. Both mutants were shown to be uracil auxotrophs.

Isolation of Spontaneous Revertants (VP-8R, VP-14R, and A9-1R). Revertants of the above mutants were isolated by exerting selective pressure favoring only growth on galactitol. The reason for this was twofold: first, to establish that the above mutations were caused by single, reversible point mutations, and second, to show that selective pressure from galactitol alone was enough to cause the reversions, establishing each gene's physiological importance in the pathway. Overnight cultures of VP-8, VP-14 and A9-1 in mineral medium plus 0.25% of D-fructose were plated at high density on mineral agar containing 0.5% galactitol (w/v). After 48 hours, revertants which appeared were isolated. It was confirmed that the cells were still auxotrophic for uracil.



Source of Materials. The following materials were obtained from the indicated sources: uracil, penicillin G, D-mannose, D-fructose, D-galactose, maltose, and lactose from Sigma Chemical Co.; ethyl methanesulfonate from Eastman Organic Chemicals; agar from Difco Laboratories; D-glucose and sucrose from Mallinckrodt Chemical Works; and L-arabinose from Pfanstiehl Laboratories.

RESULTS

Analysis of Enzyme Defects in the Mutants. The parental strain (PRL-R3 U^-), the mutants (A9-1, VP-8, and VP-14), and revertants (A9-1R, VP-8R, and VP-14R) were incubated in nutrient broth containing 0.5% galactitol for 6 hours. Cell extracts were prepared and assayed for the galactitol pathway enzymes (Table 11). Strain A9-1 was confirmed to be missing D-fructose 6-phosphate kinase and was also shown to be deficient in D-tagatose 6-phosphate kinase, whereas A9-1R regained both these activities. Mutant VP-8 was missing D-tagatose 1,6-diphosphate aldolase, whereas the revertant regained this activity. The cell extract of VP-14 lacks L-galactitol 1-phosphate dehydrogenase, but the revertant did exhibit this activity. Thus, a mutant deficient for each one of the pathway enzymes (dehydrogenase, kinase, and aldolase) has been isolated.

Growth of Mutants. The growth of the parental strain (PRL-R3 U^-) on eight different sugars, including galactitol, is shown in Figure 23. The cells were observed to exhibit a lag before growth on galactitol took place. Mutant A9-1 was seen to be affected for growth on a number of sugars, with no growth detected on galactitol (Figure 24). Growth of mutants VP-8 and VP-14--as well as revertants A9-1R, VP-8R, and VP-14R--on galactitol is seen in Figure 25. VP-8 and VP-14, like A9-1, did not grow on galactitol, but were not

Table 11. Induction of enzymes for the catabolism of L-galactitol 1-phosphate in parental and mutant strains

Strain	Units/mg protein[a]			
	L-galactitol 1-phosphate dehydrogenase	D-fructose 6-phosphate kinase	D-tagatose 6-phosphate kinase	D-tagatose 1,6-diphosphate aldolase
PRL-R3 U^-	0.101	0.158	0.095	0.217
A9-1	0.021	<0.003	<0.003	0.108
VP-8	0.019	0.136	0.089	<0.001
VP-14	<0.001	0.109	0.069	0.038
A9-1R	0.057	0.127	0.076	0.093
VP-8R	0.076	0.167	0.103	0.262
VP-14R	0.083	0.152	0.100	0.142

[a] 1 unit = 1 μmole substrate consumed per min at 30°.

