L-SORBOSE 1-PHOSPHATE REDUCTASE FROM AEROBACTER AEROGENES: ITS PURIFICATION, CHARACTERIZATION, AND ROLE IN THE METABOLISM OF L-SORBOSE AND D-FRUCTOSE

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ABSTRACT

L-SORBOSE 1-PHOSPHATE REDUCTASE FROM *AEROBACTER AEROGENES*: ITS PURIFICATION, CHARACTERIZATION, AND ROLE IN THE METABOLISM OF L-SORBOSE AND D-FRUCTOSE

By

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In *Aerobacter aerogenes* L-sorbose is metabolized via the pathway: L-sorbose → L-sorbose 1-phosphate → D-glucitol 6-phosphate → D-fructose 6-phosphate. The steps of the pathway are catalyzed by three enzymes: a phosphoenolpyruvate-dependent L-sorbose 1-phosphotransferase system; L-sorbose-1-P reductase, and D-glucitol-6-P dehydrogenase, respectively. A key reaction in this pathway is the reduction of L-sorbose-1-P (L-*xylo*-2-ketulose) with NADH or NADPH to D-glucitol-6-P by the L-sorbose-1-P reductase (L-sorbose-1-P:NAD(P) ketoreductase), which is inducible by growth on L-sorbose. The work in this dissertation covers three facets of L-sorbose-1-P reductase: (i) the isolation and identification of the product of the phosphoenolpyruvate-dependent phosphorylation of L-sorbose to demonstrate that L-sorbose-1-P is available as a substrate for the reductase in the cell; (ii) the purification and characterization of L-sorbose-1-P reductase, the first enzyme reported to catalyze the reduction of a ketohexose 1-phosphate; and (iii) the role of

this enzyme in the evolution of a novel pathway for the metabolism of D-fructose in *A. aerogenes*.

The product of the phosphoenolpyruvate-dependent phosphorylation of L-sorbose was isolated from a reaction mixture containing an extract of *A. aerogenes* supplemented with phosphoenolpyruvate and L-sorbose. The phosphorylated product (which gave a positive ketohexose test) was eluted from a Dowex-1 bicarbonate column at a concentration of $KHCO_3$ which elutes sugar monophosphates. It was identified as a ketohexose monophosphate on the basis of: (i) a ketohexose to phosphate ratio of 1:1; (ii) a rate of hydrolysis in 1N HCl that was identical with that of authentic L-sorbose-1-P but different from L-sorbose-6-P; and (iii) a mass spectrogram of the trimethylsilyl O-methyloxime derivative of the product which gave a fragmentation pattern and molecular weight predicted for a 2-ketohexose 1-phosphate. The ketohexose moiety was identified as L-sorbose by chemical, enzymatic, and chromatographic techniques.

L-Sorbose-1-P reductase was purified 41-fold by DEAE-cellulose chromatography and by affinity chromatography on NADP-sepharose using elution with various combinations of cofactor and D-fructose-1-P. The final preparation contained no more than 1% contamination by other proteins as determined by acrylamide gel electrophoresis. The L-sorbose-1-P reductase was shown to have a molecular weight of 90,000 $\pm$ 2,000 by gel filtration and ultracentrifugation and is composed of two identical subunits of 45,000 $\pm$ 1,000 molecular weight as determined by acrylamide gel electrophoresis with and without sodium dodecyl sulfate. The enzyme is an acidic protein based on the following data: (i) amino acid analysis revealed

relatively low amounts of basic amino acids; (ii) the isoelectric point is 5.0 by isoelectric focusing; (iii) the enzyme migrated toward the anode at pH 6.2; and (iv) the enzyme bound to DEAE-cellulose at pH 6.2.

Treatment of purified L-sorbose-1-P reductase with EDTA abolished catalytic activity. The activity of EDTA-treated enzyme could be restored by adding various divalent metal cations, but only Mn^{+2} could reactivate the enzyme to levels shown before EDTA-treatment. The enzyme was not capable of reducing L-sorbose, D-fructose, D-fructose-6-P, or D-fructose-1,6-P_2 at concentrations up to 10 mM. The enzyme could catalyze the reduction of either L-sorbose-1-P or D-fructose-1-P with a Km of 0.65 mM and 0.38 mM, respectively. The maximal velocity was three times as great for L-sorbose-1-P as for D-fructose-1-P. The enzyme could use either NADH or NADPH as a cofactor with a Km of about 20 μM for each.

The product of the reduction of D-fructose-1-P was identified as D-mannitol-1-P by chemical, enzymatic, and chromatographic techniques. The equilibrium constant of the reaction for the reduction of L-sorbose-1-P and D-fructose-1-P at pH 6.15 was determined to be 2.6×10^7 and 4.5×10^7 liters per mole, respectively. The pH optimum of the reaction was 6.2 in three buffers tested; the greatest activity was obtained in the presence of 2(N-morpholino)sulfonic acid buffer. The stoichiometry of the reaction revealed that 1 mole of ketohexose-1-P was reduced by 1 mole of NAD(P)H for every mole of sugar alcohol phosphate and $NAD(P)^+$ produced.

Ronald Allen Simkins

The normal pathways of exogenous and endogenous D-fructose catabolism (D-fructose → fructose-1-P → fructose-1,6-P_2 and fructose → fructose-6-P → fructose-1,6-P_2, respectively) were blocked in *A. aerogenes* by sequentially mutating the genes for D-fructose-1-P kinase and fructokinase. This fructose-negative strain would grow linearly on D-fructose if preinduced by growth on L-sorbose plus D-mannitol. By selecting from this strain a mutant (SF1) which was constitutive for L-sorbose-1-P reductase, the ability to utilize D-fructose as a sole source of carbon and energy was concomitantly gained. Mutants of SF1 missing either L-sorbose-1-P reductase or D-mannitol-1-P dehydrogenase were unable to grow on D-fructose, whereas a mutant with increased levels of D-mannitol-1-P dehydrogenase showed an increased growth rate on D-fructose. When incubated with D-fructose, the L-sorbose-1-P reductase-negative mutant accumulated D-fructose-1-P and no detectable levels of D-mannitol-1-P, whereas the D-mannitol-1-P dehydrogenase-negative mutant accumulated both D-fructose-1-P and D-mannitol-1-P at greater levels than SF1. The mutant with increased levels of D-mannitol-1-P dehydrogenase showed a proportional decrease in the levels of D-mannitol-1-P accumulated. Thus the newly evolved pathway uses the phosphoenolpyruvate:D-fructose 1-phosphotransferase system of normal exogenous D-fructose metabolism, the L-sorbose-1-P reductase of L-sorbose metabolism, and the D-mannitol-1-P dehydrogenase of D-mannitol metabolism to convert D-fructose → D-fructose-1-P → D-mannitol-1-P → D-fructose-6-P. This is a novel pathway of D-fructose metabolism which has been previously undetected in any organism.

L-SORBOSE 1-PHOSPHATE REDUCTASE FROM *AEROBACTER AEROGENES*: ITS PURIFICATION, CHARACTERIZATION, AND ROLE IN THE METABOLISM OF L-SORBOSE AND D-FRUCTOSE

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LIST OF SYMBOLS AND ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
DEAE-cellulose	Diethylaminoethyl cellulose
EDTA	(Ethylenedinitrilo) tetraacetic acid
Fru	D-fructose
D-fructose-1-P	D-fructose 1-phosphate
D-fructose-6-P	D-fructose 6-phosphate
D-fructose-1,6-P_2	D-fructose 1,6-biphosphate
D-glucitol-1-P	D-glucitol 1-phosphate
D-glucitol-6-P	D-glucitol 6-phosphate
D-glucose-6-P	D-glucose 6-phosphate
D-gulose-6-P	D-gulose 6-posphate
D-iditol-1-P	D-iditol 1-phosphate
D-mannitol-1-P	D-mannitol 1-phosphate
mercaptoethanol	2-hydroxymethylmercaptan or 2-thioethanol
MES	2[N-morpholino]ethanesulfonic acid
MES buffer	0.025 M MES (pH 6.15) containing 0.2% mercaptoethanol and 2 mM EDTA
Mtl	D-mannitol
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide

$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PEP:D-fructose PTS	phosphoenolpyruvate-dependent D-fructose 1-phosphotransferase system
PEP:L-sorbose PTS	phosphoenolpyruvate-dependent L-sorbose 1-phosphotransferase system
Scs	sucrose
SDS	sodium dodecyl sulfate
Sor	L-sorbose
D-sorbose-1-P	D-sorbose 1-phosphate
L-sorbose-1-P	L-sorbose 1-phosphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

During on-going investigations in this laboratory on the metabolism of L-sorbose (L-*xylo*-2-ketulose) in *Aerobacter aerogenes*, I became involved in the elucidation of the catabolic pathway in collaboration with Norman E. Kelker and Richard L. Anderson. In the initial stages, the elucidation of the pathway was facilitated by examining mutants defective in hexose and hexitol metabolism. In contrast to the wild-type strain, mutants missing either enzyme I of the phosphoenolpyruvate-dependent phosphotransferase system, D-glucitol-6-P dehydrogenase, or D-fructose-6-P kinase were unable to utilize L-sorbose as a sole source of carbon and energy. These findings implicated D-glucitol-6-P, D-fructose-6-P, and some phosphorylated product of a phosphoenolpyruvate-dependent L-sorbose phosphotransferase system (PEP:L-sorbose PTS) in the pathway. There was no evidence for ATP-dependent phosphorylation of L-sorbose. These data suggested that the product of the PEP:L-sorbose PTS might be L-sorbose-1-P and, consequently, that there should be a reductase capable of catalyzing the reduction of L-sorbose-1-P to D-glucitol-6-P. By using chemically synthesized substrate, a pyridine nucleotide-dependent L-sorbose-1-P reductase was in fact detected in crude extracts of cells grown on L-sorbose, but not in extracts of cells grown on D-glucose, D-glucitol, or

glycerol. The L-sorbose inducibility of the reductase was consistent with its participation in L-sorbose metabolism. This supposition was confirmed by the isolation of an L-sorbose-1-P reductase-negative mutant which simultaneously lost the ability to grow on L-sorbose, and by the isolation of a revertant of this mutant which concomitantly regained the reductase activity and the ability to utilize L-sorbose.

In crude extracts of L-sorbose-grown cells supplemented with phosphoenolpyruvate and L-sorbose, it was possible to demonstrate a precursor-product relationship between L-sorbose-1-P and D-glucitol-6-P that would be predicted by the catabolic pathway depicted in Figure 1. The first step of this pathway involves the phosphorylation of L-sorbose at carbon atom 1 by a PEP:L-sorbose PTS. This system was found to be present in crude extracts of cells grown on a variety of carbohydrates, suggesting that the system is constitutive. The second step is the reduction of L-sorbose-1-P to D-glucitol-6-P by the inducible L-sorbose-1-P reductase. The third step is the oxidation of D-glucitol-6-P to D-fructose-6-P by an inducible D-glucitol-6-P dehydrogenase. This is a unique pathway that has not been previously described in any organism.

This dissertation concerns three topics that deal directly with or were derived from the elucidation of the pathway shown in Figure 1. The first part reports the isolation and identification of the product of the PEP:L-sorbose PTS. To prove that the proposed pathway was correct it was essential to show unequivocally that L-sorbose-1-P is the first intermediate. The second part

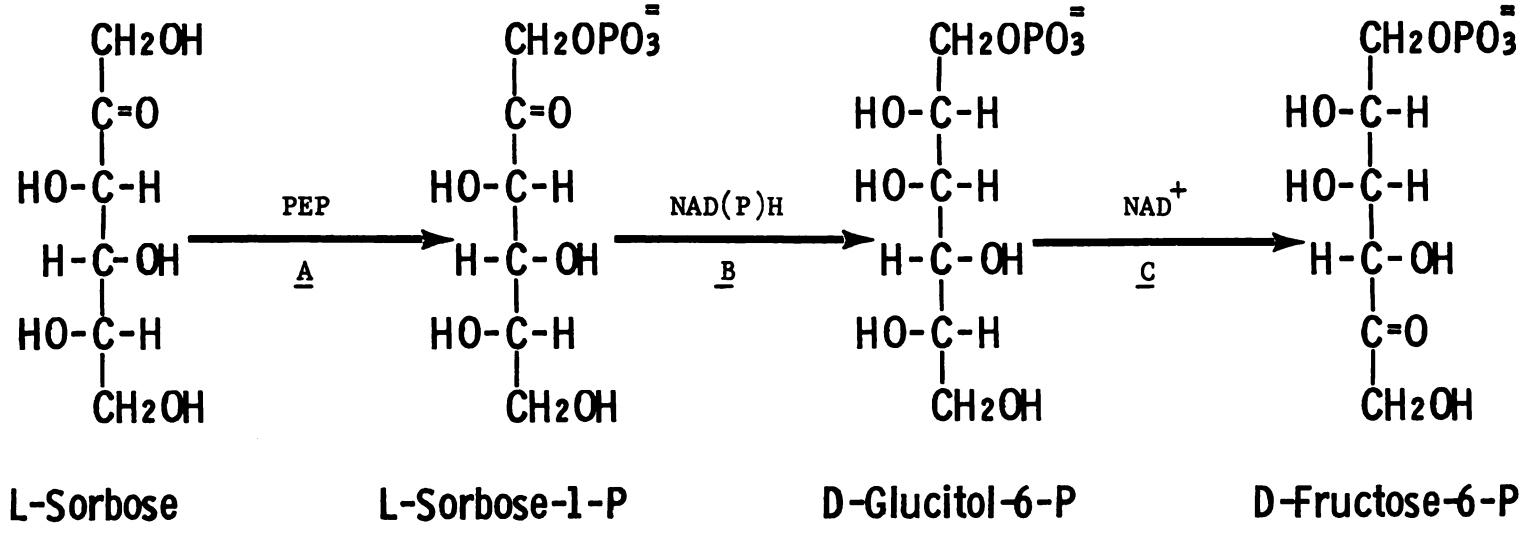


Figure 1 - Pathway of L-sorbose metabolism in Aerobacter aerogenes. Enzymes catalyzing the reactions are — A : PEP:L-Sorbose 1-Phosphotransferase System
B : L-Sorbose 1-Phosphate Reductase
C : D-Glucitol 6-Phosphate Dehydrogenase.

describes the purification and properties of the enzyme catalyzing the second step in the pathway: L-sorbose-1-P:NAD(P) ketoreductase (or trivialy, L-sorbose-1-P reductase). The third part deals with a project that arose from the determination of the substrate specificity of the reductase: the evolution of a novel pathway for the metabolism of D-fructose by conscripting enzymes normally associated with D-fructose, L-sorbose, and D-mannitol metabolism. The data from the first part of my dissertation have been published in a paper dealing with the elucidation of the pathway of L-sorbose metabolism in *A. aerogenes* (1).

LITERATURE REVIEW OF L-SORBOSE METABOLISM

Investigations of metabolic reactions of L-sorbose have been concerned primarily with those found in microorganisms. There has been little reported work with higher organisms. Although rat liver fructokinase can catalyze the phosphorylation of L-sorbose with ATP, the reaction is considered not to be of physiological significance (2).

In addition to the metabolic pathway of L-sorbose in *A. aerogenes* which has been discussed in the Introduction to this dissertation (Figure 1), two other series of reactions in which L-sorbose is modified have been proposed to occur in microorganisms (Figure 2). Early studies demonstrated that 2-keto-L-gulonate accumulated in the extracellular medium when species of *Acetobacter*, *Alcaligenes*, *Aerobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Serratia*, or *Xanthomonas* were grown in the presence of L-sorbose (3,4). Later studies by Perlman and co-workers demonstrated that cell-free extracts of *Gluconobacter melanogenus* were capable of converting L-sorbose to 2-keto-L-gulonate (5,6). A subsequent abstract suggested that the initial step in this conversion was the oxidation of L-sorbose to L-sorbosone (7). Furthermore, Makover and co-workers reported in abstract form that extracts of *A. aerogenes* and *Pseudomonas putida*

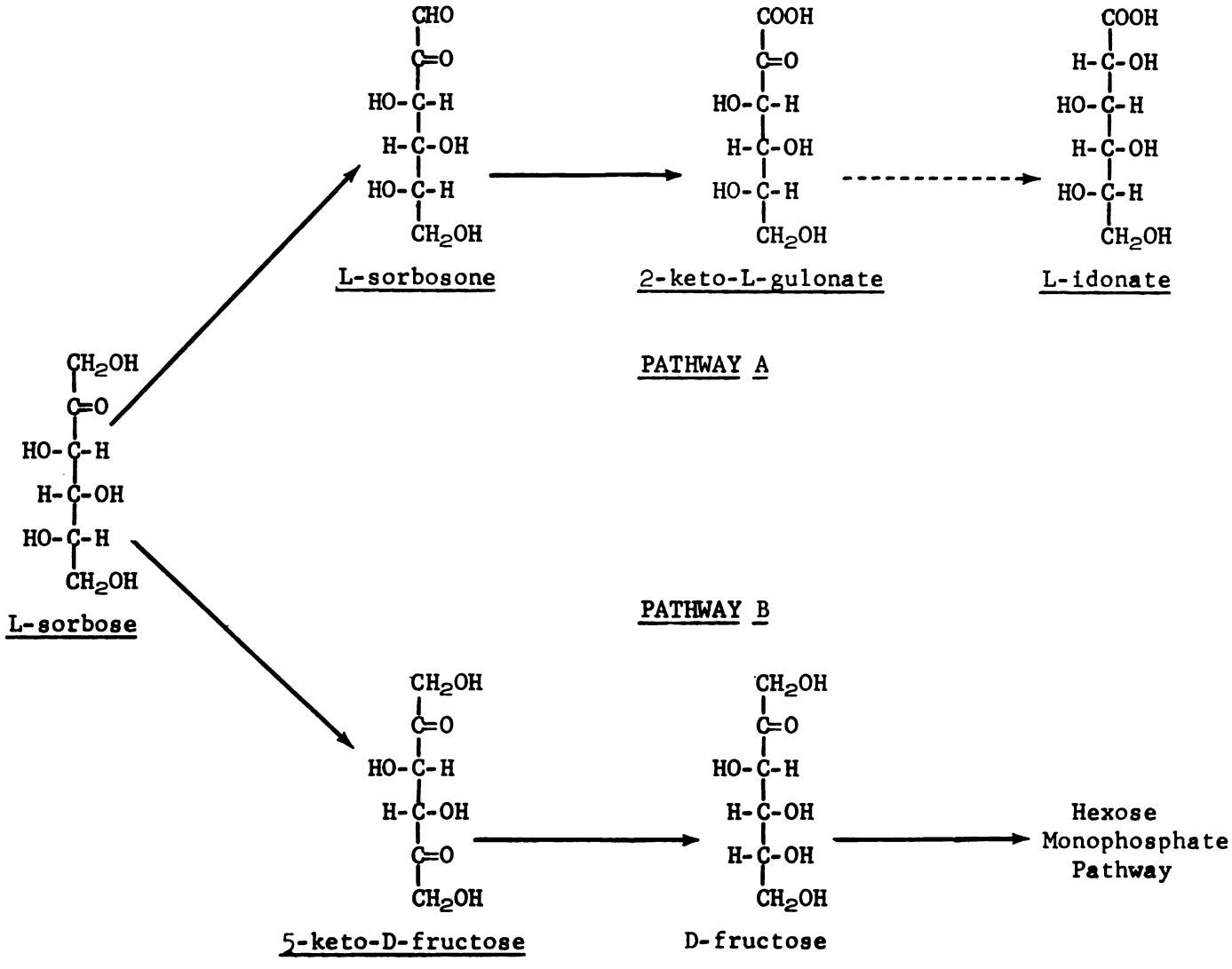


Figure 2 - Two proposed pathways by which L-sorbose may be modified in certain species of bacteria.

contained an L-sorbose oxidase which converted L-sorbose to L-sorbosone (8), and that extracts of *P. putida* and *G. melanogenus* contained an L-sorbosone oxidase which converted L-sorbosone to 2-keto-L-gulonate (9). These findings led to the proposal of pathway A in Figure 2. The latter group also reported the presence of a 2-keto-L-gulonate reductase in extracts of *P. putida* and *G. melanogenus* which converted 2-keto-L-gulonate to L-idonate (10). Since this reaction could not be reversed, an earlier proposal that L-idonate was an intermediate in 2-keto-L-gulonate formation (11) may not be correct. Due to the fact that the majority of these findings were reported only in short abstracts of verbal presentations, it is not possible to evaluate the evidence for this proposed pathway. Regardless of its possible existence, the significance and function of the pathway also remain to be established. Tsukada and Perlman (6) have reported that only 2% of the metabolized L-sorbose is converted to 2-keto-L-gulonate in *G. melanogenus*, thus leaving the fate of the remaining 98% of the metabolized L-sorbose apparently in need of further investigation.

Reports of an L-sorbose oxidase in *Trametes sanguinea* (12) and a 2,6-dichlorophenol indophenol-linked L-sorbose dehydrogenase in *Gluconobacter suboxydans* (13), both of which oxidize L-sorbose to 5-keto-D-fructose, in addition to reports on the purification and characterization of NADP-linked 5-keto-D-fructose reductases from *G. cerinus* (14) and *G. albidus* (15), led some researchers to propose that L-sorbose may be metabolized in some microorganisms by pathway B in Figure 2 (16). In order for this pathway to be accepted as an

established route for the metabolism of L-sorbose, however, it would be necessary to (i) determine the physiological electron acceptor for the L-sorbose dehydrogenase and (ii) demonstrate the presence of both the L-sorbose dehydrogenase and the 5-keto-D-fructose reductase in the same organism.

PART I

ISOLATION AND IDENTIFICATION OF THE PRODUCT OF THE PHOSPHOENOLPYRUVATE:L-SORBOSE 1-PHOSPHOTRANSFERASE SYSTEM OF *AEROBACTER AEROGENES*

Introduction

In the course of elucidating the metabolism of L-sorbose in *A. aerogenes*, L-sorbose-1-P was proposed to be the product of the phosphoenolpyruvate-dependent phosphorylation of L-sorbose in the first step of the pathway. Most of the evidence that we had accumulated for this was based on work with mutants missing various enzymes involved in the metabolism of hexoses and hexitols. The discovery of the L-sorbose-1-P reductase and the demonstration of the ordered appearance of L-sorbose-1-P and D-glucitol-6-P in crude extracts of L-sorbose-grown cells gave additional substance to our proposal that the phosphorylation was on carbon atom 1 of L-sorbose. To prove conclusively that L-sorbose-1-P is indeed the first intermediate in the proposed pathway it was necessary to isolate and characterize this compound by physical and chemical methods. In this part of my dissertation I will show that the product of the phosphoenolpyruvate-dependent L-sorbose phosphotransferase system in *A. aerogenes* is L-sorbose-1-P.

Materials and Methods

Chemicals

Authentic L-sorbose-1-P was prepared by the chemical procedure described by Mann and Lardy (17). Other chemicals were obtained from commercial sources.

Growth of Cells and Preparation of Cell Extracts

Aerobacter aerogenes strain PRL-R3 was grown at 30° overnight in 100 ml of mineral broth (63) supplemented with 0.5% D-glucose.

Cells were harvested by centrifugation at 12,000 x g for 10 min. The supernatant was discarded, the sedimented cells were suspended in 50 ml of 0.85% NaCl, and were centrifuged at 12,000 x g for 10 min. After discarding the supernatant fluid, the pellet was suspended in 3.0 ml of 0.04 M Tris-HCl buffer, pH 7.5, and the suspension was sonicated for 20 min in a Ratheon 250 W, 10 kHz sonic oscillator cooled with circulating ice water. Unbroken cells and debris were removed by centrifugation at 48,000 x g for 20 min and the supernatant, containing 6 mg of protein per ml, was removed and used for the preparation of the product of the PEP:L-sorbose PTS.

Analytical Methods

Protein was determined by the method of Lowry *et al.* (18). Total phosphate and inorganic phosphate were determined by the modified method (18a) of Fiske and SubbaRow (19). Ketohexose was determined by a modification of Roe's method as discussed by Putman (20). Qualitative determination of ketohexoses was done by the cysteine-H_2SO_4 method (21,22). Trimethylsilyl derivatives were prepared by dissolving the sodium salts of sugar phosphates in trimethylsilylating reagent as described by Sweeley, Wells, and Bentley (23); the final concentration was one mg of sugar phosphate per ml. Gas-liquid chromatography was performed on a Hewlett-Packard gas chromatograph model 402, employing a 1.2 m column of 3% OV-1 (methyl silicone) at a temperature of 190°. Trimethylsilyl O-methyloxime derivatives of authentic L-sorbose-1-P and the product were prepared and subjected to combined gas chromatography and mass spectroscopy as described by Laine and Sweeley (24).

Results

Preparation of Product of the PEP:L-sorbose PTS

The reaction mixture (10 ml final volume) contained 0.04 M Tris-HCl buffer (pH 7.5), 15 mM sodium fluoride, 0.5 mM magnesium chloride, 10 mM phosphoenolpyruvate (tricyclohexylammonium salt), 20 mM L-sorbose, and 2.0 ml of cell extract (24 mg of protein). The reaction was allowed to proceed for 3 hr at 28° and was then terminated by heating at 100° for seven minutes. Precipitated protein was removed by centrifugation for 10 min at 48,000 x g after cooling the suspension on ice.

Isolation of Product

The deproteinized reaction mixture was then applied to a 1 x 7 cm column of Dowex-1, 200-400 mesh, equilibrated in the bicarbonate form. The column was washed with distilled water until no more ketohexose was detected in the effluent. The unbound ketohexose was nonphosphorylated L-sorbose. The column was then eluted with a stepwise gradient consisting of 100 ml each of 0.15 M, 0.30 M, and 0.45 M $KHCO_3$. In this system the monophosphates were eluted from the column with 0.15 M $KHCO_3$ and the diphosphates were eluted with 0.30 M $KHCO_3$. A substance that gave a positive reaction with the Roe test was eluted only with 0.15 M $KHCO_3$, suggesting that the product was a ketohexose monophosphate. The peak fractions of this 0.15 M $KHCO_3$ wash were combined and the pH of the solution lowered to 2 by treatment with Dowex-50 (hydrogen form). The solution was then quickly frozen in a dry ice-acetone bath and lyophilized to

dryness. The resulting white powder was dissolved in 5 ml of distilled water and the pH adjusted to 7.0 with NaOH. This solution contained 24 μmoles of ketohexose phosphate.

Identification of Product

The determination of total phosphate in the product indicated that there was 0.99 μmole of phosphate for every μmole of ketohexose. This result substantiates the conclusion that the product is a monophosphate, as was suggested by the elution pattern from the Dowex-1 bicarbonate column.

The rate of hydrolysis of the product and authentic L-sorbose-1-P in 1 M HCl at 100° exhibited first-order kinetics with a 50% hydrolysis time of 16 min for each. This time is distinct from that reported for L-sorbose-6-P, for which 50% hydrolysis occurs at 60 min (25).

When the trimethylsilyl O-methyloxime derivatives of the product and L-sorbose-1-P were subjected to combined gas-liquid chromatography and mass spectrometry, identical fragmentation patterns were obtained. The fragmentation pattern for the product is shown in Figure 3. The ions occurring at m/e 103, 205, 307, 414, and 516 originate from the cleavage pattern shown on the right of the figure and are those predicted for a 2-ketohexose 1-phosphate without regard for stereochemical configuration (24). The fragments at m/e 299, 314, 315, and 387 are found in most mass spectra of sugar phosphates and are attributable to various ions of methylsilyl-substituted phosphate (26). The molecular weight of

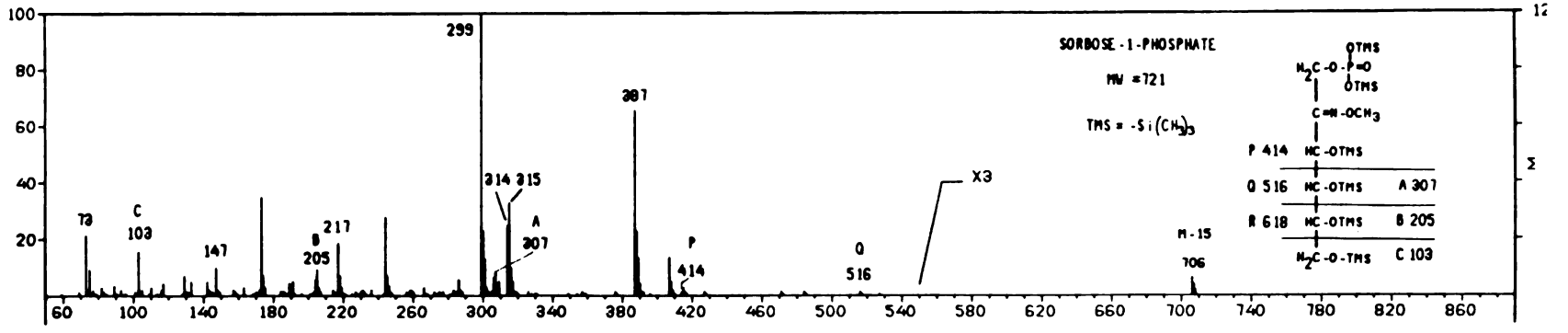


Figure 3 - Mass spectrum of the trimethylsilyl O-methyloxime derivative of the product of the phosphoenolpyruvate-dependent phosphorylation of L-sorbose. The structure shown represents the derivative of a 2-ketohexose 1-phosphate without regard to stereochemical configuration. The ordinate is relative intensity and the abscissa is m/e.

