METABOLISM OF L-MANNOSE IN *AEROBACTER AEROGENES*

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Joseph William Mayo

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This is to certify that the

thesis entitled

METABOLISM OF L-MANNOSE
IN AEROBACTER AEROGENES

presented by

Joseph W. Mayo

has been accepted towards fulfillment
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ABSTRACT

METABOLISM OF L-MANNOSE
IN AEROBACTER AEROGENES

by Joseph W. Mayo

A mutant strain of Aerobacter aerogenes PRL-R3 was isolated which, unlike the parent strain, grew readily on the unnatural hexoses, L-mannose and L-fructose, as sole carbon sources. The pathway by which L-mannose is degraded in this organism was elucidated. An isomerase catalyzes the conversion of L-mannose to L-fructose, which is phosphorylated with adenosine 5'-triphosphate by a kinase to yield L-fructose 1-phosphate. L-Fructose 1-phosphate is cleaved by an aldolase to yield dihydroxyacetone phosphate and L-glyceraldehyde. The two intermediates in the pathway, L-fructose and L-fructose 1-phosphate, were isolated and characterized.

The enzymes which degraded L-mannose also degraded the naturally occurring 6-deoxy hexose, L-rhamnose. Both L-mannose and L-rhamnose induced all three enzymes in both the wild-type and the L-mannose-positive cells; the ratio of the specific activities of the enzymes on L-mannose and L-rhamnose and their respective metabolic intermediates were the same in cells grown on either substrate. L-Mannose

was isomerized at a rate 25% that of L-rhamnose, L-fructose was phosphorylated at a rate 10% greater than L-rhamnulose, and L-fructose-1-P was cleaved at a rate 25-30% that of L-rhamnulose-1-P. Partial fractionation of the enzymes with ammonium sulfate and Sephadex G-100 failed to separate the enzymes of the L-mannose pathway from those of the L-rhamnose pathway. When the two substrates for each enzyme were mixed, the individual activities were competitive rather than additive. A mutant of A. aerogenes PRL-R3 deficient in the isomerase was unable to isomerize either L-mannose or L-rhamnose.

The mechanism by which A. aerogenes gains the ability to metabolize many of the unnatural hexoses, pentoses, and penitols has been shown by other workers to involve the selection of derepressed mutants containing higher levels of non-specific enzymes which have naturally occurring compounds as their normal substrates. In contrast to this, the gain in the ability of this organism to grow on L-mannose as a sole carbon source did not involve selection of derepressed mutants. L-Mannose inhibited the growth of both the wild type and the mutant at the onset of L-mannose utilization by the cells; only the mutant overcame this inhibition. When the inducer, L-mannose, was removed from actively growing mutant cells,

a rapid decrease in the activities of all three enzymes occurred. Also, L-mannose was not utilized by the mutant cells in the presence of either chloramphenicol or puromycin, further indicating that growth on L-mannose required the induction of these enzymes.

METABOLISM OF L-MANNOSE

IN *AEROBACTER AEROGENES*

By

Joseph William Mayo

A THESIS

Submitted to
Michigan State University
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DOCTOR OF PHILOSOPHY

Department of Biochemistry

1968

Dedicated

to my

parents

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The author wishes to express his gratitude to Dr. Richard L. Anderson for his advice and helpful criticisms throughout the course of this work. The discussions and assistance of his fellow graduate students also are deeply appreciated. Thanks are due to Mrs. Barbara Palmer and Mrs. Shirley Randall for their help in preparing this manuscript. The financial support of the National Institutes of Health also is appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Aerobacter aerogenes PRL-R3 readily gains the ability to utilize rare hexoses, pentoses, and pentitols such as L-lyxose (1), L-xylose (1,2), L-arabitol (3), xylitol (3), D-lyxose (4), and D-allose (5) as sole sources of carbon and energy. Investigations notably in the laboratories of Lin (6), Wood (7), and Mortlock (8,9) have shown that the biodegradation of these rare compounds does not involve the synthesis of new enzymes, but rather the selection of constitutive mutants containing high levels of non-specific enzymes capable of converting the uncommon sugars into readily metabolizable intermediates.

The purpose of this present investigation was to elucidate the biodegradative pathway of the unnatural hexose L-mannose in A. aerogenes PRL-R3, to characterize the enzymes involved in the pathway, and to establish the basis for the gain in the ability to grow on this hexose.

PART I

The Biodegradation of L-Mannose by *Aerobacter Aerogenes*

Aerobacter aerogenes PRL-R3 readily gains the ability to utilize L-mannose as a sole carbon source. Except for a recent report (10) that liver galactose dehydrogenase oxidizes L-mannose, the metabolism of this sugar has not been previously described for any organism. This portion of the thesis elucidates the biodegradative pathway of L-mannose in *A. aerogenes* PRL-R3.

EXPERIMENTAL PROCEDURE

Growth of Cells and Preparation of Extracts- A strain of *A. aerogenes* PRL-R3 selected for its ability to grow readily on L-mannose was used. The selection of this mutant will be described in Part III. It was grown aerobically at 30° in a medium consisting of 0.71% Na_2HPO_4, 0.15% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.01% $MgSO_4$, 0.0005% $FeSO_4 \cdot 7H_2O$, and 0.4% sugar (autoclaved separately). The sugar was L-mannose unless noted otherwise. The cells were harvested by centrifugation after 15-18 hours growth in Fernbach flasks on a rotary shaker. They were washed with water, suspended in 0.02 M tris-HCl buffer (pH 7.6), and disrupted by a 4-minute exposure in a Raytheon 10-kc sonic oscillator equipped with an ice-water cooling jacket.

The cellular debris was removed by centrifugation at 31,000 x g for 10 minutes. The resulting supernatant was the crude extract.

Analytical Procedures- Reducing sugars were determined by the method of Folin and Malmrose (11). L-Fructose and L-fructose-1-P were determined by the method of Roe (12); the molar extinction of L-fructose-1-P was 80% that of fructose. Inorganic orthophosphate was determined by the method of Fiske and SubbaRow (13), and total phosphate by the method of Umbreit, Burris, and Stauffer (14). Trioses were determined by the method of Sibley and Lehninger (15). Reducing sugars were chromatographed on Whatman No. 1 paper and developed with the following solvent systems: 1) water-saturated phenol; and 2) 2-butanone: acetic acid:water (75:25:10). The sugars were located with a bath of silver nitrate (16) and sprays of orcinol (17) and N,N-dimethyl-p-phenylenediamine monohydrochloride (18). Sugar phosphates were chromatographed on Whatman No. 1 paper (washed with 2 N HCl and water) using t-butyl alcohol: water:picric acid (80:20:2) as the developing solvent (19). The location of the compounds also was determined with a bath of silver nitrate. Glycerol and glyceric acid were chromatographed on Whatman No. 1 paper using ethyl acetate: pyridine:water (120:50:40) as the developing solvent. The spots were located by baths of benzidine hydrochloride and

of periodate (20). Protein was measured by the method of Tombs, Souter, and Maclagan (21). Trimethylsilyl derivatives of D- and L-fructose-1-P were prepared and subjected to gas chromatography by the method of Wells et al (22). Gas chromatography was performed on a Hewlett and Packard gas chromatograph, model 402. Melting points were determined with a Kofler miro-melting point apparatus. Optical rotations were made with a Zeiss photoelectric polarimeter or a Bendix digital polarimeter. Light measurements were measured on a Coleman Junior Spectrophotometer (18-mm diameter round cuvettes) or a Gilford absorbance recording spectrophotometer (1.0-cm light path) thermostated at 25°. Manometric measurements were made with a conventional Warburg respirometer.

Enzyme Assays- The reaction mixture (0.58 ml) for L-mannose isomerase contained 16 μmoles of L-mannose, 2 μmoles of $CoCl_2$, 8 μmoles of tris-HCl buffer (pH 7.6), and enzyme. The mixture was incubated at 30°, and samples were removed at time intervals and assayed for L-fructose; the values were corrected for slight interference from L-mannose. The amounts of enzyme assayed for the time periods of incubation were selected to give linear measurements. A unit of L-mannose isomerase was defined as the amount that formed 1 μmole of L-fructose per minute in

this assay.

The L-fructose kinase activity was measured spectrophotometrically at 340 nm and 25°. The reaction mixture (0.15 ml) consisted of 1.5 μmoles of L-fructose, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.5 μmole of phosphoenolpyruvate, 0.01 μmole of NADH, 2.0 μmoles of tris-HCl buffer (pH 7.6), 13 μg of lactate dehydrogenase-pyruvate kinase, and a limiting amount of L-fructose kinase preparation. A control to correct for NADH oxidase and ATPase contained all of the reaction components except L-fructose. The absence of L-fructose reductase activity in the extracts and fractions made a control without ATP unnecessary. The assay was linear with time and enzyme concentration. A unit of L-fructose kinase activity was defined as the amount that resulted in the oxidation of 1 μmole of NADH per minute in this assay.

L-Fructose-1-P aldolase activity also was measured spectrophotometrically at 340 nm and 25°. The reaction mixture (0.15 ml) consisted of 1.5 μmole of L-fructose-1-P, 0.01 μmole of NADH, 2.0 μmoles of tris-HCl buffer (pH 7.6), 1 μg of α-glycerol phosphate dehydrogenase, and a limiting amount of L-fructose-1-P aldolase preparation. A control to correct for NADH oxidase contained all of the reaction components except L-fructose-1-P. The assay was linear with time and enzyme concentration. A unit of L-

fructose-1-P aldolase was defined as the amount that resulted in the oxidation of 1 μmole of NADH per minute in this assay.

L-Glyceraldehyde reductase activity was measured spectrophotometrically at 340 nm and 25°. The reaction mixture (0.15 ml) consisted of 1.5 μmoles of L-glyceraldehyde, 0.01μmole of NADH, 2 μmoles of tris-HCl buffer (pH 7.6), and a limiting amount of reductase (45-60% $(NH_4)_2SO_4$ fraction).

Reagents- L-Mannose was prepared by the method of Sowden and Fischer (23) and L-glyceraldehyde by the method of Perlin and Brice (24). Details for the preparation of these compounds are given below. D-Fructose-1-P, D-fructose-6-P, D-fructose-1,6-diP, and α-glycerol phosphate dehydrogenase were purchased from Calbiochem, Los Angeles, California; ATP, NAD, NADH, NADP, NADPH, and phosphoenolpyruvate from P-L Biochemicals, Milwaukee, Wisconsin; lactate dehydrogenase-pyruvate kinase from Worthington Biochemical Corporation, Freehold, New Jersey; wheat germ acid phosphatase and protamine sulfate from Sigma Chemical Co., St. Louis, Missouri. L-Fructose for use as seed crystals was a generous gift from Professor M.L. Wolfrom, Ohio State University. Duolite resins C-25 (Na^+) and A-6(Cl^-) were purchased from the Diamond

Alkali Co., Redwood City, California. They were converted to the H^+ and OH^- forms respectively and mixed in a ratio of 3:5 (wet weight) cation:anion prior to preparation of the column.

Preparation of L-Mannose- L-Arabinose (150 g), 300 ml of absolute methanol, and 540 ml of mitromethane were stirred rapidly in a 3-liter, 3-necked flask fitted with a mechanical stirrer and drying tube. To this was added a solution of sodium methoxide, prepared by dissolving 32 g of sodium metal in 1050 ml of absolute methanol. The mixture was stirred approximately 20 hours during which time the reaction mixture changed from a white to yellow-brown suspension. The sodium aci-nitroalcohols were collected by suction filtration and washed with 450 ml each of cold absolute methanol and cold petroleum ether B. The nitroalcohols were dissolved in 1 liter of water at 0° and immediately were added dropwise to a stirred solution of 210 ml of sulfuric acid in 250 ml of water at room temperature. During the course of adding the nitroalcohol solution, the temperature of the acid solution did not rise above 43°. The resulting solution was diluted to 3000 ml with water and solid sodium carbonate added batchwise until the solution became neutral to congo red. The mixture then was treated with a solution containing 105 ml

of phenylhydrazine in 240 ml of glacial acetic acid and the resulting solution left for approximately 12 hours at 4°. The L-mannose phenylhydrazone which precipitated was collected by filtration, washed thoroughly with water, with 95% ethanol, and finally with anhydrous ether. The crude hydrazone amounted to 110 g, melting point 184-186°.

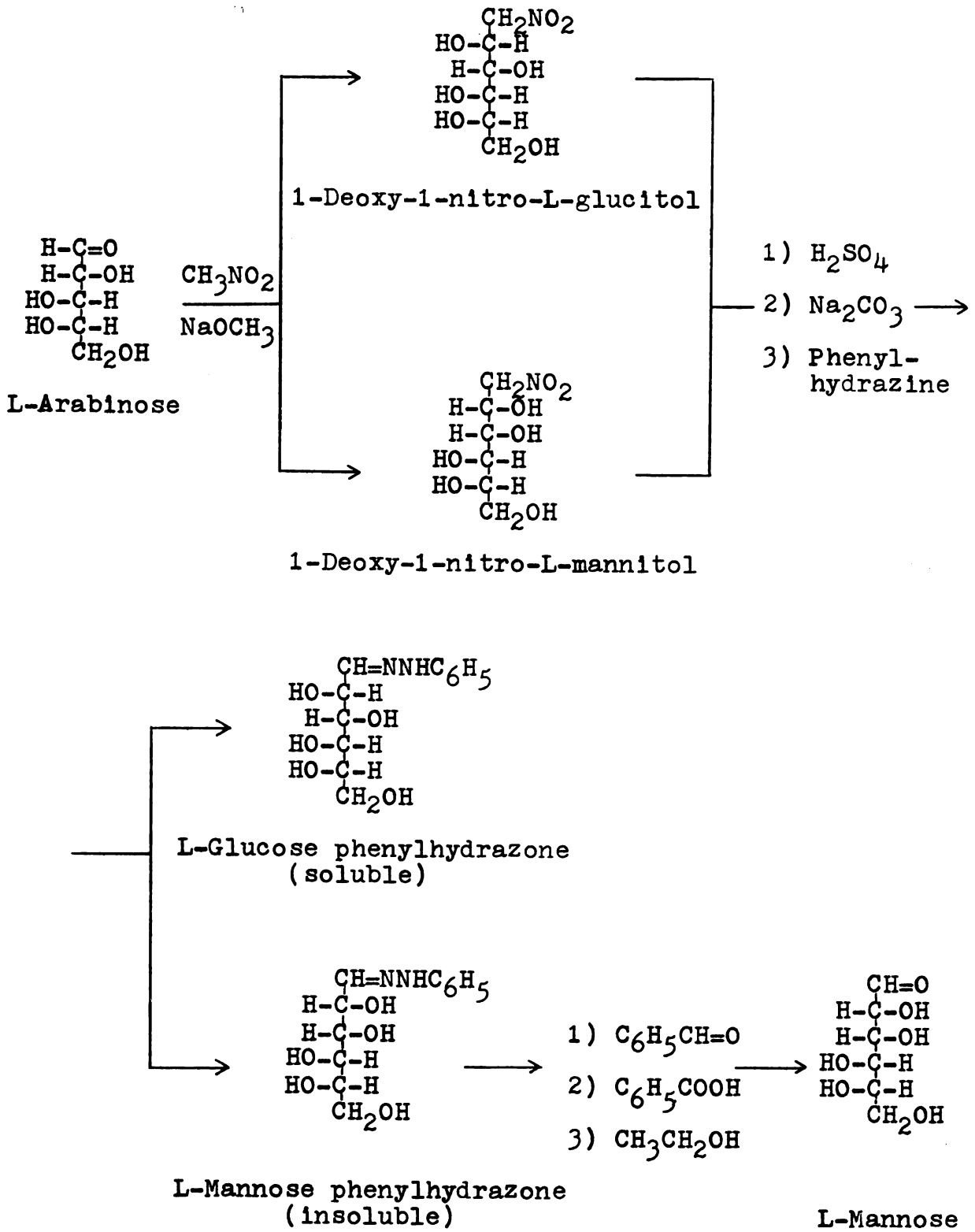
The L-mannose phenylhydrazone was suspended in a solution containing 1100 ml of water, 220 ml of ethanol, 140 ml of benzaldehyde, and 14 g of benzoic acid. The reaction mixture was refluxed for 2.5 hours, cooled to room temperature, and the aqueous phase decanted from the benzyl phenylhydrazone. The solution next was extracted three times with chloroform (500 ml of chloroform per 500 ml of solution), decolorized with Darco G-60, and concentrated in a vacuum to a syrup. To crystallize the L-mannose, the syrup was dissolved in a volume of warm absolute methanol equal to twice the volume of the syrup. Next, a volume of absolute methanol and isopropyl alcohol (50:50) equal to the previous volume of methanol was added. The solution was seeded with L-mannose and left overnight at room temperature. The L-mannose crystallized readily, but scratching the inside of the container with a glass rod hastened further crystallization. The L-mannose was

collected by suction filtration and washed with a 75:25 (v/v) solution of absolute methanol and isopropyl alcohol. The filtrate was reprocessed to obtain additional L-mannose. The final yield of L-mannose was 45-50 g (30-33%). The melting point was 124-128° and the specific rotation was $[\alpha]_{578}^{24}$ -14.2° (c 1, water). A summary of the reactions for the synthesis of L-mannose is outlined in Figure 1.

Preparation of L-Glyceraldehyde- L-Sorbose (5.0 g) was dissolved in 10 ml of water and the resulting solution diluted to 500 ml with glacial acetic acid. After the solution was cooled with an external water-bath to 17°, 28 g of lead tetraacetate was added over a period of 5-10 minutes with good stirring. During this time the temperature did not exceed 22°. Next, a solution of oxalic acid (5.0 g of anhydrous oxalic acid in 50 ml of glacial acetic acid) was added until a negative starch-iodide test was obtained. The insoluble lead oxalate was removed by filtering the solution through filter aid. The filtrate then was concentrated in a vacuum to a syrup. Small amounts of acetic acid subsequently were removed by adding 20 ml of toluene followed by concentration to a syrup in a vacuum; this operation was repeated several times. The syrup was dissolved in 25 ml of 0.1 N sulfuric acid and the resulting solution stored for 24 hours at 35°. Finally the solution

Figure 1.

SYNTHESIS OF L-MANNOSE FROM L-ARABINOSE



was passed through a column of Duolite C-25(H^+) and A-6(OH^-) and the neutral effluent concentrated in a vacuum to a syrup. The syrup was dissolved in water and chromatographed on paper using n-butanol:ethanol:water (52:32:16) as the developing solvent (25). The sugar was identified with silver nitrate and showed traces of L-sorbose. Since L-sorbose did not interfere in the enzymatic assays the crude L-glyceraldehyde was used as such.

RESULTS

Since a variety of hexoses metabolized by A. aerogenes PRL-R3 initially are phosphorylated with ATP, my initial investigations on the metabolism of L-mannose were concentrated on determining whether or not L-mannose also was phosphorylated with ATP. Preliminary investigations involving manometric, titrametric, and colorimetric techniques supported this contention. These studies are summarized below.

Apparent Phosphorylation of L-Mannose:

Manometric: L-Mannose-Stimulated CO_2 Evolution from Bicarbonate in the Presence of ATP- Wild-type and L-mannose-positive cells were grown on D-glucose and L-mannose respectively and the cell-free extracts assayed for their ability to stimulate CO_2 release from bicarbonate due to the phosphorylation of hexoses with ATP. Since the enzymes

of D-glucose metabolism are constitutive, CO_2 rapidly evolved when extracts from both wild type and mutant were incubated with D-glucose and ATP (Figure 2). On the other hand, L-mannose was metabolized rapidly only by the extract of the mutant cells grown on L-mannose, suggesting that L-mannose degradation involves a phosphorylation at some point in the pathway.