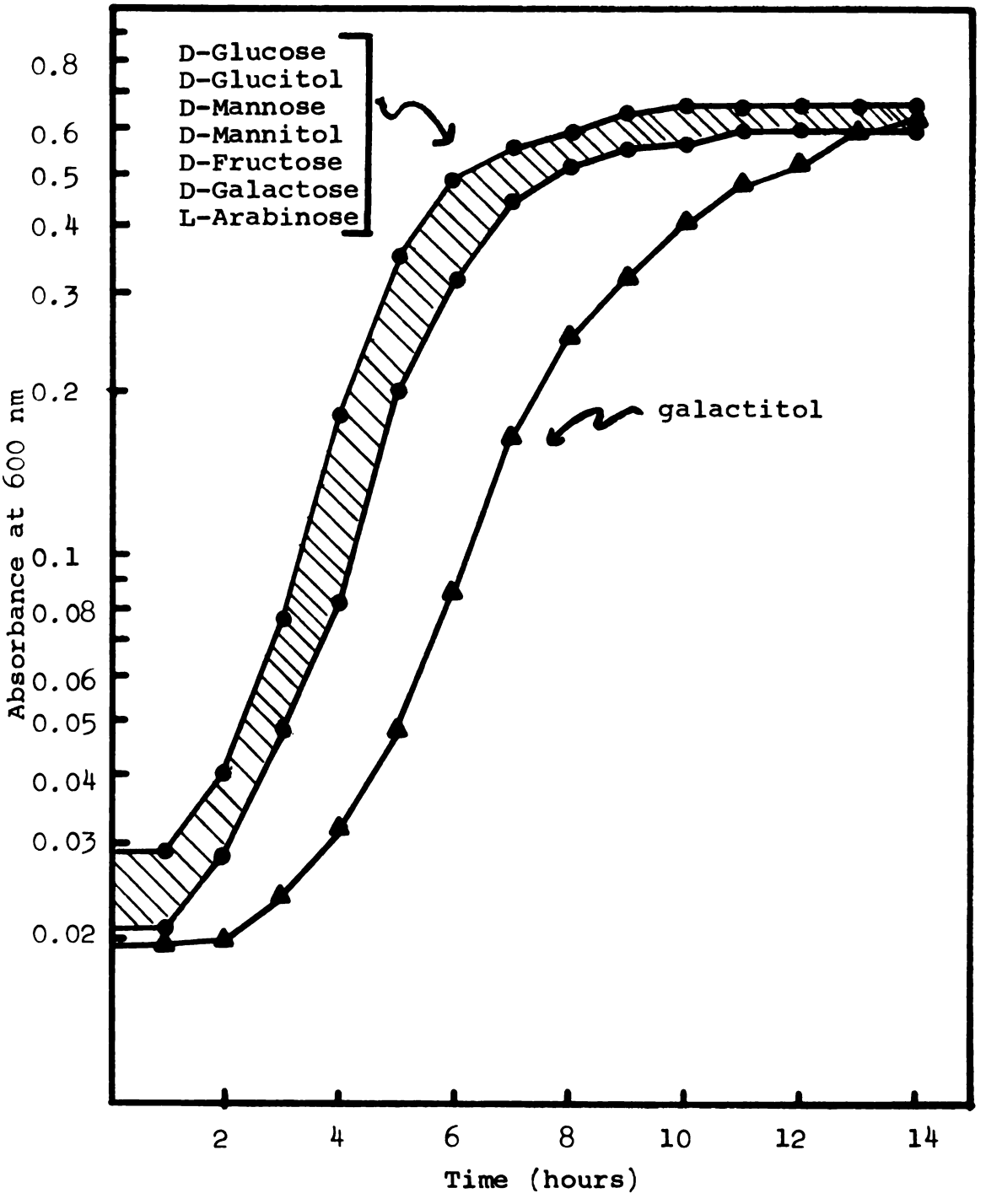


Figure 23. Growth characteristics of *K. pneumoniae* PRL-R3 (U). All carbohydrates tested were at 0.5% (w/v) concentration. The inoculum was 0.2 ml of a nutrient broth-grown culture.