721 is that predicted for the trimethylsilyl O-methyloxime derivative of 2-ketohexose-1-P.

The cysteine-H_2SO_4 method for the determination of ketose provides a means for distinguishing sorbose from fructose, psicose, and tagatose (21,22). After 15 hr in cysteine-H_2SO_4 the ratio of absorbance at 412 nm to the absorbance at 604 nm (A_{412}/A_{604}) is 0.4 for L-sorbose and greater than 3.0 for the other ketohexoses (21). When samples of the product, authentic L-sorbose-1-P, and D-fructose-1-P were treated according to this method the A_{412}/A_{604} after 15 hr were 0.41, 0.43, and 3.3, respectively. These results show that the ketohexose moiety is sorbose.

The results of the gas-liquid chromatography of the trimethylsilyl derivatives of the product and sugar phosphate standards are shown in Figure 4. The retention times and peak patterns are the same for the product (D) and authentic L-sorbose-1-P (C); there are two minor peaks at 9.5 and 11.0 min and a major peak at 13.7 min. These retention times and peak patterns are distinct from those of D-fructose-1-P (A) with major peaks at 9.5, 11.0, and 11.5 min, or of D-fructose-6-P (B) with one major peak at 10.7 min.

Discussion

These results establish unequivocally that the product of the PEP:L-sorbose PTS is L-sorbose-1-P. The elution pattern from the Dowex-1-bicarbonate column and the phosphate to ketohexose ratio show that the product is a ketohexose monophosphate. The hydrolysis rate in 1 M HCl at 100° and the mass spectrum of the trimethylsilyl

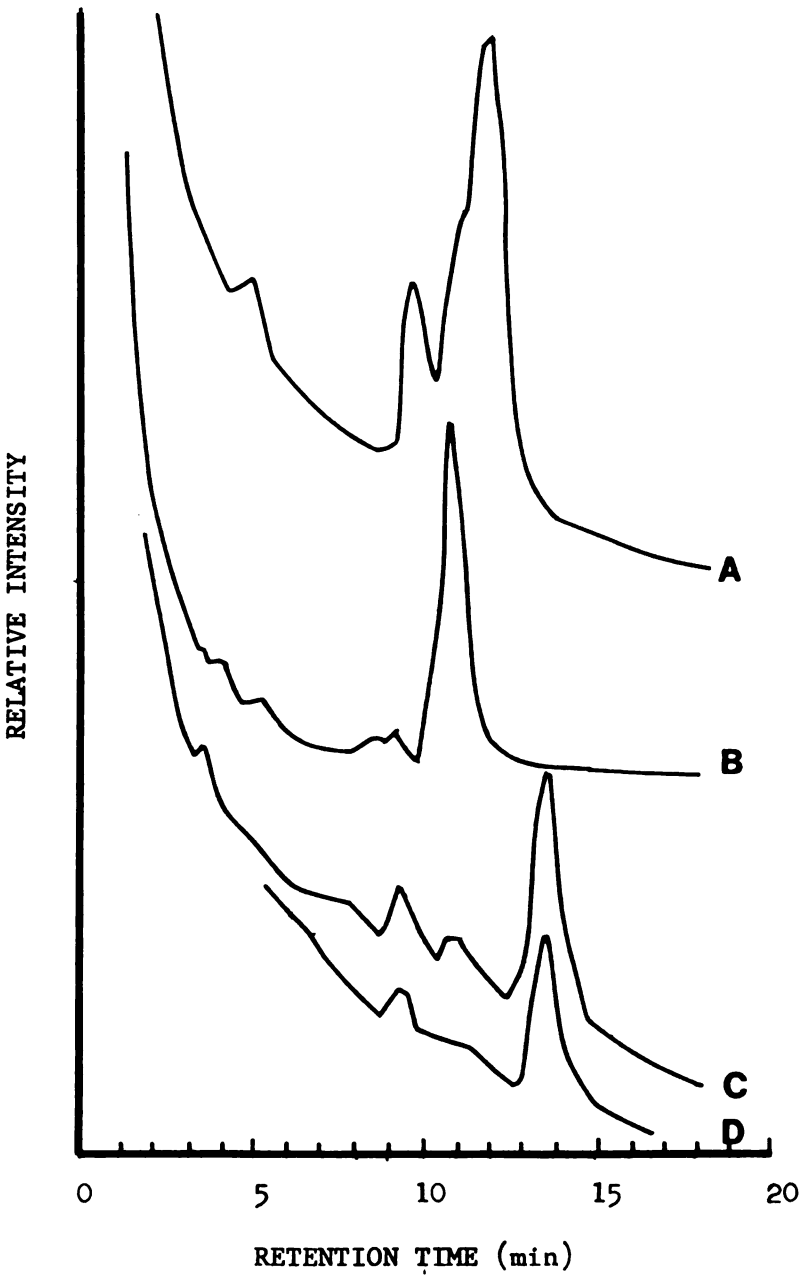


Figure 4 - Tracings of the gas-liquid chromatograms of the trimethylsilyl derivatives of: A : D-fructose-1-P; B : D-fructose-6-P; C : L-sorbose-1-P; and D : Product of the PEP:L-sorbose 1-phosphotransferase system.

O-methyloxime derivative of the product show that the phosphate is attached to carbon atom 1 of the ketohexose moiety.

The ketohexose moiety was identified as sorbose on the basis of its characteristic A_{412}/A_{604} value after 15 hr reaction with cysteine-H_2SO_4. Both the product and authentic L-sorbose-1-P gave identical peak patterns and retention times when trimethylsilylated and gas chromatographed.

The product was identified as the L isomer of sorbose-1-P on the basis of several facts: (i) the mutant studies indicated that D-glucitol-6-P and D-fructose-6-P were intermediates in the pathway; (ii) there was a precursor-product relationship between L-sorbose-1-P and D-glucitol-6-P; (iii) *in vitro* the reaction pathway of L-sorbose could be demonstrated with L-sorbose-1-P reductase, D-glucitol-6-P dehydrogenase, phosphoglucoisomerase, and D-glucose-6-P dehydrogenase to be L-sorbose-1-P ⟶ D-glucitol-6-P ⟶ D-fructose-6-P ⟶ D-glucose-6-P ⟶ D-gluconate-6-P; and (iv) if the product *were* D-sorbose-1-P the reaction for its formation from L-sorbose would require three epimerizations (at C-3, C-4, and C-5) and the product of its reduction by a D-sorbose-1-P reductase would be D-iditol-1-P, which, if oxidized by D-glucitol-6-P dehydrogenase, would form D-sorbose-6-P, which, if isomerized by phosphoglucoisomerase, would form D-gulose-6-P. It is highly unlikely that any of the four enzymes involved--the reductase, the two dehydrogenases, and the isomerase--would fortuitously use the four intermediates described. By this reasoning, and with the data previously discussed, it is established that the product of the PEP:L-sorbose 1-phosphotransferase system is L-sorbose-1-P.

PART II

PURIFICATION AND PROPERTIES OF L-SORBOSE 1-PHOSPHATE REDUCTASE

Introduction

The second reaction in the pathway of L-sorbose metabolism in *A. aerogenes* was demonstrated to be the reduction of L-sorbose-1-P to D-glucitol-6-P, catalyzed by an inducible L-sorbose-1-P:NAD(P) ketoreductase (1; see Figure 1). Since this was the first demonstration of an enzyme that catalyzes the reduction of a 2-ketohexose-1-P, a study of its properties was undertaken. In this section of my dissertation I will describe (i) the purification of this enzyme essentially to electrophoretic homogeneity, (ii) the physical, chemical, and catalytic properties of the enzyme, and (iii) the reactions catalyzed by the enzyme, particularly with respect to equilibrium and identity of products.

Materials and Methods

Chemicals

L-Sorbose-1-P was prepared as described in part I of this dissertation. D-Mannitol-1-P and D-glucitol-6-P were prepared by the procedure of Wolff and Kaplan (27), i.e., the sodium borohydride reduction of D-mannose-6-P and D-glucose-6-P, respectively. Adipic dihydrazide for affinity chromatography was prepared by refluxing diethyl adipate, hydrazine hydrate (98%), and ethanol (1:2:2, v/v/v) for 3 hr. The resulting flaky precipitate was crystallized twice from an ethanol-water mixture and dried *in vacuo*. Other chemicals were obtained from commercial sources.

All enzymes and proteins used as markers in the determinations of molecular weight were obtained from Sigma except catalase

(Worthington), D-glucose-6-P dehydrogenase (Calbiochem), and 2-keto-3-deoxy-6-phosphogluconate aldolase (a gift from W. A. Wood).

Enzyme Assays

All assays were done in 150 µl total volume. The rates of absorbance change (at 340 nm unless otherwise noted) were measured with a Gilford multiple-sample absorbance recorder thermostated at 30°.

L-Sorbose-1-P reductase. The standard assay for L-sorbose-1-P reductase contained 0.033 M MES buffer (2[N-morpholino]ethanesulfonic acid), adjusted to pH 6.15 with NaOH; 0.33 mM NADH (unless specified as NADPH); 1.0 mM $MnCl_2$; and 3.3 mM D-fructose-1-P (unless specified as L-sorbose-1-P). One unit of reductase is defined as the amount that catalyzes the oxidation of one µmole NADH per min in the standard assay.

Other enzyme assays. In the case of those assays coupled to reactions involving reduction or oxidation of nicotinamide adenine dinucleotides, the reactions were monitored for increase or decrease in absorbance at 340 nm. *Escherichia coli* alkaline phosphatase was assayed by the procedure of Garen and Levinthal (28); the assay mixture contained 1.0 mM p-nitrophenyl phosphate in 1.0 M Tris-HCl buffer, pH 8.0. The reaction was monitored by increase in absorbance at 410 nm. Lactate dehydrogenase from beef heart was assayed in a reaction mixture containing 0.067 M glycylglycine buffer (pH 7.5), 3.3 mM sodium pyruvate, and 0.33 mM NADH. Glucose-6-P dehydrogenase from yeast was assayed in a reaction mixture containing 0.067 M

glycylglycine buffer (pH 7.5), 3.3 mM D-glucose-6-P, and 0.33 mM $NADP^+$ (adjusted to pH 7.0). Yeast hexokinase was assayed in a reaction mixture containing 0.067 M glycylglycine buffer (pH 7.5), 0.33 mM $NADP^+$ (adjusted to pH 7.0), 3.3 mM D-glucose, and nonlimiting amounts of yeast D-glucose-6-P dehydrogenase.

Protein determinations. Protein was determined by the method of Lowry *et al.* (18) except in those cases where protein concentration made it more desirable to determine concentration by measuring the absorbance at 220 nm (29). Hemoglobin from sheep erythrocytes was determined by the absorbance at 410 nm.

Determination of Reaction Products

Enzymatic determination. D-Fructose-1-P was determined by a coupled end-point assay containing 0.067 M glycylglycine buffer (pH 7.5), 3.3 mM ATP, 6.6 mM $MgCl_2$, 0.33 mM NADH, nonlimiting amounts of rabbit muscle aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, and partially purified D-fructose-1-P kinase. D-Mannitol-1-P and D-glucitol-6-P were determined under identical assay conditions; the reaction mixture contained 0.067 M Tris-HCl buffer (pH 9.0), 0.33 mM NAD^+, 0.33 mM $NADP^+$, and nonlimiting amounts of phosphoglucoisomerase and D-glucose-6-P dehydrogenase. The end-point assay for D-mannitol-1-P also contained nonlimiting amounts of partially purified D-mannitol-1-P dehydrogenase (30); the end-point assay for D-glucitol-6-P also contained nonlimiting amounts of partially purified D-glucitol-6-P dehydrogenase (30). All assays were performed in a total volume of 150 µl.

Preparation of the hexaacetate derivatives of the dephosphorylated product. Approximately 50 µg of the enzymatically dephosphorylated product of the reductase-reduced D-fructose-1-P was dried by passing a stream of dry nitrogen gas over the solution containing the product. The residue was dried by suspending it in absolute ethanol and evaporating with nitrogen. The sample was then placed in a vacuum desiccator at 50° for 3 hr. After drying, the residue was dissolved in 50 µl of acetic anhydride, the container sealed, and heated in a 120° heating block for 1 hr.

Gas-liquid chromatography. Gas-liquid chromatography of the hexaacetate derivative of the dephosphorylated reaction product was performed on a Perkin Elmer 900 gas chromatograph as described by Jones and Alberscheim (31). The manifold temperature was 250°, the injection port temperature was 265°, and the column temperature was programmed from 130° to 180° at a rate of 1 degree per min. The column (1.83 m x 2 mm) consisted of a solid phase of Gas-Chrom P, 100-200 mesh with a liquid phase consisting of 0.2% ethylene glycol adipate, 0.2% ethylene glycol succinate, and 0.4% XF-115 silicone oil. The data were processed by an Autolab System 4 computer. Standards were run of the hexaacetate derivatives of rhamnitol, fucitol, arabitol, xylitol, mannitol, galactitol, glucitol, and inositol. The standards were obtained from Dr. Derek Lamport.

Preparation of NADP-Substituted Sepharose 4B for Affinity Chromatography

Activation of Sepharose 4B and coupling to adipic dihydrazide.

Sepharose 4B was activated by the method of March *et al.* (32). Sepharose 4B was washed with 100 vol of distilled water on a coarse sintered-glass funnel and suspended in two volumes of 1 M sodium carbonate by slow stirring on a magnetic stirrer. The rate of stirring was increased and 0.05 vol of a 66% (w/v) solution of cyanogen bromide in acetonitrile was added and stirred vigorously for 2 min. The slurry was then poured onto a sintered-glass funnel and washed with 5 to 10 vol each of 0.1 M sodium bicarbonate (pH 9.5), distilled water, and 0.2 M sodium bicarbonate (pH 9.5). The slurry was filtered under vacuum to a moist cake and the cake transferred to a plastic bottle containing one volume of a cold (4°) saturated solution of adipic acid dihydrazide in 0.2 M sodium bicarbonate (pH 9.5). The coupling was allowed to continue for 20 hr at 4° with gentle stirring. The coupled Sepharose-adipic acid dihydrazide was filtered on a sintered-glass funnel and alternately washed with distilled water and 0.1 M sodium chloride until the washings gave only a slight color test with 2,4,6-trinitrobenzene sulfonate (33). Samples of substituted sepharose, when subjected to this test, became deep red in color, indicating the presence of hydrazide. The gel could be stored for several days at 4° in 0.02% sodium azide.

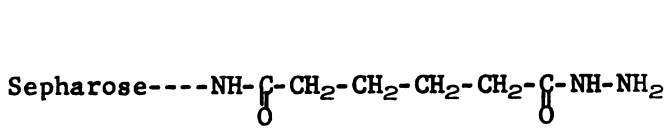
Binding of activated NADP to Sepharose-adipic hydrazide (34).

$NADP^+$ was activated by adding a cold (0°) solution of 0.02 M sodium

metaperiodate to an equal volume of cold (0°) 0.02 M $NADP^+$ adjusted to pH 7.5 with NaOH. The mixture was incubated on ice in the dark for 60 min. Sepharose-adipic acid dihydrazide (compound I in Figure 5), washed free of sodium azide and washed with 10 vol of 0.1 M sodium acetate buffer, pH 5.0, was suspended in 3.5 vol of the same buffer and 0.5 vol of the periodate-activated $NADP^+$ (Figure 5, II) added. The reaction was allowed to proceed for 3 hr with gentle stirring at 4°, at which time 7.5 vol of 2 M NaCl were added to remove uncoupled nucleotide from the gel. The mixture was stirred for an additional hour and then filtered on a coarse sintered-glass funnel. The filtrate was saved to determine the extent of coupling to the gel. The cake was washed with 5 vol of 0.02% sodium azide and stored in the same solution. Generally, there were 1.7 to 2.5 μmoles of $NADP^+$ coupled per ml of gel (Figure 5, III).

Polyacrylamide Gel Electrophoresis

General procedures. Gel tubes 0.5 cm x 10 cm were prepared by cleaning with chromic acid solution, neutralizing with sodium thiosulfate, and rinsing with distilled water. The cleaned tubes were immersed in a solution of 0.5% Photoflo for 30 min and the tubes air dried. Components of the gel mixture were degassed prior to mixing. The bottoms of the gel tubes were sealed with paraffin film. Electrophoresis was performed with a Spinco Duostat regulated D.C. power supply at constant current. After electrophoresis the gels were removed from the tubes by directing a stream of water



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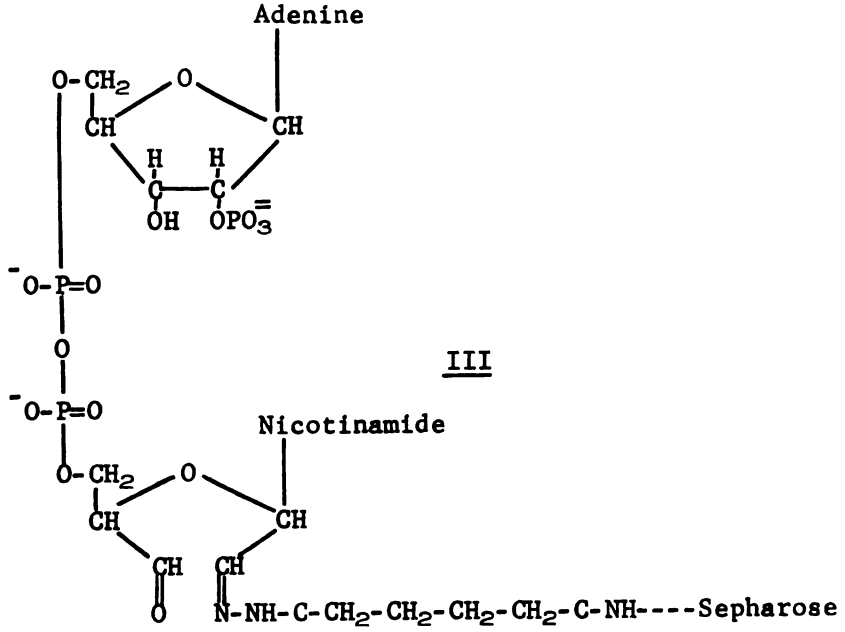
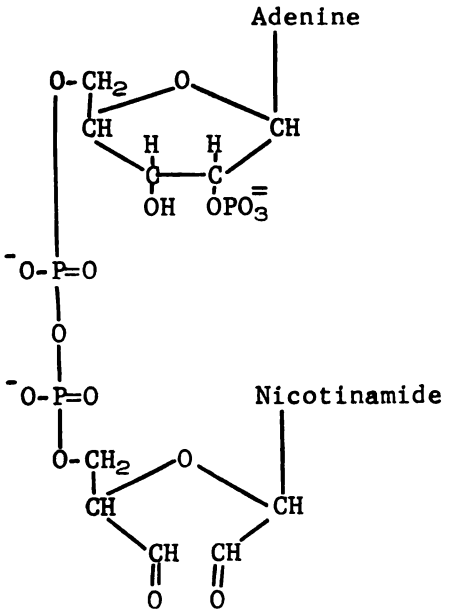


III



II

Figure 5 - Intermediates and product of the NADP-substitution of Sepharose 4B.

delivered with a 22 gauge hypodermic needle attached to a 50 ml syringe. Gels to be stained for protein were immersed in 18 ml of 12.5% trichloroacetic acid (TCA) for 20-30 min prior to the addition of the staining solution.

Preparation of native gels for determination of homogeneity. Gel tubes were filled to a height of 9 cm with a solution containing 7.5% acrylamide, 0.18% bisacrylamide (N,N'-methylenebisacrylamide), 1.25% glycerol, 0.028% TEMED (N,N,N',N'-tetramethylethylenediamine), 0.09% ammonium persulfate (prepared fresh each time), and 0.375 M Tris-HCl buffer (pH 9.0). The solution was overlaid with 50 µl of the electrophoresis buffer with the mercaptoethanol deleted and allowed to polymerize for 60 min at room temperature. The tubes were pre-electrophoresed at a constant current of 2 mA per tube at 4° for 60 min. The electrophoresis buffer contained 0.2 M glycine, 0.02 M Tris, 0.2% mercaptoethanol with a pH of 8.8. Prior to application of the protein sample, 0.05% bromphenol blue dye in 10% glycerol in a volume of 2.5 µl was applied to the gels and the gels electrophoresed for approximately 5 min. The sample was then applied in a total volume of 10 to 20 µl and the gels were electrophoresed at 2 mA per tube for 2.5 to 3 hr, at which time the tracking dye was within 1 cm of the bottom of the tube. Gels were stained for protein by adding 2 ml of an aqueous solution of 1% Coomassie Brilliant Blue R to 18 ml of 12.5% TCA and allowing to stain overnight. Destaining was achieved by immersion in 12.5% TCA with frequent changing of solution until the background was a faint blue.

To determine the location of L-sorbose-1-P reductase activity a separate series of gels was run using 0.1 M sodium phosphate buffer, pH 6.2, instead of the Tris buffer. Gels were run for 6 to 8 hr at 4 mA per tube. After gels were removed from the tubes, one set was stained for protein and the other was placed in a 1 x 10 cm glass tube, sealed at the bottom, containing 0.05 M MES buffer (pH 6.1), 3.3 mM D-fructose-1-P, 0.33 mM NADH, 1.0 mM $MnCl_2$, and 0.2% mercaptoethanol. A control tube was run with the D-fructose-1-P omitted. Gels were incubated at room temperature for 30 min and scanned at 340 nm in a 1 cm x 12 cm quartz gel-scanning cuvette on a Gilford 240 spectrophotometer with a model 2410-S linear transport attachment.

Preparation of native gels for determination of molecular weight. Molecular weight in native gels was determined by the method of Hedrick and Smith (35). Gels were polymerized and submitted to electrophoresis in the same manner as described above except that the concentration of acrylamide was varied from 6 to 10% and the bisacrylamide to acrylamide ratio was maintained at 1:30. The gel runs varied due to the change in gel concentration with each run from 1.5 hr for 6% to almost 3 hr for 10% gels. Gels were removed and stained for protein as previously described.

Preparation of SDS gels for determination of subunit molecular weight. Gels were prepared by cleaning the same glass tubes used in the native gel electrophoresis studies and soaking the tubes in 1% SDS (sodium dodecyl sulfate) for 1 hr, rinsing with

distilled water, and placing in a drying oven until dry. The gels were prepared according to the procedure of Fairbanks *et al.* (36). The gel solution contained 5.6% acrylamide, 0.21% bisacrylamide, 1% SDS, 0.15% ammonium persulfate, 0.025% TEMED, and 0.04 M Tris-0.02 M sodium acetate-0.002 M EDTA, pH 7.4. Gels were allowed to polymerize for 40 min at room temperature with an overlaid solution containing 0.15% ammonium persulfate, 0.05% TEMED, and 0.1% SDS. After polymerization the overlay solution was replaced with the electrophoresis buffer: 0.04 M Tris-0.02 M sodium acetate-0.002 M EDTA (pH 7.4) containing 1% SDS and allowed to stand at room temperature overnight. Prior to application of the sample the gels were submitted to electrophoresis for 30 min at 4 mA per tube.

Samples were prepared by dissolving 1 mg of standards or the enzyme preparation in a 1-ml solution containing 1% SDS, 10% sucrose, 10 mM Tris, 1 mM EDTA, at pH 8.0. Immediately prior to electrophoresis, mercaptoethanol was added to a final concentration of 2% and the sample heated to 100° for 15 min. Two-hundredths vol of a 10% solution of pyronin B in 5% sucrose was added to the sample and 20 µl of sample was added to each gel. Gels were subjected to electrophoresis for 4 hr at 4 mA per tube, the gels were removed from the tubes, and the dye fronts were marked by inserting a small sliver of bamboo into the center of the dye band.

Gels were stained for protein in a solution containing 10% TCA, 33% methanol, and 0.4% Coomassie brilliant blue R overnight. Destaining was performed by immersing the gels in a solution of

10% TCA and 33% methanol overnight and completing the destaining with a solution of 10% TCA.

Other Methods for Determining Molecular Weight

Sucrose density gradient centrifugation. Sucrose density gradient centrifugation was performed using 1.2 x 7.5 cm cellulose nitrate centrifuge tubes containing 4.6 ml of 5 to 20% sucrose in 0.05 M MES buffer (pH 6.1) with 0.2% mercaptoethanol. The linear gradients were poured at room temperature and then the tubes were cooled for several hours to 4°. The sample, containing partially purified L-sorbose-1-P reductase and standards of sheep erythrocyte hemoglobin and *E. coli* alkaline phosphatase, was applied in a volume of 0.1 ml in buffer containing 0.05 M MES (pH 6.1), 0.2% mercaptoethanol, and 5% sucrose. The centrifugation was performed in a model L-2 preparative Beckman centrifuge with a SW 50.1 rotor for 16 hr at 35,000 rpm and 0°. After centrifugation the tubes were punctured and 5-drop fractions collected and assayed.

Sedimentation equilibrium ultracentrifugation. Sedimentation equilibrium centrifugation of L-sorbose-1-P reductase was done by standard procedures (37,38) on a Beckman Model E ultracentrifuge equipped with Rayleigh optics. Three samples of 0.5, 1.0, and 2.0 mg protein per ml were used in standard cells oriented for 6° above, 6° below, and 0° with respect to the reference lines. The centrifugation was done for 24.5 hr at 15° at a speed of 12,573 rpm.

Amino acid analysis of L-sorbose-1-P reductase. L-Sorbose-1-P reductase which had been purified 99% (as determined by acrylamide gel electrophoresis) was carboxymethylated by the method of Crestfield as modified by Robertson *et al.* (39) to protect cysteine residues. Amino acid analysis was performed on a noncommercial amino acid analyzer (39). Tryptophan was determined by the method of Edelhoch (40).

Determination of pH. All pH measurements of buffers were determined prior to the addition of mercaptoethanol on a Beckman Zeromatic pH meter with a Sargent 30070-10 combination electrode.

Results

Purification

L-Sorbose-1-P reductase appeared at first to be relatively unstable, since most of my attempts to purify it by conventional means resulted in low recovery and little purification. Various steps which were attempted but which failed to give the desired purification and recovery were: precipitation by ammonium sulfate or by lowering the pH; ion exchange chromatography on phosphocellulose or carboxymethylcellulose; gel filtration on various commercial gels; and adsorption to calcium phosphate or bentonite. Hydroxyapatite chromatography was partially successful; however, the phosphate buffer used in the elution inhibited the enzyme activity, and the elution pattern was not reproducible.

The enzyme's stability could be increased by the addition of 0.2% mercaptoethanol and 2 mM EDTA (ethylenediaminetetraacetic

acid) to the buffer. Buffer containing 0.025 M MES (pH 6.1) and mercaptoethanol and EDTA was used in all subsequent purification steps and will be referred to as MES buffer; in cases where the MES concentration was increased to 0.05 M MES, this will be noted. The enzyme could be stored for several weeks with a minimal loss of activity in 0.05 M MES buffer containing 20% glycerol at -20°. With the exception of dialysis, any manipulative steps resulted in a loss of greater than 10% of the activity. Such steps include ultrafiltration, lyophilization, filtration on Sephadex G-25, and ammonium sulfate and pH precipitation.

Preparation of crude extracts. Cells grown on 0.5% L-sorbose in mineral broth (63) and harvested as previously described (1) were suspended in 50 ml of 0.05 M MES buffer. The cells were disrupted and the crude extract prepared as described in Section I of this dissertation. A sample was removed and the L-sorbose-1-P reductase activity determined by the standard assay described in *methods*. To get an accurate determination of reductase activity in the crude extract it was necessary to run a control with D-fructose-1-P omitted. This control measured the endogenous NADH oxidase activity present; in crude extracts of *A. aerogenes* this activity was about equal to that of the reductase. The specific activity of the L-sorbose-1-P reductase, as determined by subtracting the endogenous rate of NADH oxidation from the rate in the presence of D-fructose-1-P, varied from 0.040 to 0.075 units per mg protein in different preparations.

Chromatography on DEAE-cellulose

First DEAE-cellulose column. The 50 ml of crude extract was applied to a 1.6 x 8 cm column of diethylaminoethyl cellulose (DEAE-cellulose from Sigma, Lot No. 16C-1940). After the sample was applied, the column was washed with one column volume (16 ml) of MES buffer. An assay of the column wash-through indicated that all of the reductase activity was retained on the column but that all of the NADH oxidase activity was present in the effluent. Figure 6 is an elution profile of the first DEAE-cellulose column; the reductase activity elutes at a sodium chloride concentration of approximately 0.15 M. The total activity that could be recovered from this column was 67%; fractions 31 through 46, representing 54% of the activity applied to the column, were combined and dialyzed against MES buffer containing 20% glycerol for 18 hr at 4°. The peak fraction, number 36, represented a purification of 7.5-fold, whereas the combined fractions (31-46) represented a 5.1-fold-purification.