Titrametric: Increased Rate of Acid Production from ATP in the Presence of L-Mannose- The proton released from the utilization of ATP with L-mannose was titrated. Figure 3 shows a slightly greater increase in the activity with L-mannose compared to the endogenous ATPase. To test the validity of the phosphorylating system, D-glucose was added as a control.

Colorimetric: ATP-Stimulated Disappearance of L-Mannose- The disappearance of L-mannose in extracts of L-mannose-grown cells was stimulated by ATP, suggesting the formation of phosphorylated intermediates (Figure 4). Again, D-glucose was employed as a positive control.

Spectrophotometric Test for L-Mannokinase- Attempts to demonstrate the direct phosphorylation of L-mannose yielded negative results, but Figure 5 shows that incubating L-mannose with crude extract prior to adding ATP resulted in measurable kinase activity. This suggested that L-

Fig. 2. L-Mannose-stimulated CO_2 evolution from bicarbonate in the presence of ATP. Each Warburg vessel contained in a volume of 0.5 ml: 10 μmoles of hexose (sidearm), 5 μmoles of ATP, 25 μmoles of $MgCl_2$, 5 μmoles of NaF, 11 μmoles of $NaHCO_3$, and crude extract (3-5 mg of protein). The reaction was carried out in an atmosphere of 95% nitrogen: 5% carbon dioxide at a temperature of 30°. Hexose was omitted to measure the endogenous ATPase activity. Controls minus ATP gave no CO_2 release.

Figure 2.

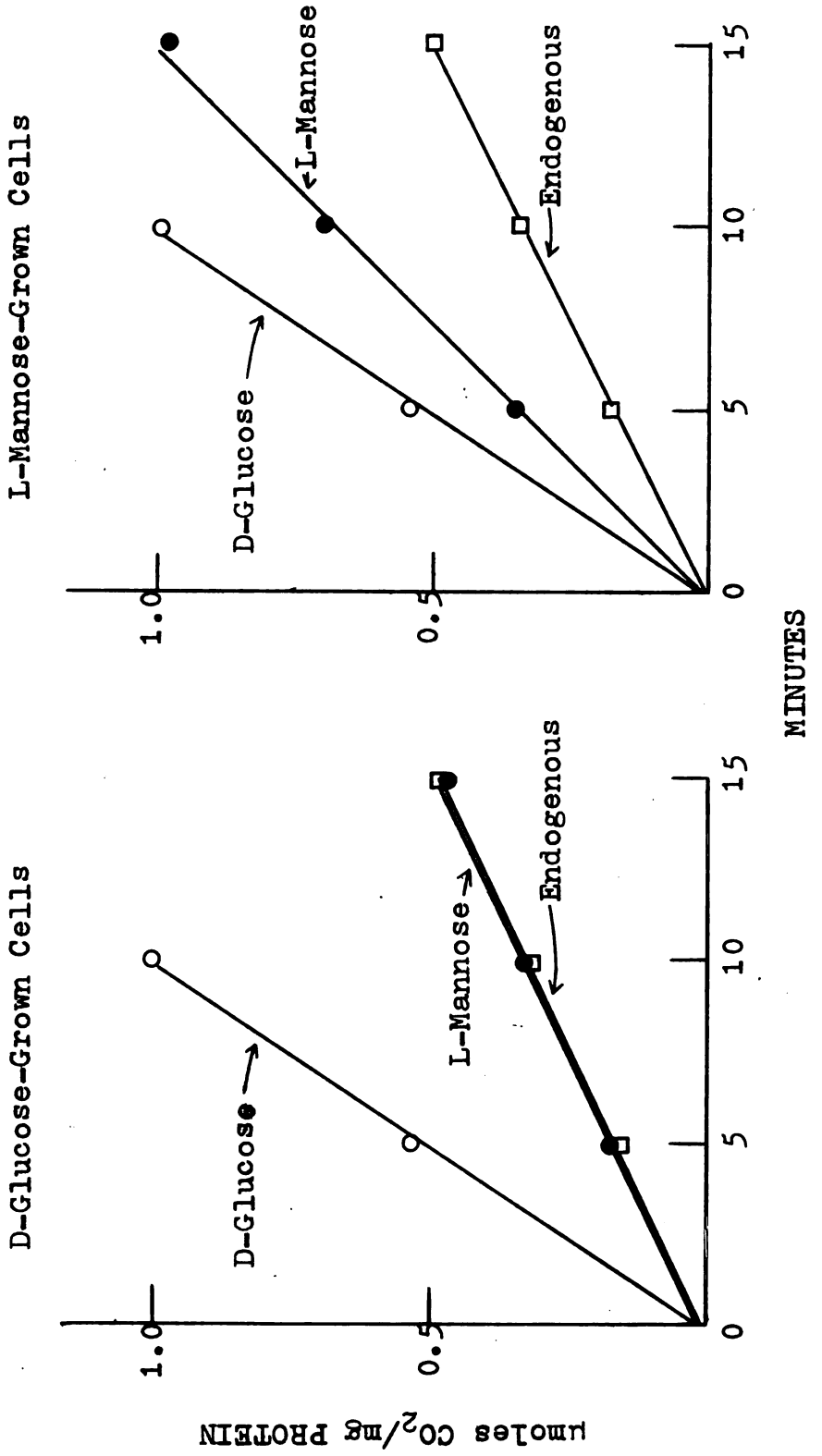

L-Mannose-Grown Cells
D-Glucose-Grown Cells
D-Glucose
L-Mannose
Endogenous
1.0
0.5
0
5
10
15
MINUTES
μmoles CO2/mg PROTEIN

Fig. 3. Titrametric measurement of acid production from ATP in the presence of L-mannose. The reaction mixture (1.5 ml) contained 20 μmoles of hexose, 25 μmoles of ATP, 50 μmoles of $MgCl_2$, and crude extract (10-15 mg of protein). The reaction was carried out at room temperature with a Beckman Zeromatic pH meter. The reaction rate was recorded by adding a measured volume of 0.1 N NaOH to the reaction at a specific time to maintain the pH at 7.5. Endogenous ATPase activity was determined by leaving out the hexose from the reaction. Controls minus ATP gave no rate.

Figure 3.

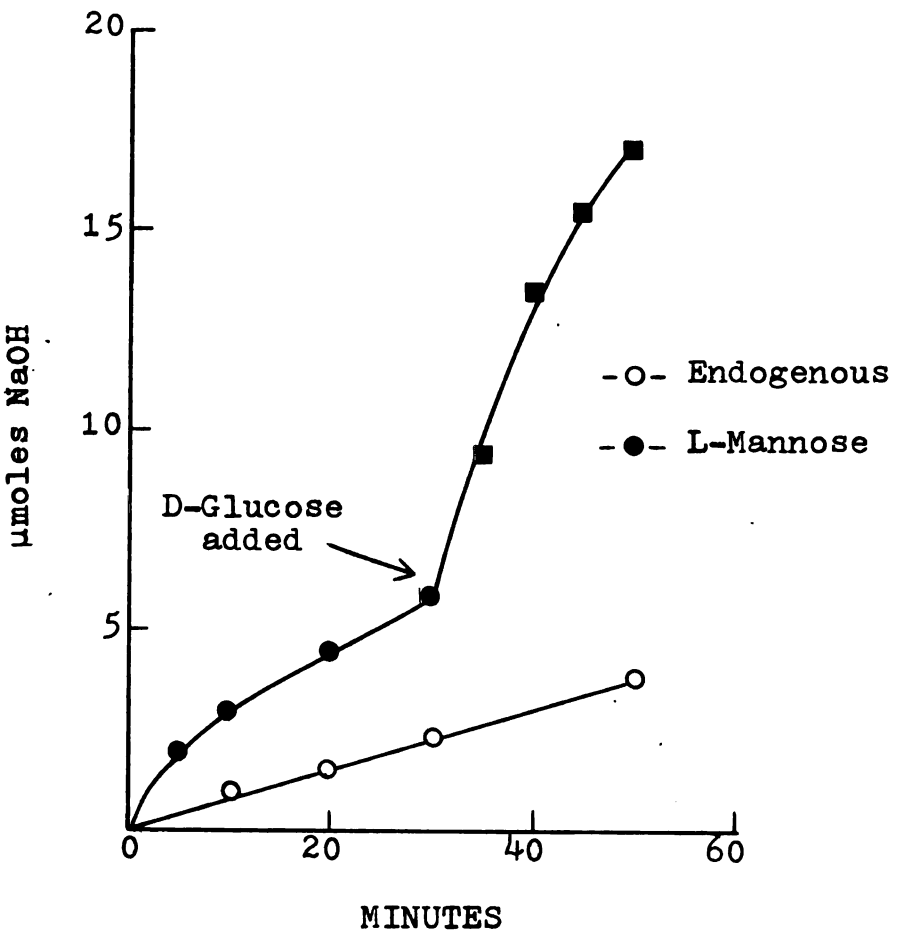

20
15
10
5
0
20
40
60
µmoles NaOH
MINUTES
D-Glucose
added
-o- Endogenous
-●- L-Mannose

Fig. 4. ATP-stimulated disappearance of L-mannose. The reaction mixture (2.0 ml) consisted of 16 μmoles of hexose, 20 μmoles of ATP, 100 μmoles of $MgCl_2$, 100 μmoles of NaF, 100 μmoles of glycylglycine buffer, pH 7.5, and crude extract (20-25 mg of protein). The reaction was incubated at 30° and 0.3 ml aliquots removed at time intervals and added immediately to 0.3 ml of 5% $ZnSO_4$ followed by the addition of 0.3 ml of 0.3 N $Ba(OH)_2$. The insoluble $BaSO_4$ was removed by centrifugation and 50 μl of the supernatant assayed for remaining reducing sugar.

Figure 4.

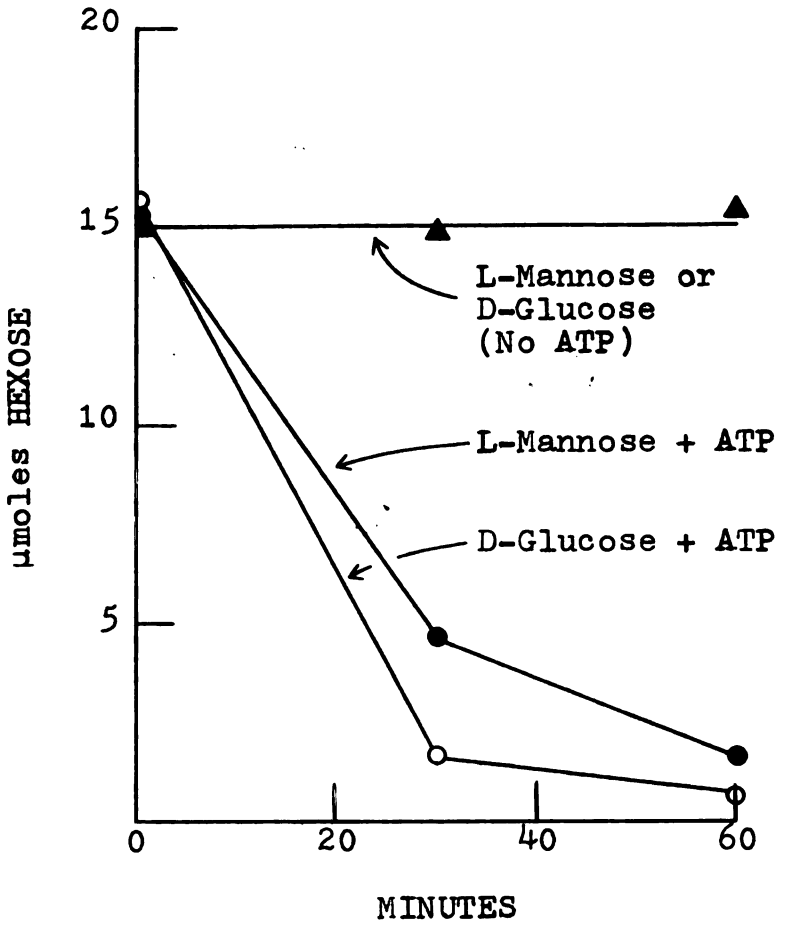
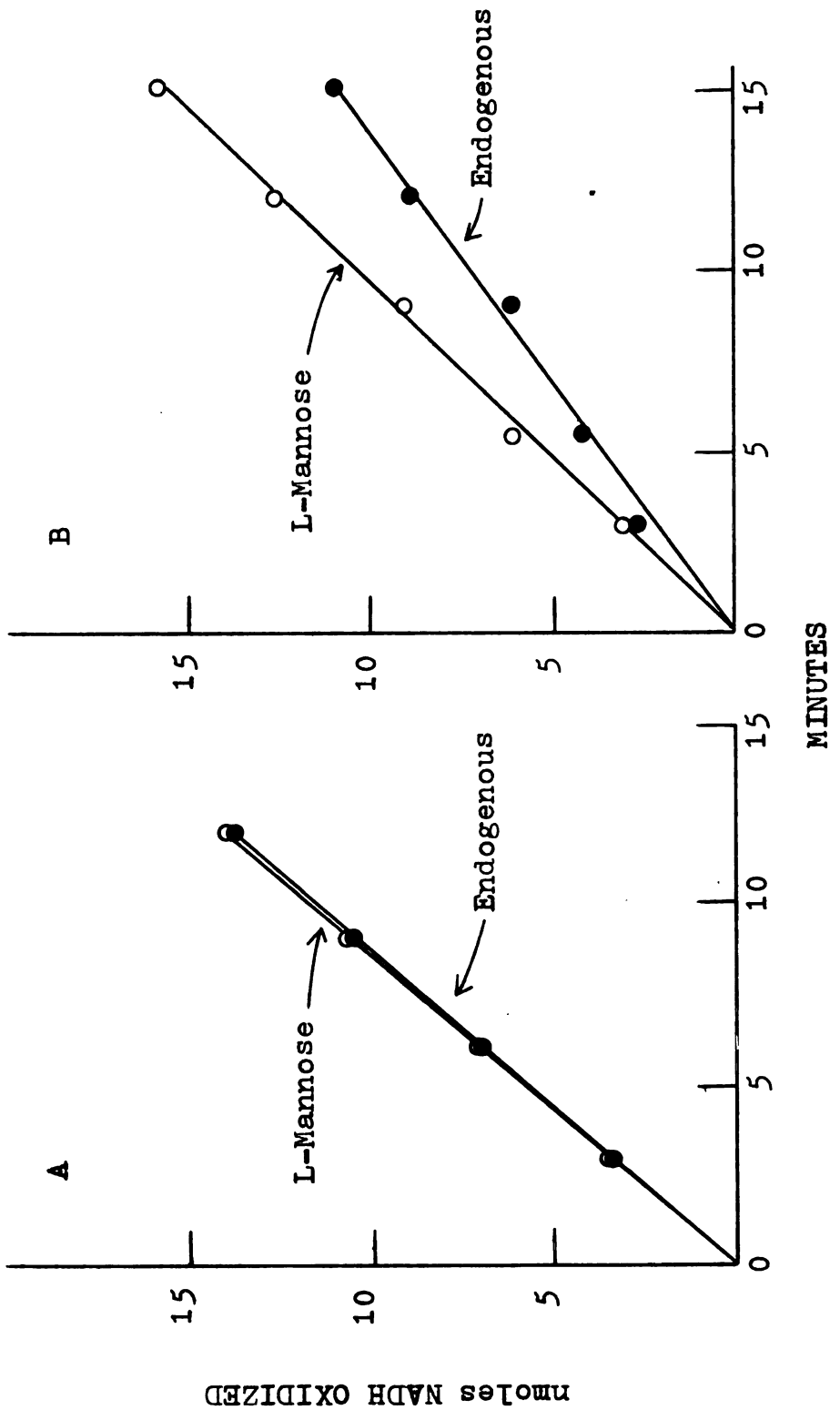

20
15
10
5
0
20
40
60
µmoles HEXOSE
MINUTES
L-Mannose or
D-Glucose
(No ATP)
L-Mannose + ATP
D-Glucose + ATP

Fig. 5. Lactate-dehydrogenase-pyruvate kinase-linked assay for the apparent phosphorylation of L-mannose with ATP. (Refer to Assay section on L-fructose kinase for details). Reaction mixtures (70 µl) containing 2.5 µmoles of L-mannose, 1 µmole of tris-HCl buffer (pH 7.6), 1 µmole of $MgCl_2$, and crude extract (0.024 mg of protein), were incubated for 1 hour at room temperature, after which ATP, lactate dehydrogenase and NADH were added and activity recorded (B). The left portion of the graph (A) depicts a control in which ATP and NADH were added at zero time.

Figure 5.


nmoles NADH OXIDIZED
A
B
L-Mannose
Endogenous
L-Mannose
Endogenous
0
5
10
15
MINUTES

mannose was converted to another intermediate prior to phosphorylation. Such a conversion might conceivably involve a phosphotransferase reaction as demonstrated by Kamel and Anderson (26). They reported that when D-mannose was incubated with crude extracts, D-glucose accumulated due to phosphorylation of D-mannose with endogenous D-glucose-6-P. In the present investigation, however, L-mannose was found to be isomerized to L-fructose.

Isomerization of L-Mannose to L-Fructose- L-Mannose was incubated with crude extracts and the mixture chromatographed on paper. Figure 6 shows the accumulation of a spot which co-chromatographed with authentic D-fructose and gave a positive orcinol test suggesting that L-mannose undergoes an isomerization to L-fructose. The preparation and isolation of the product of L-mannose isomerization is described below.

Enzymic Preparation of L-Fructose- The reaction mixture (75 ml) consisted of the following: 28 mmoles of L-mannose, 5 mmoles of $CoCl_2$, 12 mmoles of tris-HCl buffer (pH 7.6), and cell-free extract (300-350 mg of protein). The reaction was incubated at 30° for 2-3 hours or until equilibrium was established. At equilibrium, the ratio of L-mannose:L-fructose was 38:62 (Figure 7). The reaction

Fig. 6. Chromatography of the products of L-mannose isomerization. The reaction mixture (1.4 ml) consisted of 20 μmoles of L-mannose, 100 μmoles of glycylglycine buffer (pH 7.5), and a volume of crude extract containing 20 mg of protein. The reaction was incubated at 30° and 0.6 ml aliquots were removed at time intervals (t), heated in a boiling water-bath, and the denatured protein removed by centrifugation. The supernatant was concentrated in a vacuum to about 50 μl and a volume of this solution containing about 0.5 μmole of hexose was spotted on paper and then developed in water-saturated phenol for 24 hours.

Figure 6.