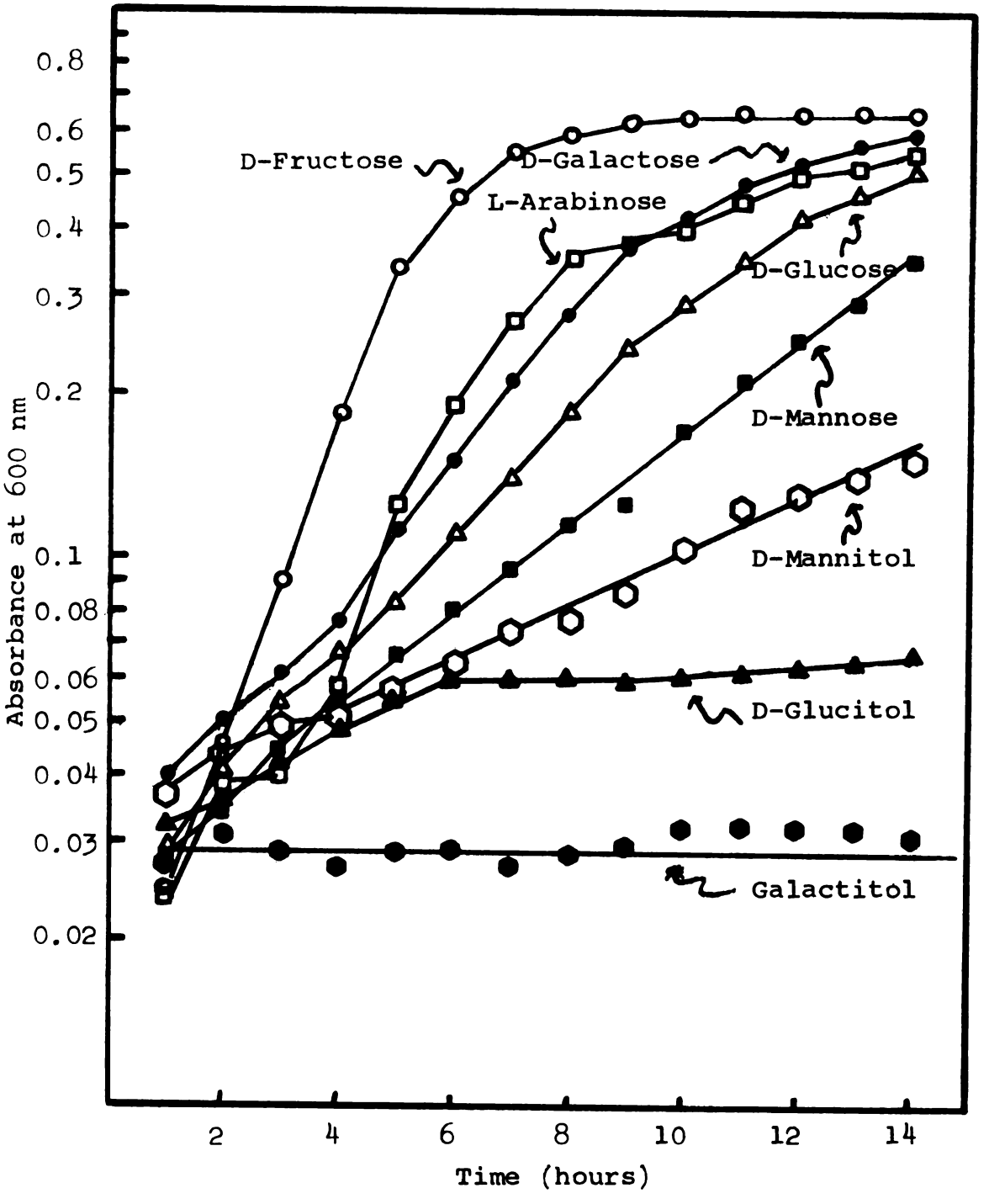


Figure 24. Growth characteristics of strain A9-1. Carbohydrate concentrations were 0.5% (w/v) and the inoculum was 0.2 ml of a nutrient broth-grown culture of A9-1.