Second DEAE-cellulose column. The dialyzed fractions from the first DEAE-cellulose purification step were applied to a 1.2 x 8 cm column of DEAE-cellulose and washed with one volume (9 ml) of MES buffer containing 0.05 M NaCl. There was no detectable reductase activity in the wash-through. Bound proteins were eluted with a 0.05 M to 0.25 M NaCl gradient (90 ml total volume) in MES buffer. Fractions of 1.4 ml were collected and assayed for protein and L-sorbose-1-P reductase activity (Figure 7). The activity peak

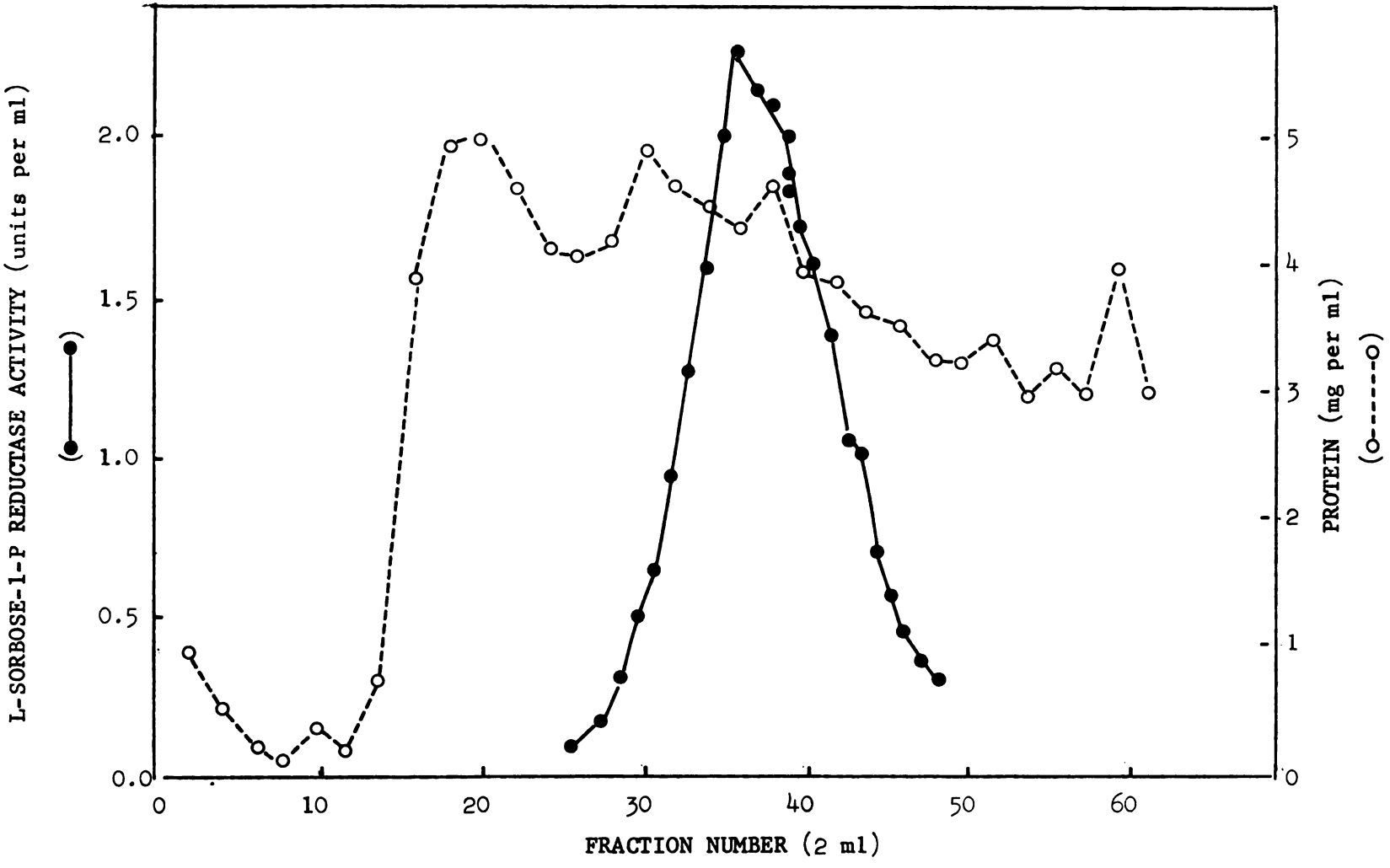


Figure 6 - Elution profile of first DEAE-cellulose column. Elution was with a 160-ml gradient of 0 to 0.3 M NaCl in MES buffer.

Figure 7. Elution profile of second DEAE-cellulose column. The column was eluted with a 90-ml gradient of NaCl from 0.05 to 0.25 M in the same buffer for the first column. Fractions (1.4 ml) were collected, and fractions 29 through 42 were combined for further purification.

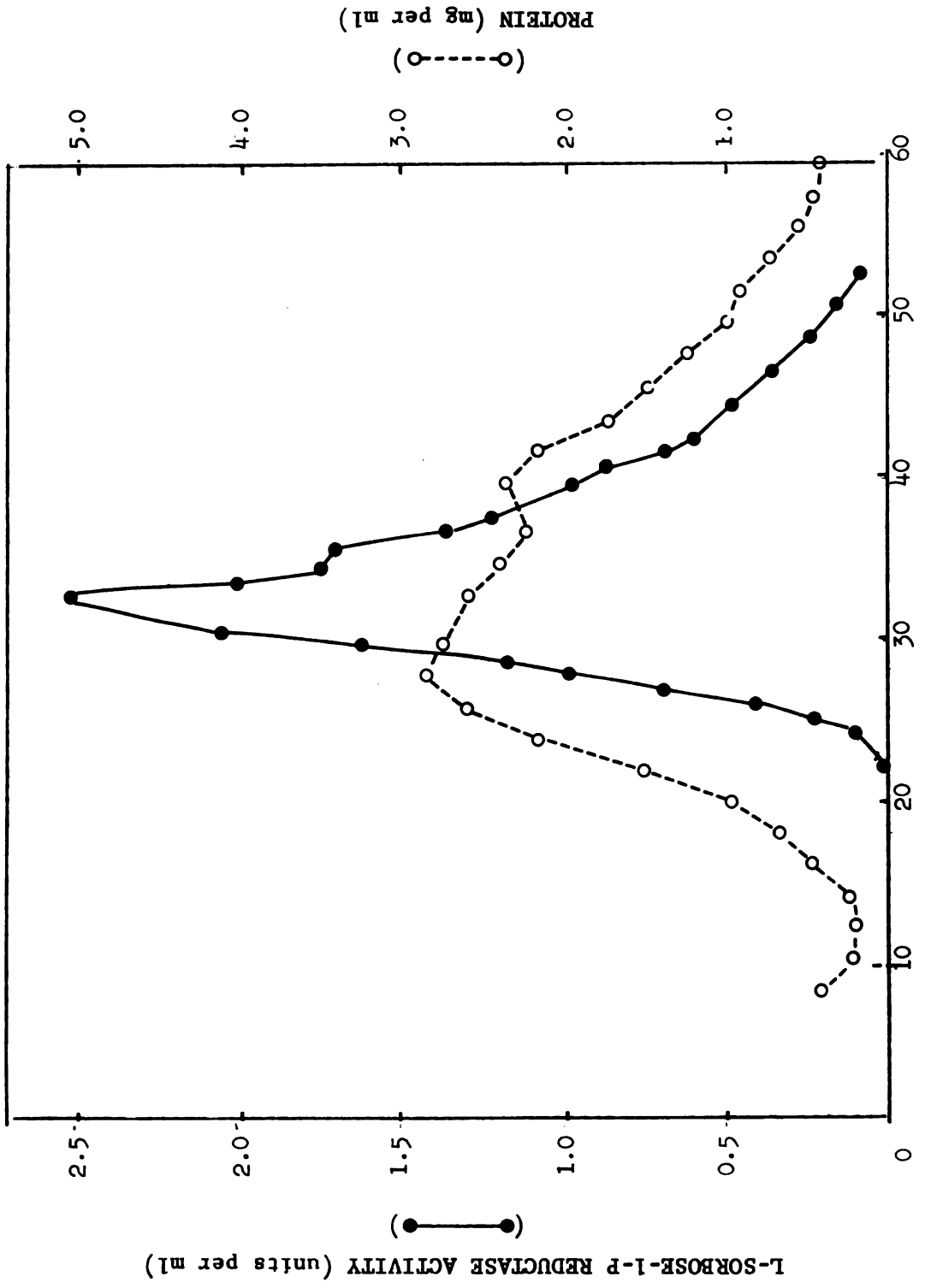

PROTEIN (mg per ml)
(o-----o)
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
60
FRACTION NUMBER (1.4 ml)
2.5
2.0
1.5
1.0
0.5
(●——●)
L-SORBOSE-1-P REDUCTASE ACTIVITY (units per ml)

Figure 7

eluted from the column at a concentration of approximately 0.15 M NaCl and represented an overall purification of 13.7-fold. Fractions 25 through 38, representing 74% of the activity applied to the column, were combined and dialyzed overnight against MES buffer containing 20% glyercol. Only 5% of the activity was lost in the dialysis step.

Affinity chromatography on NADP-substituted Sepharose

Previous studies with NAD or NADP covalently attached to immobilized supports such as Sepharose have revealed that many dehydrogenases exhibit a certain specificity in binding towards the cofactor (41-43). Those enzymes which require NADP(H) seem able to bind to either NAD- or NADP-substituted sepharose. This specificity suggested that an NADP-sepharose column should bind L-sorbose-1-P reductase (since it could use either NADH or NADPH as a cofactor) while allowing all enzymes which do not bind NADP to wash through. Elution with NAD^+ or NADH should then separate those enzymes binding more tightly to NADP from those which might use either cofactor.

Preliminary studies; elution with NaCl. Initial experiments involving a NADP-substituted sepharose column were directed towards determining whether L-sorbose-1-P reductase would bind to such a column, whether the enzyme could be recovered quantitatively from the column, and whether this type of purification step would enable the reductase to be greatly purified. Figure 8A shows the results of one of these early studies. The column was a 0.8 x 8 cm column of NADP-sepharose and the gradient was 0.0 to 0.5 M NaCl

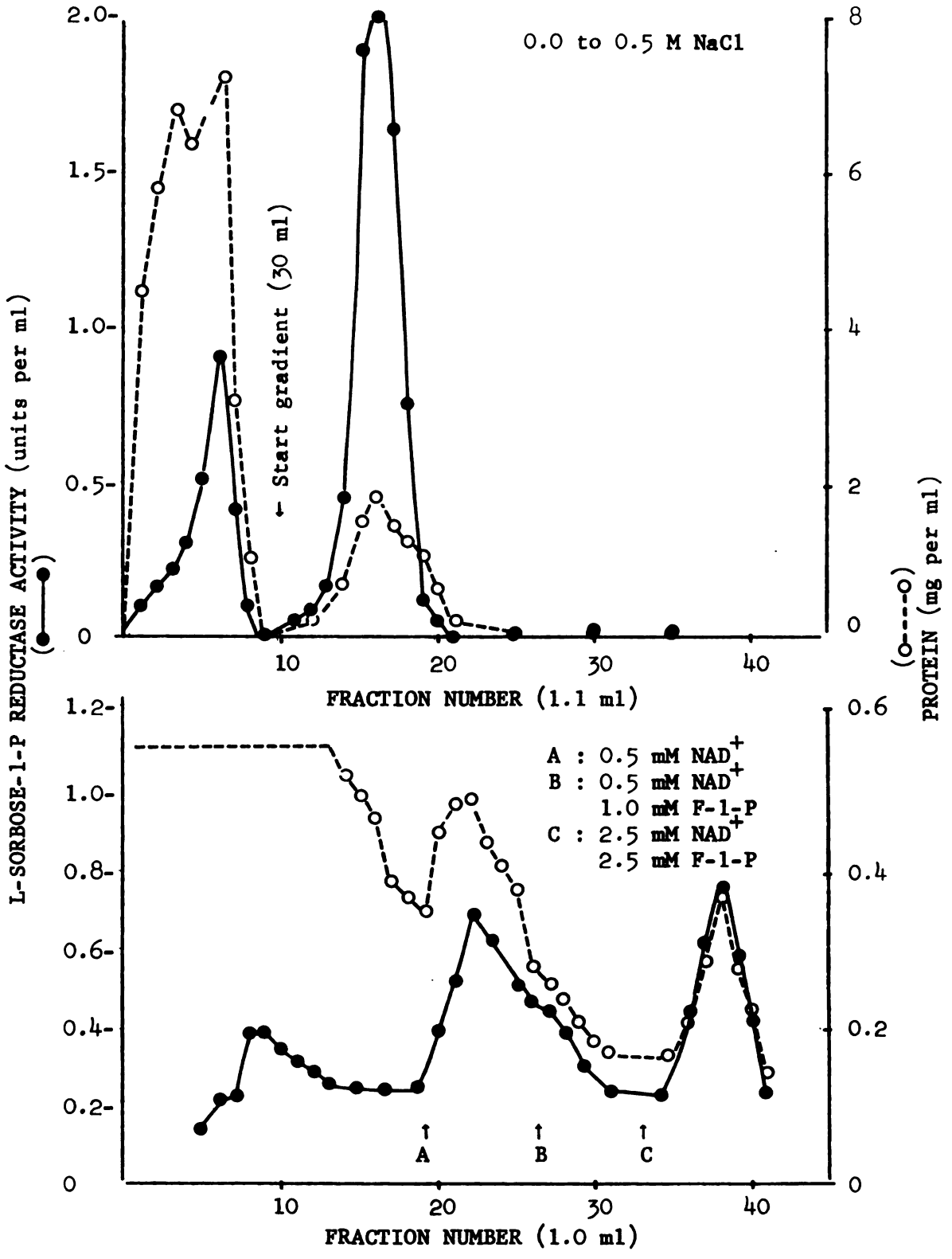


Figure 8 - Affinity chromatography of L-sorbose-1-P reductase. A : Elution with NaCl; B : Elution with various concentrations of NAD^+ and D-fructose-1-P (F-1-P). Elutions were performed in MES buffer.

in MES buffer with EDTA omitted (30 ml volume). The sample (6 ml of a preparation from the first DEAE-cellulose step) was applied to the column and washed with 6 ml of buffer. In this case 25% of the reductase activity failed to bind to the column and 81% of the protein did not bind. Elution with NaCl gave a recovery of 64.5% of the activity applied to the column or 85% of the reductase that bound. The protein eluted at the same concentration of NaCl as the reductase and represented 17% of the protein applied to the column. The purification for the combined activities (fractions 13-19) was 4.5-fold or a 13.4-fold purification overall. This purification was not as great as the purification from later columns where the elution was made more specific by eluting with NAD^+ or NADH instead of NaCl.

Elution with NAD^+ and D-fructose-1-P. Previous studies had shown that some enzymes could be eluted from affinity columns by the presence of substrate, cofactor, or both in the elution buffer (41-43). It was reasoned that since L-sorbose-1-P reductase could use either NADH or NADPH, elution in the presence of NADH or NAD^+ would give additional specificity in elution since this should cause only those enzymes which have an affinity for both NADP and NAD^+ to elute. Attempts to elute with NAD^+ alone gave no better purification than using NaCl and, in fact, gave multiple bands when examined on acrylamide gels. Elution was tried in the presence of D-fructose-1-P but this resulted in a very slow leak of reductase from the column at such low levels that it was deemed impractical for the purposes of purification. Ohlsson *et al.* (41) showed that

lactate dehydrogenase could be selectively eluted from an AMP-sepharose column by the formation of a ternary complex of the enzyme with pyruvate and the oxidized form of its cofactor, NAD^+. This approach was attempted with L-sorbose-1-P reductase by elution with NAD^+ and D-fructose-1-P. Figure 8B shows the elution pattern from such an experiment. A preparation from a first DEAE-cellulose column (10 ml) was applied to a 1 x 6.4 cm column of NADP-sepharose; 25% of the activity and 54% of the protein did not bind to the column. Elution with 0.5 mM NAD^+ resulted in a recovery of 23% of the reductase activity and 5% of the protein with the peak purification of 5.5-fold in fraction number 24. Elution with 0.5 mM NAD^+ and 1.0 mM D-fructose-1-P gave a recovery of an additional 18% of the reductase activity and 3% of the protein, whereas when 2.5 mM NAD^+ and 2.5 mM D-fructose-1-P was used to elute the column, 22% of the reductase activity and 2.5% of the protein were recovered. The remaining protein could be eluted with a 0.5 M solution of NaCl in buffer; there was no detectable reductase activity. The peak fraction, 38, represented an 8.3-fold purification or a 30-fold purification overall. Acrylamide gel electrophoresis of the fractions that eluted with 2.5 mM NAD^+ and 2.5 mM D-fructose-1-P revealed a major band (which activity gels revealed as possessing the reductase activity) and three contaminants. Since this procedure resulted in the loss of 66% of the activity which bound to the column, further studies were made to determine if a better procedure could be found.

Elution with NADH. Attempts were made to determine if NADH would prove to be more specific than NAD^+ in eluting the reductase from the sepharose. A preparation from a first DEAE-cellulose column was applied to a 1 x 6.4 cm column of NADP-sepharose; 33% of the activity applied did not bind and 73% of the protein remained unbound. A 40-ml gradient of 0 to 50 μM NADH was applied to the column. The results of the elution are shown in Figure 9. The initial peak in the figure represents part of the reductase activity from the buffer wash representing an additional 13.6% of the activity applied, and 1.8% of the protein. The gradient started at fraction 10 and the majority of the activity and protein binding to the column was eluted with 10 to 20 μM NADH. The peak fraction, number 14, represented a purification of 11.7-fold or 33-fold overall. The total activity recovered from the column by elution with NADH was 45.3% or 85% of the activity which bound to the column. Attempts to increase purification by the presence of D-fructose-1-P revealed that no activity was eluted from the column in the presence of both NADH and D-fructose-1-P although some protein was. These results suggested a modification of procedure to take advantage of this property.

Elution with NADH and D-fructose-1-P. The final procedure for the affinity chromatographic purification of L-sorbose-1-P reductase rested on a series of observations: (i) L-sorbose-1-P reductase actually seemed to bind tighter to NADP-sepharose in the presence of both D-fructose-1-P and NADH; (ii) metal ion such as Mn^{+2} was necessary for the enzyme's catalytic activity when it

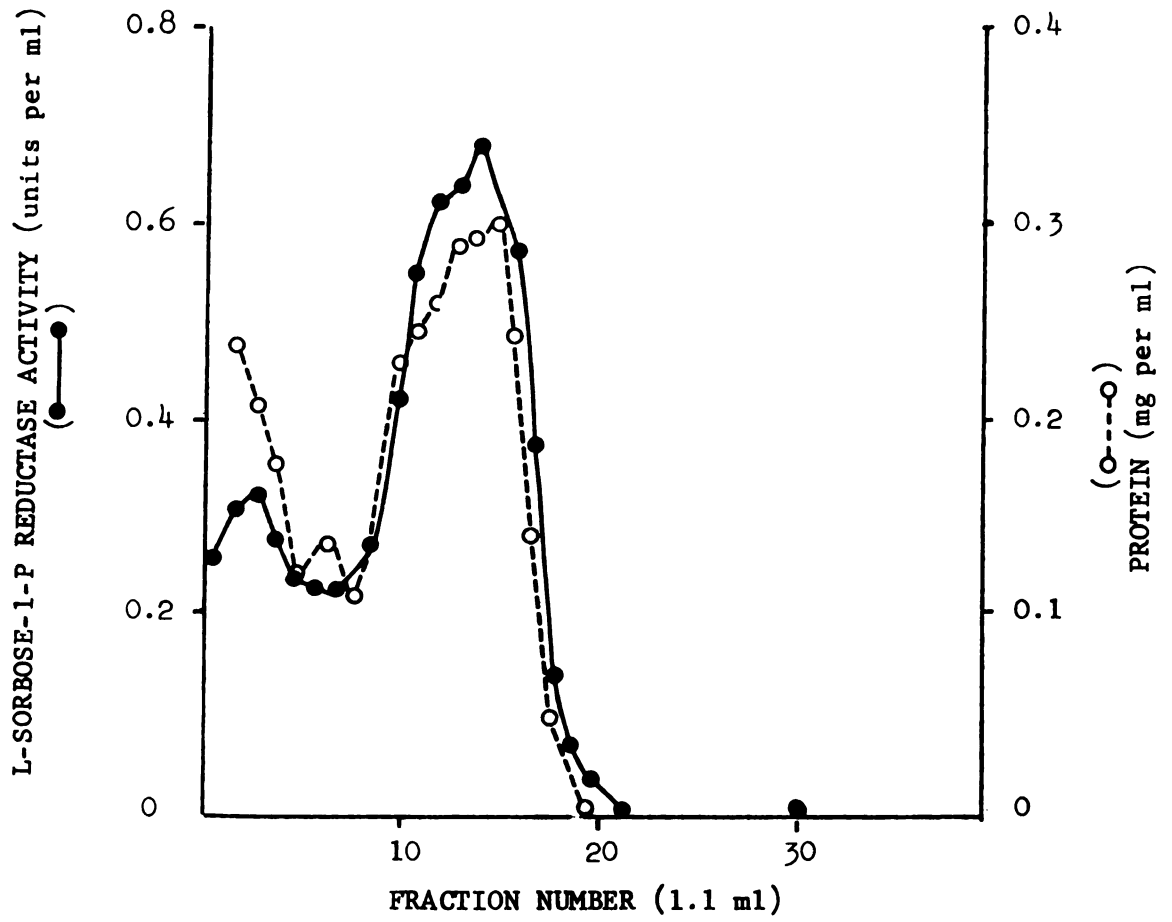


Figure 9 - Affinity chromatography of L-sorbose-1-P reductase: elution with NADH. Column was eluted with a 50-ml gradient of 0 to 50 μM NADH.

had been treated with EDTA; (iii) Mn^{+2} was not necessary for the binding of the enzyme to NADP since EDTA-treated enzyme would bind to NADP-sepharose; and (iv) with EDTA-treated enzyme in the absence of Mn^{+2} the D-fructose-1-P had no effect on the binding of the enzyme to NADP-sepharose. These properties of the enzyme suggested that elution of the column in the presence of D-fructose-1-P, $MnCl_2$, and NADH would remove those enzymes binding to NADP-sepharose which have no affinity for D-fructose-1-P, while leaving the reductase tightly bound. Elution with NADH alone should then allow the specific elution of the reductase. Figure 10 shows the results of such a procedure. The dialyzed combined fractions from the second DEAE-cellulose step were applied to a 1 x 6.3 cm column of NADP-sepharose in a buffer containing 0.025 M MES (pH 6.1), 0.2% mercaptoethanol, 2 mM EDTA, and 20% glycerol. The column was then washed with the same buffer with EDTA and glycerol omitted. The wash-through contained 58% of the protein and less than 1% of the reductase activity. When the column was eluted with the same buffer used for the wash containing 1 mM $MnCl_2$, 2 mM D-fructose-1-P, and 20 mM NADH, 3.2% of the reductase activity and 5.9% of the protein were eluted from the column. The column was then eluted with the wash buffer containing only 2 mM EDTA and 20 mM NADH, giving a recovery of 75.5% of the activity and 23% of the protein. When these fractions were combined, they represented a 32-fold purification overall. Acrylamide gel electrophoresis of a lyophilized preparation of these fractions revealed two minor contaminants comprising less than 5% of the total protein (Figure 11). This

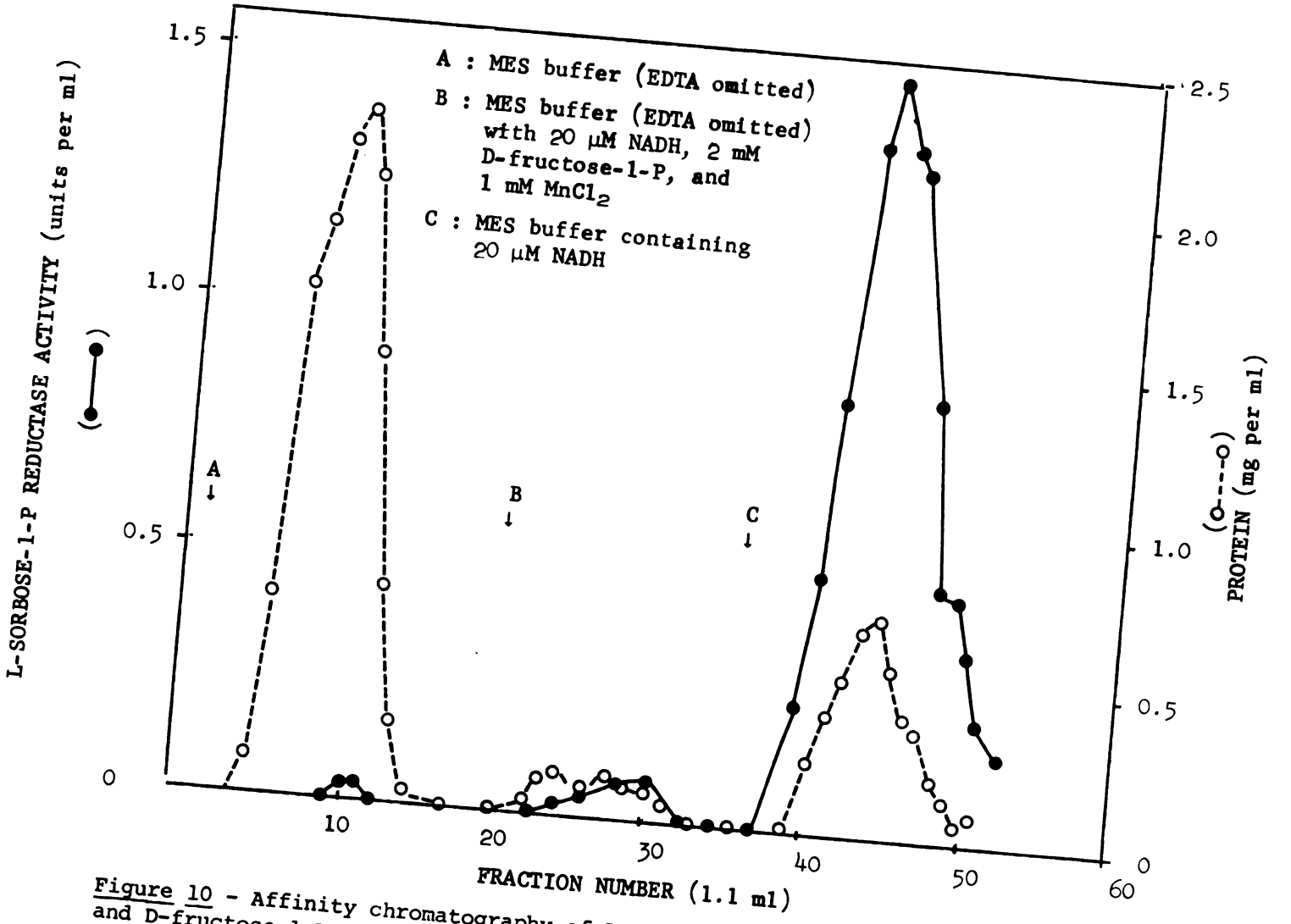


Figure 10 - Affinity chromatography of L-sorbose-1-P reductase; elution with NADH and D-fructose-1-P.

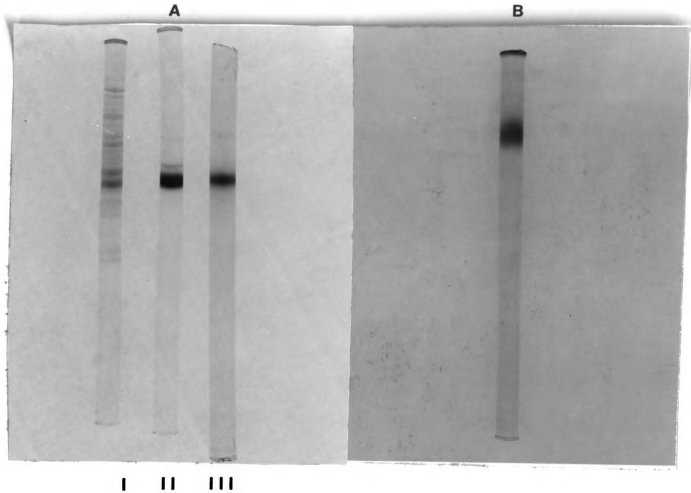


Figure 11 - Polyacrylamide gels of L-sorbose-1-P reductase at various stages of purification. A : Gels run at pH 8.8 in Tris-glycine buffer; I - Sample from second DEAE-cellulose column (120 µg protein applied); II - Sample from first affinity column (120 µg protein applied); III - Sample from second affinity column (60 µg protein applied). B : Gel run at pH 6.2 in sodium phosphate buffer - same sample as A - III.

combined eluent was used for most of the characterization studies. Where a purer preparation was required the combined fractions from this step were applied to a second affinity column as described below.