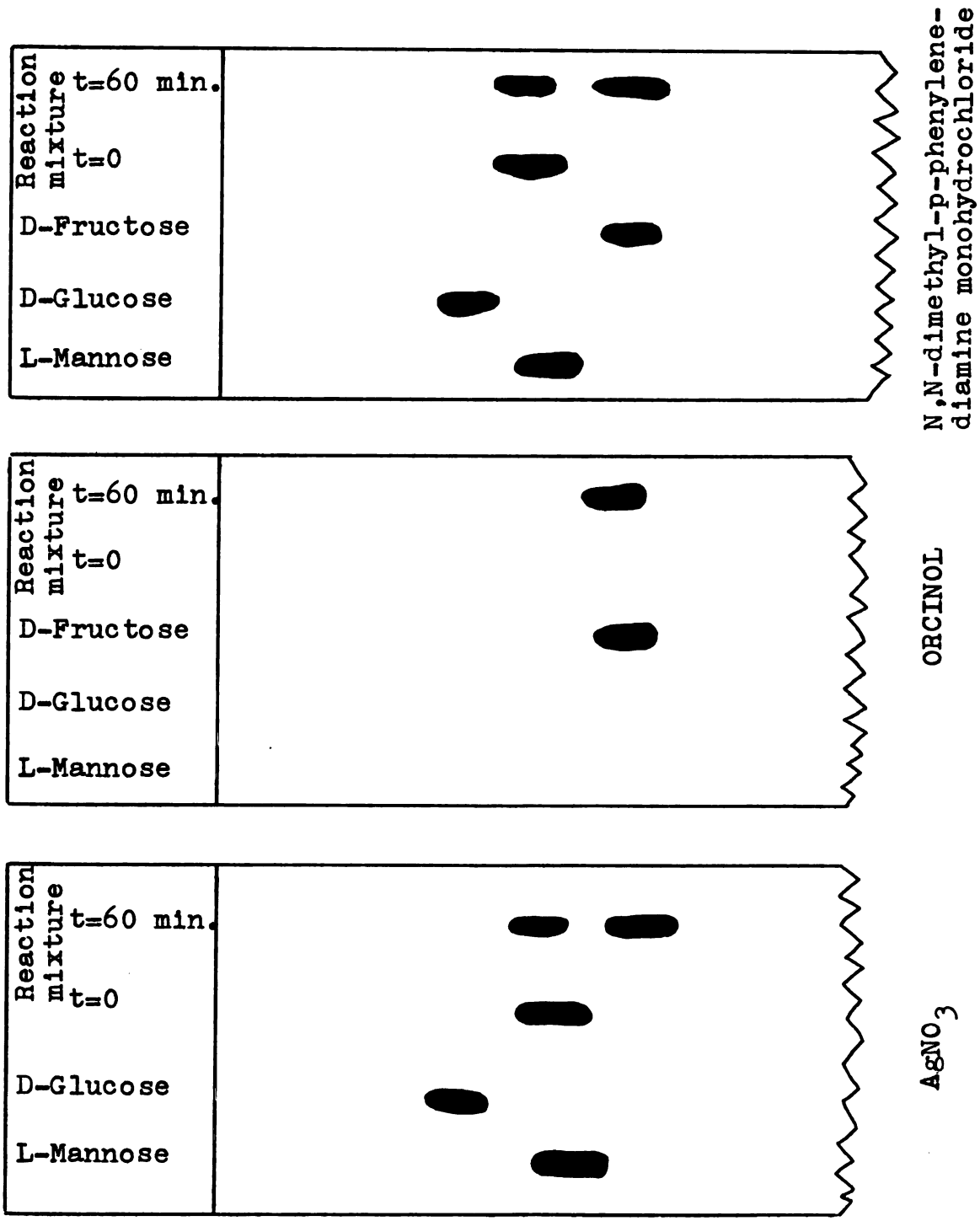
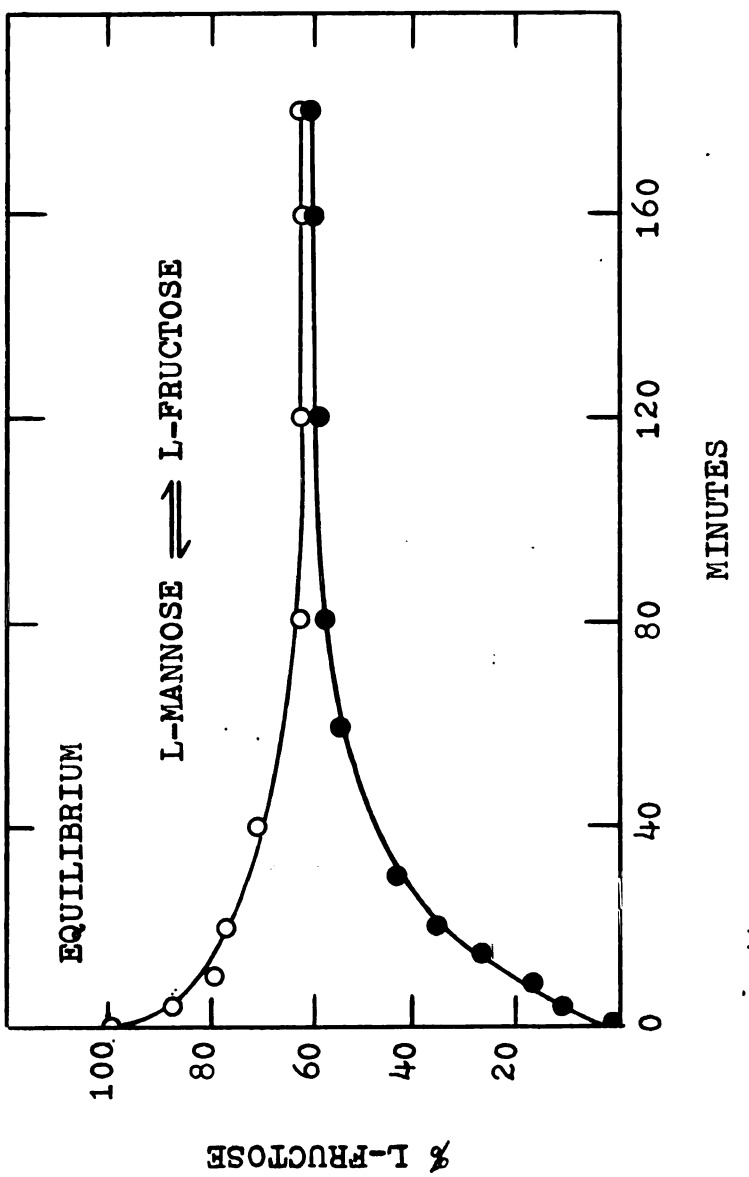

Reaction mixture
t=60 min.
t=0
D-Fructose
D-Glucose
L-Mannose
N,N-dimethyl-p-phenylene-diamine monohydrochloride
Reaction mixture
t=60 min.
t=0
D-Fructose
D-Glucose
L-Mannose
ORCINOL
Reaction mixture
t=60 min.
t=0
D-Glucose
L-Mannose
AgNO3

Fig. 7. Equilibrium of the L-mannose ⇄ L-fructose interconversion. The reaction mixture (1.16 ml) consisted of 10 μmoles of L-mannose or L-fructose, 6 μmoles of $CoCl_2$, 20 μmoles of tris-HCl buffer (pH 7.6), and crude extract (4.4 mg of protein). The mixture was incubated at 30°, and at time intervals samples were removed and assayed for fructose.

Figure 7.


EQUILIBRIUM
L-MANNOSE ⇌ L-FRUCTOSE
% L-FRUCTOSE
100
80
60
40
20
0
40
80
120
160
MINUTES

mixture then was heated in a boiling water-bath for several minutes, and the denatured protein removed by centrifugation at 32,000 x g for 15 minutes. Next, L-mannose was separated from L-fructose by adding a solution containing 3.1 g of phenylhydrazine in 7.0 ml of glacial acetic acid and leaving the resulting mixture overnight at 4°. The L-mannose phenylhydrazone which crystallized was removed by suction filtration. The filtrate then was reduced under vacuum to about 50-60 ml and stored an additional 2-3 hours at 4°. The precipitate that formed was removed by suction filtration. Free L-fructose was regenerated from the phenylhydrazone by refluxing the filtrate for 2-3 hours with 13 ml of ethanol, 8 ml of benzaldehyde, and 0.8 g of benzoic acid. The liquid phase, after being cooled to room temperature, was decanted from the insoluble benzyl phenylhydrazone, washed with three 100-ml portions of chloroform, and decolorized with Darco G-60. This solution (about 60 ml) was deionized by passage through a column of mixed-bed resins consisting of Duolite C-25 (H^+) and A-6 (OH^-) (2.7 cm x 30 cm). About 500 ml were collected, which yielded a 96% recovery of L-fructose. The neutral effluent was reduced in a vacuum to a syrup. L-fructose was crystallized as fine needles by dissolving the syrup

in warm absolute alcohol, seeding with crystals of L-fructose, and leaving at room temperature for 24 hours. The crystals were collected by gravity filtration and the mother liquor reprocessed to give additional product. The overall yield of L-fructose was 1.4-1.6 g (28-32%), m.p. 93-95°, $[\alpha]_{5578}^{24}$ + 91.0° (c 2.5, water) (27). Figure 8 is a photograph of the L-fructose crystals and Figure 9 is an outline of the procedure for the preparation of L-fructose.

Preparation of L-Glucose Phenylosazone- Both fructose and glucose produce the same osazones when heated with excess phenylhydrazine. The osazone derivative was prepared by the procedure of Garard and Sherman (28). The reaction mixture (10.0 ml) consisted of 0.1 g of L-fructose, 1 g of phenylhydrazine, 1.25 g of glacial acetic acid, and 0.68 g of sodium acetate. The pH of the resulting mixture was 4.0-4.2. The reaction mixture was heated in a boiling water-bath for 30 minutes during which time bright yellow needles of L-glucose phenylosazone appeared. The product was collected by gravity filtration, washed with five 10-ml portions of water, and dried in an oven at 100°. This same procedure also was used to make D-glucose phenylosazone from D-fructose and D-glucose. All of the products gave a melting point

Fig. 8. Crystals of L-fructose. The crystals were obtained from the first crop crystallized from absolute alcohol. They were photographed under an AO Spencer phasestar microscope fitted with an ortho-illuminator. They were photographed with Kodak 35 mm Panatomic-x film through the 43x objective, giving a magnification of 860x on the film. Final magnification on the photograph is 3,600x.

Figure 8.

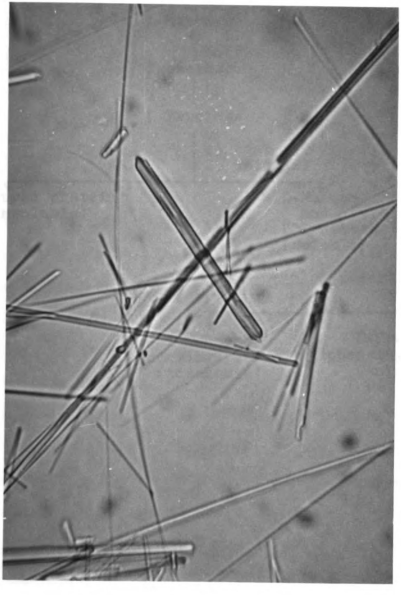
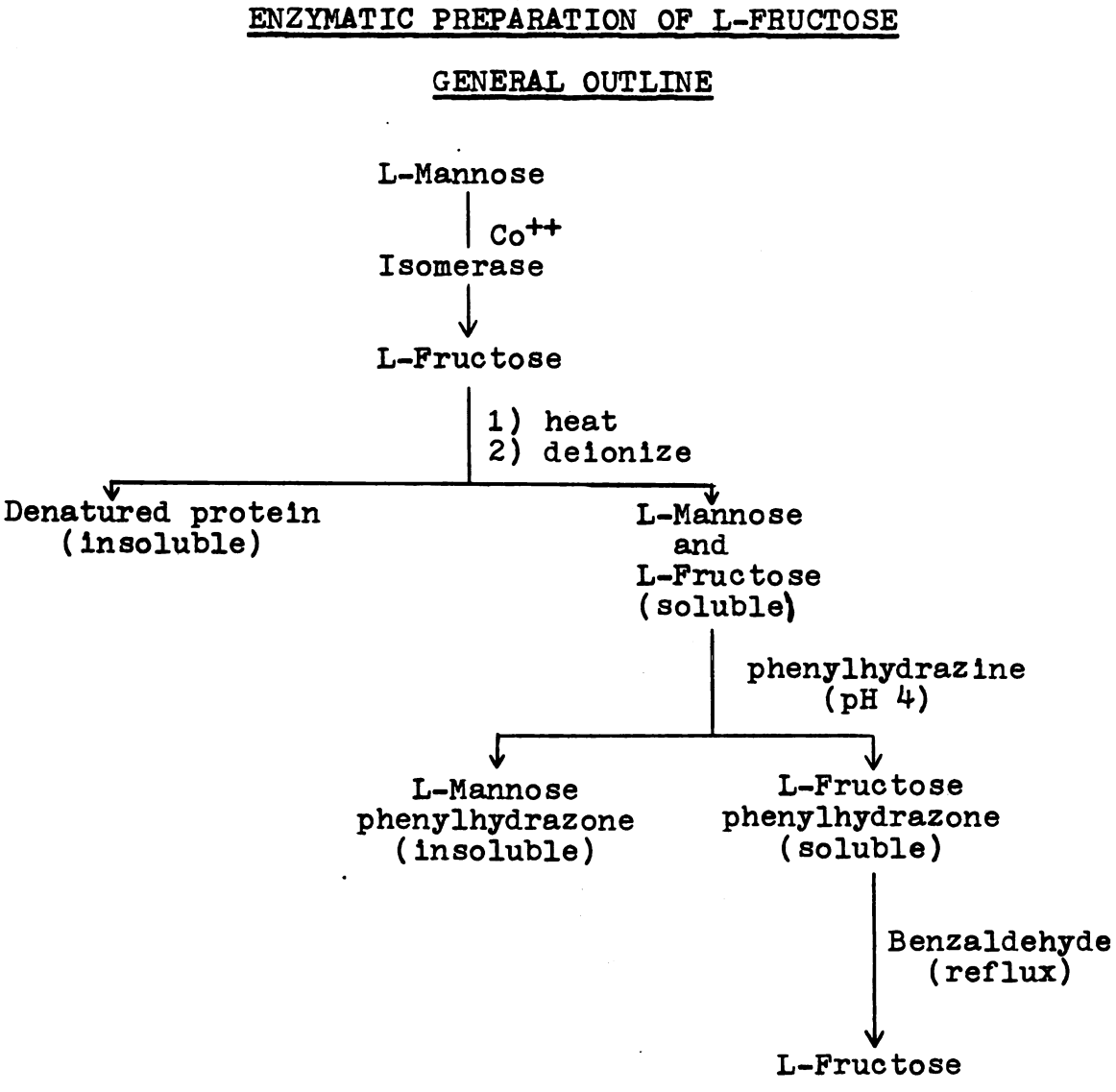


Figure 9.


ENZYMATIC PREPARATION OF L-FRUCTOSE
GENERAL OUTLINE
L-Mannose
Co++
Isomerase
L-Fructose
1) heat
2) deionize
Denatured protein
(insoluble)
L-Mannose
and
L-Fructose
(soluble)
phenylhydrazine
(pH 4)
L-Mannose
phenylhydrazone
(insoluble)
L-Fructose
phenylhydrazone
(soluble)
Benzaldehyde
(reflux)
L-Fructose

of 205-208° in good agreement with the reported values.

Preparation of Phenyl-L-glucosotriazole- The osazones previously prepared were converted to the triazole derivative by the method of Hann and Hudson (29). The reaction mixture consisted of 0.1 g of L-glucose phenylosazone, 0.3 g of copper sulfate, 0.5 cc of 0.5 N H_2SO_4, 6 ml of methyl alcohol, and 9 ml of water. The mixture was refluxed for 2 hours during which time the color of the reaction changed from a deep red-brown to yellow-green. The solution was cooled and then concentrated on a steam-bath to about 4-5 ml. After cooling in the refrigerator for 3 hours a brown precipitate formed which was collected by gravity filtration, washed thoroughly with water, and then dissolved in 15 ml of hot water. The solution was decolorized by Darco G-60, filtered hot, and left at 4° overnight. The white needles which crystallized were collected by gravity filtration and dried in an oven at 90°. The phenyl-L-glucosotriazole had a melting point of 193-197° and a $[\alpha]^{24}_{578} = +80.0°$ in good agreement with reported values.

Water Content of L-Fructose- The enzymatically prepared L-fructose was essentially anhydrous as was also reported by Wolfrom (30). Table I summarizes the per cent loss in weight of water from L-fructose, and commercial D-fructose and L-rhamnose (monohydrate) in

TABLE I

Water content of L-fructose

The water content of L-fructose was determined in an Abderhalden drying apparatus. D-Fructose (Pfanstiehl) and L-rhamnose (General Biochemicals) were used as standards. The solvents, t-butyl alcohol (b.p. 81°) and toluene (b.p. 110°), were Mallinckrodt reagent grade chemicals. The contents of the tube were heated at solvent temperatures for two hours. The samples then were cooled to room temperature and weighed on a Mettler analytical balance. None of the samples possessed hygroscopic properties.

Solvent	Compound	Initial Weight (mg)	Final Weight (mg)	Loss in Weight (mg)	% Loss in Weight
t-butyl alcohol	L-fructose	98.9	97.6	1.3	1.3
	D-fructose	197.9	197.9	0	0
	L-rhamnose (monohydrate)	200.2	190.8	9.4	4.7
toluene	L-fructose	81.0	79.8	1.2	1.5
	L-rhamnose (monohydrate)	199.2	187.6	11.6	5.7

the presence of two different boiling-point solvents.

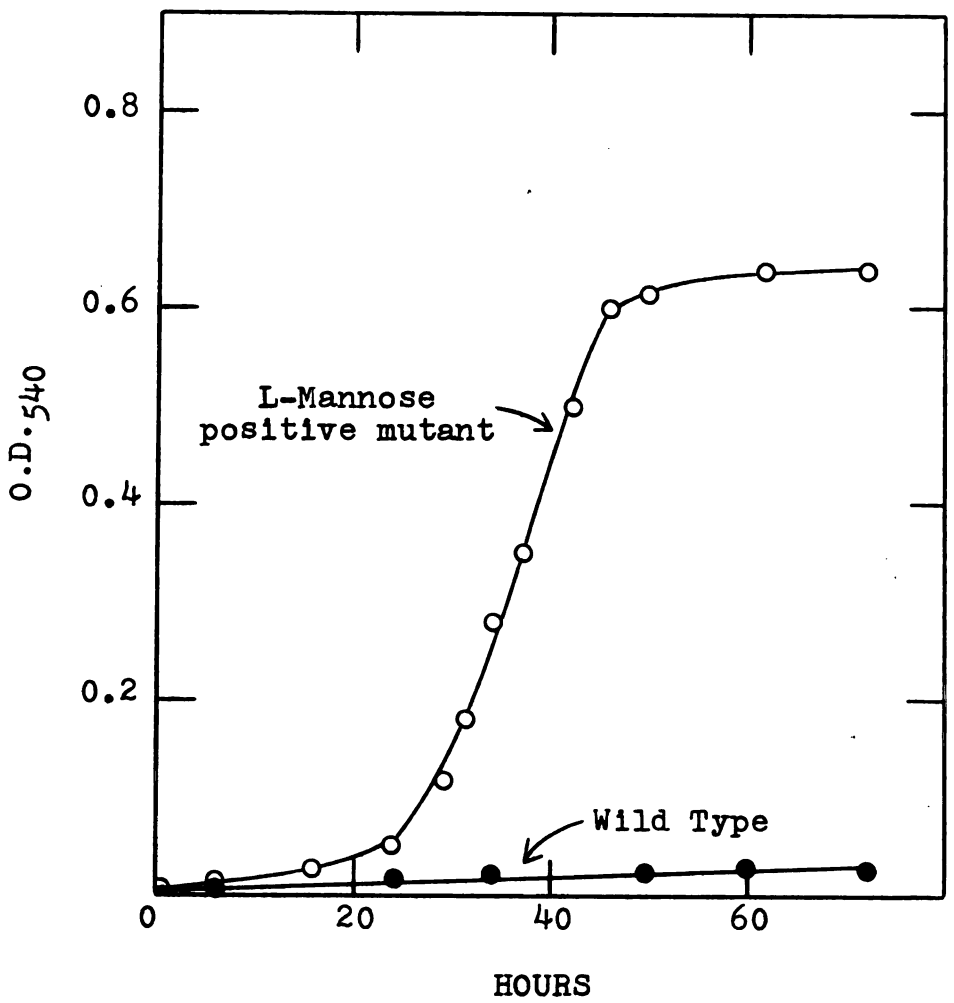
The water content of fructose influences its crystalline form. D-Fructose crystallizes as fine needles of the hemihydrate which, when vigorously shaken at 0°, is converted to prisms of D-fructose dihydrate (31). Seeding the hemihydrate above 20° with anhydrous D-fructose changes the needles to prisms of the anhydrous form (32). Although anhydrous D-fructose does not appear to crystallize as needles, anhydrous L-fructose does (30).