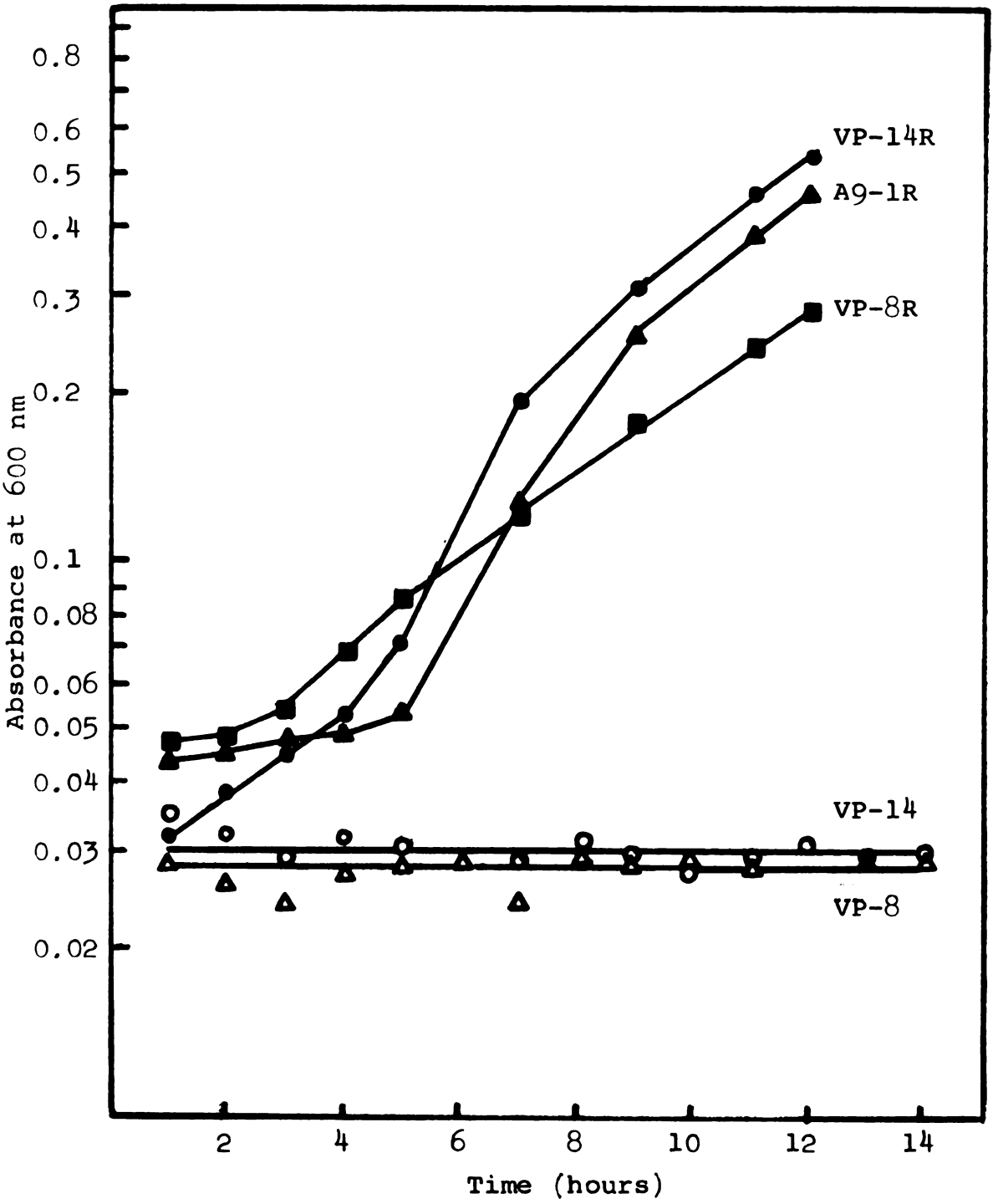


Figure 25. Growth of strains VP-8, VP-14, A9-1R, VP-8R, and VP-14R on galactitol. An inoculum of 0.2 ml of a nutrient broth-grown culture was used. Growth for all strains on 0.5% (w/v) D-glucose, D-glucitol, D-mannose, D-mannitol, D-fructose, D-galactose, and L-arabinose was the same as for PRL-R3 (U^-) in Figure 23.

affected for growth on any other carbohydrate tested. All three revertants regained the ability to grow on galactitol, and A9-1R no longer exhibited slow growth on D-glucitol, D-mannitol, D-glucose, or other sugars tested.

Specificity of Induction. The parent strain (PRL-R3 U^-) was grown on a variety of carbohydrates to show the induction pattern of the dehydrogenase, kinase, and aldolase (Table 12). These data demonstrate that the kinase activity is constitutive as previously reported (106), being present in extracts of cells grown on nutrient broth and all the carbohydrates tested. The dehydrogenase and aldolase, on the other hand, are specifically induced by growth on galactitol. Growth on any of the other 12 carbon sources failed to induce the activities.

Table 12. Induction of the galactitol pathway enzymes in *K. pneumoniae* grown on a variety of carbohydrates

Carbon source	Units/mg protein[a]		
	Dehydrogenase[b]	Kinase[c]	Aldolase[d]
Nutrient broth	<0.002	0.112	<0.005
D-glucose	<0.002	0.167	<0.005
D-mannose	<0.002	0.103	<0.005
D-mannitol	<0.002	0.216	<0.005
D-glucitol	<0.002	0.226	<0.005
D-fructose	<0.002	0.209	<0.005
D-galactose	<0.002	0.150	<0.005
L-arabinose	<0.002	0.133	<0.005
Maltose	<0.002	0.137	<0.005
Lactose	<0.002	0.119	<0.005
Sucrose	<0.002	0.156	<0.005
Glycerol	<0.002	0.190	<0.005
Galactitol	0.299	0.128	0.239

[a] 1 unit = 1 μmole of substrate used per min at 30°.