Second affinity purification step. A second affinity purification was performed by applying the 32-fold purified preparation from the first affinity step to the same NADP-sepharose column and eluting with NAD^+ and D-fructose-1-P as described on pages 38 and 39. Figure 12 shows the elution profile of this column. Since the preparation had been lyophilized, some of the reductase was inactivated (the specific activity had decreased to 1.21) and so 68% of the protein applied did not bind to the column and 18% of the reductase activity did not bind. In the presence of 1 mM NAD^+ 12.5% of the reductase activity and 14% of the protein eluted from the column. Increasing the NAD^+ concentration to 2.5 mM and adding 2.5 mM D-fructose-1-P to the MES buffer (with 1 mM $MnCl_2$) allowed a recovery of 49% of the reductase activity and 20% of the protein applied to the column, and gave an overall purification of 41-fold. This preparation was lyophilized and applied to acrylamide gels (Figure 11). One minor contaminant was present representing less than 1% of the total protein. This preparation was used in the amino acid analysis. A summary of the purification data is presented in Table 1.

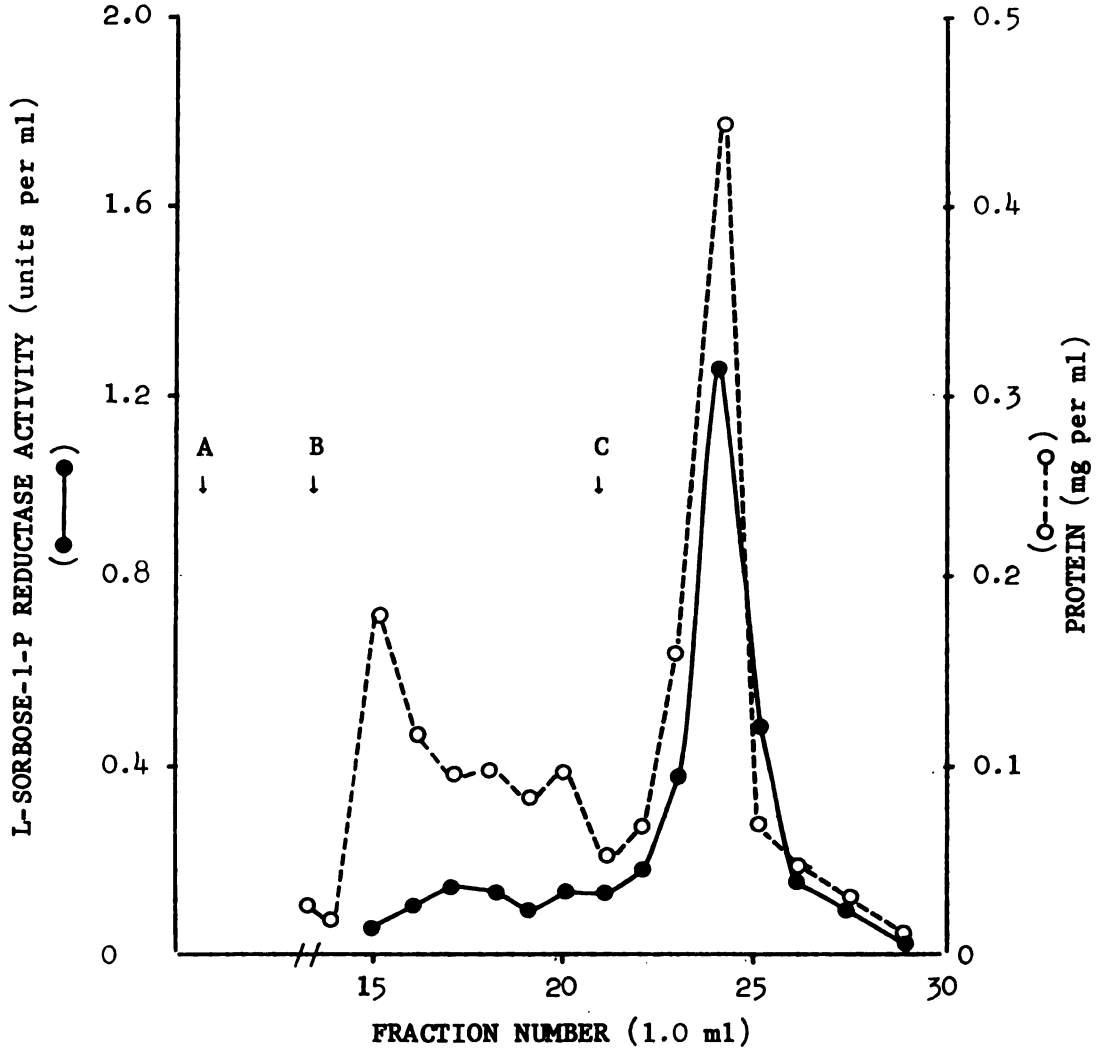


Figure 12 - Second affinity chromatography step. Column was eluted with:

A : MES buffer
B : MES buffer plus 1 mM NAD^+
C : MES buffer (EDTA omitted) plus 2.5 mM NAD^+, 2.5 mM D-fructose-1-P, and 1 mM $MnCl_2$.

Table 1. Summary of purification of L-sorbose-1-P reductase

Purification step	Volume (ml)	Total units[1] (μmoles/min)	Total protein (mg)	Specific activity (units/mg prot.)	Percent recovery	Fold purified
Crude extract, pH 6.1	53.0	76.5	1083	0.071	100	1
1st DEAE column, fract. no. 31-46	17.8	41.3	114	0.363	54	5.1
2nd DEAE column, fract. no. 25-38	18.8	29.3	44	0.661	38	9.3
NADP affinity col.,[2] 20 μM NADH elut.	26.0	23.4	9.9	2.264	28	32.3
NADP affinity col. F-1-P & NAD^+ elut.[3]	26.0	11.2	3.8	2.946	14	41.5

[1]Units reported as moles of D-fructose-1-P reduced per minute, pH 6.1, 30°.

[2]Column eluted with 20 μM NADH; values reported are adjusted to correspond with values which would be obtained had all the units from the previous step been subjected to this step.

[3]Column eluted with 2.5 mM NAD^+ and 2.5 mM D-fructose-1-P. Values are adjusted as for the first NADP affinity column step.

Properties

Determination of molecular weight

Native acrylamide gel electrophoresis. When a graph of the log of protein mobility (relative to a marker dye at a constant pH) versus the percentage of acrylamide in the gels is plotted, a series of lines is obtained. The slope of each of these lines is directly proportional to the molecular weight of the protein producing the line (35). This type of molecular weight determination was performed for L-sorbose-1-P reductase using three marker proteins: bovine erythrocyte carbonic anhydrase, MW 29,000 (44); bovine serum albumin, MW 65,000 (45); and *P. putida* 2-keto-3-deoxy-6-phosphogluconate aldolase, MW 73,000 (46). Figure 13 shows the relationships obtained from such a molecular weight determination. Part A of Figure 13 is a graph of the log of relative mobility versus the percentage of acrylamide in the gel at pH 8.8. The slopes of the lines were determined by the method of the least squares and these slopes plotted against the molecular weight in Part B of Figure 13. The accuracy of the slope for the carbonic anhydrase (Figure 13A) could be questioned due to the scatter in the points; however, Figure 13B shows that the slope determined by statistical methods must be correct or the line would not have intersected at the origin, as it must in this type of graph. When the slope of L-sorbose-1-P reductase is plotted on the standard curve in Part B of the figure, a molecular weight of 44,000 $\pm$ 2,000 is obtained. As will be seen below, this value actually represents the subunit molecular weight.

Figure 13. Determination of the molecular weight of L-sorbose-1-P reductase by acrylamide gel electrophoresis. A shows the relationship between the relative mobility and the concentration of acrylamide in the gel. B shows the relationship between the slope (as determined by the method of least squares) of the lines from part A and the molecular weight of three standards and L-sorbose-1-P reductase. Proteins used were: carbonic anhydrase (◆); bovine serum albumin (●); 2-keto-3-deoxy-6-phosphogluconate aldolase from *P. putida* (◇); and L-sorbose-1-P reductase (o).

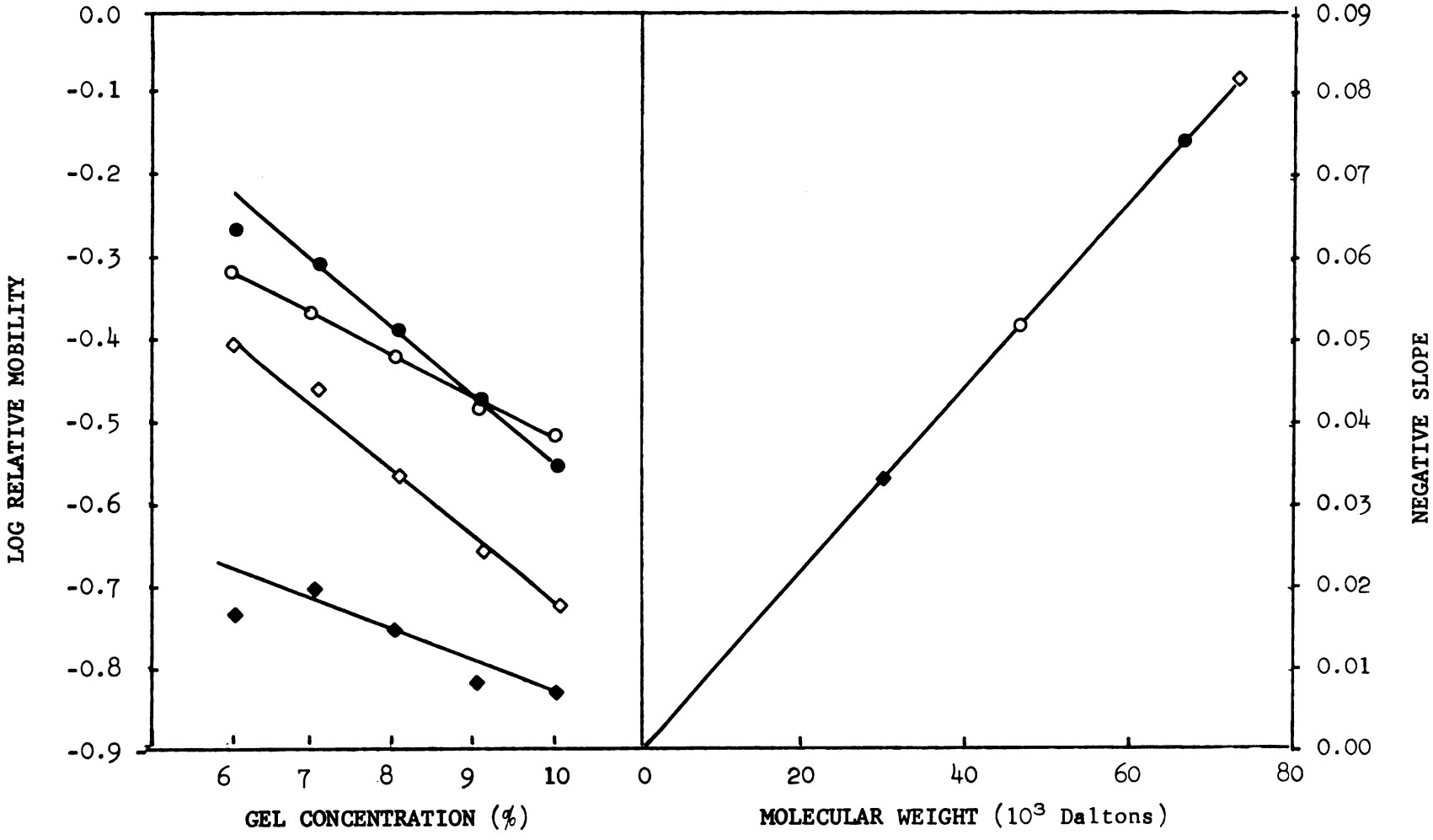

0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
LOG RELATIVE MOBILITY
6
7
8
9
10
GEL CONCENTRATION (%)
0
20
40
60
80
MOLECULAR WEIGHT (10^3 Daltons)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
NEGATIVE SLOPE

Figure 13

Subunit molecular weight determined by SDS acrylamide gel electrophoresis. Electrophoresis in the presence of 1% SDS was performed as described in *methods* for L-sorbose-1-P reductase and the following standards: bovine serum albumin, MW 65,000 (45); beef liver catalase, MW 60,000 (47); ovalbumin, MW 45,000 (48); yeast alcohol dehydrogenase, MW 37,000 (49); and bovine erythrocyte carbonic anhydrase, MW 29,000 (44). The data obtained by this method are presented as a graph (Figure 14) of the MW on a log scale versus the relative mobility (expressed as the distance traveled from the origin divided by the length of the gel). Each point was determined by averaging three values for each protein. Using the standard deviation of the relative mobility of L-sorbose-1-P reductase as a source of error, the molecular weight of the subunit of L-sorbose-1-P reductase was determined to be 46,000 $\pm$ 1,000.

Gel filtration on Sephadex G-100. Gel filtration was performed on a 1.5 x 57 cm column of Sephadex G-100. The sample in a volume of 2 ml contained L-sorbose-1-P reductase and the following standards: beef heart lactate dehydrogenase, MW 131,000 (50); yeast D-glucose-6-P dehydrogenase, MW 12,000 (51); bovine serum albumin, MW 65,000 (45); yeast hexokinase, MW 50,000 (52); and ovalbumin, MW 45,000 (48). The column was eluted with MES buffer with EDTA omitted and fractions of 1.1 ml collected and assayed for enzyme activities and protein. The elution pattern from the column is presented in Figure 15. From this pattern the standard curve (Figure 16) of the molecular weight (plotted on a

Figure 14. Determination of the subunit molecular weight of L-sorbose-1-P reductase (SR) by SDS acrylamide gel electrophoresis. Standards used were: bovine serum albumin (BSA); beef liver catalase (CAT); ovalbumin (OA); yeast alcohol dehydrogenase (ADH); and bovine erythrocyte carbonic anhydrase (CA).

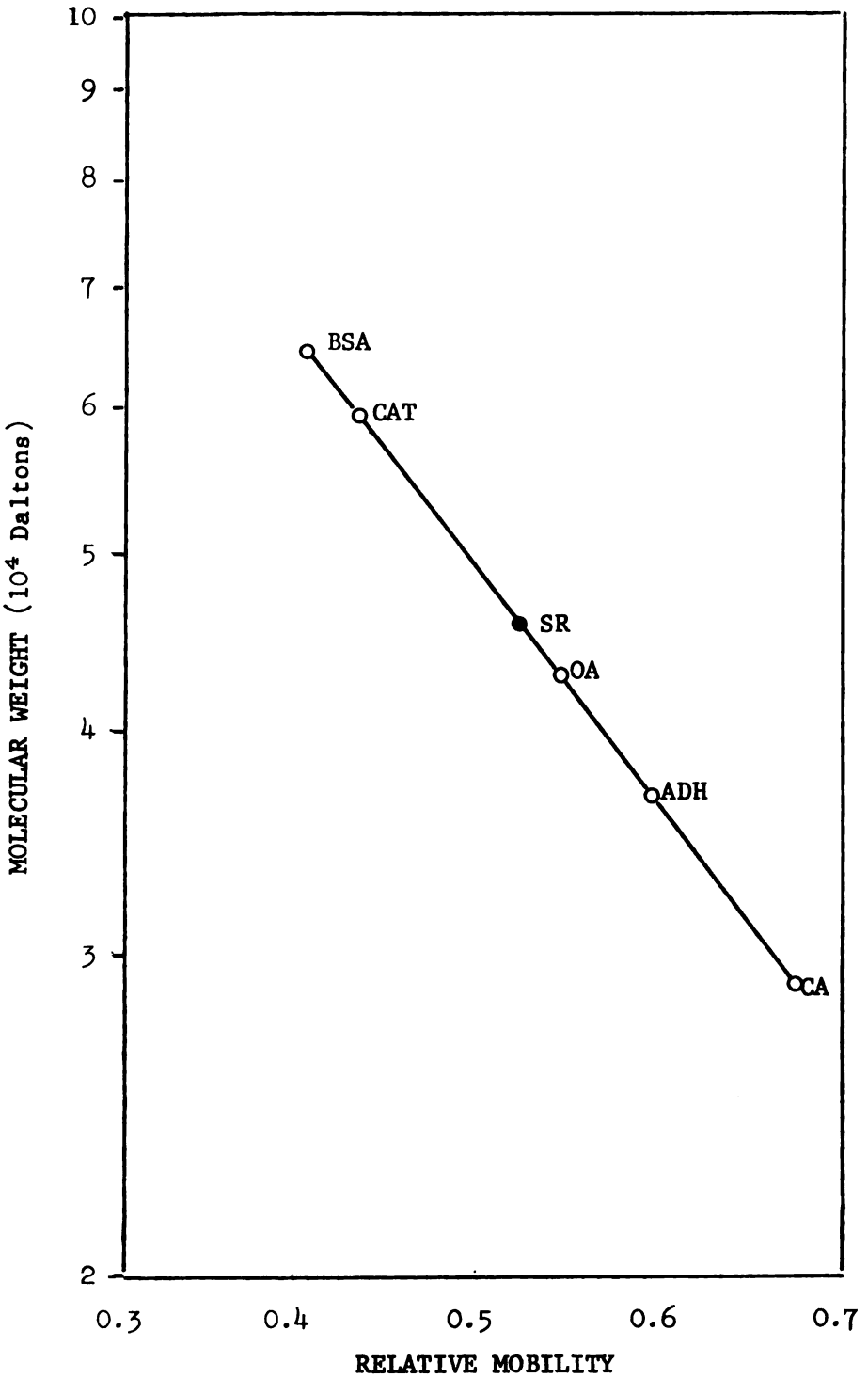

10
9
8
7
6
5
4
3
2
MOLECULAR WEIGHT (10^4 Daltons)
BSA
CAT
SR
OA
ADH
CA
0.3
0.4
0.5
0.6
0.7
RELATIVE MOBILITY

Figure 14

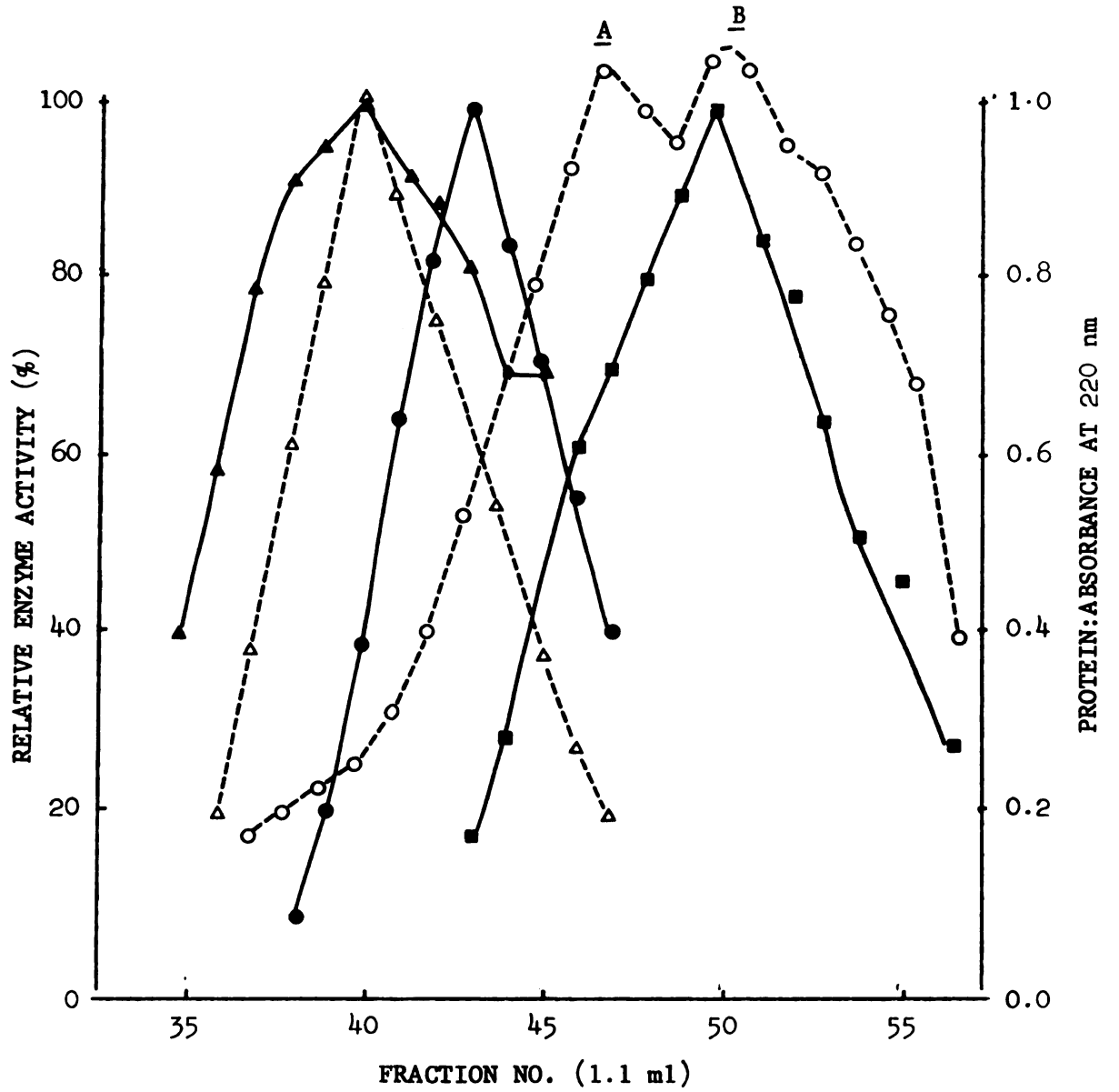


Figure 15 - Elution profile of Sephadex G-100 column. Marker proteins used were: lactate dehydrogenase (△); D-glucose-6-P dehydrogenase (▲); hexokinase (■); bovine serum albumin (peak A) and ovalbumin (peak B) as determined by protein absorbance at 220 nm (o). L-sorbose-1-P reductase is shown by ●

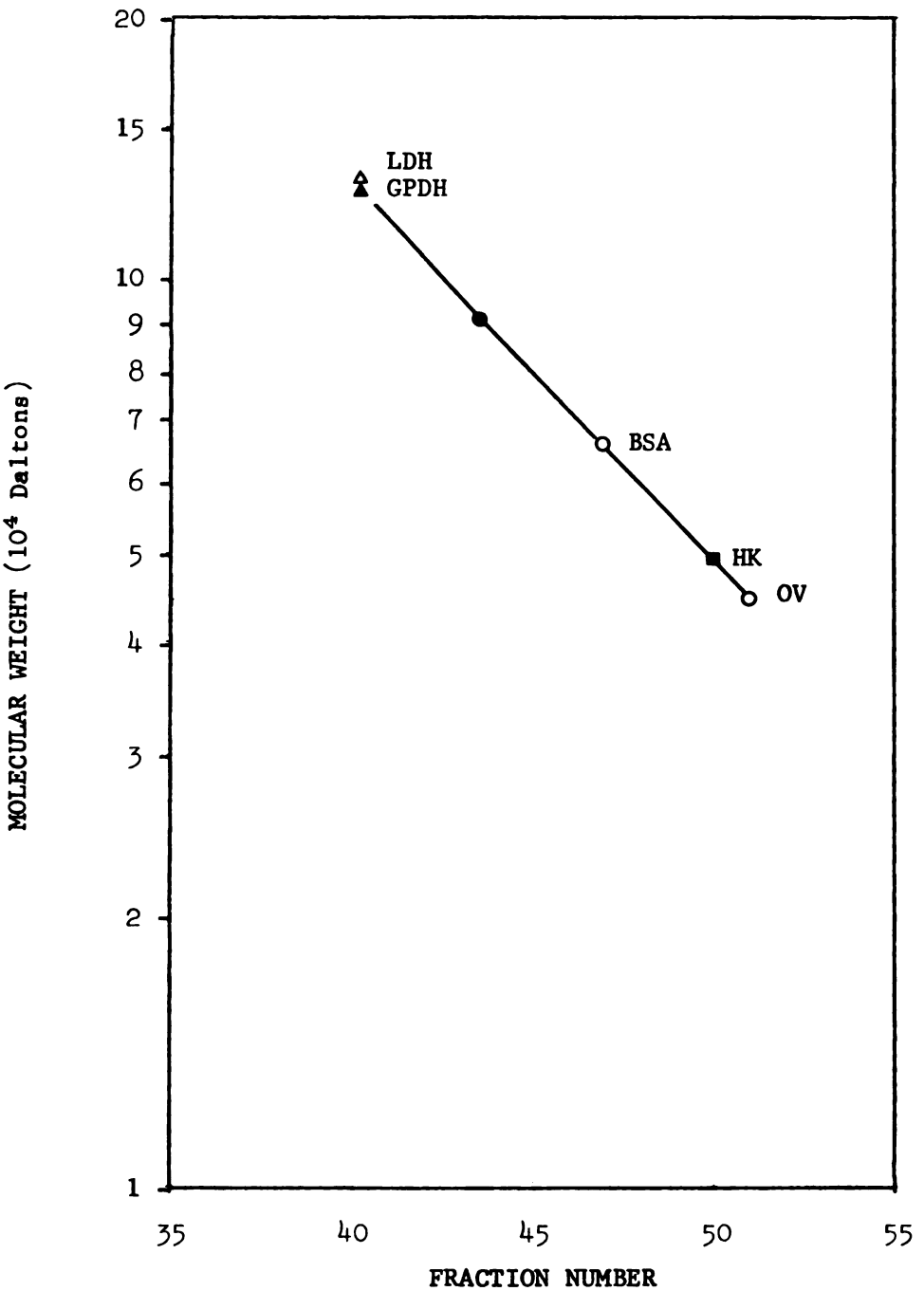


Figure 16 - Determination of molecular weight of L-sorbose-1-P reductase (●) using data obtained from Figure 15. Abbreviations used are: LDH : lactate dehydrogenase; GDPH : D-glucose-6-P dehydrogenase; BSA : bovine serum albumin; HK : hexokinase; and OV : ovalbumin.

log scale) versus the fraction number was obtained. Allowing that the activity peak of the L-sorbose-1-P reductase may be off by one half of a fraction either way, the molecular weight was determined as 92,000 $\pm$ 2,000.