Growth of A. aerogenes on L-Fructose- The L-mannose-positive strain, unlike the wild-type, is capable of growing on L-fructose as the sole carbon source (Figure 10).

Phosphorylation of L-Fructose to L-Fructose 1-Phosphate- The L-fructose enzymatically prepared from L-mannose was used to determine the next reaction in the pathway. Since the utilization of ATP in crude extracts still had not been explained, investigations were conducted to determine if L-fructose was the phosphorylated intermediate. The results showed that crude extracts from L-mannose-grown cells possessed a kinase which phosphorylated L-fructose with ATP. The isolation and characterization of the phosphorylated product is described below.

Fig. 10. Growth of A. aerogenes PRL-R3 on L-fructose. A suspension (0.05 ml) of either wild-type or L-mannose-positive cells previously grown in nutrient broth were inoculated into 7.0 ml of mineral medium supplemented with L-fructose (0.28%). The cells were allowed to grow at 30° on a reciprocating shaker and growth was recorded by measuring the increase in the turbidity of the culture at 540 nm with a Coleman Junior Spectrophotometer.

Figure 10.


0.8
0.6
0.4
0.2
O.D.540
L-Mannose
positive mutant
Wild Type
0
20
40
60
HOURS

Enzymic Preparation of L-Fructose 1-Phosphate- L-Fructose 1-phosphate was prepared by the following procedure: the reaction mixture (100 ml) consisted of 1.0 mmole of L-fructose, 1.2 mmoles of ATP, 1.2 mmoles of $MgCl_2$, and 7-fold-purified L-fructokinase (8.2 mg of protein); details of the purification procedure are outlined in Part II of this thesis. The reaction was carried out at 22° and at a pH of 7.5. The extent of phosphorylation was determined by automatic titration (Sargent recording pH-stat) with 0.5 N NaOH. After 8 hours the reaction was complete. The resulting mixture was chilled in ice, 2.4 mmoles of barium acetate were added, and the resulting mixture was left an additional 30 minutes in the ice bath. The precipitate that formed was removed by centrifugation. The pH of the supernatant was then brought to pH 2.0 with hydrobromic acid and the 260 nm-absorbing material removed by treatment with Darco G-60. The pH was increased to 8.0 with NaOH and the solution cooled in an ice bath. Cold ethanol then was added to a final concentration of 80% and the resulting solution left at 4° overnight, during which time a white flocculent precipitate of barium L-fructose 1-phosphate formed. The precipitate was washed with 80%, 90%, 95%, and absolute alcohol and then with anhydrous

ether. The final yield was 322 mg (81%). Figure 11 outlines the procedure.

Identification of L-Fructose 1-phosphate:

(a) Paper Chromatography- The barium salt of the product was deionized by Dowex 50W-X8(H^+) and the free sugar-phosphate chromatographed on paper. The product co-chromatographed with D-fructose 1-phosphate and was distinguishable from both D-fructose 6-phosphate and D-fructose 1,6-diphosphate.

(b) Fructose to Phosphorus Ratio- The fructose to phosphorus ratio was 1 to 1 and the amount of phosphate released after 80 minutes hydrolysis in 1 N H_2SO_4 was equivalent to that released when the sugar-phosphate was hydrolyzed by 10 N H_2SO_4 (Table II).

(c) Polarimetry- The product had a specific rotation of $+50.7^{\circ}$ compared to -51.0° for D-fructose 1-phosphate.

(d) Wheat Germ Acid Phosphatase- When the product was dephosphorylated by wheat germ acid phosphatase and chromatographed on paper, a single spot appeared which co-chromatographed with authentic D-fructose in two solvent systems.

(e) Acid Labile Phosphate- Figure 12 compares the rate of phosphate released when the product was hydrolyzed by 1 N H_2SO_4. Both L- and D-fructose 1-phosphate are

Figure 11.

ENZYMATIC PREPARATION OF L-FRUCTOSE-1-PHOSPHATE

GENERAL OUTLINE

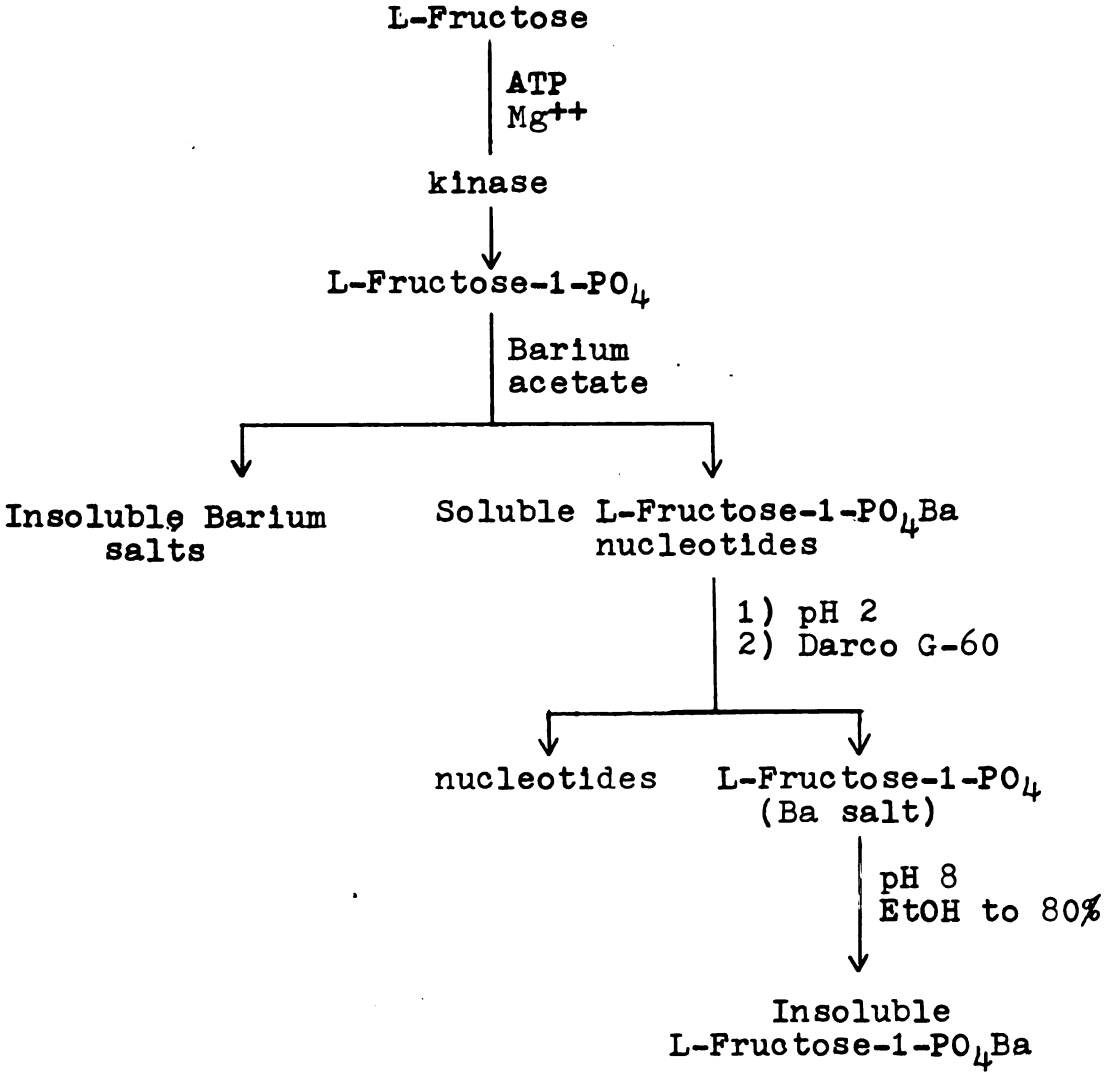


TABLE II

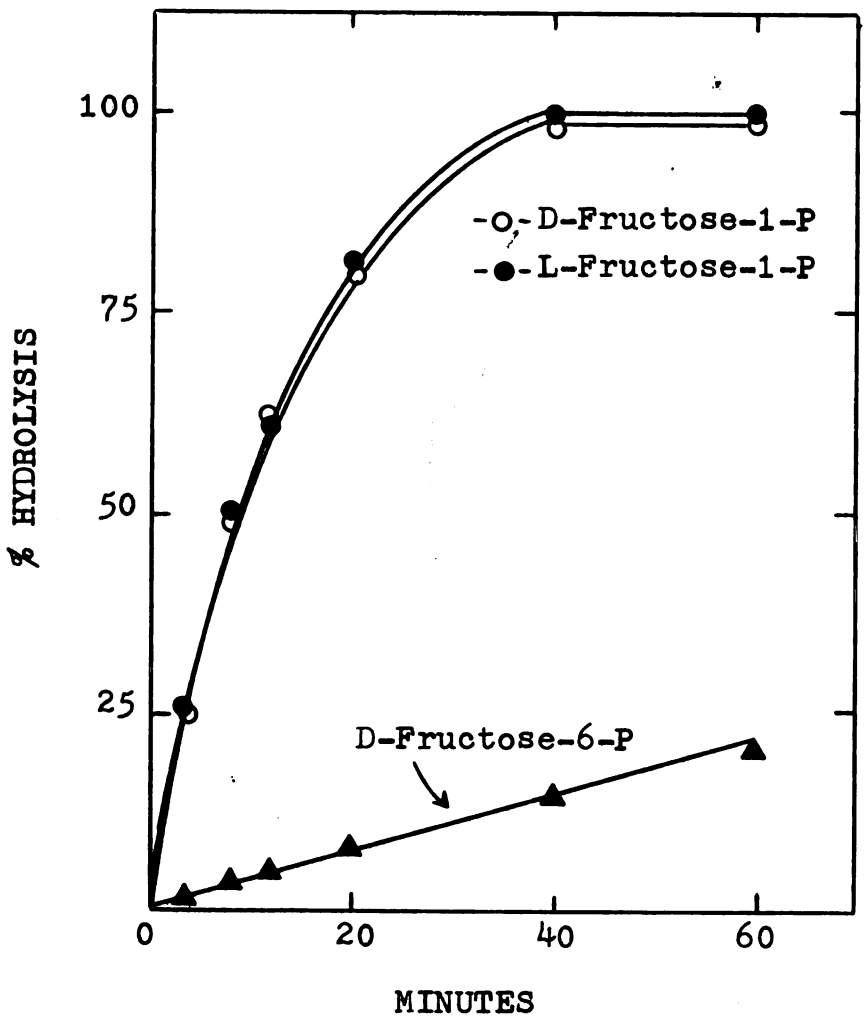
Phosphate to fructose ratio of L-fructose-1-P

Aliquots of L-fructose 1-phosphate were added to 4.0 ml of 1 N H_2SO_4 and the solution placed in a boiling water-bath for 80 minutes. The solution was cooled and assayed for total free fructose and phosphorus. Fructose was determined by the Roe test. Inorganic phosphate was measured by a modified procedure of Fiske and SubbaRow.

Compound assayed	Amount
	μmoles
Before dephosphorylation:	
L-Fructose-1-P	0.88
Total Phosphate	0.86
After dephosphorylation:	
L-Fructose	0.86
Inorganic Orthophosphate	0.85

Fig. 12. Acid hydrolysis of D-fructose-1-P, D-fructose-6-P, and the product of L-fructose phosphorylation. Tubes containing 1.0 μmole of the indicated fructose ester in 4.0 ml of 1 N H_2SO_4 were heated in a boiling water-bath. At time intervals, they were removed and the contents assayed for inorganic orthophosphate.

Figure 12.


100
75
50
25
% HYDROLYSIS
-o- D-Fructose-1-P
-●- L-Fructose-1-P
D-Fructose-6-P
0
20
40
60
MINUTES

hydrolyzed at the same rate. D-Fructose 6-phosphate hydrolyzes at a slower rate, and on this basis the C-1 phosphate is distinguishable from the C-6 phosphate. The half-life (50% hydrolysis) of both the product and D-fructose 1-phosphate was 8 minutes.

(f) Gas Chromatography- The gas chromatography patterns of the trimethysilyl derivatives of D- and L-fructose-1-P are similar (Figure 13).

Enzymic Cleavage of L-Fructose 1-Phosphate- The metabolism of L-fructose 1-phosphate undergoes a direct cleavage to dihydroxyacetone phosphate and L-glyceraldehyde. Figure 14 shows a direct relation between the rate of cleavage of L-fructose 1-phosphate and the rate of formation of trioses. Dihydroxyacetone phosphate was identified by coupling to NADH linked α-glycerol phosphate dehydrogenase. The rate at which dihydroxyacetone phosphate forms also is an indication of the aldolase activity (see assay section on aldolase). The other cleavage product has not been specifically identified but would be expected to be L-glyceraldehyde. Cleavage of L-fructose-1-P by either crude extracts or the partially purified aldolase fraction could not be detected in the absence of a coupling system to remove one of the products, indicating that the equilibrium of the reaction

Fig. 13. Gas chromatography: preparation of the trimethylsilyl derivatives of fructose-1-P. A solution containing 600 μg of the phosphorylated product of L-fructose was concentrated in a vacuum and the residue dissolved in 2.0 ml of methanol and diluted with 2 ml of diethyl ether. The phosphoric acid group was esterfied with diazomethane generated from N-methyl-N-nitroso-p-toluenesulfonamide. The resulting mixture was concentrated in a vacuum and the residue dissolved in 10 ml of pyridine. Trimethysilylation was carried out by adding 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. About 3 μg of the product was used for a sample run (33).

Figure 13.

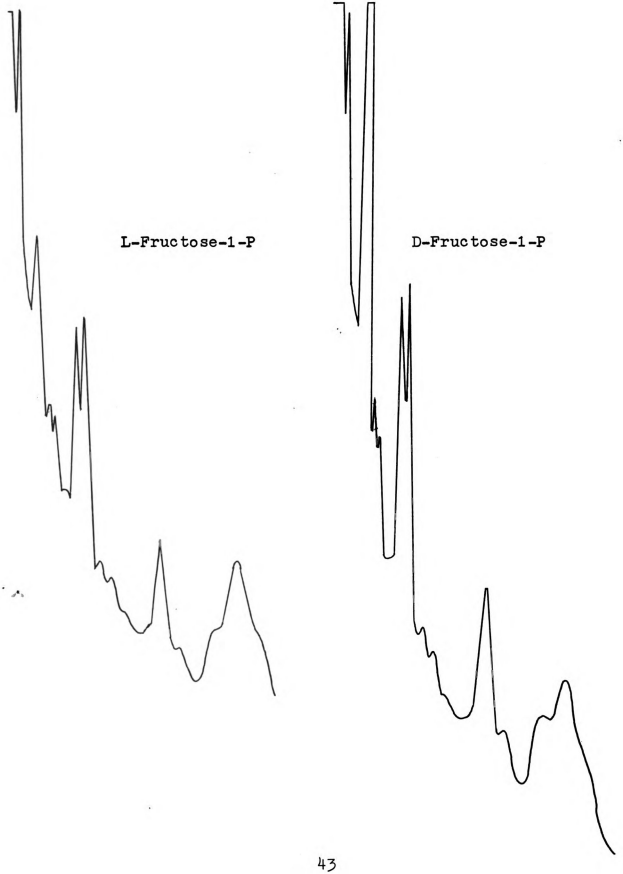
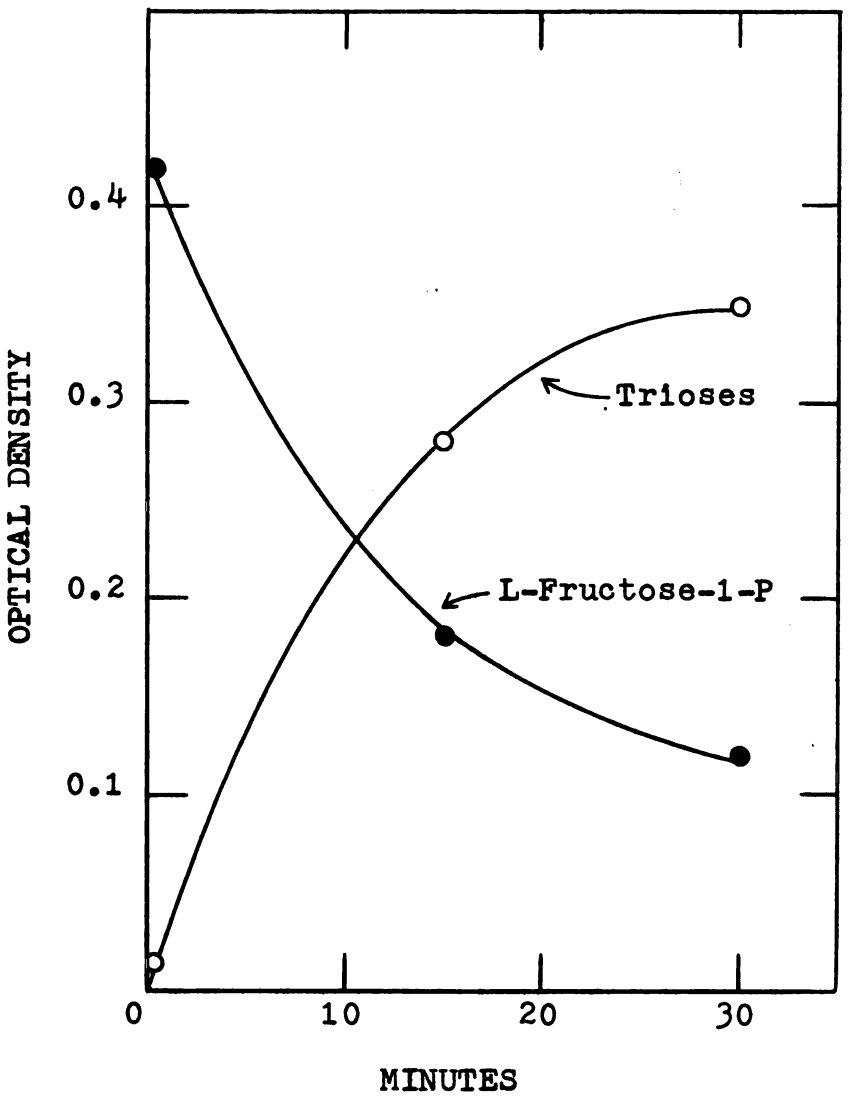


Fig. 14. Triose formation from L-fructose-1-P. The reaction mixture (0.9 ml) consisted of 3.5 μmoles of L-fructose 1-phosphate, 20 μmoles of hydrazine sulfate, 80 μmoles of glycylglycine buffer, pH 7.5, and crude extract (10-12 mg of protein). The reaction was incubated at 30° and at time intervals 0.1 ml aliquots were removed and assayed for L-fructose 1-phosphate ($O.D._{520}$) (11) and trioses ($O.D._{540}$) (14).

Figure 14.


OPTICAL DENSITY
0.4
0.3
0.2
0.1
0
10
20
30
MINUTES
Trioses
L-Fructose-1-P

lies in the direction of synthesis. That the equilibrium strongly favors the fructose ester also has been noted for D-fructose-1-P aldolase (34).

Test for Possible Epimerization- The possibility that epimerization of L-fructose-1-P to some other ketose-1-P preceded cleavage was ruled out as follows. L-Fructose-1-P (10 μmoles) was incubated at 25° with 1.0 ml of partially purified L-fructose-1-P aldolase (2.4 mg of protein) in 0.02 M tris-HCl buffer (pH 7.6). After 2.5 hours, H_2SO_4 was added to a final concentration of 1 N, and the precipitated protein was removed by centrifugation. The supernatant solution was heated in a boiling water-bath for 1 hour, cooled, and deionized by passage through a mixed-bed of Duolite C-25(H^+) and A-6(OH^-) (35). The neutral effluent was concentrated *in vacuo* and chromatographed on paper. Visualization of the spots with orcinol revealed a single spot which co-chromatographed with fructose but not with tagatose, sorbose, or psicose. Thus, it may be concluded that L-fructose-1-P is the substrate for the aldolase and is not epimerized to some other ketohexose-1-P prior to cleavage.