[b] L-Galactitol 1-phosphate dehydrogenase.

[c] D-Fructose 6-phosphate (D-tagatose 6-phosphate) kinase. Assayed with D-fructose 6-phosphate.

[d] D-Tagatose 1,6-diphosphate aldolase.

DISCUSSION

The data of this section of the dissertation revealed that mutant strains of *K. pneumoniae* missing L-galactitol 1-phosphate dehydrogenase, D-tagatose 6-phosphate kinase, or D-tagatose 1,6-diphosphate aldolase fail to grow on galactitol. Revertants of these strains regained the ability to grow on galactitol, as well as the enzyme that was formerly missing. Also, the aldolase and dehydrogenase activities were specifically induced by growth on galactitol, while the kinase was a constitutive activity.

Failure of mutants missing any of the enzymes of the pathway to grow on galactitol is evidence that this is the sole pathway of physiological importance for galactitol catabolism in this organism. The ability to obtain revertants of the mutants by plating on galactitol-containing medium suggests that they were all single point-mutations, and affirms the physiological importance of the gene products. Selective induction of the dehydrogenase and aldolase confirms the high substrate specificities observed in Section 2, indicating that these enzymes are unique to this pathway. The growth of mutant VP-14, missing L-galactitol 1-phosphate dehydrogenase, on D-glucitol substantiates that this activity is not merely a manifestation of D-glucitol 6-phosphate dehydrogenase (47). These data also confirm that D-tagatose 6-phosphate kinase and D-fructose 6-phosphate kinase activities are manifestations of the same protein.

Mutant A9-1 was selected to be missing D-fructose 6-phosphate kinase without respect to its growth characteristics on galactitol, and was found to be also deficient for D-tagatose 6-phosphate kinase. This mutant is unable to grow on galactitol, or a number of other sugars. When a revertant is obtained from galactitol plates, both kinase activities are restored, and growth on all sugars is comparable to the parent strain. Thus, data presented here and in Section 2 (co-purification, substrate competition, coincident thermal inactivation, etc.) establish that the constitutive D-fructose 6-phosphate kinase also catalyzes the phosphorylation of D-tagatose 6-phosphate to D-tagatose 1,6-diphosphate.

This section of the dissertation has, therefore, established the following: (i) D-tagatose 6-phosphate kinase is a constitutive activity, (ii) L-galactitol 1-phosphate dehydrogenase and D-tagatose 1,6-diphosphate aldolase are specifically induced by growth on galactitol, (iii) a single gene product is responsible for the D-fructose 6-phosphate kinase and D-tagatose 6-phosphate kinase activities, and (iv) these reactions constitute the sole pathway of physiological importance for galactitol catabolism in this organism.

SUMMARY

A review of the literature has shown that although many species utilize galactitol, a pathway for its metabolism has never been established. The purpose of this dissertation was to determine the catabolic reaction sequence that allows *Klebsiella pneumoniae* to make use of galactitol as a carbon source for growth. It was previously reported that in *K. pneumoniae* galactitol is phosphorylated by a PEP:galactitol phosphotransferase, yielding L-galactitol 1-phosphate. The data presented in this dissertation demonstrate that in this organism, L-galactitol 1-phosphate is further catabolized through a previously unreported pathway.

The evidence on which I have based these conclusions is as follows: (i) the demonstration and partial purification of L-galactitol 1-phosphate dehydrogenase, D-tagatose 6-phosphate (D-fructose 6-phosphate) kinase, and D-tagatose 1,6-diphosphate aldolase; (ii) the chromatographic, enzymatic, and chemical identification of the reaction products of these reactions; (iii) the galactitol-specific induction of the dehydrogenase and aldolase; and (iv) the selection and isolation of mutant strains unable to utilize galactitol and which are also missing one of these three enzymes. That this is the sole physiological pathway of galactitol catabolism is demonstrated by the failure of these mutants to grow

on galactitol, and the lack of a dehydrogenase, kinase, or epimerase in wild-type cells capable of modifying the free hexitol.

Thus, in *K. pneumoniae*, galactitol is catabolized as follows: galactitol ⟶ L-galactitol 1-phosphate ⟶ D-tagatose 6-phosphate ⟶ D-tagatose 1,6-diphosphate ⟶ dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate.

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