Sedimentation equilibrium ultracentrifugation. Ultracentrifugation was performed as described in *methods*. Three concentrations of L-sorbose-1-P reductase were used: 0.5 mg/ml, 1.0 mg/ml, and 2.0 mg/ml. After 24.5 hr only the 0.5 mg/ml sample appeared to have reached equilibrium and all measurements were obtained from the Rayleigh interferogram of this concentration. The interferogram of this sample appears as the upper image in the photograph appearing to the left in Figure 17. Measurements were made by standard methods (37,38) and plotted (Figure 17) in a graph of three plus the logarithm of the vertical displacement from the meniscus versus the square of the distance from the center of rotation. The slope of the resulting straight line was substituted in the equation.

$$M_z = \frac{2\ R\ T\ (2.303)}{(1 - \bar{v}\rho)\ \omega^2} \times \text{slope from Figure 17,}$$

where M_z is the z-average molecular weight, R is the gas constant (8.314×10^7 erg°K^{-1}mol^{-1}), T is the absolute temperature of the centrifuge run (288°), ρ is the density of the solvent at the given temperature (0.999), ω is the speed of rotation of the run in radians per sec, and $\bar{v}$ is the partial specific volume of the protein, which was assumed to be 0.733 g/ml^3 (that for an "average" protein). Most of the error in this method comes from the value

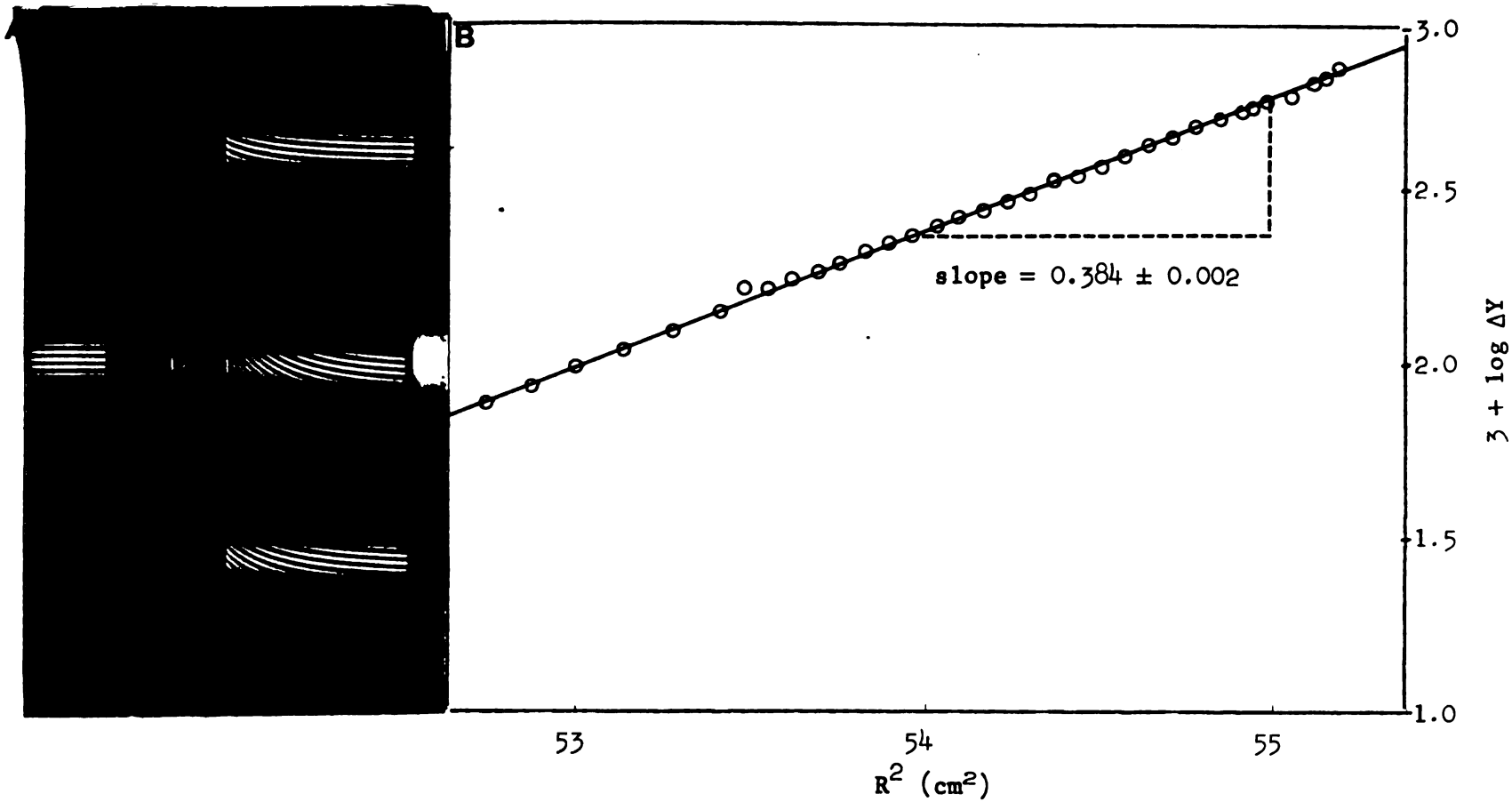


Figure 17 - Sedimentation equilibrium ultracentrifugation of L-sorbose-1-P reductase to determine molecular weight. A : Photography of Rayleigh interferograms from centrifuge run (12,573 rpm) at 14° after 24.5 hr in an AN-G rotor. Concentrations used were: 0.5 mg protein/ml (upper image); 1.0 mg protein/ml (lower image); and 2.0 mg protein/ml (center image). B : Plot of data obtained from interferograms in A showing the relationship between a log function of the vertical displacement and the square of the distance from the center of rotation.

used for the partial specific volume; an error of 1% in $\bar{v}$ will result in a 3% error in the molecular weight (37). From the equation the molecular weight of L-sorbose-1-P reductase was calculated to be 87,900 $\pm$ 2,600.

Determination of Svedberg coefficient from sucrose density gradient centrifugation. Centrifugation was performed as described in *methods* using a 0.1-ml sample containing L-sorbose-1-P reductase and two marker proteins: sheep hemoglobin, 4.13 S (53) and *E. coli* alkaline phosphatase, 6.10 S (28). A profile of the protein peaks obtained from the centrifugation is shown in Figure 18; the dotted lines are extensions to determine the distance that the markers and the reductase traveled from the origin. Since this distance is inversely proportional to the S-value, then the average calculated from each marker for L-sorbose-1-P reductase is 5.54 $\pm$ 0.04 x 10^{13} sec.

Summary of molecular weight determinations. The results of the various methods by which the molecular weight of L-sorbose-1-P reductase was determined is summarized in Table 2. The molecular weight of the native enzyme as determined by gel filtration and ultracentrifugation correlated well with the molecular weight of the enzyme calculated from the values of the subunit molecular weight. The average molecular weight determined by all of the methods is calculated to be 90,000 $\pm$ 2,000. The average molecular weight of the subunit molecular weight is calculated to be 45,000 $\pm$ 1,000, indicating that the enzyme is a dimer in the native state.

Figure 18. Sucrose density gradient determination of Svedberg coefficient of L-sorbose-1-P reductase. Gradient was 4.7 ml of 5.0% to 20% sucrose in 0.050 M MES buffer (pH 6.1) and 0.2% β-mercaptoethanol. Tubes (1.2 cm x 7.5 cm) were run at 0° for 16 hrs at 35,000 rpm in a SW 50.0 swinging bucket rotor. Markers used were: sheep hemoglobin (4.13 S) determined by absorbance at 410 nm (□——□); and *E. coli* alkaline phosphatase (6.10 S) determined by rate of hydrolysis of p-nitrophenylphosphate in 1.0 M Tris-HCl buffer (pH 8.0) as measured by increase in A_{410} per minute per ml (o——o). L-Sorbose-1-P reductase is shown as μmoles NADH oxidized per minute per ml (●——●).

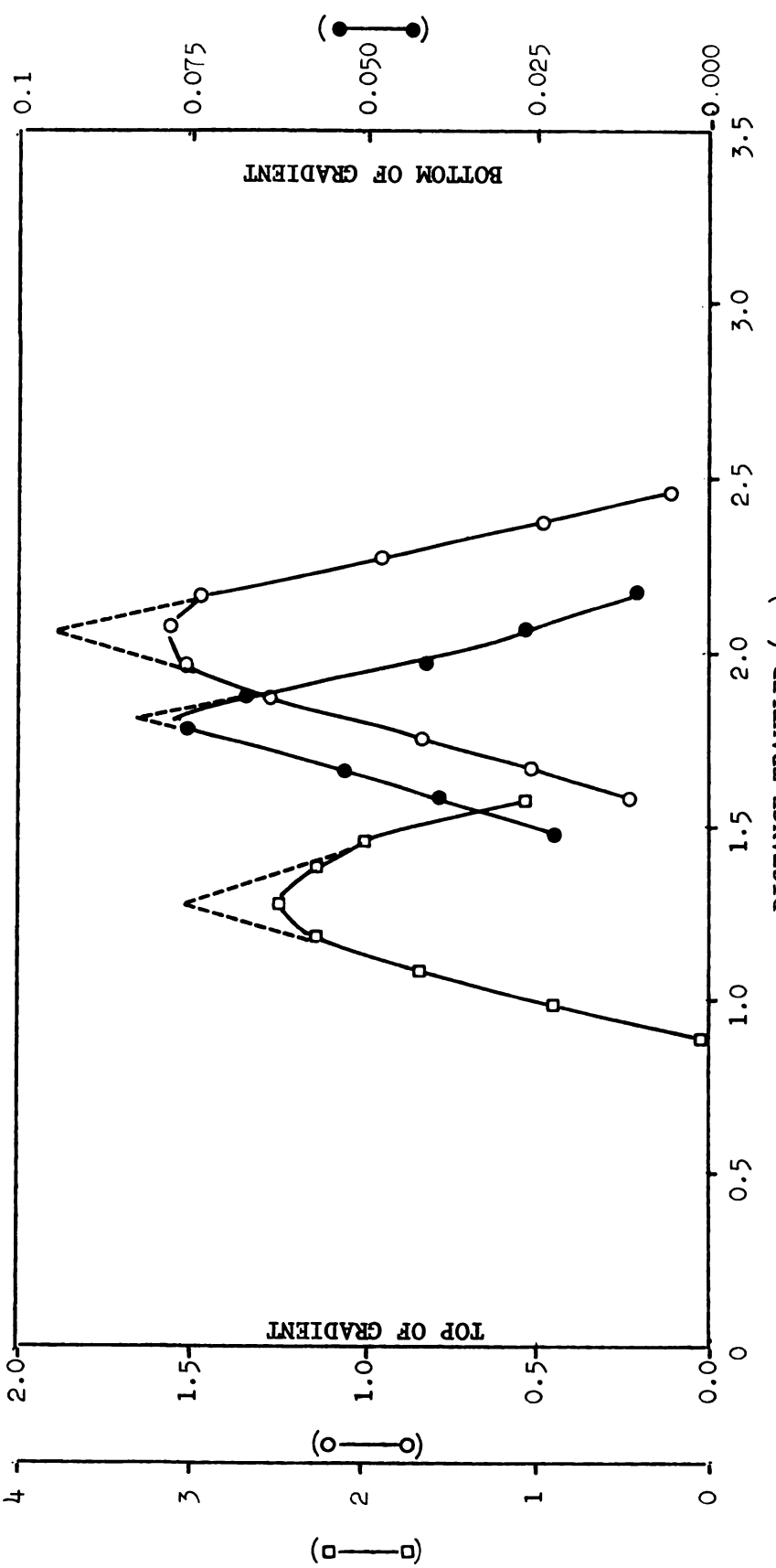

TOP OF GRADIENT
BOTTOM OF GRADIENT
DISTANCE TRAVELED (cm)
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
0.000
0.025
0.050
0.075
0.1
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Figure 18

Table 2. Summary of molecular weight determination of L-sorbose-1-P reductase

Method of determination	Molecular weight		Sedimentation coefficient (Svedbergs)
	Subunit	Native	
"Native" acrylamide gel electrophoresis	44,000 ± 2,000	88,000 ± 4,000*	---
SDS acrylamide gel electrophoresis	46,000 ± 1,000	92,000 ± 2,000*	---
Gel filtration on Sephadex G-100	---	92,000 ± 2,000	---
Sedimentation equilibrium centrifugation	---	87,900 ± 2,600	---
Sucrose density gradient centrifugation	---	---	5.54 ± 0.04

*Calculated by assuming two subunits per native enzyme.

Catalytic properties

Effect of pH. The effect of pH on the relative activity of L-sorbose-1-P reductase in MES, maleate-NaOH, and citrate-phosphate buffers is shown in Figure 19. The pH optimum in all three buffers is 6.2, but the velocity is greatest in MES.

Metal ion requirements. Monovalent metal cations such as Na^+, K^+, NH_4^+, and Li^+ have an inhibitory effect on the activity of L-sorbose-1-P reductase; in concentrations above 10 mM, the velocity is inhibited approximately 50%. In the presence of EDTA all L-sorbose-1-P reductase activity is abolished, indicating the divalent metal cations are necessary for the enzyme's activity. In order to identify the divalent metal ion requirements, EDTA-treated L-sorbose-1-P reductase [enzyme which had been purified in 0.025 M MES buffer (pH 6.15) containing 0.2% mercaptoethanol and 2 mM EDTA] was passed over a Biogel P-6 column equilibrated with 0.025 M MES buffer (pH 6.15) containing 0.2% mercaptoethanol. Fractions containing protein were combined and assayed; they exhibited no L-sorbose-1-P reductase activity in the absence of divalent metal cation.

Assay mixtures (with various divalent metal cations) were prepared containing all components of the standard mixture but one and incubated for 5 min at 30°. The reaction was initiated by adding the missing component to the mixture. The relative activity of L-sorbose-1-P reductase in the presence of different metal ions is shown in Table 3; the four columns represent the effect of

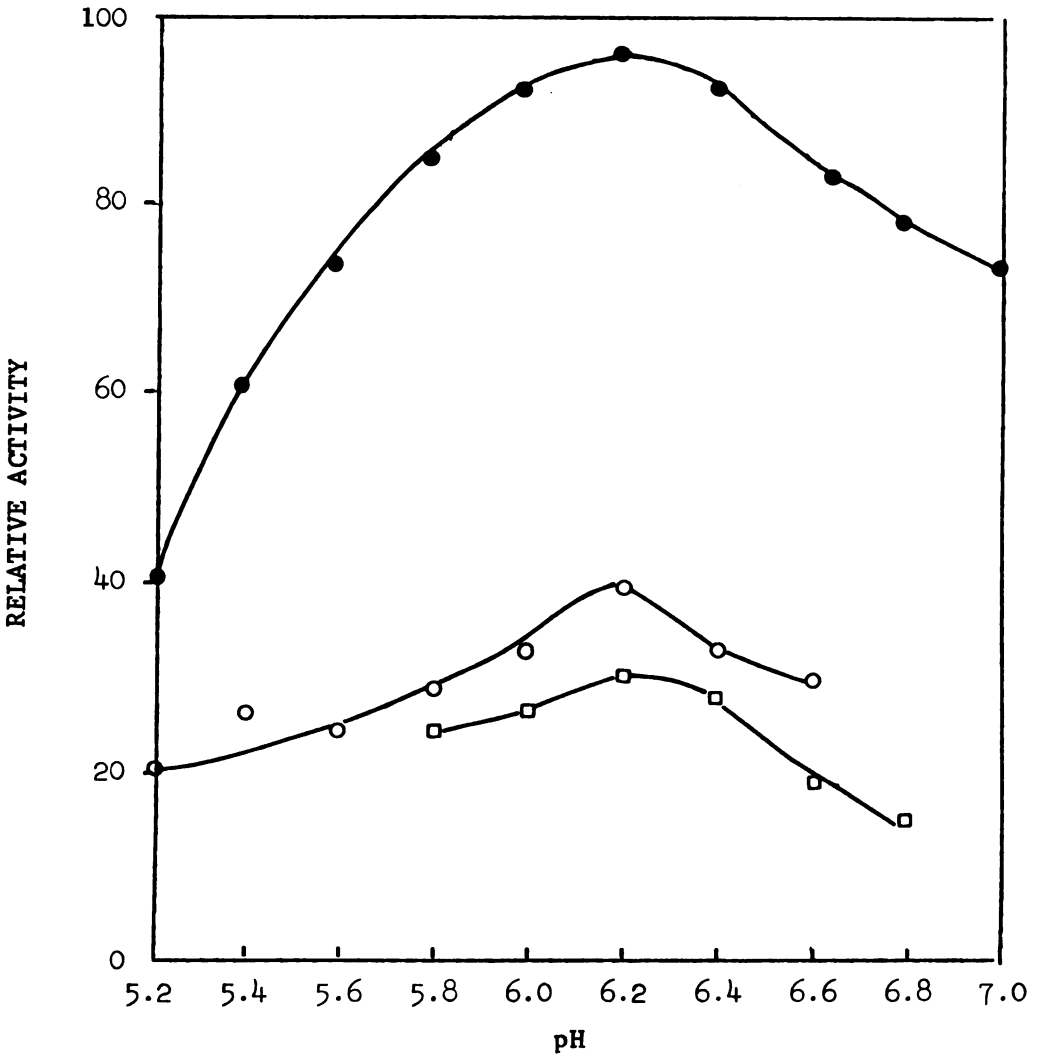


Figure 19 - Relative activity of L-sorbose-1-P reductase as a function of pH and buffer. Relative rates in 0.067 M MES buffer (●) represent the average of four determinations at each pH. The pH was determined for each complete assay mixture after the rates were determined. The relative rates for 0.067 M maleate-NaOH (□) and 0.067 M citrate-phosphate (○) represent one determination at each pH value.

Table 3. The effects of various divalent metal cations on the activity of EDTA-treated L-sorbose-1-P reductase. The assay conditions and methodology are described in the text.

Metal added (1.0 mM)	Activity expressed as percent activity with $MnCl_2$			
	Component of assay mixture used to initiate rxn			
	D-fru-1-P†	Metal ion	NADH	Enzyme
$BaCl_2$	3	2	3	8
$CaCl_2$	30	38	23	41
$CoCl_2$	225	††	294	††
$FeSO_4$	94	95	86	83
$MnCl_2$	100[a]	100[b]	100[c]	100[d]
$MgCl_2$	73	4	3	77
$NiCl_2$	63	54	55	80
$ZnSO_4$	40	37	35	36

†D-fructose-1-P

††Rates were nonlinear

[a]0.511 nmoles NADH oxidized per min.

[b]0.445 nmoles NADH oxidized per min.

[c]0.445 nmoles NADH oxidized per min.

[d]0.507 nmoles NADH oxidized per min.

initiating the reaction with various components of the assay mixture. The addition of Mn^{+2} reactivated the enzyme to the same rates obtained before the EDTA-treatment. Ferrous ion produced rates closest to those obtained with Mn^{+2}. Neither Ba^{+2}, Ca^{+2}, nor Zn^{+2} produced rates greater than one half those obtained in the presence of the same amount of Mn^{+2}. Ni^{+2} produced intermediate rates of activation (i.e., between 50 and 100%). Mg^{+2} produced significant activation only in those cases where D-fructose-1-P and the enzyme were not present together in the preincubation mixture. Co^{+2} was a better activator (i.e., gave greater initial velocities) of reductase than was Mn^{+2}. However, the reaction rates were nonlinear when either the enzyme or Co^{+2} were used to initiate the reaction.

Attempts at determining the optimal concentration of divalent metal cation necessary for optimal activation of the reductase produced variable results (Table 4). Ba^{+2} and Ca^{+2} were unable to activate the enzyme at concentrations below 0.1 mM. Zn^{+2} activated the reductase optimally at a concentration of 0.01 mM and the activity was comparable to that obtained with Mn^{+2} at this concentration. All other divalent metals produced optimal activity at a concentration of 0.1 mM except Mg^{+2} (which activated maximally at 10 mM).

Substrate specificity. Preliminary investigations of the substrate specificity of less purified preparations of L-sorbose-1-P reductase revealed that D-fructose-1-P would serve as a substrate for the enzyme nearly as well as L-sorbose-1-P.

Table 4. Effects of varying concentrations of divalent metal cations on the activity of L-sorbose-1-P reductase. The reaction conditions were the same as in Table 3 except that the reaction was initiated by the addition of enzyme to the assay mixture.

Metal ion	Activity (nmoles NADH oxidized per min per ml) Concentration (mM)			
	10	1.0	0.1	0.01
$BaCl_2$	0.074	0.018	0.000	0.000
$CaCl_2$	0.160	0.064	0.000	0.000
$MnCl_2$	0.288	0.322	0.346	0.346
$MgCl_2$	0.105	0.090	0.090	0.035
$NiCl_2$	0.136	0.234	0.293	0.254
$FeSO_4$	ppt*	0.244	0.351	0.293
$ZnSO_4$	0	0.156	0.302	0.351

*Formed a precipitate at this concentration.

A graph of reaction velocity versus amount of purified enzyme added to the assay revealed that at concentrations above 1.2 µg (0.008 mg/ml in the assay mixture), the reaction velocity did not increase at the same rate as below this concentration (Figure 20). A double reciprocal plot (Figure 21) shows that at higher concentrations of enzyme the Km for D-fructose-1-P and L-sorbose-1-P increases from 0.38 mM to 1.2 mM and from 0.65 mM to 1.73 mM, respectively. The Vmax for D-fructose-1-P and L-sorbose-1-P increases from 0.61 nmole/min to 2.04 nmole/min and from 1.83 nmole/min to 6.08 nmole/min, respectively. Since all assays and other kinetic determinations in this dissertation were done at enzyme concentrations below 1.2 µg, this phenomenon was not studied further.

A study of the Km and Vmax determined for L-sorbose-1-P and D-fructose-1-P from Figure 21 reveals that although the Km of the reductase for L-sorbose-1-P is 1.7 times greater than that for D-fructose-1-P, the Vmax of the enzyme is three times greater with L-sorbose-1-P than with D-fructose-1-P. The specificity of the enzyme was limited to these two ketohexose 1-phosphates; L-sorbose, D-fructose, D-fructose-6-P, and D-fructose-1,6-P_2 would not serve as substrates for the enzyme at concentrations of up to 10 mM.

Determination of the Km and Vmax of the enzyme for both NADH and NADPH (Figure 22) indicated that the values were nearly identical for both and were comparable to the levels of NADH needed to elute the reductase from an NADP-sepharose column (20 µM).

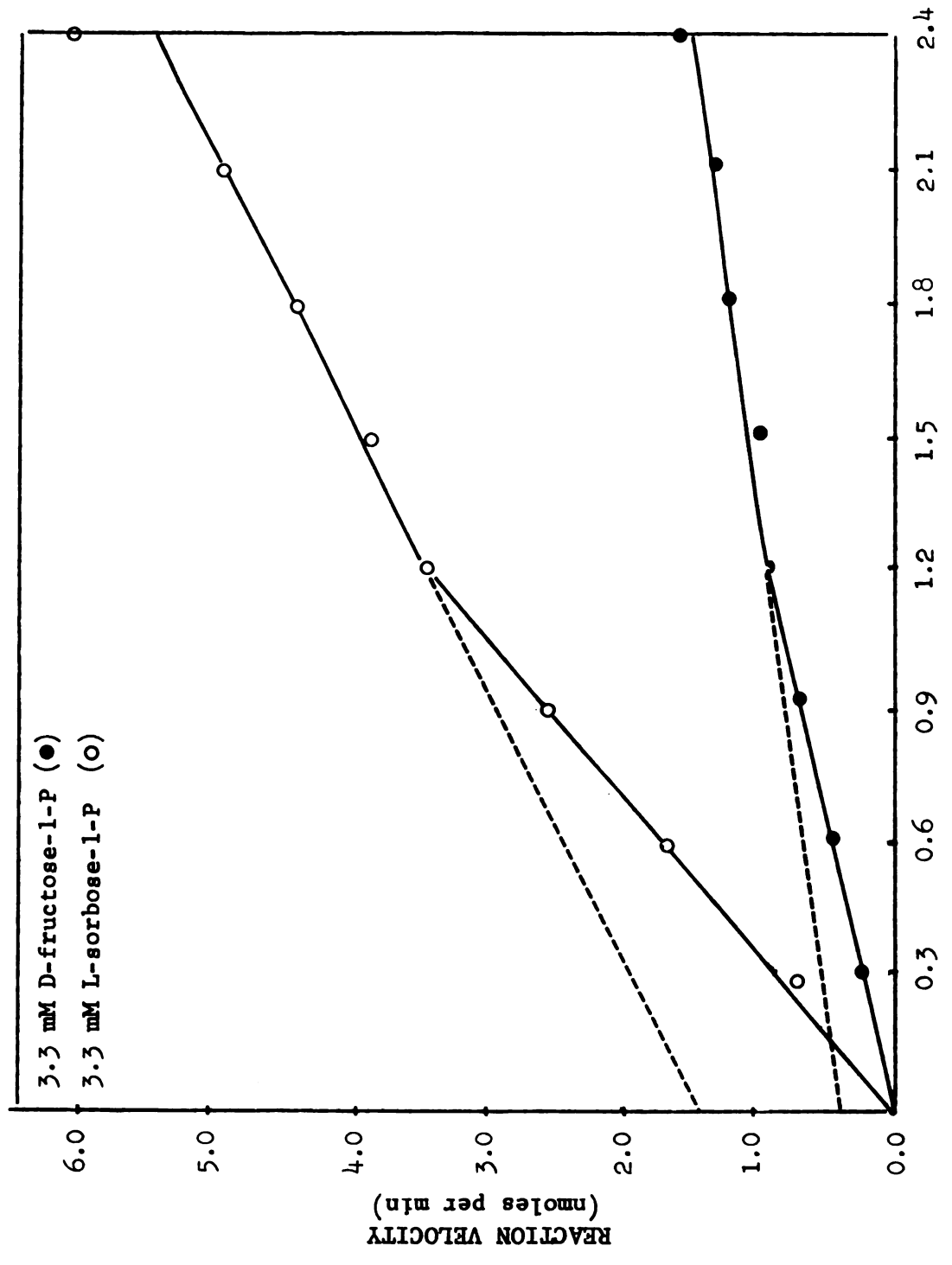


Figure 20 - Relationship between L-sorbose-1-P reductase concentration and the velocity of the reaction. A preparation from the first affinity chromatographic purification step was used.

Figure 21. Double reciprocal plot showing the relationship between D-fructose-1-P or L-sorbose-1-P concentration and the reaction velocity using two concentrations of enzyme. Either 0.65×10^{-3} units (o,●) or 1.66×10^{-3} units (△,▲) (as measured by the reduction of D-fructose-1-P with NADH) of a preparation of L-sorbose-1-P reductase with a specific activity of 0.866 units per mg protein were used.

Substrate	Km	Vmax
D-Fructose-1-P (○)	0.38 mM	0.61 nmole/min
D-Fructose-1-P (△)	1.20 mM	2.04 nmole/min
L-Sorbose-1-P (●)	0.65 mM	1.83 nmole/min
L-Sorbose-1-P (▲)	1.73 mM	6.08 nmole/min

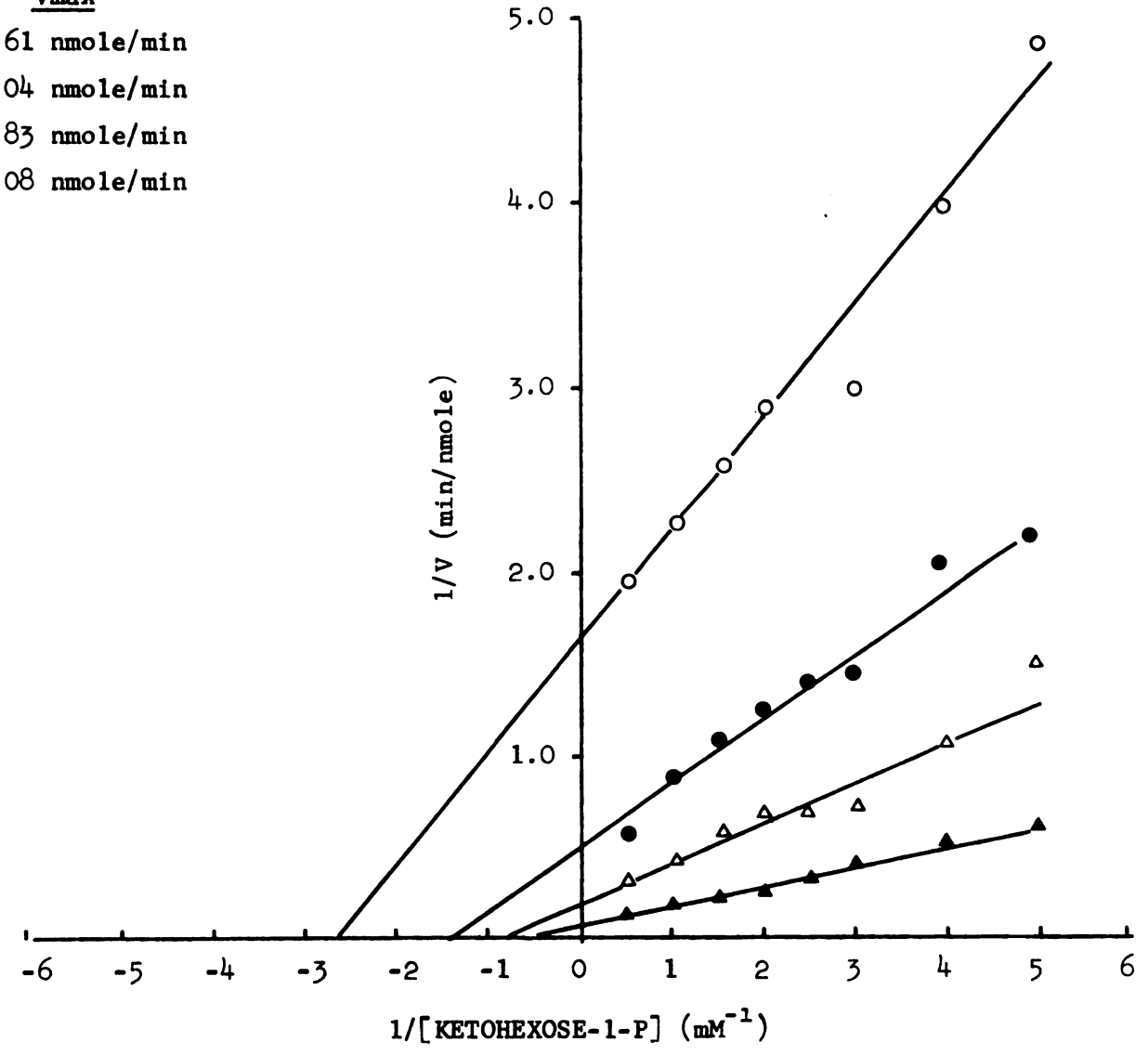


Figure 21

Figure 22. Double reciprocal plot showing the relationship between NADH or NADPH concentration and reaction velocity. Each assay contained 3.3 μM D-fructose-1-P and 0.474×10^{-3} unit of L-sorbose-1-P reductase activity (as measured by the reduction of D-fructose-1-P with NADH) of a preparation with a specific activity of 1.56 units per mg protein.