A. aerogenes degrades L-mannose into products which the cell can readily use for energy and growth. Presumably dihydroxyacetone phosphate is further metabolized

via triose phosphate isomerase. On the other hand, L-glyceraldehyde was degraded by at least two distinct pathways: 1) reduction to glycerol; and 2) oxidation to glyceric acid.

Reduction of L-Glyceraldehyde to Glycerol- An enzyme was detected in ammonium sulfate fractions of the crude extract which reduced L-glyceraldehyde to glycerol. A large scale incubation mixture (0.8 ml) consisting of 20 μmoles of L-glyceraldehyde, 30 μmoles of NADH, and about 10 mg of protein from the 40-60% $(NH_4)_2SO_4$ fraction was incubated at 30°. At zero time and at 60 minutes, incubation samples were withdrawn and placed in a boiling water-bath. The denatured protein was removed by centrifugation and the supernatant concentrated in a vacuum to about 50-100 μliters. Aliquots were spotted on paper and the chromatogram developed (See EXPERIMENTAL PROCEDURE). In the reaction mixture incubated for 60 minutes, three spots appeared which co-chromatographed with glycerol, glyceraldehyde, and glyceric acid.

Oxidation of L-Glyceraldehyde to Glyceric Acid- The presence of glyceric acid as another product of the reaction indicated the presence of another enzyme in the ammonium sulfate fraction which oxidized L-glyceral-

dehyde to L-glyceric acid. To further show that glyceric acid was also a product of L-glyceraldehyde metabolism a large scale incubation mixture containing L-glyceraldehyde, NAD, and enzyme was incubated as previously described. The results showed that when NAD was present, glycerol and glyceric acid accumulated, indicating a dismutation existed in the metabolism of L-glyceraldehyde in which NADH and NAD were interconverted by the enzymic oxidation and reduction of L-glyceraldehyde.

DISCUSSION

The pathway of L-mannose metabolism in A. aerogenes as deduced from experiments presented in Part I of this thesis is depicted in Figure 15. L-Mannose is initially isomerized to L-fructose, which is phosphorylated with ATP by a kinase to L-fructose 1-phosphate. L-Fructose 1-phosphate is then cleaved by the action of an aldolase to yield dihydroxyacetone phosphate and L-glyceraldehyde.

The sequence of reactions reported here is similar to the sequences involved in the degradation of the naturally occurring 6-deoxy hexoses, L-rhamnose and L-fucose, in that they too undergo sequential isomerization, phosphorylation at carbon atom one, and cleavage (36-43). However, no underived aldohexose other than L-mannose has been reported to be metabolized through such a sequence

Figure 15.

PATHWAY OF L-MANNOSE DEGRADATION IN AEROBACTER AEROGENES

in any organism.

The enzymic isomerization of L-fructose from L-mannose is a convenient method for the preparation of this rare ketohexose. L-Fructose first was synthesized by Fischer (44), who treated α-acrose with phenylhydrazine. The resulting D,L-glucose plenylosazone was isolated, hydrolyzed to the osone, and then reduced to D,L-fructose. This subsequently was digested by yeast to yield a solution of L-fructose which was identified by its L-glucose phenylosazone, but was not, itself, isolated in crystalline form. L-Fructose was later synthesized by Wolfrom and Thompson (30) by the following sequence of reactions: L-arabonic acid tetraacetate → L-arabonyl chloride tetraacetate → 1-diazo-1-deoxy-keto-L-fructose tetraacetate → keto-L-fructose pentaacetate → L-fructose. The L-fructose was crystallized and identified by polarimetry, derivatives, and elemental analysis. To date this procedure is the only satisfactory method available for the synthesis of L-fructose.

The enzymic method for the preparation of L-fructose described here in this thesis is considerably shorter than the chemical method previously described (30). It involves only the isomerization of L-mannose, a compound which can be readily prepared or purchased;

the enzyme does not need to be purified, but can be used directly as it occurs in the cell-free extracts. Unreacted L-mannose may be recovered from the equilibrium mixture by regeneration from its phenylhydrazone. The yield of L-fructose (28-32%) is equal to or better than that obtained with the chemical method starting from L-arabonic acid tetraacetate (30).

Although the isomerization of L-mannose to L-fructose represents a new enzymic reaction, the isolation of L-fructose is an adaptation of a standard procedure. Mannose phenylhydrazone is well known for its low solubility in water; thus, it has been used to separate mannose quantitatively from other hexoses. Sowden and Fischer succeeded in separating L-mannose from L-glucose through their corresponding phenylhydrazones (33), and I have succeeded in separating L-mannose from L-fructose by the same means. Two precautions might specifically be noted. First, a high excess of phenylhydrazine should be avoided because L-fructose and L-fructose plenylhydrazone readily react with the excess reagent to produce insoluble L-glucose phenylosazone (45), thus decreasing the yield of L-fructose. Second, mixed-bed resins possessing strong anionic groups should not be used for deionizing solutions of fructose because they cause partial isomerization to glucose.

Since the extent of isomerization is based solely on establishing equilibrium, the amount of L-fructose that can be obtained is limited only by the amount of L-mannose available; the time required to reach equilibrium is inversely proportional to the amount of enzyme. Thus, larger quantities of L-fructose could be prepared simply by increasing the initial reaction components.

L-Fructose 1-phosphate is the intermediate in L-fructose metabolism. This is the first report of this sugar phosphate as an intermediary metabolite. On the other hand, A. aerogenes metabolizes D-fructose differently. Hansen and Anderson have demonstrated a four-component system in which D-fructose is phosphorylated with phosphoenolpyruvate at carbon atom one (45a). A second phosphorylation with ATP and an induced kinase yields D-fructose 1,6-diphosphate (46) and this can be metabolized by known reactions.

The metabolism of L-glyceraldehyde in bacteria has not been previously reported; however, its metabolism by A. aerogenes is similar to pathways determined in mammalian tissue. Holldorf and coworkers (47) reported an aldehyde dehydrogenase which oxidized L-glyceraldehyde to L-glyceric acid. Dawkins and Dickens (48) later showed that L-glyceric acid is oxidized further to hydroxypyru-

vate, a precursor to L-serine (49). Glyceric acid accumulated in the growth media from both wild type and L-mannose positive cells which utilized L-mannose. Whether or not some of the glyceric acid was metabolized by the cells is not known. The significance of glyceric acid formation during the utilization of L-mannose by A. aerogenes is considered in more detail in Part III of this thesis.

The reduction of L-glyceraldehyde to glycerol has been demonstrated previously in both mammalian and bacterial sources. Hadorn et al (50) showed in the reverse reaction that horse liver dehydrogenase oxidized glycerol to mainly L-glyceraldehyde and only a small amount of the D-isomer. Burton (51) reported that glycerol dehydrogenase from A. aerogenes reduced glyceraldehyde at a rate 14% that of glycerol, but he did not specify which enantiomorph of glyceraldehyde was reduced. Lin and Magasanik (52, 53) later reported the properties of glycerol dehydrogenase isolated from a capsulated strain of A. aerogenes. In this case, however, specificity studies revealed that the enzyme did not reduce DL-glyceraldehyde. My results showed that L-glyceraldehyde is reduced to glycerol in fractionated extracts of A. aerogenes PRL-R3. The reduction of dihydroxyacetone also has been demonstrated

in these same extracts, suggesting that glycerol is reoxidized to dihydroxyacetone which can be phosphorylated with ATP to yield dihydroxyacetone phosphate. The phosphorylation of glycerol could not be demonstrated in these studies.

It is of interest that L-mannose has not yet been found to occur naturally. Kleczkowski and Wierzchowski (54) reported that Bacillus krzemienlewski (Bacillus circulans (55)) formed a polysaccharide consisting of L-mannosyl residues, but Forsyth and Webley (56) later reported that D-mannose was the correct enantiomorph. Although L-mannose does not occur naturally, it was prepared initially by the Kiliani-Fischer cyanohydrin synthesis (57, 58). However, it can be prepared more conveniently by the method of Sowden and Fischer (22).

L-Fructose, also, has not yet been found to occur naturally. Ahmed, Rizk, and Hammouda (59) have reported that L-fructose is a constituent of several species of Egyptian Plantago; however, because the sugar was identified only by chromatography on paper, a procedure which does not distinguish between the D and L enantiomorphs, evidence for the natural occurrence of L-fructose must be considered nil. There also are reports that L-fructose is phosphorylated with ATP by cell extracts of trypanosomes (60, 61), but this seems to be in error; a

close inspection of the data reveals that D-fructose must have been intended.

PART II

Common Identity of the Enzymes of L-Mannose and L-Rhamnose Metabolism

Part I of this thesis showed that in A. aerogenes PRL-R3 L-mannose was metabolized by sequential isomerization, phosphorylation at carbon atom one, and cleavage. This sequence of reactions is similar to the sequence by which L-rhamnose (6-deoxy-L-mannose) has shown to be degraded in Pasteurella pestis (36), Lactobacillus plantarum (37, 38), and Escherichia coli (39-41). In each of these cases, however, the enzymes involved in the degradation of L-rhamnose either did not degrade L-mannose or the investigators did not use L-mannose or its metabolic intermediates as test substrates. Part II of this thesis describes the characteristics of the first three enzymes of the L-mannose degradative pathway in A. aerogenes PRL-R3 and confirms that in this organism both L-mannose and L-rhamnose are metabolized by the same enzymes.

EXPERIMENTAL PROCEDURE

Growth of Cells and Preparation of Extracts- Unless otherwise stated, the cells were grown aerobically 10-12 hours on mineral medium containing 0.4% L-rhamnose. The cell extracts were prepared as described in Part I. The

isomeraseless mutant (B-22), obtained by mutagenesis with ethyl methanesulfonate, was provided by Dr. R.L. Anderson.

Analytical Procedures- L-Rhamnulose was determined by the method of Dische and Borenfreund (62); 0.1 μmole of L-rhamnulose in 7.4 ml of solution gave a $O.D._{540}$ = 0.28 (18-mm light path) after 10 minutes incubation at 37°. Protein was determined in crude extracts as described in Part I and in fractionated extracts was determined by the ratio of the absorbances at 260 nm and 280 nm (63).

Enzyme Assays- The enzymatic assays were the same as those for L-mannose metabolism, as described in Part I, except for the following changes: for L-rhamnose isomerization the assay contained 16 μmoles of L-rhamnose and 2 μmoles of $MnCl_2$ in place of L-mannose and $CoCl_2$; the kinase assay contained 0.5 μmole of L-rhamnulose in place of L-fructose; and the aldolase assay contained 1.0 μmole of L-rhamnulose 1-phosphate in place of L-fructose 1-phosphate.

Partial Purification of L-Fructose Kinase and L-Fructose-1-P Aldolase- All procedures were carried out at 0-4°. The crude extract was diluted with 0.02 M tris-HCl buffer (pH 7.6) to give a protein concentration of 10 mg/ml. Crystalline ammonium sulfate was added to a

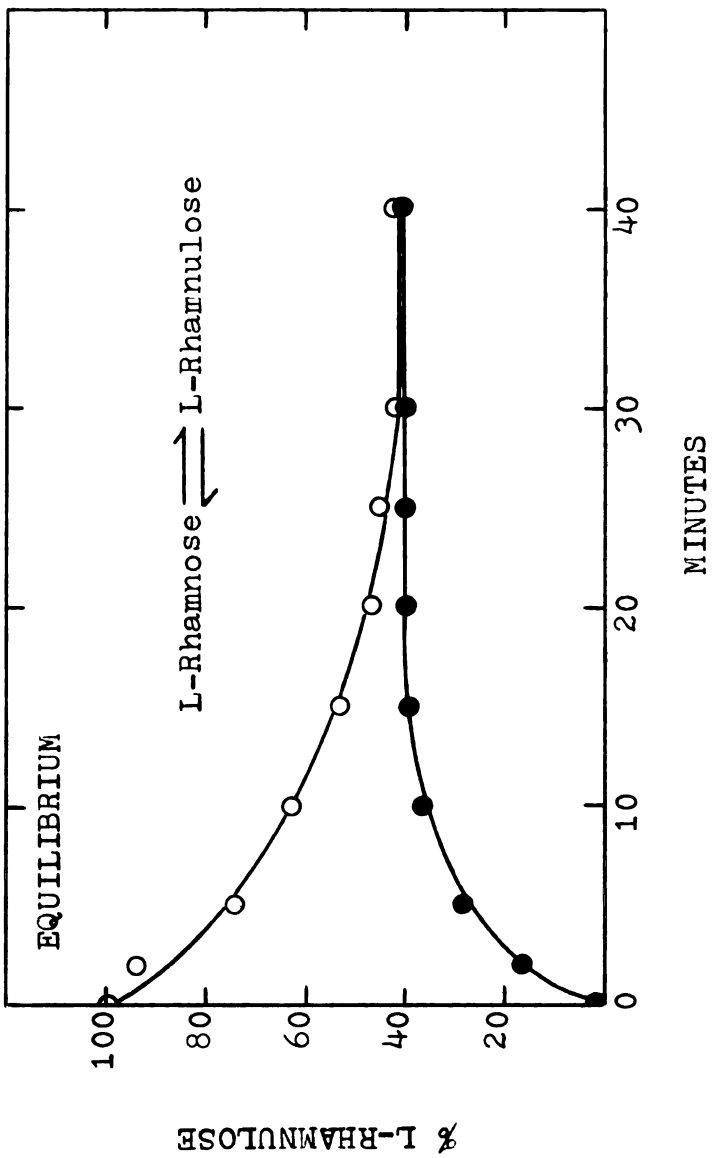
concentration of 0.1 M. A 2% solution of protamine sulfate (pH 7.6) then was added slowly with stirring in an amount equalling 20% (v/v) of the extract. After 10 minutes the precipitate was removed by centrifugation and discarded, and the supernatant solution was fractionated with crystalline ammonium sulfate. The protein precipitating between 40% and 60% saturation was dissolved in 0.02 M tris-HCl buffer (pH 7.6) and was chromatographed on a column of Sephadex G-100 equilibrated with the same buffer. The fractions containing the highest specific activity of each enzyme were combined. Both enzymes were purified about 7-fold with a 20% recovery.

Reagents- L-Rhamnose was purchased from General Biochemicals. Protamine sulfate was purchased from Sigma Chemical Co. L-Glucose was prepared by the modified procedure of Hudson (64) and Frush and Isbell (65). L-Rhamnulose and L-rhamnulose-1-P were prepared enzymatically by the following procedures:

Preparation of L-Rhamnulose- L-Rhamnulose was prepared enzymatically from L-rhamnose. The reaction mixture (40 ml) consisted of 2.8 mmoles of L-rhamnose, 0.07 mmole of $MnCl_2$, and a volume of crude extract containing about 200 mg of protein. The reaction mixture was incubated at 45° until equilibrium was established (Figure 16),

Fig. 16. Equilibrium of the L-rhamnose $\rightleftarrows$ L-rhamnulose interconversion. The reaction mixture (0.6 ml) consisted of 2 μmoles of L-rhamnose or L-rhamnulose, 3 μmoles of $MnCl_2$, 8 μmoles of tris-HCl buffer (pH 7.6), and crude extract (1.1 mg of protein). The mixture was incubated at 30° and at time intervals samples were removed and assayed for rhamnulose.

Figure 16.


% L-RHAMNULOSE
100
80
60
40
20
0
10
20
30
40
MINUTES
EQUILIBRIUM
L-Rhamnose
L-Rhamnulose

after which the solution was heated in a boiling water-bath for several minutes and the denatured protein removed by centrifugation. The supernatant was decolorized by treatment with Darco G-60 and concentrated in a vacuum to about 3.0 ml.

The L-rhamnulose was separated from L-rhamnose by paper chromatography as follows: 13-15 μmoles of crude L-rhamnulose were streaked across the base line of Whatman No. 3 paper, then developed 32 hours in n-butanol: ethanol:water (52:32:16). Strips were cut from the sides and center of the paper and after identifying the L-rhamnulose with silver nitrate (16), the L-rhamnulose band was removed and washed from the paper with water. About 90% of the L-rhamnulose, which was free from L-rhamnose, was recovered by this procedure.

Preparation of L-Rhamnulose-1-Phosphate- L-Rhamnulose-1-P was prepared by the method of Chiu and Feingold (66). The reaction mixture (40 ml) consisted of 0.5 mmole of L-rhamnulose, 0.7 mmole of ATP, 0.25 mmole of $MgCl_2$, and about 8 mg of partially purified kinase (refer to enzyme purification for details). The reaction was performed at room temperature on a Sargent recording pH-Stat; the pH of the reaction was maintained at 7.5 by the addition of 0.2 N NaOH. When phosphorylation

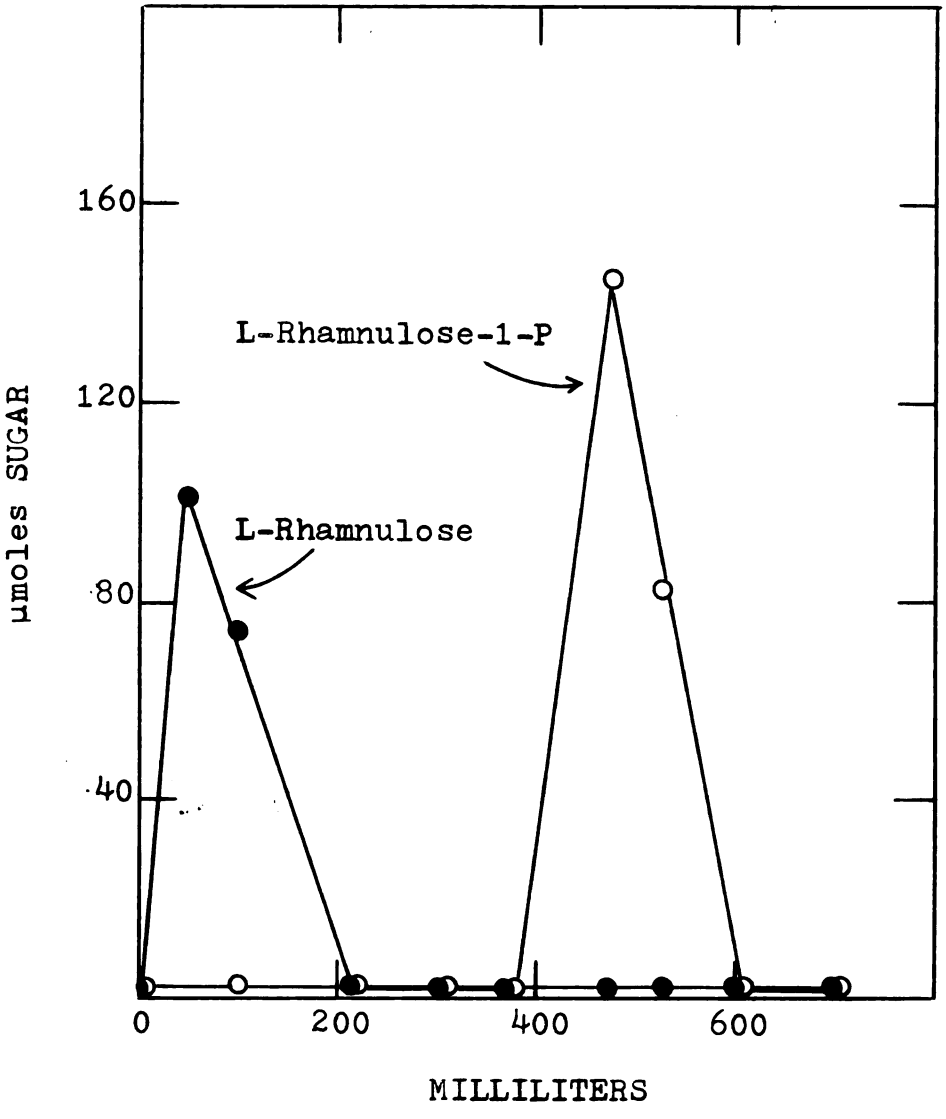
was completed, L-rhamnose was separated from L-rhamnulose-1-P by placing the reaction mixture on a Dowex-1-formate column (24 cm x 1.5 cm). The column was developed by gradient elution with 1 liter of a solution containing 0.4 N formic acid and 0.1 N sodium formate in the reservoir connected to a mixing chamber containing 200 ml of water. 50-ml fractions were collected and assayed for both L-rhamnulose (62) and L-rhamnulose-1-P (measured by the inorganic orthophosphate released (13)). Figure 17 is the elution pattern of L-rhamnulose and L-rhamnulose-1-P from the column. The fractions containing the sugar-phosphate were pooled and concentrated to about 40 ml. The pH of the solution was adjusted to 6.4 with saturated barium hydroxide, after which 400 ml of ethyl alcohol was added [80% (v/v)] and the resulting solution left overnight at 4°. The precipitate that formed was centrifuged, washed with 80% ethyl alcohol, and anhydrous ether, then dried in a vacuum. The final yield of barium L-rhamnulose-1-P was 175 mg.