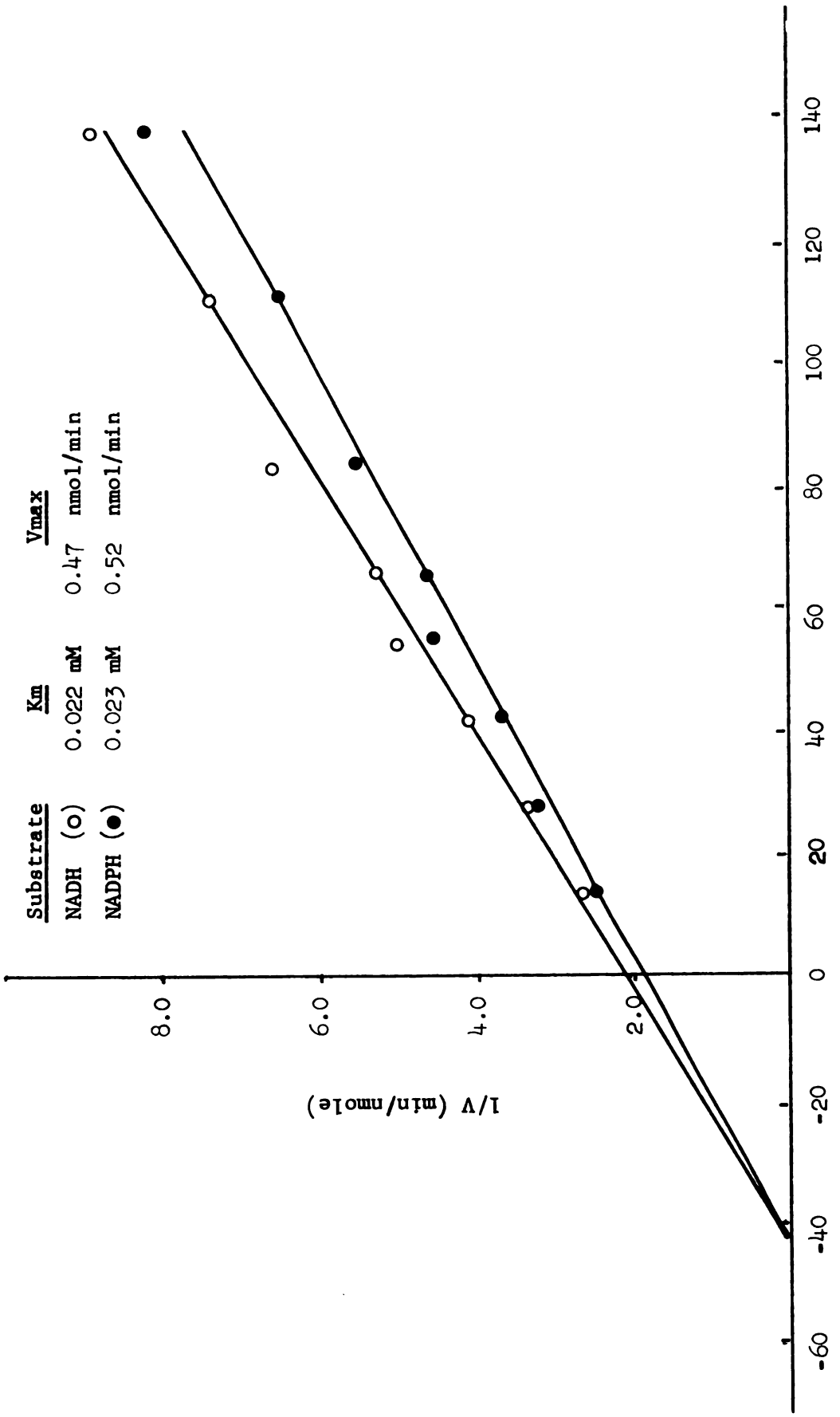

Substrate Km Vmax
NADH (o) 0.022 mM 0.47 nmol/min
NADPH (●) 0.023 mM 0.52 nmol/min
1/V (min/nmole)
8.0
6.0
4.0
2.0
0
20
40
60
80
100
120
140
-20
-40
-60
1/[NAD(P)H] (mM-1)

Figure 22

Physical and chemical properties

Amino acid composition. Determination of the amino acid composition of L-sorbose-1-P reductase purified from a second affinity step (99% pure by electrophoretic criteria) was performed as described in *methods*. The results of this determination are summarized in Table 5. L-Sorbose-1-P reductase is rather low in the basic amino acids--histidine, arginine, and lysine--and contains relatively high amounts of aspartate-asparagine (Asx) and glutamate-glutamine (Glx). Such a distribution of amino acids suggests that L-sorbose-1-P reductase is an acidic enzyme at neutral pH and has an acidic isoelectric point.

Isoelectric point. In order to determine the isoelectric point of L-sorbose-1-P reductase, duplicate gels, composed as described by Wrigley (53a), were subjected to 4 hr of focusing at 300 V constant voltage. For these studies a preparation from a first affinity step was used since purity was not being determined. The activity peak focused at a pH of 5.0 in the gradient and was the same place as the protein peak (Figure 23). This isoelectric point is in agreement with the prediction that the L-sorbose-1-P reductase is acidic because it contains few basic amino acid residues, migrates towards the anode when subjected to electrophoresis at pH 6.2, and binds to DEAE-cellulose at pH 6.15. Early studies with acid precipitation showed that the L-sorbose-1-P reductase precipitated only at pH values below 5.0, which is also in agreement with the other data.

Table 5. Amino acid analysis of L-sorbose-1-P reductase

Amino acid	No. residues per subunit of enzyme	Amino acid	No. residues per subunit of enzyme
Ala	26.2	Lys	11.0
Arg	7.4	Met	3.2
Asx	35.0	Phe	7.6
Cys[1]	3.2	Pro	15.2
Glx	63.7	Ser	58.1
Gly	104.6	Thr	18.9
His	12.2	Trp[2]	26.2
Ile	9.8	Tyr	13.1
Leu	17.0	Val	13.1

[1]Cysteine determined as carboxymethyl derivative.

[2]Tryptophan determined spectrophotometrically as described in text.

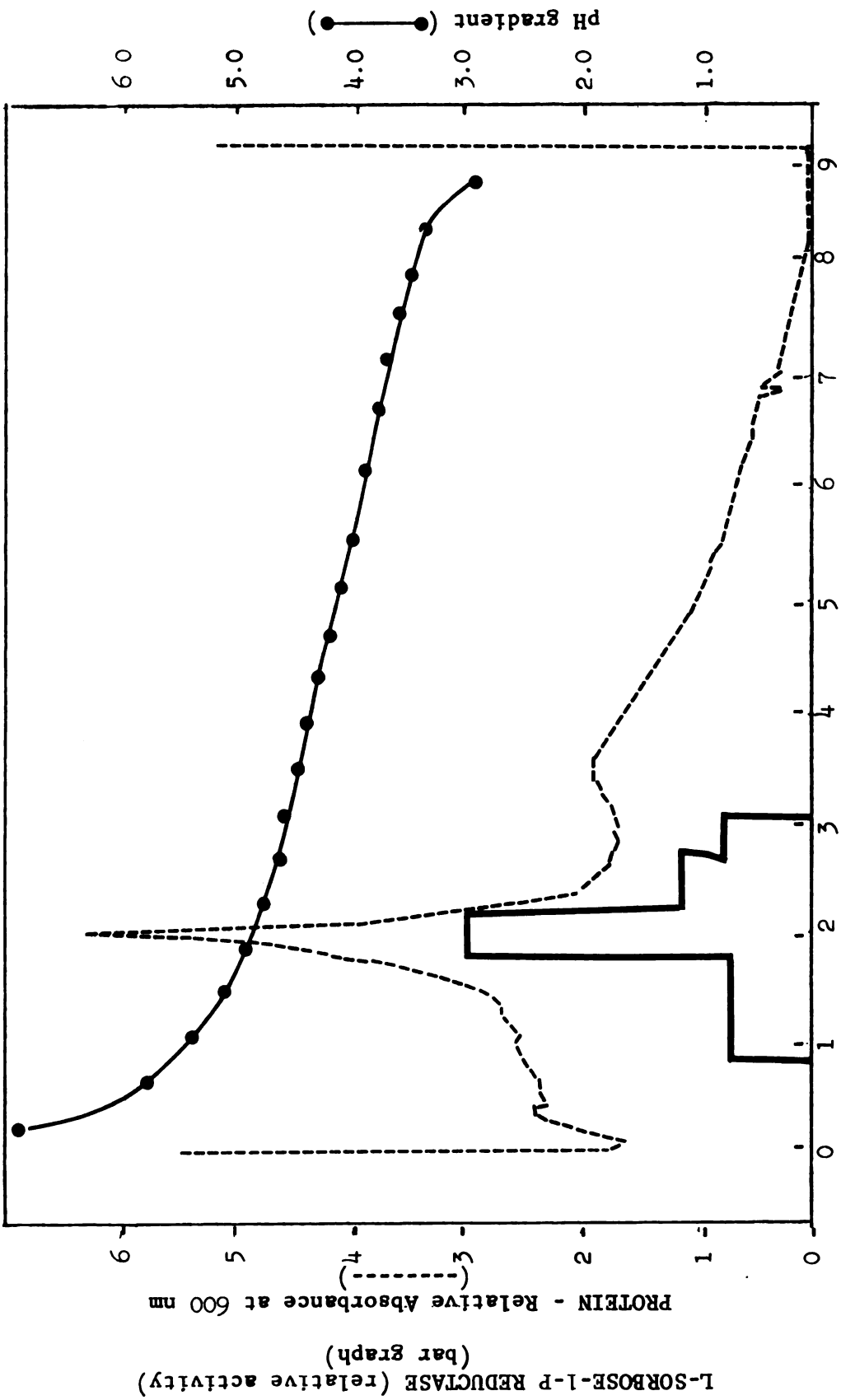


Figure 23 - Isoelectric focusing on an acrylamide gel from pH 3.0 to 6.0 of a partially purified preparation of L-sorbose-1-P reductase. Duplicate gels were run at constant voltage (300 V) for 4 hr after which gels were assayed for enzyme activity, pH, or stained for protein.

Characterization of the Reaction

Identification of the product of the reduction of D-fructose-1-P.

Reduction of D-fructose-1-P at the keto group can produce either D-mannitol-1-P or D-glucitol-1-P. By analogy with the reduction of L-sorbose-1-P by L-sorbose-1-P reductase to form D-glucitol-6-P (1), D-mannitol-1-P would be the expected product of the reduction of D-fructose-1-P by L-sorbose-1-P reductase. To identify the product of the reduction a scaled up reaction mixture was prepared containing 0.067 M MES buffer (pH 6.1), 0.2% mercaptoethanol and 10% glycerol (to stabilize the enzyme during the extended incubation period), 2 mM D-fructose-1-P, 2 mM NADH, and 1 mM $MnCl_2$ in a total volume of 2 ml. The reaction was started by adding 0.1 ml of partially purified L-sorbose-1-P reductase (0.75 total units of activity and 0.48 mg total protein). The reaction was allowed to proceed for 3 hr at 30° at which time it was terminated by making the mixture 2.5% (w/v) trichloroacetic acid. The acidified solution was clarified by centrifugation for 20 min at 48,000 x g, extracted three times with 1 volume of ethyl ether (to remove the TCA), and the pH adjusted to 8.0 with 10% ammonium hydroxide. The mixture was placed on a Dowex-1 column (bicarbonate) as described in part I of this dissertation (p. 12). The column was eluted with 0.5 M $KHCO_3$ so that all phosphorylated products could be analyzed. The product isolated in this manner served as a substrate for D-mannitol-1-P dehydrogenase but not for D-glucitol-6-P dehydrogenase when added to a coupled assay containing phosphoglucoisomerase and D-glucose-6-P dehydrogenase. The same assay

mixture with phosphoglucoisomerase omitted produced only half the amount of reduced nicotinamide adenine dinucleotide cofactor. Dephosphosphorylation of the product was performed as described in *methods* and the dephosphorylated mixture was assayed for inorganic phosphate after 30 min. There was 1 µmole of inorganic phosphate present for every µmole of D-mannitol-1-P present in the initial mixture prior to dephosphorylation (as determined by the D-mannitol-1-P dehydrogenase assay) and there were no detectable levels of product capable of being used as a substrate for the coupled D-mannitol-1-P dehydrogenase assay. When the dephosphorylated product was acetylated as described in *methods* and subjected to gas-liquid chromatography there was one major peak (representing 99% of the detectable peaks) with the same retention time (40.7 min) as the hexaacetate derivative of authentic D-mannitol in a mixture of sugar alcohol standards (Table 6). The results of these experiments indicate that the product of the reduction of D-fructose-1-P by L-sorbose-1-P reductase is D-mannitol-1-P as shown chemically, enzymatically and chromatographically.

Equilibrium and stoichiometry of the reaction. The equilibrium of the reduction reaction was determined under standard assay conditions. The concentration of NADH present initially was determined by measuring the absorbance at 340 nm, the amount of NADH oxidized was determined from the decrease in absorbance at 340 nm when the mixture had reached equilibrium (i.e., when there was no longer a decrease in absorbance at 340 nm). Controls were run with no Mn^{+2} present. After equilibrium was attained the reaction was terminated

Table 6. Gas-liquid chromatography of the hexaaacetate derivative of the dephosphorylated reduction product of D-fructose-1-P*

Hexaacetate standards	RT (min)	Dephosphorylated D-fructose-1-P reduction product	
		% peak height	RT (min)
Rhamnitol	16.4		
Fucitol	18.0		
Arabitol	24.3		
Xylitol	29.9		
Mannitol	41.1	99	40.7
Galactitol	44.1		
Glucitol	45.9		
Inositol	50.5		

*Product was prepared as described in *methods*. Retention times (RT) are compared to hexaacetate derivatives of sugar alcohol standards. Chromatography was performed on a 0.002 x 1.83 m column of 0.2% polyethyleneglycol adipate, 0.2% polyethyleneglycol succinate, and 0.4% XF-115 silicone oil with a Perkin Elmer 900 gas chromatograph programmed to run from 130° to 180° at a rate of 1° per min.

by adding trichloroacetic acid so that the final concentration in the mixture was 0.2 M and the acidified solution was placed on ice for 30 min at which time the acid was neutralized by adding an appropriate amount of NaOH. Samples were removed and D-fructose-1-P and D-mannitol-1-P were determined by coupled assays as described in *methods*. The results of these determinations indicated that the D-fructose-1-P levels at equilibrium were too low to permit a very accurate determination; however, since 0.96 μmoles of D-mannitol-1-P was produced for every μmole of D-fructose-1-P present initially (as determined from the control mixture) the difference between these values (D-fructose-1-P minus D-mannitol-1-P) was taken to represent the lvel of D-fructose-1-P present at equilibrium. By using these values and the initial and final determinations of NADH concentration, it was determined that the ratios of NADH oxidized to D-fructose-1-P initially present to D-mannitol-1-P formed was 1.2:1.0:0.96. Any errors in these values may be the results of the manipulations performed on the sample after equilibrium was reached. The equilibrium constant (K_{eq}) for the reaction at pH 6.15 was determined from the equation:

$$K_{eq} = \frac{[\text{D-mannitol-1-P}]\ [NAD^+]}{[\text{D-fructose-1-P}]\ [NADH]\ [H^+]}$$

where the bracketed values are the concentration of the various species present at equilibrium. The K_{eq} calculated in this manner is 4.5×10^7 liters per mole.

The equilibrium for L-sorbose-1-P was determined in the same manner as for D-fructose-1-P except that the amount of L-sorbose-1-P

present at equilibrium could not be determined. The amount of L-sorbose-1-P added initially was determined as organically bound phosphate (using alkaline phosphatase as described for dephosphorylation of the reduction product of D-fructose-1-P). The ratio of NADH oxidized to L-sorbose-1-P initially present to the amount of D-glucitol-6-P formed (as determined in a coupled assay with D-glucitol-6-P dehydrogenase) was 1.2:1.0:0.93. The equilibrium constant at pH 6.15 was determined by substituting the appropriate equilibrium concentrations into the equation

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

The K_{eq} calculated in this manner is 2.6×10^7 liters per mole. The equilibrium constants for the reduction of D-fructose-1-P and L-sorbose-1-P indicate that the reaction catalyzed by the reductase under the conditions used lies approximately 95% in the direction of the formation of the sugar alcohol phosphate products.

Discussion and Summary

L-Sorbose-1-P reductase was purified 41-fold by DEAE-cellulose chromatography and by affinity chromatography using selective elution with combinations of cofactor and D-fructose-1-P. Acrylamide gel electrophoresis indicated that the purified enzyme contained no more than 1% contamination by other proteins.

An examination of the physical properties of the purified enzyme revealed that the native enzyme has a molecular weight of 90,000 (by ultracentrifugation and gel filtration) and consists of two subunits of identical molecular weight, i.e., 45,000 (by

electrophoresis on native and SDS acrylamide gels). Although native gels are normally used to determine the molecular weight of native enzymes, the conditions under which my determination was performed (pH 8.8 in Tris-glycine buffer) appeared to cause the reductase to dissociate into its subunits. This dissociation allowed the conclusion that the subunits were not only molecular weight-isomers but also charge-isomers (35), thereby providing further evidence that the subunits are identical monomers. The value of the Svedberg coefficient (determined by sucrose gradient centrifugation) was 5.54×10^{13} sec and is similar to those values obtained for other proteins possessing a molecular weight of around 90,000 (54).

Several sets of data indicate that the L-sorbose-1-P reductase is an acidic protein: (i) the amino acid analysis reveals that the enzyme has relatively low amounts of basic amino acids (Table 5); (ii) isoelectric focusing shows that the isoelectric point is 5.0 (Figure 23); (iii) the enzyme precipitates in acidic solutions only when the pH is lowered to below 5.0; (iv) the reductase migrates towards the anode during electrophoresis at pH 6.2 (Figure 12); and (v) the enzyme will bind to DEAE-cellulose at pH 6.15.

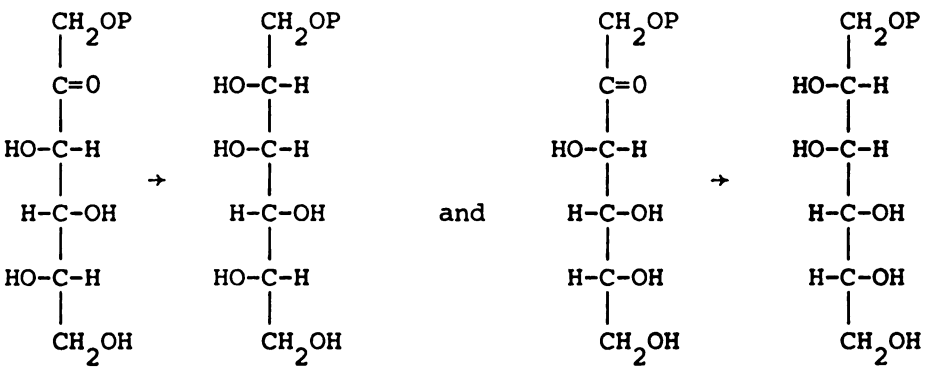
The pH optimum is 6.2 in the three buffers tested, and the highest activity was obtained in MES buffer. The enzyme requires a divalent metal cation for catalytic activity. Treating L-sorbose-1-P reductase with EDTA abolishes the ability of the enzyme to reduce L-sorbose-1-P and D-fructose-1-P, but the activity can be restored by the addition of an appropriate divalent metal ion. EDTA-treated enzyme will bind to NADP-sepharose but divalent metal

ion (Mn^{+2}) is required for the enzyme to bind more tightly in the presence of D-fructose-1-P. Manganese appears to be the metal normally associated with the enzyme since the presence of this metal restores the enzyme to the same levels of activity possessed prior to treatment with EDTA. The best reactivation of enzyme occurs when the metal ion is preincubated in the presence of either D-fructose-1-P without enzyme present, or enzyme without D-fructose-1-P present (Table 3). In the instances in which the sugar phosphate and enzyme are allowed to form complexes either before or at the same time as the metal ions are added, the activation of the enzyme is not as high. The aforementioned results and observations are consistent with a model in which the metal ion binds to the enzyme to facilitate the binding of the sugar phosphate in a complex with the correct conformation to allow catalysis to occur. The exact nature of the interactions involved remains to be determined but several types of enzyme--metal ion--substrate interactions have been discussed by Dwyer (55).

L-Sorbose-1-P reductase is capable of catalyzing the reduction of either L-sorbose-1-P or D-fructose-1-P with either NADH or NADPH to form D-glucitol-6-P or D-mannitol-1-P, respectively. The Michaelis constants for either ketohexose-1-P are similar (Figure 21) but the maximal velocity for the reduction of L-sorbose-1-P is three times that for the reduction of D-fructose-1-P. The Michaelis constant and maximal velocity of the reaction are nearly identical for either NADH or NADPH and agree with the concentration of NADH required to elute the enzyme from an NADP-sepharose

affinity column. The product of the reduction of D-fructose-1-P was identified as D-mannitol-1-P on the basis of chromatographic and enzymatic analysis. The equilibrium of the reduction of L-sorbose-1-P and D-fructose-1-P lies far in the direction of the formation of the sugar alcohol phosphate products (K_{eq} = 2.6 x 10^7 and 4.5 x 10^7 liters/mole, respectively), and the stoichiometry of the reaction shows that one molecule of ketohexose-1-P is reduced by one molecule of NAD(P)H for every molecule of hexitol phosphate and $NAD(P)^+$ formed.

Studies of the substrate specificity reveal that L-sorbose, D-fructose, D-fructose-6-P, and D-fructose-1,6-P_2 will not serve as substrates for the enzyme. This specificity plus the fact that the only two reactions that I was able to demonstrate to be catalyzed by L-sorbose-1-P reductase were



suggests that the specificity of the enzyme is for those ketohexose 1-phosphates which possess structures which are the same as L-sorbose-1-P at C-1 through C-4. The reduction involves the formation of a hydroxyl group at the second carbon atom to make an

erythro structure for the hexitol phosphate at the carbon atoms which correspond to C2 and C3 of the ketohexose-1-P.

L-Sorbose-1-P reductase from *A. aerogenes* is the first ketohexose-1-P reductase to be reported for any organism. Since the enzyme is induced in this organism only by growth on L-sorbose, its main role is in the metabolism of L-sorbose (1). Although the enzyme is capable of reducing D-fructose-1-P to D-mannitol-1-P, it normally serves no function in the metabolism of D-fructose: the enzyme is not present when the wild-type strain is grown on D-fructose and D-fructose has been shown to be metabolized almost exclusively via two alternative pathways in the wild-type organism (76). However, the presence of D-mannitol-1-P dehydrogenase in cells uninduced by growth on D-mannitol coupled with the ability of L-sorbose-1-P reductase to convert D-fructose-1-P to D-mannitol-1-P suggested that under certain conditions, D-fructose might be metabolized in *A. aerogenes* by a novel pathway in which L-sorbose-1-P would play a key role. The evolution of such a pathway for the metabolism of D-fructose in *A. aerogenes* will be demonstrated in Part III of this dissertation.

PART III

EVOLUTION OF A NOVEL PATHWAY FOR THE METABOLISM OF D-FRUCTOSE BY *AEROBACTER AEROGENES*

Introduction

There are several routes by which D-fructose is known to be metabolized in various organisms. The classical studies were done with yeast, for which it was shown that D-fructose is phosphorylated with ATP by hexokinase to yield D-fructose-6-P (56).

In mammals the main site of D-fructose metabolism is the liver, wherein: (i) D-fructose is phosphorylated to D-fructose-1-P by an ATP-dependent 1-fructokinase; (ii) D-fructose-1-P is cleaved by an aldolase into dihydroxyacetone phosphate and D-glyceraldehyde; and (iii) D-glyceraldehyde is phosphorylated to D-glyceraldehyde-3-P by an ATP-dependent triokinase (57).

In certain pseudomonads, such as *Gluconobacter cerinus*, carbohydrates are metabolized almost exclusively via the hexose monophosphate pathway (58). It has been established that D-fructose also plays a key role in the regeneration of the $NADP^+$ that is reduced during the oxidation of D-glucose-6-P when *G. cerinus* is grown on D-fructose (59,60). D-Fructose is oxidized by a particulate, respiratory-chain-linked fructose 5-dehydrogenase to yield 5-keto-D-fructose (61), which is then reduced by an NADPH-dependent 5-keto-D-fructose reductase (14), thereby regenerating both D-fructose and $NADP^+$.

Another major pathway for the metabolism of exogenously supplied D-fructose in microorganisms was first elucidated in *A. aerogenes* by Anderson and co-workers (62-65). It was shown that: (i) D-fructose is simultaneously transported and phosphorylated by an inducible P-enolpyruvate-dependent phosphotransferase system;

(ii) the phosphorylation is at carbon atom 1 of D-fructose; and (iii) D-fructose-1-P is then phosphorylated with ATP by a specific, inducible kinase to form D-fructose-1,6-P_2. This pathway was subsequently found in *Escherichia coli* (66-68), *Clostridium pasteuranium* (69-71), *C. roseum* (71), *C. rubrum* (71), *C. butyricum* (71), *C. thermocellum* (72), *Arthrobacter pyridinolis* (73), and *Rhodopseudomonas capsulata* and other species of phototrophic bacteria (74). One of the enzymes, D-fructose-1-P kinase, has been reported to occur in *Bacteroides symbiosis* (75) and in *Clostridium formicoaceticum* (71).

Endogenous D-fructose is utilized in *A. aerogenes* by a different route. Sucrose is transported into the cell as the free sugar, where a hydrolase cleaves the disaccharide into its component monosaccharides, D-fructose and D-glucose. This intracellular D-fructose undergoes an ATP-dependent phosphorylation at carbon atom 6 by means of a sucrose-induced, specific D-fructokinase (76). D-Fructokinase activity is also present in very low amounts in cells grown on carbohydrates other than sucrose, but even these low amounts are sufficient for mutants missing D-fructose-1-P kinase to grow slowly on D-fructose.

This section of my dissertation will describe the evolution of a novel pathway for the metabolism of D-fructose. Previous work on the evolution of new pathways in microorganisms has been concerned almost exclusively with the metabolism of "xenobiotics", i.e., compounds normally not found in the natural environment of the cell (77,78). Acquisition of the ability to grow on xenobiotics

has been found to occur by mutation in one of three ways: (i) constitutive production of enzymes for which the novel compounds normally serve as fortuitous substrates, but which are normally induced only by natural substrates; (ii) mutation in a structural gene which allows the new enzyme to also use the novel compound as a substrate; or (iii) formation of a new enzyme which has no known function other than to facilitate the metabolism of the novel compound. The discovery (part II of this dissertation) that L-sorbose-1-P reductase would also reduce D-fructose-1-P to D-mannitol-1-P suggested the possibility of genetically manipulating *A. aerogenes* to allow it to utilize D-fructose for growth by a totally novel pathway. This would be the first report of the evolution of a new metabolic pathway for a compound which is commonly found in the milieu of the cell.

It was known that D-mannitol-1-P dehydrogenase is present in relatively high levels even in cells grown on substrates other than D-mannitol. By using a double mutant (strain 14) missing both D-fructose-1-P kinase and D-fructokinase, and which is consequently unable to grow on D-fructose, it was possible to select for a mutant which was constitutive for L-sorbose-1-P reductase. This mutant is able to utilize D-fructose as a sole source of carbon and energy by conscripting enzymes from three normally separate metabolic pathways. D-Fructose is transported and converted to D-fructose-1-P by the PEP:D-fructose phosphotransferase system of the normal D-fructose pathway. The D-fructose-1-P is then reduced at the keto group by L-sorbose-1-P reductase. The product,

D-mannitol-1-P, is then oxidized at carbon atom 5 by D-mannitol-1-P dehydrogenase from the D-mannitol pathway. The bacterium is then able to metabolize the resulting D-fructose-6-P via the same pathways as in the wild-type strain.