RESULTS

Whole Cell Fermentation- Cells grown on L-rhamnose or L-mannose readily oxidized both of these hexoses at approximately equivalent rates, whereas cells grown on D-glucose or nutrient broth did not oxidize either L-

Fig. 17. Fractionation of L-rhamnulose-1-P on Dowex-1-formate. L-Rhamnulose-1-P was purified by fractionation on a column of Dowex-1-formate. It was eluted from the column by a formate-formic acid gradient. Details of the procedure are given in the text.

Figure 17.


160
120
80
40
µmoles SUGAR
L-Rhamnulose-1-P
L-Rhamnulose
0
200
400
600
MILLILITERS

hexose (Figures 18 and 19).

Induction of Enzymes by L-Mannose and L-Rhamnose- The isomerase, kinase, and aldolase activities were determined in crude extracts obtained from cells grown either on L-mannose or L-rhamnose (Table III). The ratio of the rates of L-rhamnose to L-mannose isomerization was 4:1 in crude extracts of cells grown on either L-hexose. The ratio of the rates of L-rhamnulose to L-fructose phosphorylation was 1:1.1, and the ratio of the rates of L-rhamnulose-1-P to L-fructose-1-P cleavage was about 3:1. These enzymes were not detected in cells grown on D-glucose or nutrient broth.

Properties of L-Mannose (L-Rhamnose) Isomerase:

(a) Activity in Crude Extracts- The rates of isomerization of L-mannose and L-rhamnose were compared as a function of isomerase concentration in crude enzyme extracts from both the wild-type and mutant cells (Figure 20).

(b) Partial Fractionation- The L-mannose and L-rhamnose isomerase activities did not separate when crude extracts were fractionated with ammonium sulfate, Sephadex G-100 (Figure 21), and DEAE-sephadex (Figure 22). On Sephadex G-100 the highest isomerase activity appeared in the highest protein fraction for both L-mannose and

Fig. 18 Whole cell fermentation. Each Warburg vessel contained, in a total volume of 0.55 ml, 10 μmoles of hexose (sidearm), 180 μmoles of potassium hydroxide (center well), and a volume of cells (2-3 mg dry weight) suspended in 0.2 M potassium phosphate buffer, pH 7.0. Water was used in place of the hexose to determine the endogenous activity. The temperature was 30° and the gas phase was air.

Figure 18.

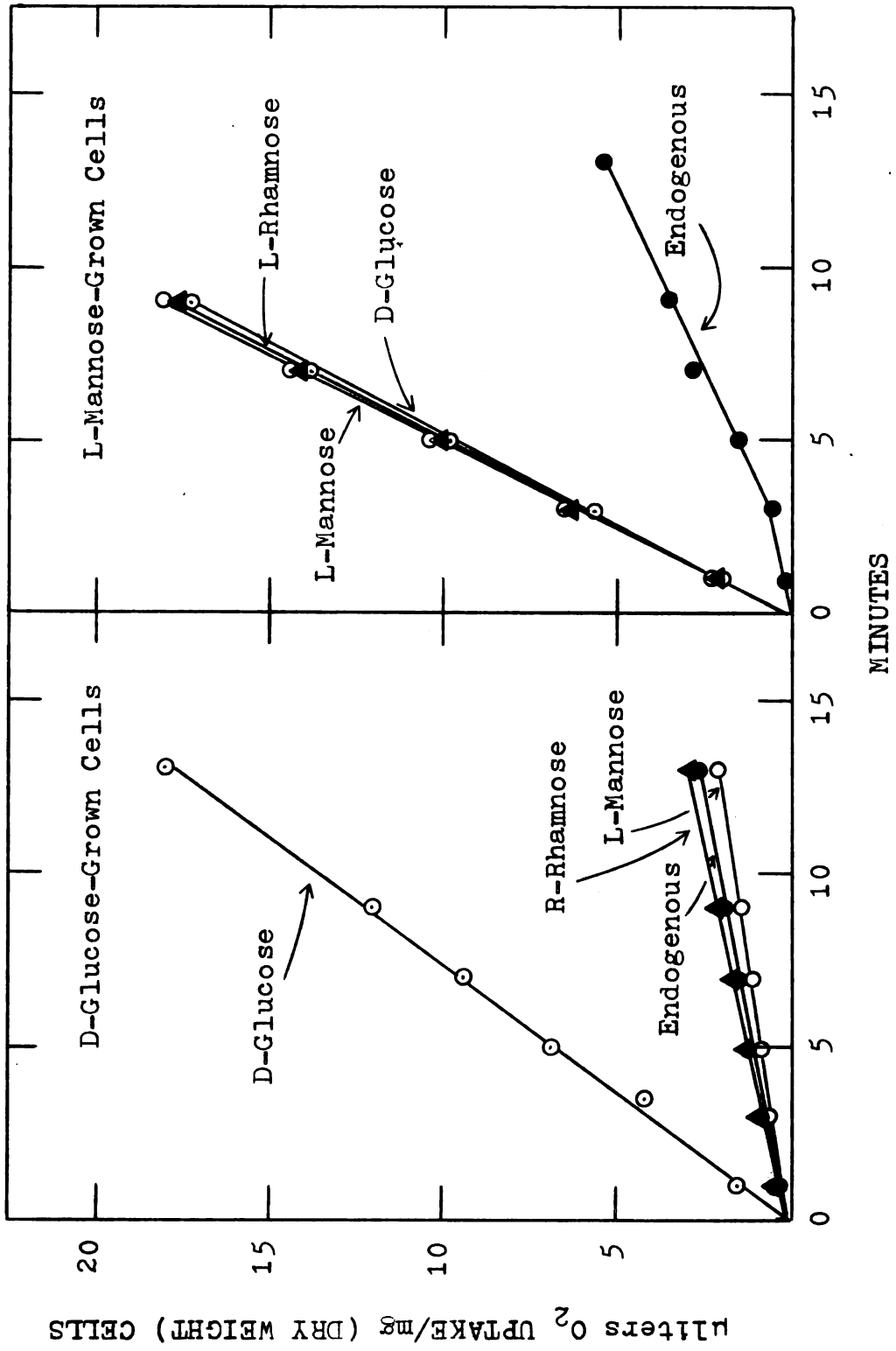

L-Mannose-Grown Cells
L-Rhamnose
D-Glucose
L-Mannose
Endogenous
D-Glucose-Grown Cells
D-Glucose
R-Rhamnose
L-Mannose
Endogenous
MINUTES
0
5
10
15
20
microliters O_2 UPTAKE/mg (DRY WEIGHT) CELLS

Fig. 19. Whole cell fermentation of D-glucose, L-mannose, and L-rhamnose. Details are given in Figure 18.

Figure 19.

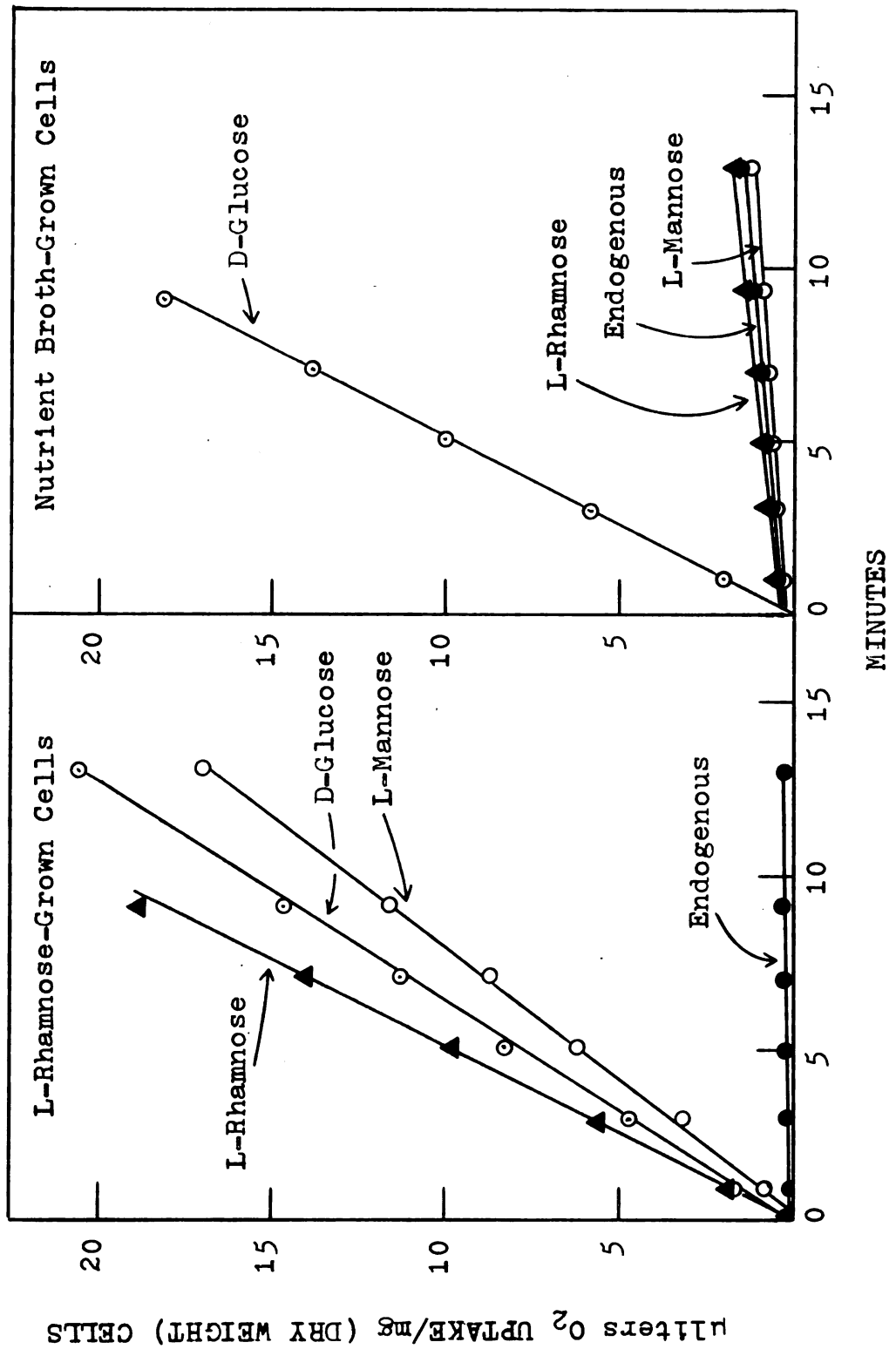

L-Rhamnose-Grown Cells
Nutrient Broth-Grown Cells
L-Rhamnose
D-Glucose
L-Mannose
Endogenous
20
15
10
5
0
5
10
15
MINUTES
microliters O2 UPTAKE/mg (DRY WEIGHT) CELLS

TABLE III

Induction of L-mannose and L-rhamnose enzymes in Aerobacter aerogenes

The cells were grown on either L-mannose, L-rhamnose, or nutrient broth according to procedures outlined in the text. Activity was based on enzymes present in the crude extracts.

Growth Substrate	Isomerase		Kinase		Aldolase	
	L-rhamnose	L-mannose	L-rhamnulose	L-fructose	L-rhamnulose-1-P	L-fructose-1-P
L-mannose	1.06	0.25	0.15	0.17	0.07	0.02
L-rhamnose	0.81	0.18	0.26	0.28	0.08	0.02
Nutrient broth	0*	0*	0*	0*	0*	0*

* < 0.002

Results are expressed as μmoles of product/min/mg of protein.

Fig. 20. Activity of the isomerase in crude extracts. The routine isomerase assay was used except that the protein concentration was varied.

Figure 20.

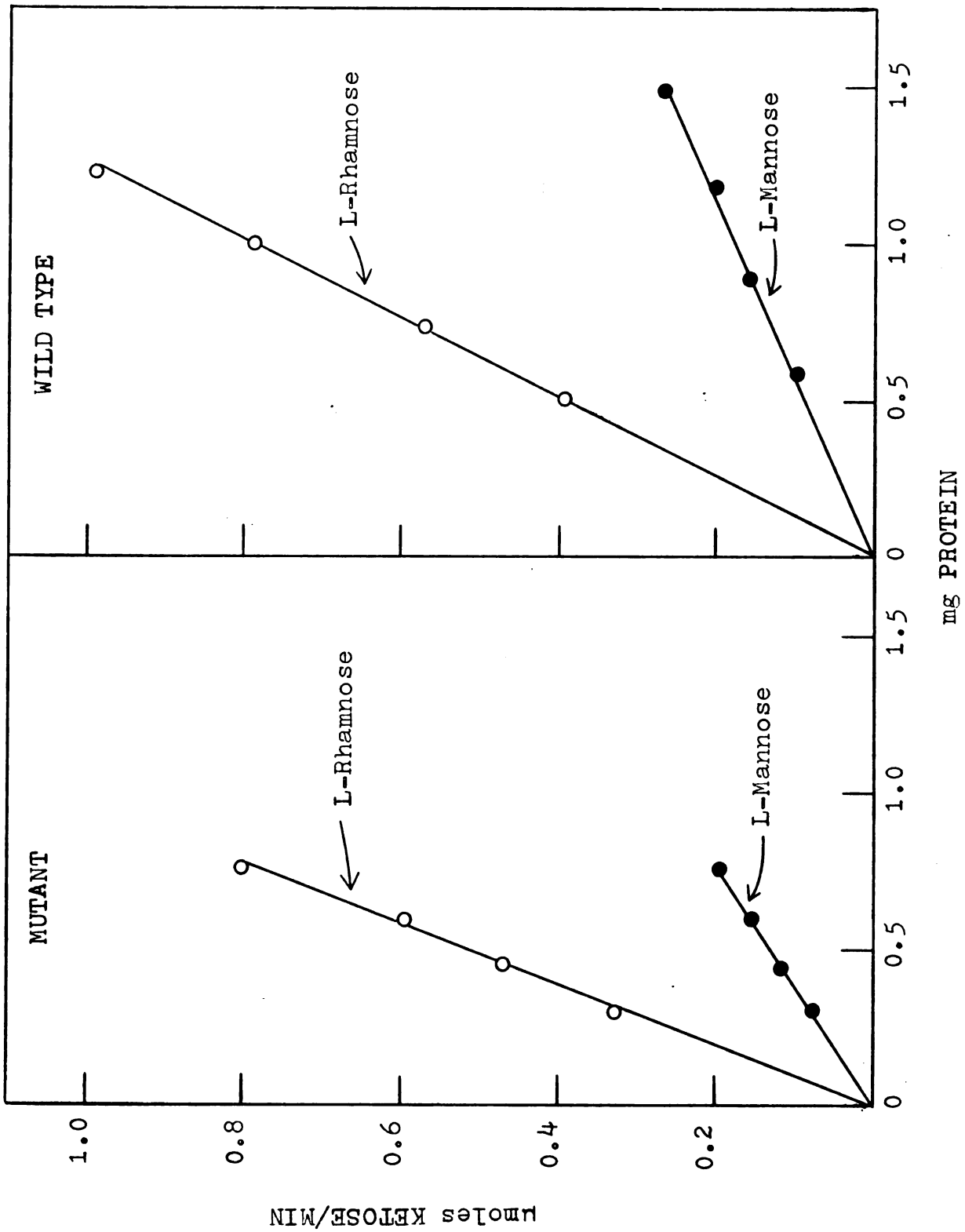

WILD TYPE
MUTANT
L-Rhamnose
L-Mannose
L-Rhamnose
L-Mannose
0
0.5
1.0
1.5
0.2
0.4
0.6
0.8
1.0
μmoles KETOSE/MIN
mg PROTEIN

Fig. 21. Fractionation of the isomerase on Sephadex G-100. Details are in the text. The volume of the fractions collected was 5 ml.

Figure 21.