Materials and Methods

Isolation of Mutants--General Procedures

The phenotype and genotype of the parental and mutant strains used or developed in this study are shown in Table 7. Parental strains were inoculated into culture tubes containing 7 ml of nutrient broth and grown overnight at 30°. The fully grown cultures were transferred to a sterile 12.5-ml centrifuge tube and the cells were washed by alternatively centrifuging for 5 min at 12,000 x g and suspending the pelleted cells in 7 ml of sterile mineral broth. After three such washes a 2-ml sample of suspended cells was transferred to a sterile 5-ml test tube and 30 µl of ethyl methane sulfonate was added. The tube was incubated with shaking for 2 hr at 30°. The mutagenized sample was transferred to a sterile 12.5-ml centrifuge tube, 7 ml of sterile mineral broth was added, and the tube was centrifuged for 5 min at 12,000 x g. The cells were washed as described above and the final suspension in mineral broth was transferred to a sterile culture tube containing 0.2 ml of a 17.5% sterile carbohydrate solution. After the cells were allowed to grow for four generations (one generation was considered to be one doubling in absorbance at 520 nm), a 1-ml portion was transferred to a fresh tube of 0.5% carbohydrate in

Table 7. Genotype and phenotype of mutants used in this study

Strain	Parent	Genotype[†]	Phenotype[††]	Reference
PRL-R3	---	Wild-type	Fru^{+}_{+}, Mtl^{+}, Sor^{+}, Scs^{+}	
DD31	PRL-R3	fru-31	Fru^{s}_{+}, Mtl^{+}, Sor^{+}, Scs^{+} (FlPK-negative)	76
14	DD31	fru-31, scs-14	Fru^{-}_{+}, Mtl^{+}, Sor^{+}, Scs^{+} (FlPK-neg.; FK-neg.)	76
SF1	14	fru-31, scs-14, sor-1	Fru^{+}_{+}, Mtl^{+}, Sor^{+}, Scs^{+} (FlPK-neg.; FK-neg.; SR-constructive)	this dissertation
SF1-S5	SF1	fru-31, scs-14, sor-1, sor-5	Fru^{-}, Mtl^{+}, Sor^{-}, Scs^{s} (FlPK-neg.; FK-neg.; SR-neg.)	this dissertation
SF1-S5R	SF1-S5	Revertant to SF1	Fru^{+}_{+}, Mtl^{+}, Sor^{+}, Scs^{+} (same as SF1)	this dissertation
SF1-MA2	SF1	fru-31, scs-14, sor-1, mtl-2	Fru^{-}, Mtl^{-}, Sor^{+}, Scs^{s} (FlPK-neg.; FK-neg.; SR-const.; MlPDH-neg.)	this dissertation
SF1-MA2R	SF1-MA2	Revertant to SF1	Fru^{+}_{+}, Mtl^{+}, Sor^{+}, Scs^{+} (same as SF1)	this dissertation
SF1-MC1	SF1	fru-31, scs-14, sor-1, mtl-1	Fru^{+}_{+}, Mtl^{+}, Sor^{+}, Scs^{+} (FlPK-neg.; FK-neg.; SR-const.; MlPDH-2 to 3 times higher)	this dissertation

[†]Fru = D-fructose; Mtl = D-mannitol; Sor = L-sorbose; Scs = sucrose.

[††]+ = able to grow; - = unable to grow; s = able to grow slowly; FlPK = D-fructose-1-P kinase; FK = D-fructokinase; SR = L-sorbose-1-P reductase; MlPDH = D-mannitol-1-P dehydrogenase.

mineral broth and allowed to grow for three generations. This transfer was repeated once more, the culture was allowed to grow fully, and was selected for the appropriate phenotype as described in the Results section.

Analytical Procedures

Enzyme assays. All assays were performed in a total volume of 0.150 ml and the rates monitored by change in absorbance at 340 nm on a Gilford multiple-sample absorbance-recording spectrophotometer thermostated at 30°. The assay for L-sorbose-1-P reductase has been described in part II of this dissertation. The assay for D-mannitol-1-P dehydrogenase contained 0.067 M Tris-HCl buffer (pH 9.0), 3.3 mM D-mannitol-1-P, 0.33 mM NAD^+ (adjusted to pH 7.0 with NaOH), 0.33 mM $NADP^+$ (adjusted to pH 7.0 with NaOH), and nonlimiting amounts of phosphoglucoisomerase and D-glucose-6-P dehydrogenase. Controls were run with D-mannitol-1-P omitted. Rates were constant for the initial 2 min of the assay. The assay for D-fructose-1-P kinase contained 0.067 M glycylglycine buffer (pH 7.5), 3.3 mM D-fructose-1-P (sodium salt), 3.3 mM ATP, 6.6 mM $MgCl_2$, 67 mM KCl, 0.33 mM NADH, and nonlimiting amounts of rabbit muscle aldolase and α-glycerophosphate dehydrogenase-triose phosphate isomerase. Controls were run with ATP omitted. The assay for D-fructokinase contained 0.067 M glycylglycine buffer (pH 7.5), 3.3 mM D-fructose, 3.3 mM ATP, 6.6 mM $MgCl_2$, 0.33 mM $NADP^+$ (adjusted to pH 7.0 with NaOH), and nonlimiting amounts of phosphoglucoisomerase and D-glucose-6-P dehydrogenase. Controls were run with ATP omitted.

Determination of intracellular metabolite levels in mutant strains. Each strain was grown overnight in 100 ml of nutrient broth at 30°. Cells were washed by alternately centrifuging and suspending in 14 ml of sterile mineral broth a total of three times. Cells were suspended finally in 50 ml of sterile mineral broth and this was added to 75 ml of mineral broth and D-fructose so that there was a final concentration of 0.5% D-fructose in 100 ml of mineral broth. Cells were incubated for 3 hr at 30° after which they were washed as before with mineral broth. The pellet containing the cells from the final centrifugation was weighed and then suspended in 1 ml of mineral broth. D-Fructose (80 µmoles) was added to this suspension and the cells incubated for 10 min at room temperature. The incubation was terminated by the addition of 0.1 ml of 70% perchloric acid and mixing periodically for 5 min at room temperature. The tubes were placed on ice for 20 min and the solution then neutralized with approximately 0.5 ml of 2.5 M potassium bicarbonate. Precipitated protein and cellular debris were removed by centrifugation at 12,000 x g for 10 min. The supernatant was removed and the pH adjusted with $KHCO_3$ to neutrality if needed. A control to determine the extent of leakage of metabolites from the cells during incubation and subsequent manipulations was performed. A sample of cells incubated for three hr in the presence of 0.5% D-fructose was separated into two portions and incubated with 80 µmoles of D-fructose, as described above, for 5 min at room temperature. The two portions were centrifuged at 12,000 x g at 22°. The samples were then divided as

follows: one sample was divided into supernatant and pellet; the other sample was treated *in toto*. The three resulting fractions were treated with perchloric acid and potassium bicarbonate as described above. After assaying for the metabolite levels as described below, there were no detectable metabolites in the supernatant fraction and the metabolites present in the cells or the cells plus the supernatant were the same.

IMViC tests. The IMViC tests were performed according to standard procedures (80,81) and modified as described below.

For the *indole test*, cultures of *E. coli* and *A. aerogenes* strains were grown at 37 and 30 degrees, respectively, in culture tubes containing 7 ml of 1% tryptone broth. Inocula were allowed to grow for 24 hr without shaking at which time 0.2-0.3 ml of reagent were added. The reagent was composed of 5 gm p-dimethylaminobenzaldehyde in 75% n-amyl alcohol-25% concentrated HCl. The test was considered to be positive if a deep purple color formed at the interface between the medium and the air.

The *methyl red* and *Voges-Proskauer* tests were performed on cultures grown on mineral broth supplemented with 0.5% D-glucose. After 24 and 48 hr 1-ml samples were removed and tested for the accumulation of acetoin (3-hydroxy-2-butanone) in the medium. The Voges-Proskauer test for acetoin consists of adding to the 1-ml sample, 0.6 ml of 5% α-naphthol in absolute ethanol followed by 0.2 ml of 0.3% creatine in 40% KOH. After mixing a red color developing within a few minutes constitutes a positive test. The same cultures, allowed to incubate without shaking for 5 days, were

used for the methyl red test for acid production. To the cultures 5 drops of 0.3% methyl red in 50% ethanol were added. A red color persisting for several hours was considered a positive test.

Results

Linear Growth of Strain 14 on D-Fructose after Induction with D-Mannitol and L-Sorbose

The feasibility of genetically producing an organism with the proposed pathway for D-fructose metabolism (see Introduction to part III of this dissertation) was examined in a mutant of *A. aerogenes* in which both the normal pathways for the metabolism of D-fructose were genetically blocked. The levels of L-sorbose-1-P reductase and/or D-mannitol-1-P dehydrogenase were temporarily increased in this mutant (strain 14) by growth on L-sorbose, D-mannitol, or a mixture of both. Figure 24A shows that merely increasing the level of D-mannitol-1-P dehydrogenase by growth on D-mannitol alone is insufficient to allow strain 14 to grow on 0.3% D-fructose in mineral broth. When strain 14 is grown on L-sorbose, the L-sorbose-1-P reductase which is induced is sufficient to allow limited linear (not logarithmic) growth on D-fructose (Figure 24B). Cells in which both the level of L-sorbose-1-P reductase and D-mannitol-1-P dehydrogenase have been temporarily increased by growth on L-sorbose and D-mannitol (Figure 24C) grow 7.5 times as fast as those with elevated levels of L-sorbose-1-P reductase alone. Such results suggest in these studies that the level of D-mannitol-1-P dehydrogenase is the limiting factor in the metabolism of D-fructose by the new pathway. The linear growth

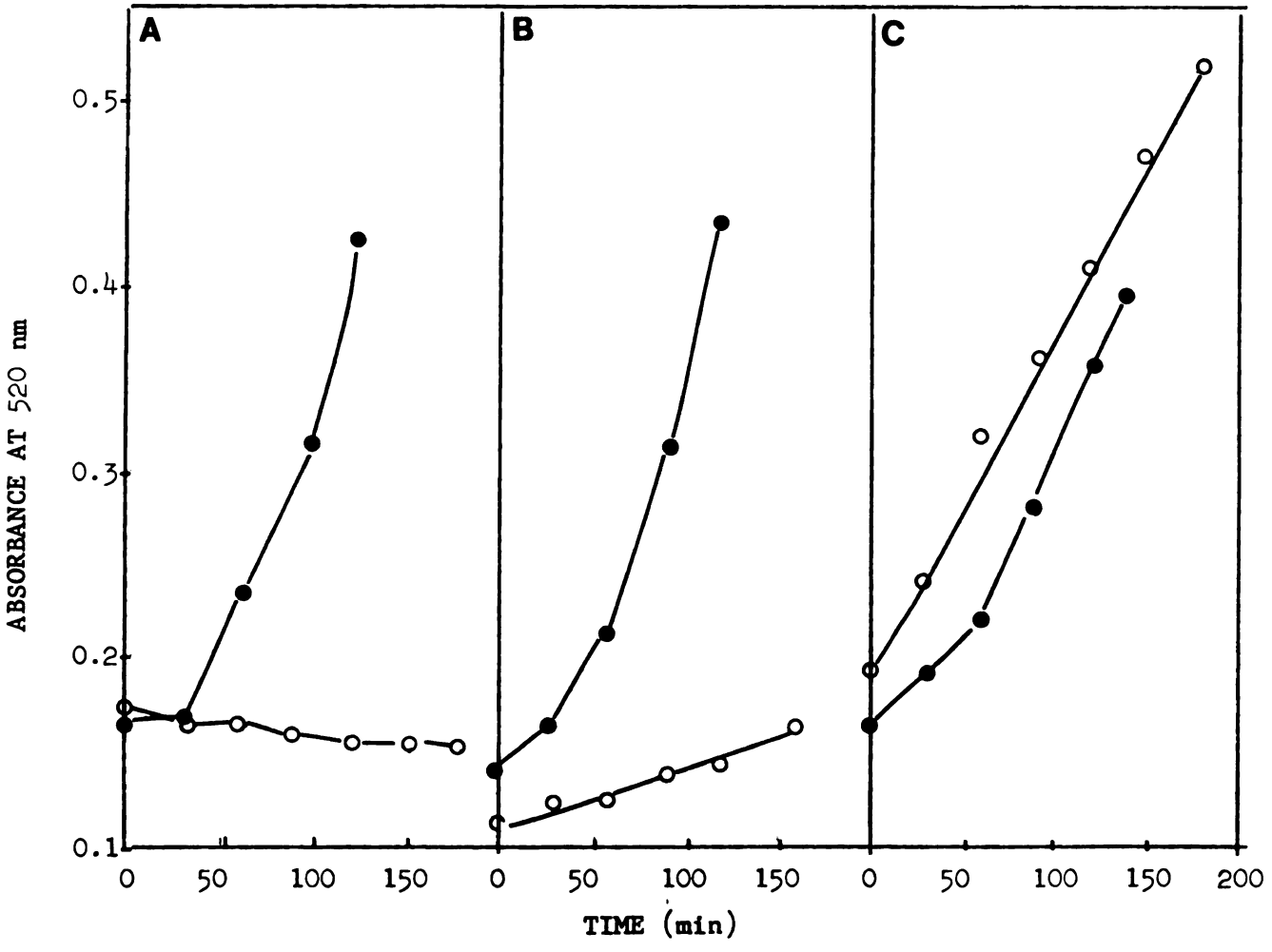


Figure 24 - Growth of strain 14 on D-glucose (●) and D-fructose (○) after preinduction by growth on D-mannitol and L-sorbose. Growth was determined on 0.3% of each sugar. A : Preinduction on 0.3% D-mannitol; B : Preinduction on 0.3% L-sorbose; C : Preinduction on 0.15% D-mannitol plus 0.15% L-sorbose.

rates evinced in Figure 24 are those expected in the case of a constant amount of enzyme being present in the whole inoculum of cells. The logarithmic growth curves shown by the cells grown on D-glucose are those expected when each cell synthesizes new enzyme as it grows.

Selection of Mutants of L-Sorbose-1-P Reductase and D-Mannitol-1-P Dehydrogenase

To develop and elucidate the new pathway of D-fructose metabolism, mutants with altered levels and/or regulation of L-sorbose-1-P reductase and D-mannitol-1-P dehydrogenase were selected as described below. It was unnecessary in this study to select mutants in the PEP:D-fructose 1-phosphotransferase system since its role in the transport and phosphorylation of D-fructose in *A. aerogenes* had already been established (63,65). The genealogy and description of the mutants used in this study was summarized in Table 7.

Mutants constitutive for L-sorbose-1-P reductase. Strain 14, a double mutant missing both D-fructose-1-P kinase and D-fructokinase (Table 7), was mutagenized as described in *methods* and grown on 0.5% L-sorbose as the carbohydrate. One milliliter of the fully grown culture was transferred to a culture tube containing 7 ml of mineral broth with 16.7 mM (0.3%) L-sorbose and allowed to grow for three generations. A 1-ml sample was inoculated into a culture tube containing 7 ml of mineral broth with 16.7 mM (0.3%) D-glucose. After growth proceeded for 3 to 4 generations, a 0.2-ml sample was transferred to a culture tube containing 16.7 mM L-sorbose in mineral

broth and was incubated at 30° for 3 to 4 generations. Sterile D-glucose was added to a final concentration of 27 mM (0.5%) and the cells were allowed to grow at 30° overnight. This alternate transfer between 16.7 mM L-sorbose and 27 mM D-glucose was considered to be one cycle of enrichment. After 31 cycles, samples were removed, serially diluted, and plated on MacConkey agar supplemented with 1.0% D-fructose. Mutants were selected as red colonies appearing after incubation of the plates at 30° for 24 to 36 hr. Individual colonies were selected and transferred to a culture tube containing 7 ml of nutrient broth. After cultures were fully grown, crude extracts were prepared as described in part II, *methods*, and assayed for L-sorbose-1-P reductase and D-fructose-1-P kinase as described in *methods*. Mutants obtained in this manner either possessed very low constitutive levels of the reductase (0.007 to 0.010 units per mg protein in crude extracts) or were revertants possessing D-fructose-1-P kinase. Unexpectedly, the mutants with low constitutive levels of reductase were found *not* to grow on mineral broth supplemented with 0.3% D-fructose. At this point I modified both the enrichment and selection procedures. Mutagenized cells of strain 14 were cycled between 0.1 mM (0.018%) L-sorbose and the same concentration (16.7 mM) of D-glucose. By recycling between 0.1 mM L-sorbose and 16.7 mM D-glucose I reasoned that there would be a doubly selective advantage. As in the former enrichment procedure, cycling between L-sorbose and D-glucose would enrich for those mutants with a constitutive reductase because they would not require the normal two hour lag period to induce the

reductase and, hence, would start growing before the wild-type cells. By lowering the L-sorbose concentration I thought that those mutants with higher constitutive levels of the reductase would have a selective advantage because they would grow at the normal induced rate initially and thereby deplete the medium of the limited amount of L-sorbose before mutants with lower levels of constitutive reductase could be induced to the normal levels of the enzyme for faster growth. This method would allow the enrichment of the higher constitutive mutants at a faster rate than the old method. The selection procedure was now modified because of the necessity to distinguish between those mutants able to metabolize D-fructose sufficiently to appear as red colonies on MacConkey agar and those mutants truly able to utilize D-fructose as a sole source of carbon and energy. To select for the latter mutants, samples from various cycles of enrichment were plated onto mineral agar supplemented with 1.0% D-fructose. With the modified procedure one would predict that the number of mutants with increased levels of reductase, appearing as red colonies on MacConkey agar supplemented with 1.0% D-fructose, would increase with the number of cycles. At the same time those mutants with the higher levels of constitutive reductase (and according to my theory able to utilize D-fructose as a sole source of carbon and energy) should also increase, but at a rate greater than the increase in constitutive mutants in general. This prediction was substantiated as shown in Figure 25. Note that the total number of constitutive reductase mutants is initially greater than those with constitutive levels high enough to permit growth on

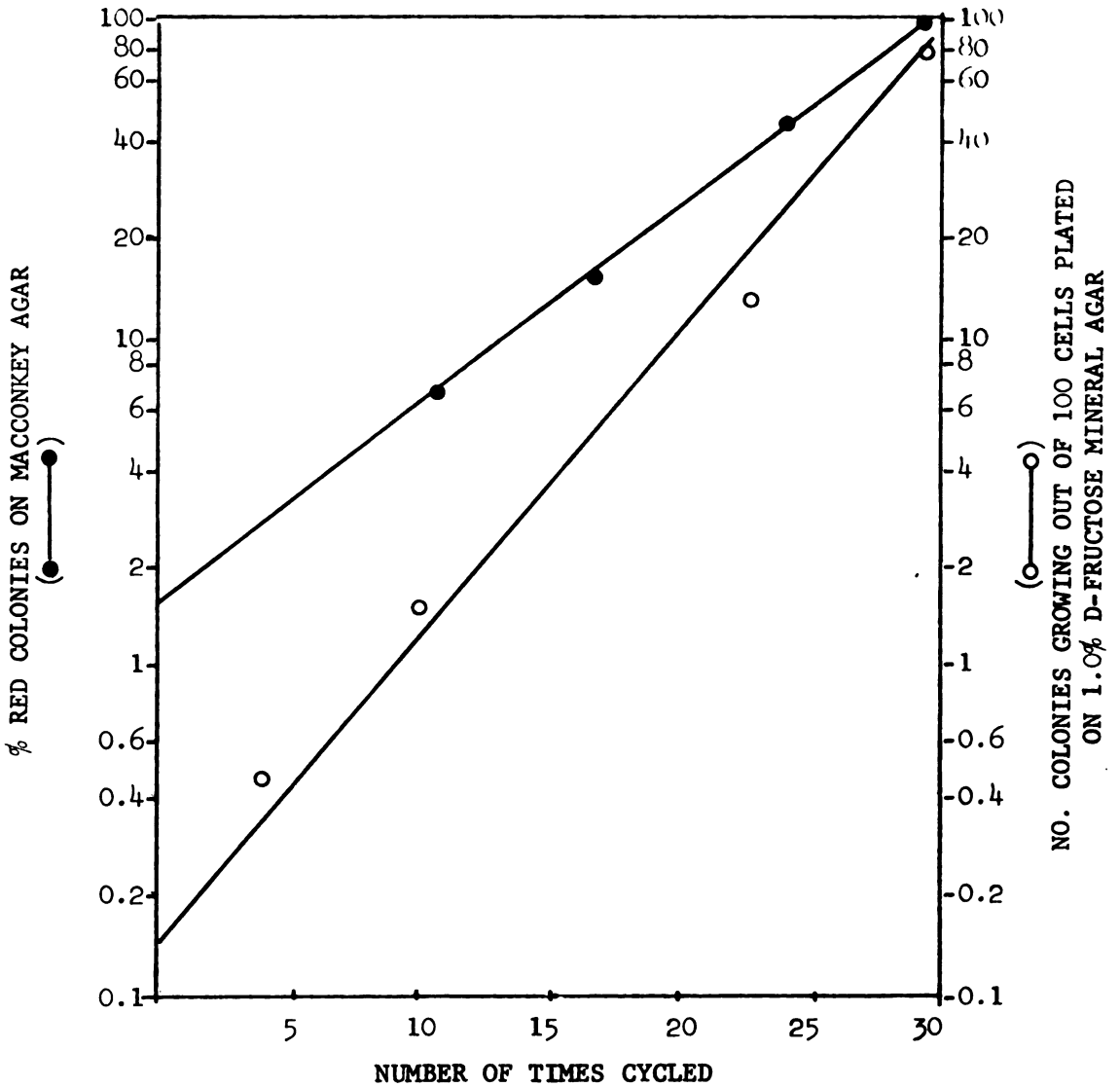


Figure 25 - Ability of mutants of A. aerogenes to utilize D-fructose as a function of enrichment for constitutivity of growth on L-sorbose. Mutants were cycled on 0.1 mM L-sorbose and 16.7 mM D-glucose as described in the text.

D-fructose alone. By the end of 30 cycles 90% of the constitutive mutants are those which can utilize D-fructose as a sole source of carbon and energy. Controls which were recycled on 0.1 mM D-mannitol and 16.7 mM D-glucose showed no increase in the number of mutants able to grow on D-fructose and those that did at no time exceeded 0.8%. SF1 was selected as a mutant growing on mineral agar supplemented with 1.0% D-fructose as was used in further characterization studies.

Mutants negative for L-sorbose-1-P reductase. SF1 was mutagenized and grown on D-glucose as the carbohydrate as described in *methods*. Mutants missing either D-mannitol-1-P dehydrogenase or Enzyme I or HPr of the P-enolpyruvate-dependent phosphotransferase system were removed by growth overnight on 0.5% D-mannitol in mineral broth. Cells fully grown on D-mannitol were washed as described in *methods* and suspended finally in 2 ml of mineral broth. Samples of this suspension were added to a culture tube containing mineral broth so that the initial absorbance at 520 nm was 0.075 when determined in a 1.8-cm culture tube with a Model 6A Coleman Jr. spectrophotometer. A sterile solution of 17.5% L-sorbose was added to this culture and the cells grown at 30° until the absorbance at 520 nm had reached 0.30, at which point 8 mg of penicillin G (benzylpenicillin) was added. The tube was vortexed and the incubation continued for 4 hr, at which time the absorbance at 520 nm was 0.075. Cells were washed by alternate centrifugation and suspension in mineral broth three times. A sterile 17.5% solution (0.2 ml) of D-mannitol was added to the

final mineral broth suspension and the cells allowed to grow at 30° overnight. The fully grown culture was serially diluted and 10^2 cells plated on MacConkey agar supplemented with 1.0% L-sorbose. After incubation for 24 hr at 30°, 28 pale colonies were selected, inoculated into culture tubes containing 7 ml of nutrient broth, grown overnight at 30°, and streaked on MacConkey agar plates containing 1.0% D-glucose, D-mannitol, L-sorbose, or D-fructose. Eighteen of these mutants grew as pale colonies on L-sorbose and D-fructose and as red colonies on D-mannitol and D-glucose. Four of these, SF1-S5, SF1-S10, SF1-S15, and SF1-S20, were negative for L-sorbose-1-P reductase when crude extracts were tested. SF1-S5 was selected for further use in the study.

Mutants negative for D-mannitol-1-P dehydrogenase. Selection procedures paralleled those of the previous section except that D-mannitol and L-sorbose in the media were interchanged. However, mutants which were unable to metabolize D-fructose and D-mannitol were also unable to use L-sorbose or D-glucose, appearing as pale colonies on MacConkey agar containing any of these four sugars. The procedure was then modified in the following manner: instead of just penicillin G, penicillin G plus 0.1 M cycloserine (79) was added to growing cultures on 0.5% D-mannitol and the incubation continued for 6 hr, at which point the final absorbance at 520 nm was 0.070. Cells were washed as described in *methods*, and colonies appearing pale on MacConkey agar supplemented with 1.0% D-mannitol were selected. All such mutants appeared as pale colonies on MacConkey agar supplemented with 1.0% of either D-fructose,

D-glucose, or L-sorbose, indicating that the mutants possessed a mutation or mutations affecting the general growth characteristics of the cell. Since it was possible that D-mannitol might cause growth inhibition of cells unable to utilize D-mannitol, mutants from the penicillin-cycloserine procedure were plated onto mineral agar containing 0.5% D-mannitol and 0.005% D-glucose. After incubation for 24 hr at 30° pinpoint-size colonies were selected, grown on nutrient broth, and plated on MacConkey agar containing 1.0% D-glucose, L-sorbose, D-fructose, or D-mannitol. Five mutants appeared as pale colonies on D-mannitol or D-fructose and red on D-glucose or L-sorbose. These mutants were grown on nutrient broth and crude extracts were prepared and assayed for the presence of D-mannitol-1-P dehydrogenase. SF1-MA2 has the lowest level of dehydrogenase and was selected for further characterization.

Revertants of SF1-S5 and SF1-MA2. Revertants were selected to show that SF1-S5 and SF1-MA2 were the result of single point mutations. After several days of incubation in 0.5% L-sorbose mineral broth and 0.5% D-mannitol mineral broth, respectively, growth appeared in the cultures. Samples were taken, serially diluted, and plated on MacConkey agar supplemented with 1.0% L-sorbose for SF1-S5 revertants or 1.0% D-mannitol for SF1-MA2 revertants. Colonies appearing red on these plates after 24 hr incubation at 30° were selected, grown on nutrient broth, and streaked on MacConkey plates containing D-fructose, L-sorbose, D-mannitol, or D-glucose. Revertants were selected as those that

gave red colonies on all four sugars. One mutant each (SF1-S5R and SF1-MA2R) was selected for further characterization.

Mutants with elevated levels of D-mannitol-1-P dehydrogenase. Mutants of SF1 were mutagenized and treated as described for the selection of L-sorbose constitutive mutants except that the cells were cycled with 0.1 mM D-mannitol substituting for 0.1 mM L-sorbose. After 30 cycles the culture was serially diluted and plated on mineral agar containing 1.0% D-fructose. After incubation for 24 hr at 30° the four largest colonies were selected for assay of D-mannitol-1-P dehydrogenase. SF1-MC1 had the highest levels of dehydrogenase present in crude extracts of mineral broth-grown cells.

Diagnostic Tests to Demonstrate the Common Ancestry of the Mutants

In all of these tests the *Escherichia coli* control was the strain B/r ara-2.

IMViC tests. The IMViC tests, which are used to differentiate between *Aerobacter aerogenes* and *Escherichia coli* (80,81), were deemed useful markers for confirming that the mutants were derivatives of *A. aerogenes* and not contaminants. When mutants of *A. aerogenes* and an *E. coli* control were subjected to these tests the data summarized in Table 8 were obtained. All of the *A. aerogenes* strains produced indole when grown on 1% tryptone broth, gave a negative methyl red test, and could utilize citrate as a sole source of carbon and energy. The Voges-Proskauer test for

Table 8. Results of IMViC tests on wild-type and mutant strains of *A. aerogenes* and on an *E. coli* control

Strain	Indol test[1]	Methyl red test[2]	Voges-Proskauer test[3]	Growth on citrate[4]
E. coli				
B/r ara-2	pos.	pos.	+	neg.
A. aerogenes				
PRL-R3	pos.	neg.	+++++	pos.
14	pos.	neg.	+++	pos.
SF1	pos.	neg.	++	pos.
SF1-S5	pos.	neg.	++	pos.
SF1-MA2	pos.	neg.	++	pos.
SF1-S5R	pos.	neg.	+	pos.
SF1-MA2R	pos.	neg.	+	pos.
SF1-MC1	pos.	neg.	++++	pos.

[1]pos. = deep purple color forming at interface of medium and air.

[2]pos. = red color persisting for several hours.

[3]+ = relative intensity of red color developing after several minutes.

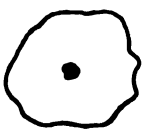
[4]pos. = extensive growth on 0.5% sodium citrate-mineral broth within 24 hr.
neg. = no detectable growth after 24 hr.

acetoin production was variable. The reason for the variability was not pursued, but it could be related to the rate of utilization of acetoin as well as to the rate of its formation. In light of the other data this was not considered to militate against the common ancestry of the strains.

Colony characteristics on eosin-methylene blue agar. When appropriate dilutions of *A. aerogenes* mutants and the *E. coli* control were plated on eosin-methylene blue agar, the colony characteristics described in Table 9 resulted. The *E. coli* colonies were distinct from those of the *A. aerogenes* strains. Although all of the colonies of the *A. aerogenes* mutants were large and slightly irregular in shape, the colonies formed by strains 14, SF1-S5, and SF1-MA2 lacked the dark center which was apparent in the other mutants and the wild-type strain. This may be explained by the fact that current commercial preparations of eosin-methylene blue agar (Difco) contain 0.5% sucrose. The three strains of different morphology grew slowly on sucrose (Table 7), probably due to inhibition of growth by an accumultion of intracellular D-fructose. This same inhibition could have affected the acid production necessary for the formation of the metallic green product (80).