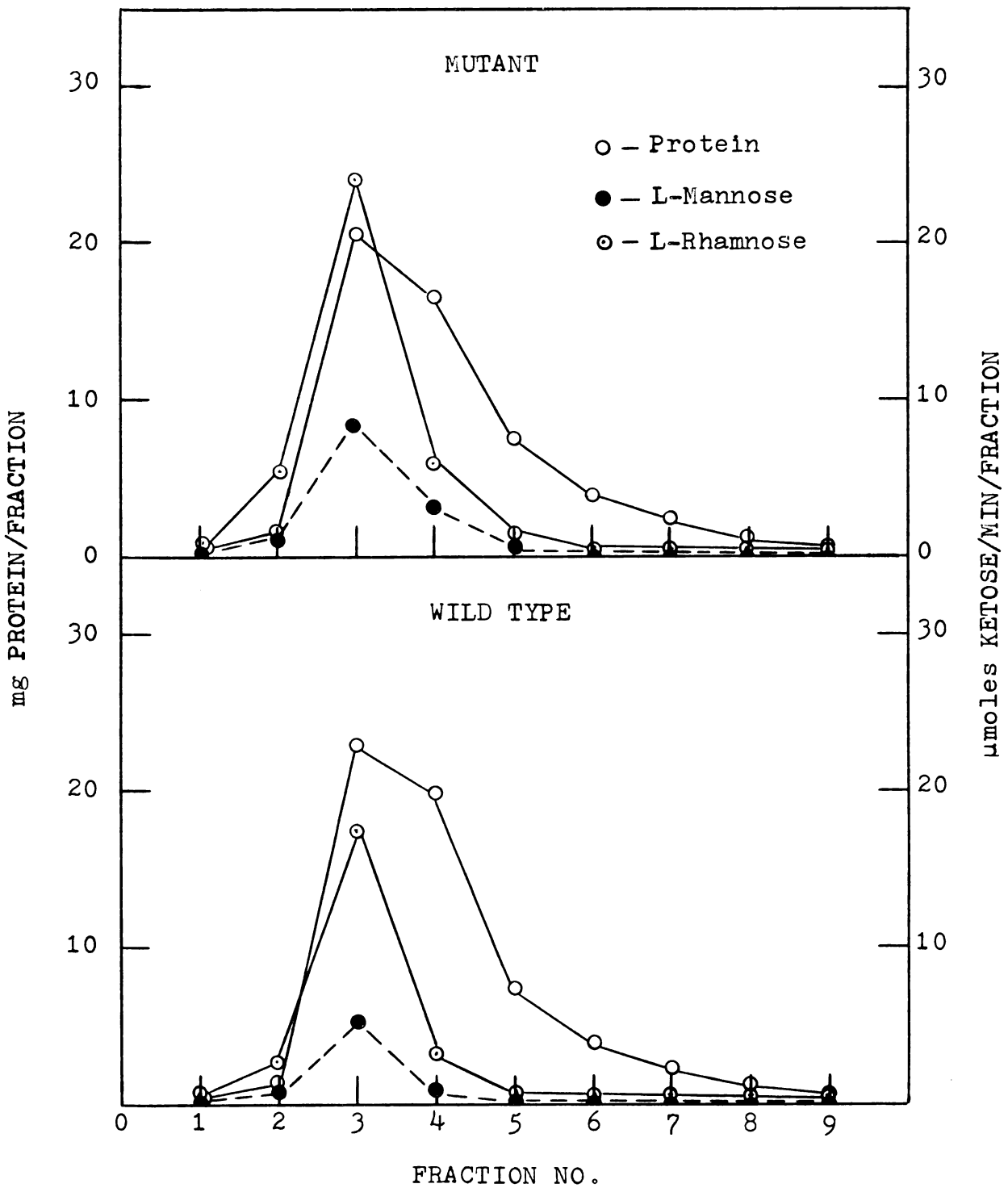
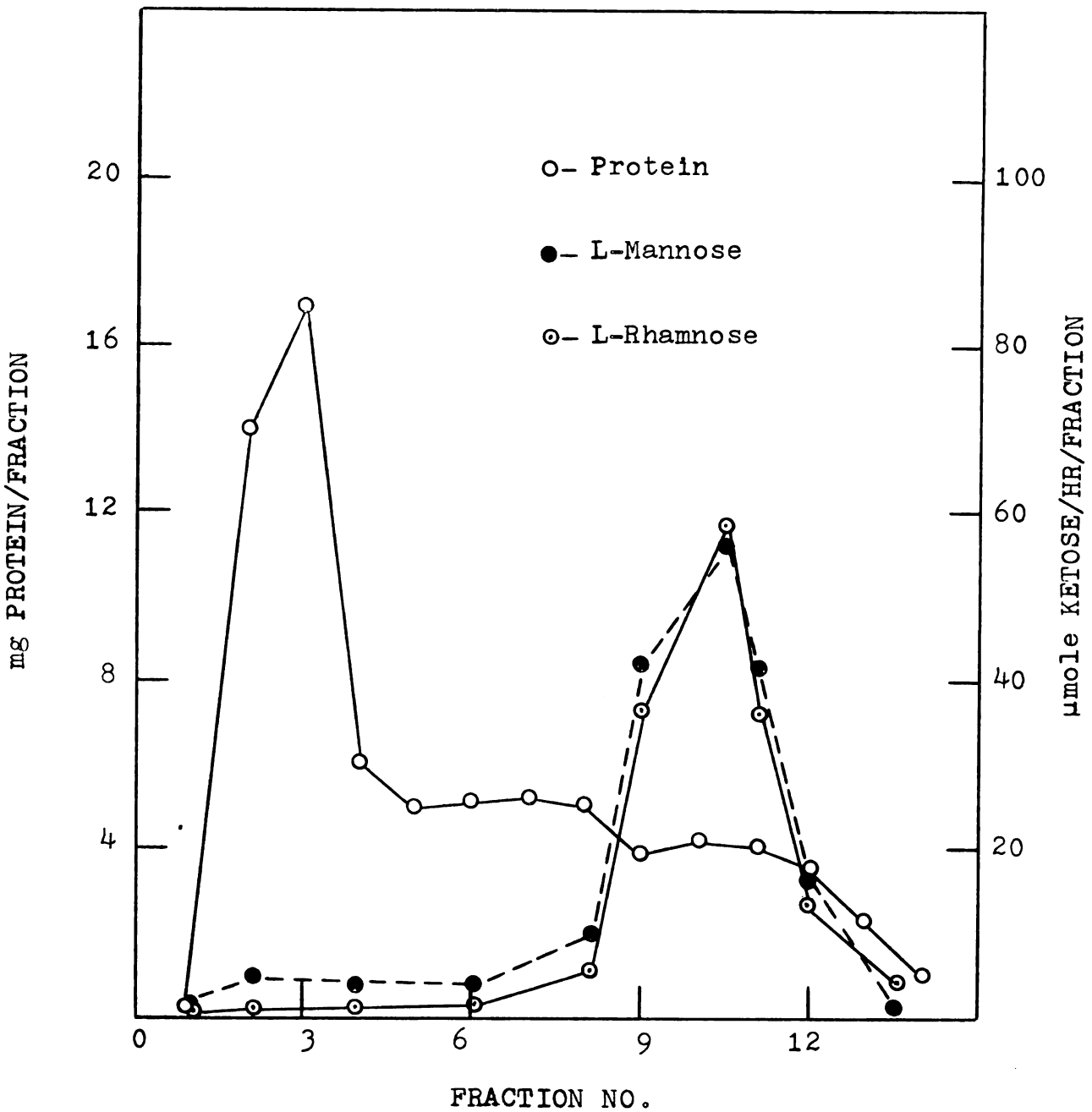

MUTANT
WILD TYPE
○ – Protein
● – L-Mannose
⊙ – L-Rhamnose
mg PROTEIN/FRACTION
µmoles KETOSE/MIN/FRACTION
FRACTION NO.
0
10
20
30
1
2
3
4
5
6
7
8
9

Fig. 22. Fractionation of the isomerase on DEAE-Sephadex. A volume of isomerase previously fractionated on Sephadex G-100 and containing 110 mg of protein was placed on a column of DEAE-Sephadex (75 mm x 15 mm) which was equilibrated with 0.05 M phosphate buffer (pH 7.0). The column was developed with a sodium chloride gradient ranging in concentration from 0 to 0.6 M. The volume of the fractions collected was 10 ml. For determining isomerase activity the reaction mixture (1.0 ml) was incubated 1 hour at 30° and the amount of ketose produced was determined.

Figure 22.


o- Protein
●- L-Mannose
⊙- L-Rhamnose
mg PROTEIN/FRACTION
µmole KETOSE/HR/FRACTION
FRACTION NO.
20
16
12
8
4
100
80
60
40
20
0
3
6
9
12

L-rhamnose. DEAE-sephadex inactivated the isomerase activity 90%. This high inactivation of the isomerase coupled to the prolonged incubation periods in the assay to measure the remaining isomerase activity resulted in a further decrease in enzyme activity. Thus, the activity on L-mannose and L-rhamnose as depicted in Figure 22 was not calculated in the linear range of the assay but rather on the amount of ketose which accumulated after a given time interval.

(c) Substrate Specificity- Only L-mannose and L-rhamnose were significantly isomerized; L-ribose showed about 1-2% activity and D-arabinose and L-fucose were isomerized about 1% that of L-rhamnose in wild-type extracts (Table IV).

(d) Metal Ion Activation: Effect of Mn^{++} and Co^{++}- Mn^{++} was the only cation that stimulated L-rhamnose isomerization (Table V). All other ions either inhibited or did not affect isomerase activity. On the other hand, only Co^{++} stimulated L-mannose isomerization (Table VI). All other ions either inhibited or showed no effect toward the enzyme. Co^{++} and Mn^{++} exerted a mutual antagonistic effect on the isomerization of L-mannose and L-rhamnose. Co^{++} stimulated the isomerization of L-mannose by 40% and Mn^{++} inhibited it by 75%. On the other hand,

TABLE IV

Substrate specificity of the isomerase

The isomerase activity was determined in crude extracts by the standard assay. For the other substrates (0.028 M) 5 to 10 times as much enzyme was employed and the incubation time was extended from 10 to 30 minutes. Except for D- and L-glucose, in which the Roe test was employed, activity on the substrates was measured with cysteine-carbazole.

Substrate	L-Rhamnose-Grown Cells Comparative Rate (%)	L-Mannose-Grown Cells Comparative Rate (%)
L-Rhamnose	100	100
L-Mannose	25	25
D-Mannose	0	0
L-Fucose	1	0
D-Fucose	0	0
L-Glucose	0	0
D-Glucose	0	0
L-Galactose	0	0
D-Galactose	0	0
D-Ribose	1.5	2
L-Arabinose	0	0
D-Arabinose	1	0
L-Xylose	0	0
D-Xylose	0	0

TABLE V

Effect of various metal ions on the isomerization of L-rhamnose

Refer to Table V for details.

Cation	L-Rhamnose-Grown Cells Comparative Rate (%)	L-Mannose-Grown Cells Comparative Rate (%)
None	100	100
Mn^{++}	135	135
Co^{++}	65	70
Mg^{++}	100	100
Fe^{+++}	90	85
Ni^{++}	20	16
Ca^{++}	35	25
Zn^{++}	0	0
Cu^{++}	0	0
Na^{+}	100	100
K^{+}	100	100
NH_4^{+}	100	100

TABLE VI

Effect of various metal ions on the isomerization of L-mannose

The routine isomerase assay was employed in the crude enzyme extract. The concentration of each cation was 3.4×10^{-3} M. The chloride salt of each cation was used in each case.

Cation	L-Rhamnose-Grown Cells Comparative Rate (%)	L-Mannose-Grown Cells Comparative Rate (%)
None	100	100
Mn^{++}	25	25
Co^{++}	135	140
Mg^{++}	100	100
Fe^{+++}	30	35
Ni^{++}	10	15
Ca^{++}	12	15
Zn^{++}	0	0
Na^{+}	100	100
K^{+}	100	100
NH_4^{+}	100	100

Mn^{++} stimulated L-rhamnose isomerization by 35% and Co^{++} inhibited it by 35%.

The effects of Co^{++} and Mn^{++} concentrations on L-mannose and L-rhamnose isomerase activities are shown in Figure 23. The data suggest that the enzyme binds Mn^{++} more strongly than Co^{++}. However, it should be kept in mind that crude extracts contain nucleotides and other proteins which also may bind these metals, thus affecting their availability to the isomerase.

When Mn^{++} and Co^{++} were mixed together, Co^{++} exerted a greater effect on the isomerization of L-rhamnose (Table VII). By increasing the concentration of Mn^{++} up to 3 times that of Co^{++} no significant increase in the activity occurred. With L-mannose as the substrate, increasing the concentration of Co^{++} increased L-mannose isomerization and increasing the concentration of Mn^{++} decreased it.

(e) Effect of Mixing Substrates- Isomerase activity on mixtures of L-mannose and L-rhamnose was less than on L-mannose alone (Table VIII).

(f) An Isomeraseless Mutant (B-22)- Table IX shows that both L-mannose and L-rhamnose induced all three enzymes in the wild-type cells. In the B-22 mutant only the kinase and the aldolase were induced; the isomerase

Fig. 23. Effect of varying the Mn^{++} and Co^{++} concentrations on isomerase activity. The routine isomerase assay was used to determine activity in crude extracts. Only the concentrations of Mn^{++} and Co^{++} were varied. As a control the isomerase activity was measured in the absence of the cations.

Figure 23.

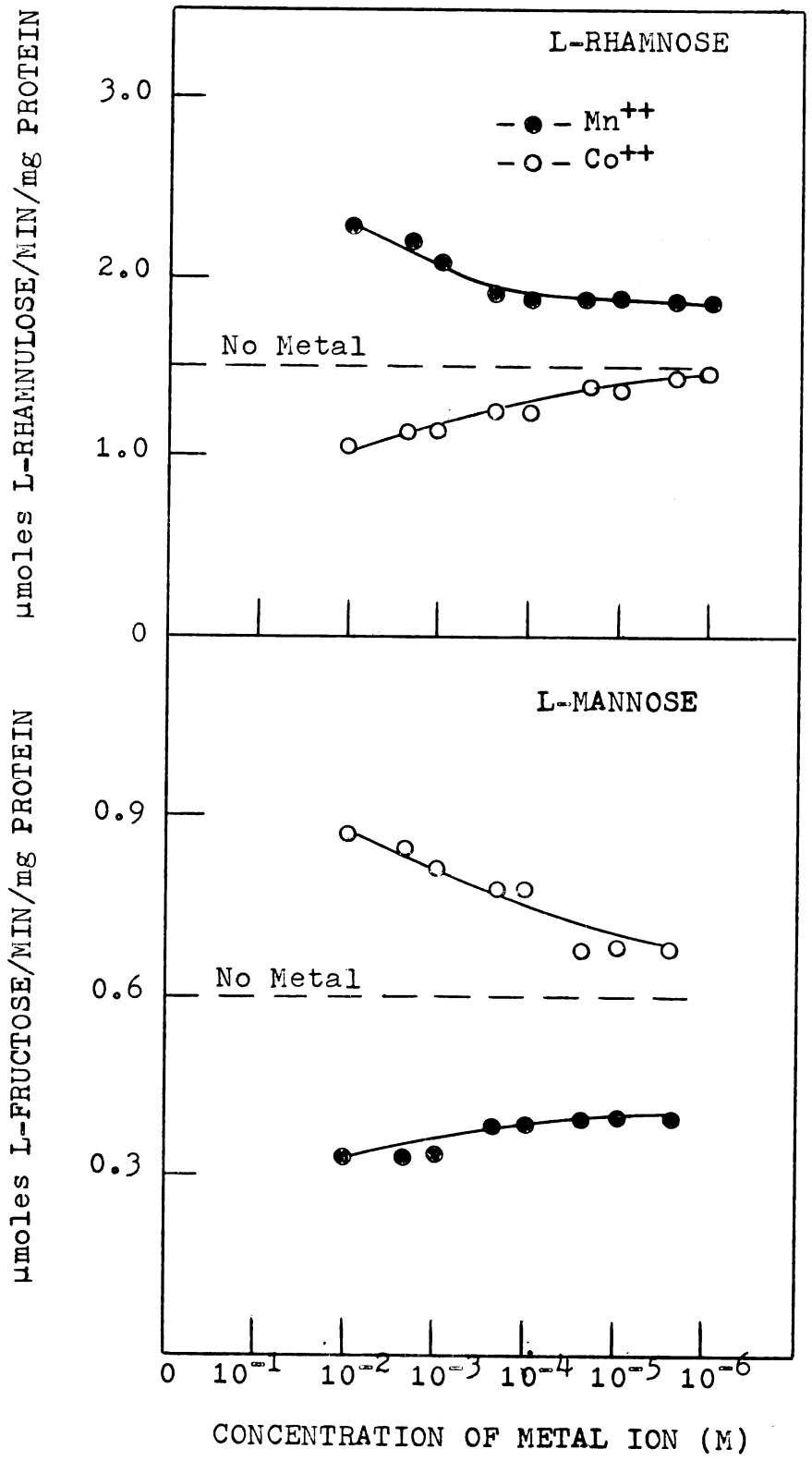

L-RHAMNOSE
Mn^{++}
Co^{++}
No Metal
3.0
2.0
1.0
0
µmoles L-RHAMNULOSE/MIN/mg PROTEIN
L-MANNOSE
No Metal
0.9
0.6
0.3
µmoles L-FRUCTOSE/MIN/mg PROTEIN
0 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
CONCENTRATION OF METAL ION (M)

TABLE VII

Effect of Mn^{++} and Co^{++} on the isomerization of L-rhamnose and L-mannose

The standard isomerase assay was employed. The cells were grown on L-rhamnose and the crude extracts were used. The assay concentrations for Mn^{++} and Co^{++} were 3.4×10^{-3} M and increases in the concentrations of each cation were made according to the desired ratio.

Substrate	Metal Ions (Ratio)	Specific Activity μmoles ketose/min/mg protein
L-Rhamnose	none	0.44
	Mn^{++}	0.60
	Co^{++}	0.29
	Co^{++}:Mn^{++} (1:1)	0.25
	Co^{++}:Mn^{++} (1:2)	0.24
	Co^{++}:Mn^{++} (1:3)	0.23
L-Mannose	none	0.23
	Mn^{++}	0.08
	Co^{++}	0.44
	Co^{++}:Mn^{++} (1:1)	0.27
	Co^{++}:Mn^{++} (3:1)	0.36
	Co^{++}:Mn^{++} (1:2)	0.24
	Co^{++}:Mn^{++} (1:4)	0.14

TABLE VIII

Effect of mixing substrates on isomerase activity

The Roe Test (12) was employed to determine activity in crude extracts on L-mannose and mixtures of L-rhamnose and L-mannose. The concentration of each substrate was the same as described in the routine assay except that no metal ions were included.

Substrate		$O.D._{520}$/20 min
L-Mannose		0.290
L-Rhamnose		0.085
L-Mannose + L-Rhamnose		0.070
Controls (no enzyme)	**Sample (μmoles)**	**$O.D._{520}$**
L-Fructose	0.23	0.330
L-Rhamnulose	0.30	0.050
L-Rhamnose	0.30	0.015
L-Fructose + L-Rhamnose	0.23 + 0.30	0.345
L-Fructose + L-Rhamnulose	0.23 + 0.30	0.390

TABLE IX

Enzyme activities in extracts of wild type (PRL-R3) and a L-mannose/L-rhamnose-negative mutant

Wild type and B-22 cells were grown in 500 ml of nutrient broth supplemented with either L-rhamnose or L-mannose (350 mg each). L-Mannose was added to the growth medium at the same time the cells were inoculated, and the cells were harvested when L-mannose utilization had begun. L-Rhamnose was added 6 hours after inoculation and after an induction time of 70 minutes the cells were harvested.

		Isomerase		Kinase*		Aldolase*	
Strain	Inducer	L-Rhamnose	L-Mannose	L-Rhamnulose	L-Fructose	L-Rhamnulose -1-P	l-Fructose -1-P
PRL-R3 (parent)	L-Rhamnose	0.24	0.07	1.25	1.40	0.11	0.03
	L-Mannose	0.11	0.03	1.10	1.30	0.09	0.02
B-22 (mutant)	L-Rhamnose	<0.001	<0.001	1.10	1.30	0.08	0.02
	L-Mannose	<0.001	<0.001	0.80	0.91	0.03	0.01

Results are expressed as μmoles of product/min/mg of protein.

*Activity in an ammonium sulfate fraction of the cell extract.

was not induced by either L-mannose or L-rhamnose, and no isomerase activity was detected on either of these two substrates.

(g) pH Optimum- The pH optimum of the isomerase was determined with both L-mannose and L-rhamnose as substrates and in the presence and absence of their activating metals (Figure 24). In the presence of Co^{++}, L-mannose was isomerized maximally over a pH range of 7.5-7.7, and without Co^{++} over a pH range of 7.4-8.5. The decrease in the pH optimum in the presence of Co^{++} probably was due to cobalt hydroxide formation.

The shift in the pH optimum was more clearly defined when L-rhamnose was the substrate. In the presence of Mn^{++}, L-rhamnose was isomerized at a maximum rate when the pH was 7.3-7.7 and in the absence of Mn^{++} a pH shift to 8.7-9.0 occurred. The rapid decrease in L-rhamnose isomerization after pH 7.7 probably was due to manganese dioxide formation.

(h) Temperature Optimum- With L-rhamnose as the substrate and Mn^{++} as the activating metal, the isomerase exhibited a temperature optimum of 60-65° compared to 55-60° in the absence of Mn^{++} (Figure 25). With L-mannose as substrate the isomerase in the presence or absence of Co^{++} had a temperature optimum of 50-55°.

Fig. 24. The pH optimum of the isomerase. The standard assay for measuring isomerase activity was employed. The crude extract was fractionated on Sephadex G-100 and the fraction having the highest isomerase activity was used. The amount of protein per assay was 0.52 mg. Each of the buffers employed was 0.02 M and had the following pH ranges: sodium phosphate (pH 6.7-7.5), tris-HCl (pH 7.4-8.8), glycine-NaOH (pH 8.9-9.4), and carbonate-bicarbonate (pH 9.8-10.1). The pH of the reaction was recorded at the end of the incubation period.

Figure 24.