Lysis by a bacteriophage specific for *A. aerogenes*. The various mutant strains of *A. aerogenes* and an *E. coli* control were exposed to infection by a bacteriophage discovered in our laboratory (Hanson, T. E., Ph.D. dissertation, 1969, Michigan State University, p. 327). This phage lyses *A. aerogenes* PRL-R3 but will

Table 9. Colony characteristics of wild-type and mutant strains of *A. aerogenes* and an *E. coli* control on eosin-methylene blue agar plates

Strain	Colony characteristics	Appearance
E. coli		
B/r ara-2	Small dark colonies appearing with a green metallic sheen when viewed by reflected light	
A. aerogenes		
PRL-R3	Large pale colonies, irregular with small dark centers appearing with a green metallic sheen when viewed by reflected light	
14	Large pale colonies, irregular with light concave center and no metallic green sheen	
SF1	Same as PRL-R3	Same as PRL-R3
SF1-S5	Same as 14	Same as 14
SF1-MA2	Same as 14	Same as 14
SF1-S5R	Same as PRL-R3	Same as PRL-R3
SF1-MA2R	Same as PRL-R3	Same as PRL-R3
SF1-MC1	Same as PRL-R3	Same as PRL-R3

not lyse various strains of *E. coli*, *Pseudomonas*, or other common bacteria which might be contaminants in our laboratory. Figure 26 shows that all mutant strains used in this study were lysed when the bacteriophage was added to rapidly growing cultures in nutrient broth. The lysis was similar to that observed with the wild-type parent, PRL-R3, whereas *E. coli* control was unaffected by addition of the phage.

Additional evidence that all mutants developed for this study had a common ancestry is provided by growth patterns on various carbohydrates discussed below.

Growth Response on Carbohydrates

Growth response on carbohydrates other than D-fructose. All the mutant strains of *A. aerogenes* grow on mineral broth supplemented with 0.5% each of D-glucose, D-galactose, D-mannose, L-rhamnose, D-ribose, L-arabinose, D-xylose, maltose, melibiose, cellobiose, or glycerol. All strains grow slowly on lactose. Growth response on D-mannitol and L-sorbose is summarized in Table 7. Only the mutants missing enzymes in the pathways for D-mannitol or L-sorbose metabolism fail to grow on D-mannitol or L-sorbose: SF1-S5, the L-sorbose-1-P reductase-negative mutant, is unable to utilize L-sorbose for growth and SF1-MA2, the mutant with very low levels of D-mannitol-1-P dehydrogenase, will not grow on mineral broth supplemented with D-mannitol.

Figure 26. Lysis of wild-type and mutant strains of *A. aerogenes* by a bacteriophage. Phage was added at 0 min to actively growing cultures of *A. aerogenes* and *E. coli* in nutrient broth. *Escherichia coli* was grown at 37°; all other strains were grown at 30°.

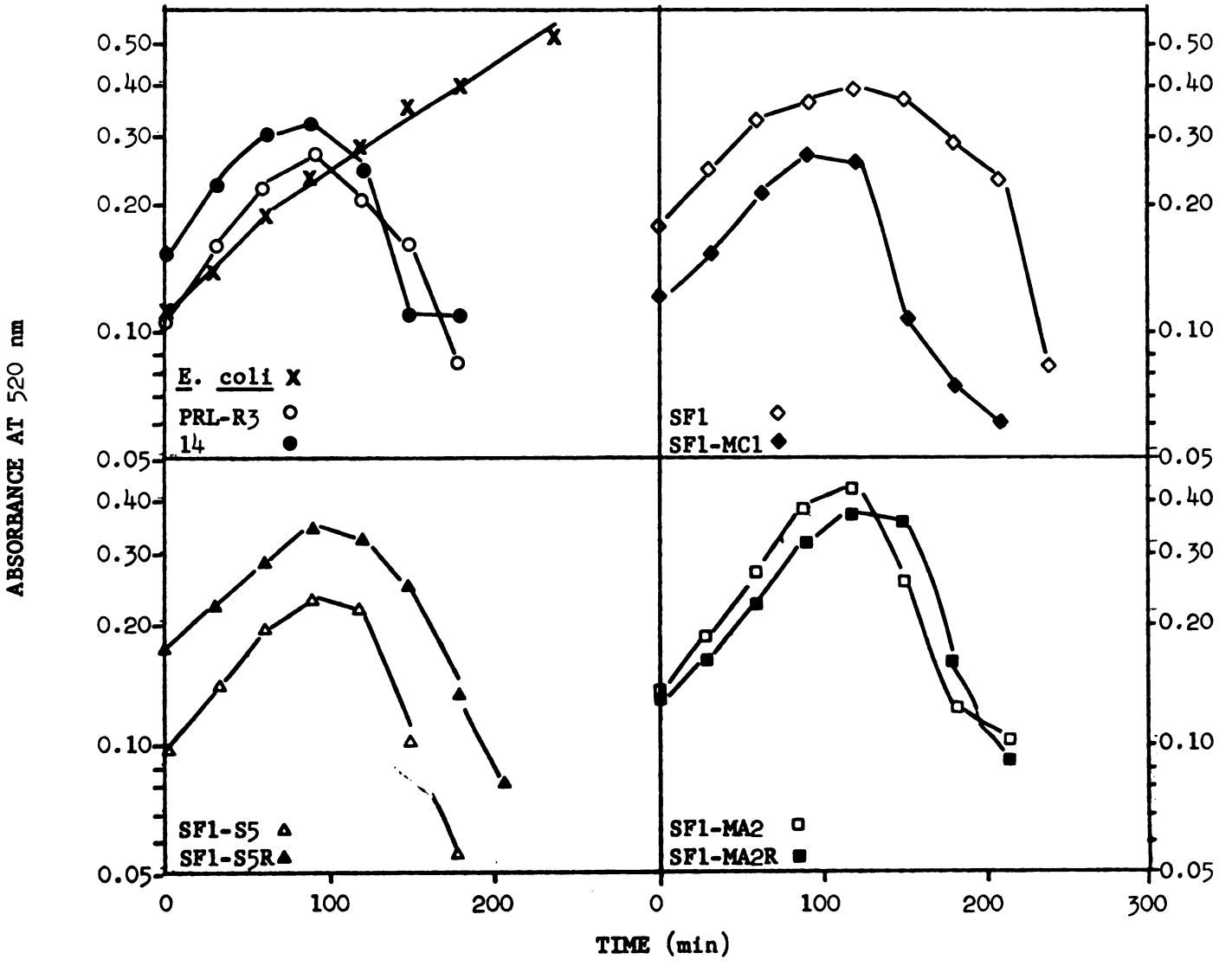

ABSORBANCE AT 520 nm
0.50
0.40
0.30
0.20
0.10
0.05
E. coli X
PRL-R3 O
14 ●
SF1 ◇
SF1-MC1 ◆
SF1-S5 △
SF1-S5R▲
SF1-MA2 □
SF1-MA2R ■
0
100
200
300
TIME (min)

Figure 26

Growth on D-fructose. Although strain 14 is unable to utilize D-fructose as a sole source of carbon and energy, the L-sorbose-1-P reductase-constitutive mutant, SF1, is able to grow on 0.3% D-fructose in mineral broth at a rate which is 38% that of the wild-type, PRL-R3 (Figure 27). The earlier studies on the effect of preinducing strain 14 on L-sorbose and D-mannitol (Figure 24) suggested that the D-mannitol-1-P dehydrogenase might be the limiting factor for optimal utilization of D-fructose by the new pathway. This speculation is supported by the fact that a mutant with increased levels of D-mannitol-1-P dehydrogenase, SF1-MC1, is able to grow on D-fructose at a rate which is 78% that of PRL-R3 and twice that of SF1. Thus the levels of D-mannitol-1-P dehydrogenase (Table 11) correlate with the rate of growth on D-fructose.

Not only is strain 14 unable to grow on D-fructose, but its growth rate on nutrient broth is inhibited 83% by the presence of 0.5% D-fructose in the medium (Table 10). In contrast to strain 14, SF1 is inhibited only 13% when grown on nutrient broth in the presence of D-fructose. SF1-MC1, which uses the new pathway to metabolize D-fructose at a faster rate than SF1, not only is uninhibited but grows slightly faster when nutrient broth is supplemented with 0.3% D-fructose. Mutants missing either of two enzymes of the new pathway, L-sorbose-1-P reductase (SF1-S5) or D-mannitol-1-P dehydrogenase (SF1-MA2), are inhibited by D-fructose to nearly the same extent as is strain 14.

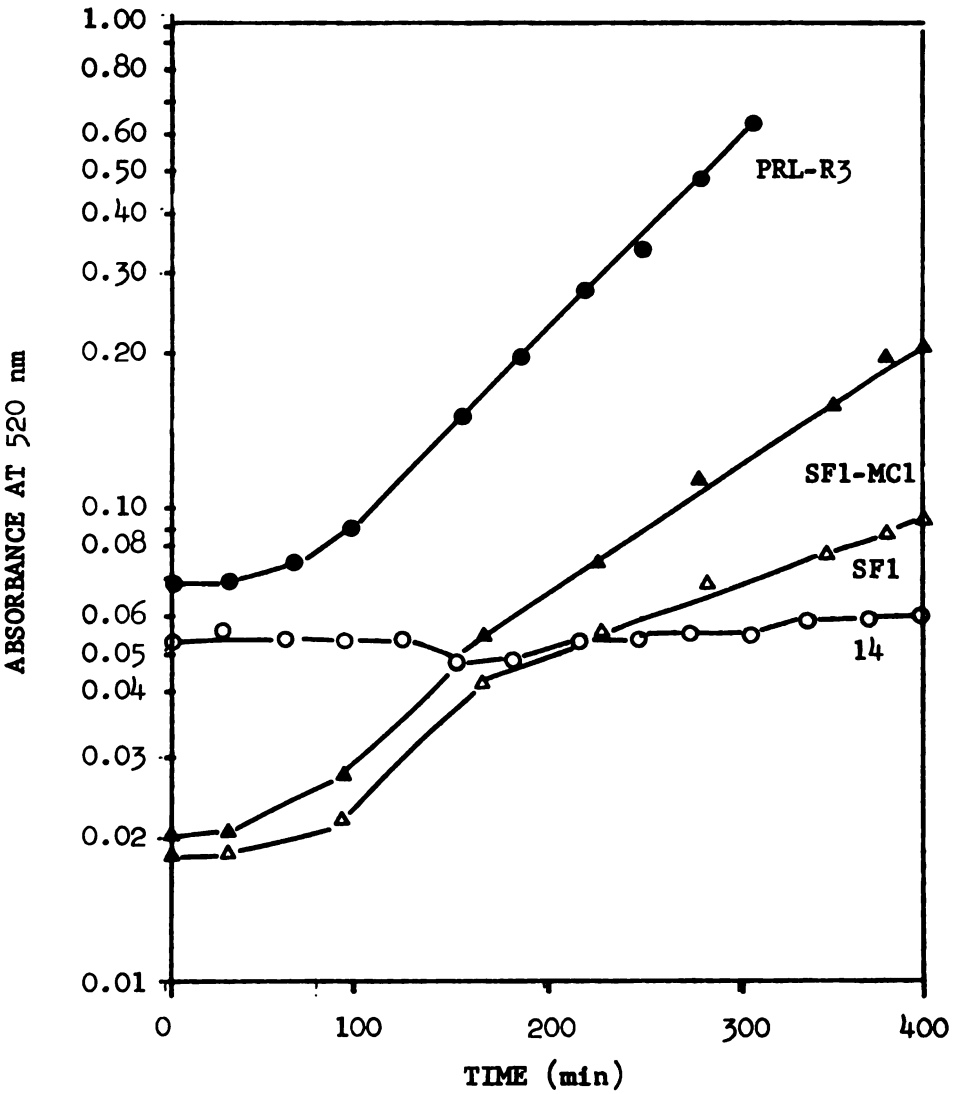


Figure 27 - Growth curves of wild-type and mutant strains of A. aerogenes on 0.3% D-fructose in mineral broth at 30°.

Table 10. Effect of the presence (+) of D-fructose on the growth rate of mutants of *A. aerogenes* on nutrient broth

Strain	D-fructose	Growth rate (generations/hr)	Percent inhibition of growth rate on nutrient broth by D-fructose
PRL-R3	-	1.5	0
	+	1.5	
14	-	1.1	83
	+	0.18	
SF1	-	0.92	13
	+	0.80	
SF1-S5	-	0.97	71
	+	0.27	
SF1-MA2	-	0.97	71
	+	0.27	
SF1-MC1	-	1.0	-10
	+	1.1	

Enzyme Levels in Mutants of *A. aerogenes*

Table 11 summarizes the specific activities of L-sorbose-1-P reductase and D-mannitol-1-P dehydrogenase found in crude extracts of various mutants of *A. aerogenes* grown on L-sorbose, D-mannitol, D-fructose, or glycerol. There were no detectable levels of D-fructose-1-P kinase in any of the strains tested. D-Fructokinase activity was below 0.008 units per mg protein and was apparently too low to allow utilization of D-fructose for growth (76).

Strain 14, the mutant blocked in the normal pathways for D-fructose metabolism, has detectable levels of L-sorbose-1-P reductase only when grown on L-sorbose. All mutants which can utilize D-fructose as a sole source of carbon and energy for growth (i.e., SF1, SF1-S5R, SF1-MA2R, and SF1-MC1) possess reductase activity regardless of the carbon source on which they are grown. A mutant missing the reductase completely, SF1-S5, also loses the ability to grow on D-fructose. Thus the presence of constitutive levels of L-sorbose-1-P reductase correlates with the ability to grow on D-fructose.

D-Mannitol-1-P dehydrogenase is present in all strains except SF1-MA2, which possesses only 10-20% the normal uninduced levels of this enzyme. Growth on D-mannitol increases the levels of D-mannitol-1-P dehydrogenase dramatically in all mutants except SF1-MA2. SF1-MC1, the mutant selected by cycling an 0.1 mM D-mannitol and 16.7 mM D-glucose, has at least twice the uninduced levels of dehydrogenase found in other strains and at least one and one-half times the levels of dehydrogenase when grown on

Table 11. Specific activities of L-sorbose-1-P reductase and D-mannitol-1-P dehydrogenase in crude extracts of mutants of *A. aerogenes* grown on various carbohydrates in mineral broth

Strain	Specific Activity (μmoles NAD^+ or NADH produced per min per mg protein)							
	glycerol		L-sorbose		D-mannitol		D-fructose	
	SR[1]	DH[2]	SR[1]	DH[2]	SR[1]	DH[2]	SR[1]	DH[2]
14	0.000	0.106	0.073	0.073	0.000	0.787	NG	NG
SF1	0.040	0.108	0.069	0.087	0.020	0.922	0.082	0.089
SF1-S5	0.000	0.084	NG[3]	NG	0.000	0.619	NG	NG
SF1-S5R	0.046	0.123	0.046	0.070	0.025	0.890	0.053	0.106
SF1-MA2	0.029	0.015	0.023	0.012	NG	NG[4]	NG	NG
SF1-MA2R	0.040	0.092	0.050	0.071	0.036	0.236	0.040	0.064
SF1-MC1	0.011	0.305	0.031	0.200	0.012	1.419	0.027	0.234

[1]SR - L-sorbose-1-P reductase determined by the reduction of D-fructose-1-P with NADH.

[2]DH - D-mannitol-1-P dehydrogenase determined by the oxidation of D-mannitol-1-P with NAD^+.

NG = no detectable growth after 24 hr.

[3]NG - cells grown on nutrient broth and induced on 0.3% L-sorbose had no detectable levels of L-sorbose-1-P reductase in crude extracts.

[4]NG - cells grown on nutrient broth and induced on 0.3% D-mannitol gave a specific activity of 0.005 μmoles NAD^+ reduced per min per mg protein in crude extracts.

D-mannitol. These increased levels of dehydrogenase are not due to a mutation affecting the affinity of the enzyme for D-mannitol-1-P (Figure 28) but are due to changes in the level of activity of the enzyme. The data from Table 11 indicate two conditions which are necessary for a mutant blocked in the normal pathways of D-fructose metabolism to utilize D-fructose as a sole source of carbon and energy for growth. The first condition is that there be constitutive levels of D-mannitol-1-P dehydrogenase present in the cell. The second condition is that constitutive levels of L-sorbose-1-P reductase must approach those found in cells induced by growth on L-sorbose.

Intracellular Metabolite Levels in *A. aerogenes* Mutants

The intracellular accumulation of D-fructose-1-P and D-mannitol-1-P in mutants of *A. aerogenes* correlates in the predicted manner with the levels of L-sorbose-1-P reductase and D-mannitol-1-P dehydrogenase and with the rate of growth on D-fructose (Table 12). PRL-R3, which has D-fructose-1-P kinase when induced on D-fructose, has on the order of 1% the level of D-fructose-1-P found in the mutants which are missing this enzyme. Strains PRL-R3 and 14 possess L-sorbose-1-P reductase only when grown on L-sorbose (Table 11) and hence lack the ability to convert D-fructose-1-P to D-mannitol-1-P. Those mutants missing enzymes in the new D-fructose pathway (strains 14, SF1-S5, and SF1-MA2) accumulate greater amounts of D-fructose-1-P than those mutants with a functional pathway. Strains 14 and SF1-S5, both lacking the reductase, accumulate no detectable levels of D-mannitol-1-P as would be

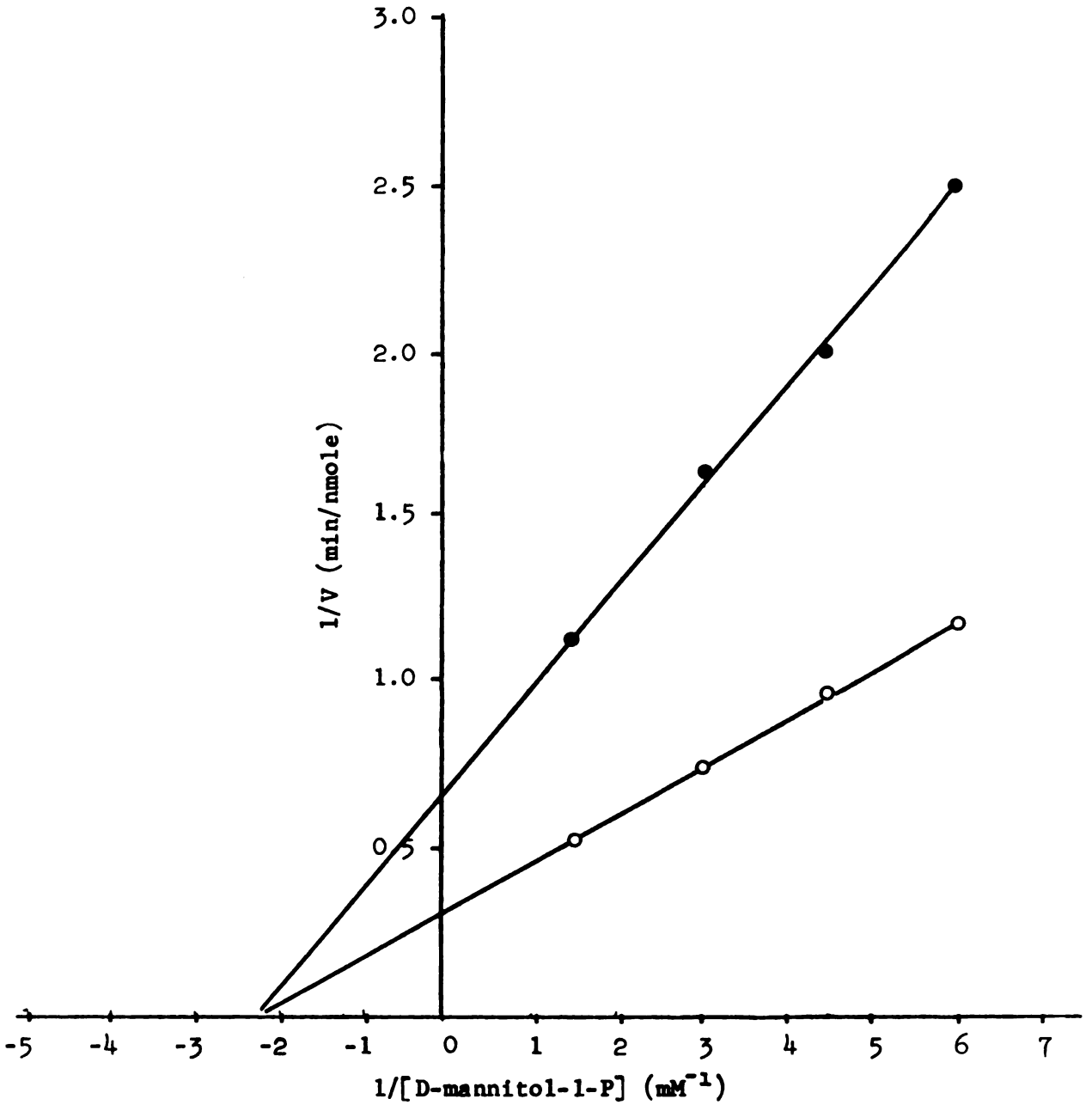


Figure 28 - Double reciprocal plot showing the relationship between D-mannitol-1-P concentration and reaction velocity for D-mannitol-1-P dehydrogenase from PRL-R3 and SF1-MC1. Assays were performed with crude extracts of nutrient broth-grown cells of A. aerogenes strains PRL-R3 (●) and SF1-MC1 (○).

Table 12. Intracellular metabolite levels in wild-type and mutant strains of *A. aerogenes* induced for 5 hr on 0.5% D-fructose as compared to enzymes present and growth rate on 0.3% D-fructose

Strain	Enzymes present after 5 hr incubation in presence of 0.5% D-fructose[1]	Metabolite levels[2] (μmoles/gram cells) F-1-P[3]	Mtl-1-P[4]	Growth rate on 0.3% D-fructose (generations/hr)
PRL-R3	FK^-, $F\text{-}1\text{-}PK^+$, $S\text{-}1\text{-}PR^-$, $M\text{-}1\text{-}PDH^+$	0.024	0.005	0.77
14	FK^-, $F\text{-}1\text{-}PK^-$, $S\text{-}1\text{-}PR^-$, $M\text{-}1\text{-}PDH^+$	4.1	0.005	0.00
SF1	FK^-, $F\text{-}1\text{-}PK^-$, $S\text{-}1\text{-}PR^+$, $M\text{-}1\text{-}PDH^+$	3.4	6.9	0.29
SF1-S5	FK^-, $F\text{-}1\text{-}PK^-$, $S\text{-}1\text{-}PR^-$, $M\text{-}1\text{-}PDH^+$	4.4	0.005	0.00
SF1-S5R	Same as SF1	2.1	6.6	0.21
SF1-MA2	FK^-, $F\text{-}1\text{-}PK^-$, $S\text{-}1\text{-}PR^+$, $M\text{-}1\text{-}PDH^-$	5.1	12.6	0.00
SF1-MA2R	Same as SF1	3.0	5.5	0.23
SF1-MC1	FK^-, $F\text{-}1\text{-}PK^-$, $S\text{-}1\text{-}PR^+$, $M\text{-}1\text{-}PDH^{++}$	3.1	2.3	0.55

[1] Enzymes present (+), absent (-), or present at increased levels (++).

[2] There were less than 0.005 μmoles per gram of cells (wet weight) of D-fructose-6-P and D-fructose-1,6-P_2. PRL-R3 contained 0.083 μmoles of D-fructose-1,6-P_2 per gram of cells.

[3] D-Fructose-1-P determined with D-fructose-1-P kinase (see *methods* for details).

[4] D-Mannitol-1-P determined with D-mannitol-1-P dehydrogenase (see *methods* for details).

predicted according to the order of steps in the new pathway. SF1-MA2, which has greatly reduced levels of D-mannitol-1-P dehydrogenase, accumulates twice the amount of D-mannitol-1-P and, as a consequence, increased amounts of D-fructose-1-P. SF1-MC1, with twice the level of dehydrogenase, accumulates half the level of D-mannitol-1-P, and grows at twice the rate of mutants with normal levels of dehydrogenase on D-fructose.

Discussion and Summary

This section of my dissertation has demonstrated the evolution of a novel pathway for the metabolism of D-fructose in *A. aerogenes*. By using a mutant (strain 14) blocked in the two pathways by which D-fructose is normally metabolized, I derived a mutant capable of metabolizing D-fructose via the pathway shown in Figure 29.

The studies presented in Figure 24 indicated that the presence of L-sorbose-1-P reductase within the bacterium is sufficient to allow the bacterium to grow on D-fructose when the normal pathways of D-fructose are blocked. These same studies also showed that D-mannitol-1-P dehydrogenase is the limiting enzyme for growth on D-fructose when the reductase is also present. Since the P-enolpyruvate-dependent phosphotransferase system for L-sorbose appears to be constitutive (1) and D-glucitol-1-P dehydrogenase is induced by D-glucitol-6-P (1), then enriching for mutants constitutive for growth on L-sorbose should consequently select mutants constitutive for L-sorbose-1-P reductase. With the modified procedure described in *Results*, those mutants with the higher levels of constitutive

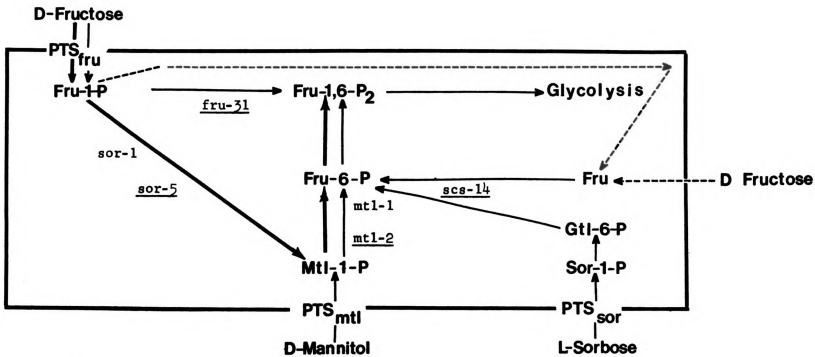


Figure 29 - The metabolic pathways of D-fructose, L-sorbose, and D-mannitol in A. aerogenes. (-------) Possible sources of intracellular D-fructose; (———) Normal metabolic pathways; (———) New pathway for metabolism of D-fructose; Lower case letters, i.e., sor-1 are mutations for constitutive or increased levels of enzyme; Underlined lower case letters, i.e., sor-5 are mutations for decreased or no detectable levels of enzyme. PTS : PEP-dependent phosphotransferase system; fru : D-fructose; sor : L-sorbose; mtl : D-mannitol; gtl : D-glucitol; and scs : sucrose.

reductase should have a selective advantage over those mutants with lower constitutive levels of reductase. The results shown in Figure 25 confirmed this prediction. One of the mutants selected in this manner, SF1, possesses constitutive levels of reductase comparable to the levels of reductase induced in the parental mutant, strain 14, when grown on L-sorbose (Table 11).

Further evidence that the new pathway is as depicted in Figure 29 was provided by the isolation of two D-fructose-negative mutants derived from SF1. These mutants, missing either L-sorbose-1-P reductase (SF1-S5) or D-mannitol-1-P dehydrogenase (SF1-MA2), lost the ability to utilize D-fructose as a sole source of carbon and energy, whereas their revertants, SF1-S5R and SF1-MA2R, concomitantly regained the missing enzyme activities and the ability to grow on D-fructose at the same rate as SF1.

The accumulation of intracellular metabolite levels of D-fructose-1-P and D-mannitol-1-P correlates in a predictable fashion with the enzymes present or absent in the various mutants (Table 12). The data for SF1-MC1 show that this mutant has about 2.5 times the level of dehydrogenase present in SF1, SF1-S5R, and SF1-MA2R and also grows on D-fructose at a rate which is 2.3 times the rate of these three mutants. The levels of D-mannitol-1-P accumulated in SF1, SF1-S5R, SF1-MA2, SF1-MA2R, and SF1-MC1 are inversely proportional to the levels of dehydrogenase present in crude extracts of these bacteria.

By these results I have demonstrated that it is possible to genetically manipulate *A. aerogenes* strains lacking the ability

to utilize D-fructose via the normal metabolic pathways, to produce a mutant possessing a novel pathway for the metabolism of D-fructose. This pathway is: D-fructose → D-fructose-1-P → D-mannitol-1-P → D-fructose-6-P. The genetic manipulations resulted in a mutant which possesses this pathway by conscripting enzymes normally associated with three separate metabolic pathways: (i) the PEP:D-fructose 1-phosphotransferase system of D-fructose metabolism; (ii) the L-sorbose-1-P reductase of L-sorbose metabolism; and (iii) the D-mannitol-1-P dehydrogenase of D-mannitol metabolism. This is a unique pathway previously unreported in any other organism and is the first new pathway evolved in the laboratory for the metabolism of a normal substrate.

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