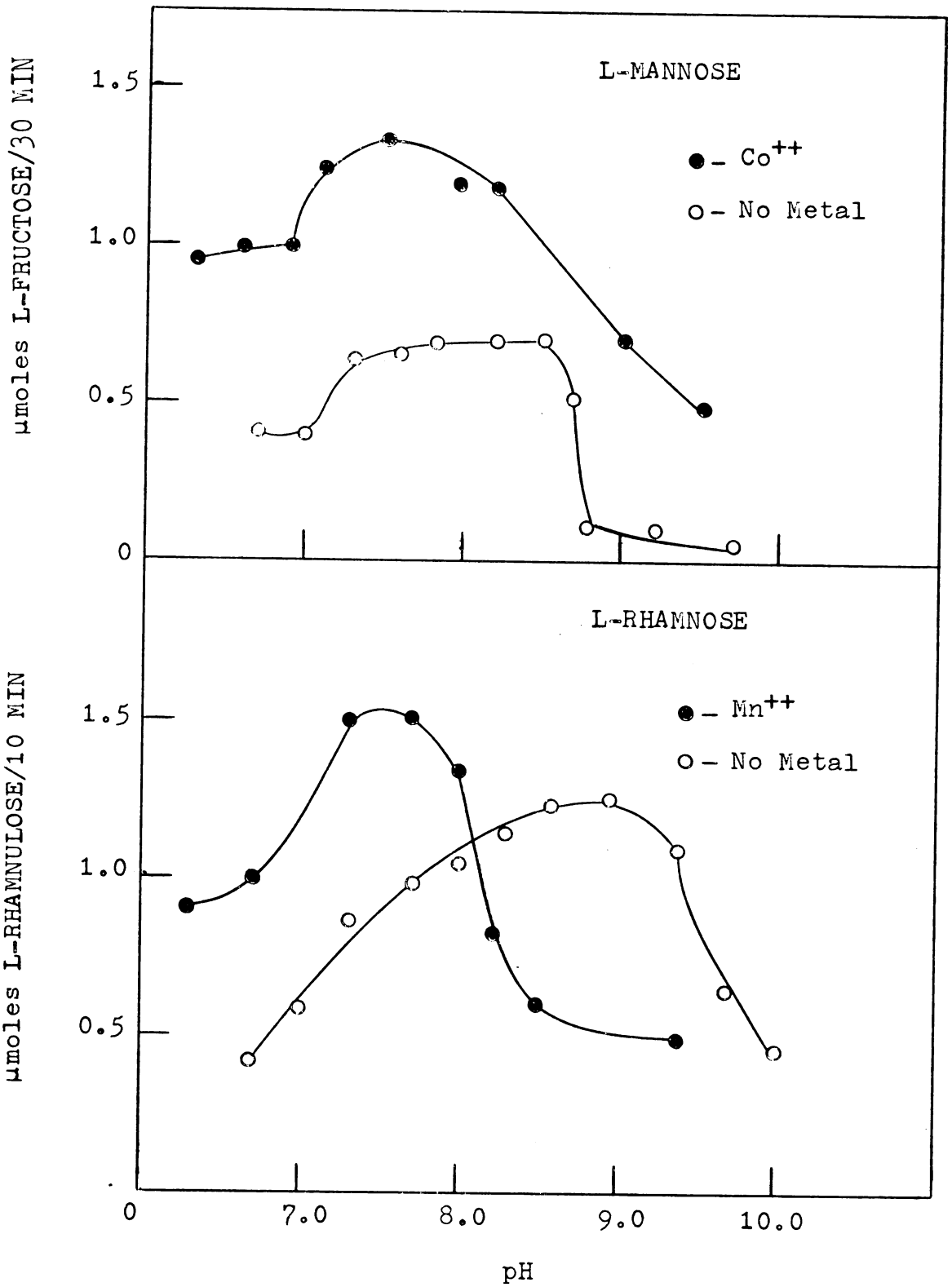
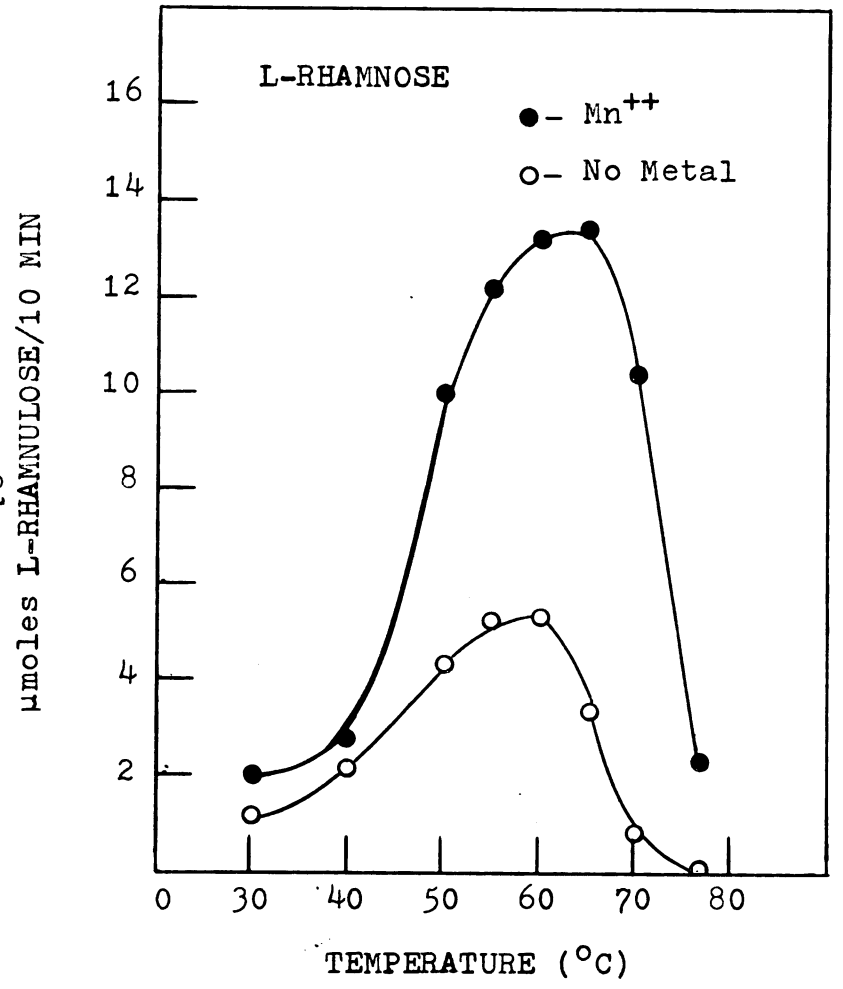

L-MANNOSE
● – Co++
○ – No Metal
µmoles L-FRUCTOSE/30 MIN
1.5
1.0
0.5
0
L-RHAMNOSE
● – Mn++
○ – No Metal
µmoles L-RHAMNULOSE/10 MIN
1.5
1.0
0.5
0
7.0
8.0
9.0
10.0
pH

Fig. 25. Temperature optimum of the isomerase. The enzyme was fractionated on Sephadex G-100 before use. To measure isomerase activity the assay mixture containing all the components except the substrate was heated for five minutes at a specific temperature, after which the substrate was added to initiate the reaction. When L-rhamnose was the substrate, the assay contained 0.17 mg of protein; when L-mannose was the substrate, the reaction mixture contained 0.52 mg of protein.


L-RHAMNOSE
● - Mn++
○ - No Metal
µmoles L-RHAMNULOSE/10 MIN
16
14
12
10
8
6
4
2
0
30
40
50
60
70
80
TEMPERATURE (°C)

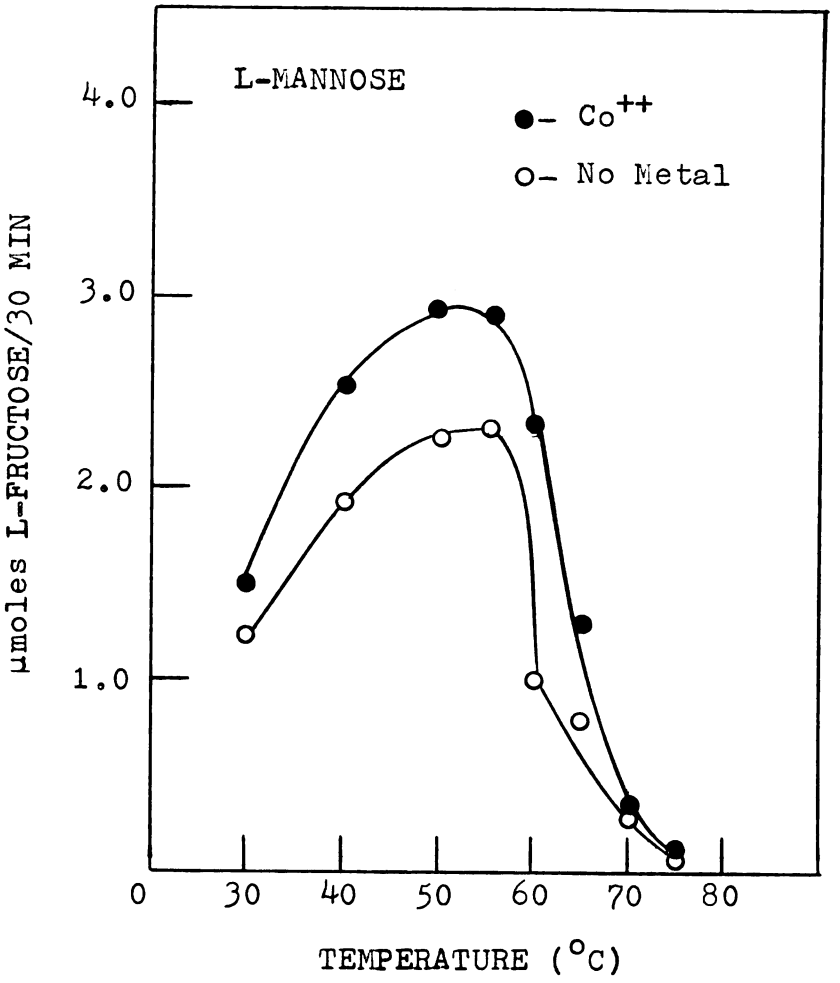

L-MANNOSE
● - Co++
○ - No Metal
µmoles L-FRUCTOSE/30 MIN
4.0
3.0
2.0
1.0
0
30
40
50
60
70
80
TEMPERATURE (°C)

Figure 25.

Properties of L-Fructose (L-Rhamnulose) Kinase:

(a) Activity of the Kinase in Crude Extracts- The rates of phosphorylation of L-fructose and L-rhamnulose were compared as a function of kinase concentration in crude extracts of both wild-type and mutant cells (Figure 26). Although the kinase activity varied in each extract, the ratio of the activities of L-fructose to L-rhamnulose remained constant.

(b) Partial Purification of the Kinase- The kinase was purified about 7-fold with a 20% recovery of activity (Table X). Fractionation with ammonium sulfate and Sephadex G-100 failed to separate the L-fructose from the L-rhamnulose kinase activity (Figure 27).

(c) Km of L-Fructose (L-Rhamnulose) Kinase- The Km's for both L-fructose and L-rhamnulose were the same for partially purified kinase obtained from both wild type and mutant cells (Figures 28 and 29). The kinase bound L-rhamnulose more strongly than L-fructose. The Km value for L-rhamnulose was 0.05 mM compared to 1.7 mM for L-fructose.

(d) Substrate Specificity- The kinase was specific for L-fructose and L-rhamnulose (Table XI). Activity on D-fructose was attributed to D-fructose kinase.

(e) Effect of Mixing Substrates- Kinase activity on L-fructose and L-rhamnose was not additive (Table XII)

Fig. 26. Activity of L-fructose (L-rhamnulose) kinase in crude extracts. The standard kinase assay was employed except for the change in protein concentration.

Figure 26.

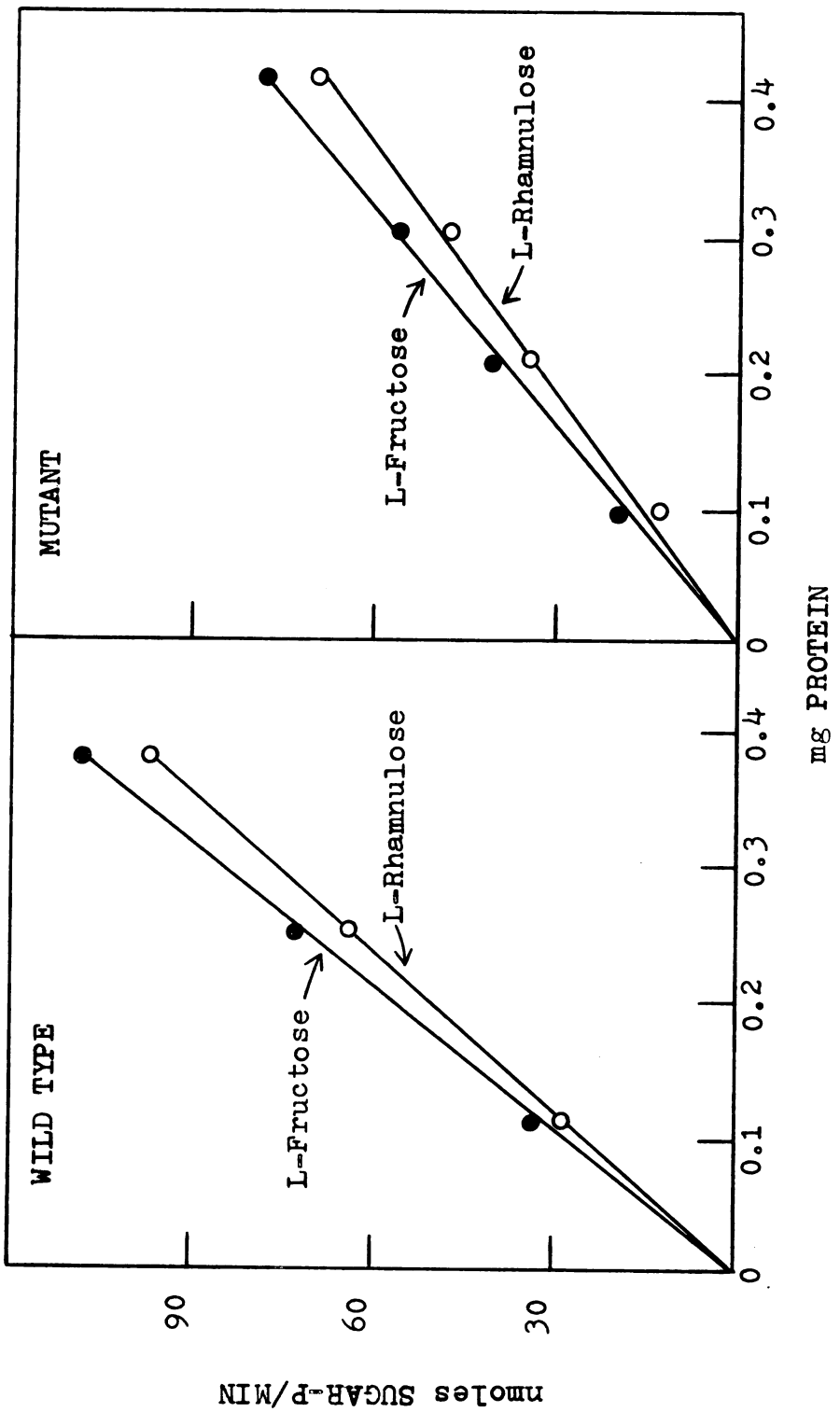

WILD TYPE
MUTANT
L-Fructose
L-Rhamnulose
L-Fructose
L-Rhamnulose
nmoles SUGAR-P/MIN
mg PROTEIN
0
30
60
90
0.1
0.2
0.3
0.4

TABLE X

Partial purification of L-fructose (L-rhamnulose) kinase from wild-type cells

Fraction	Total Activity		Recovery	Specific Activity	
	Units*		%	Units/mg protein	
	L-fructose	L-rhamnulose		L-fructose	L-rhamnulose
Crude extract	74	66	100	0.30	0.27
Ammonium sulfate	52	42	62-70	0.81	0.66
Sephadex G-100	15	14	21	2.28	2.21

* Micromoles of L-fructose or L-rhamnulose phosphorylated per minute.

Fig. 27. Fractionation of the kinase on Sephadex G-100. Details of the procedure are in the text. Each fraction was assayed for kinase activity on L-fructose and L-rhamnulose.

Figure 27.

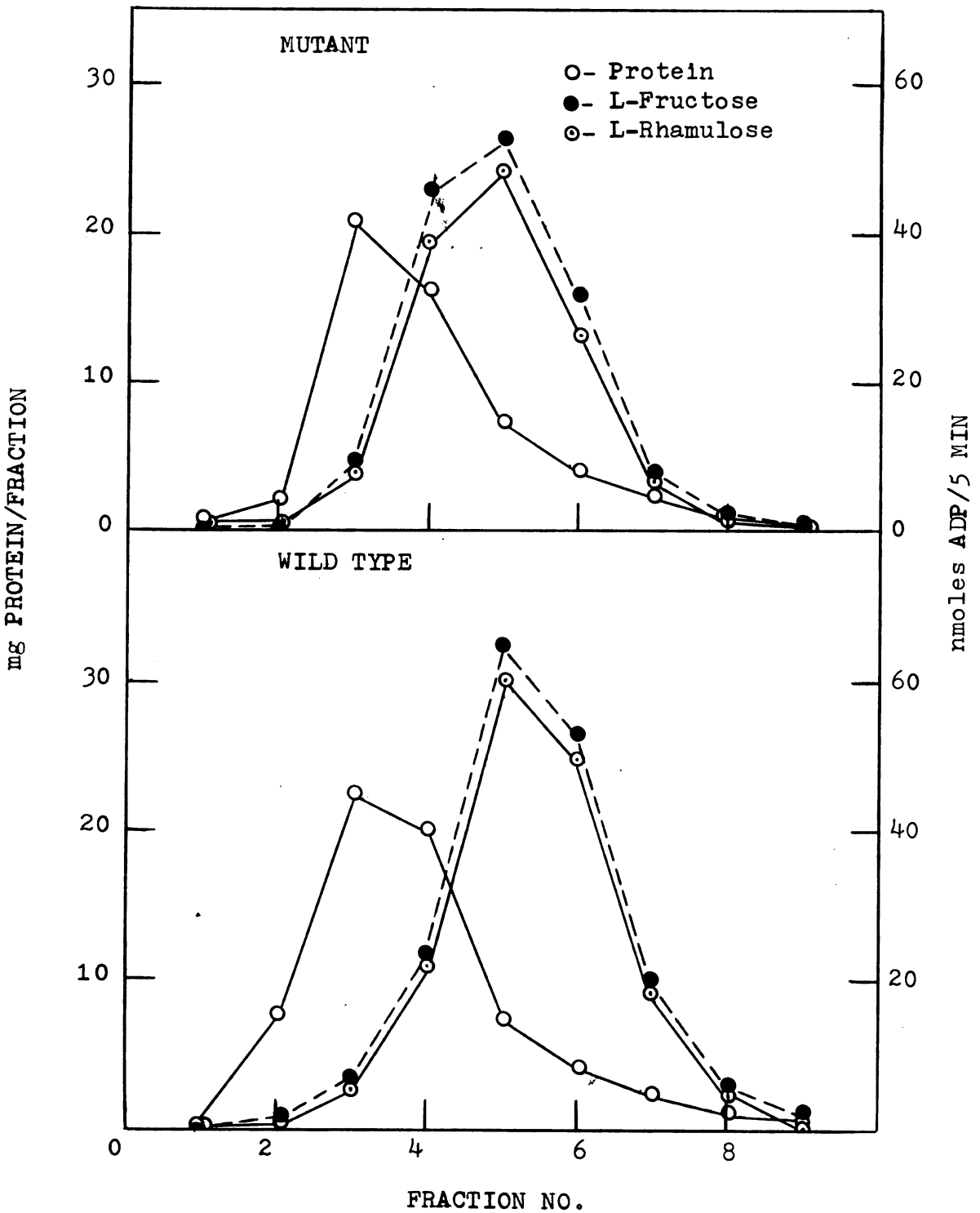
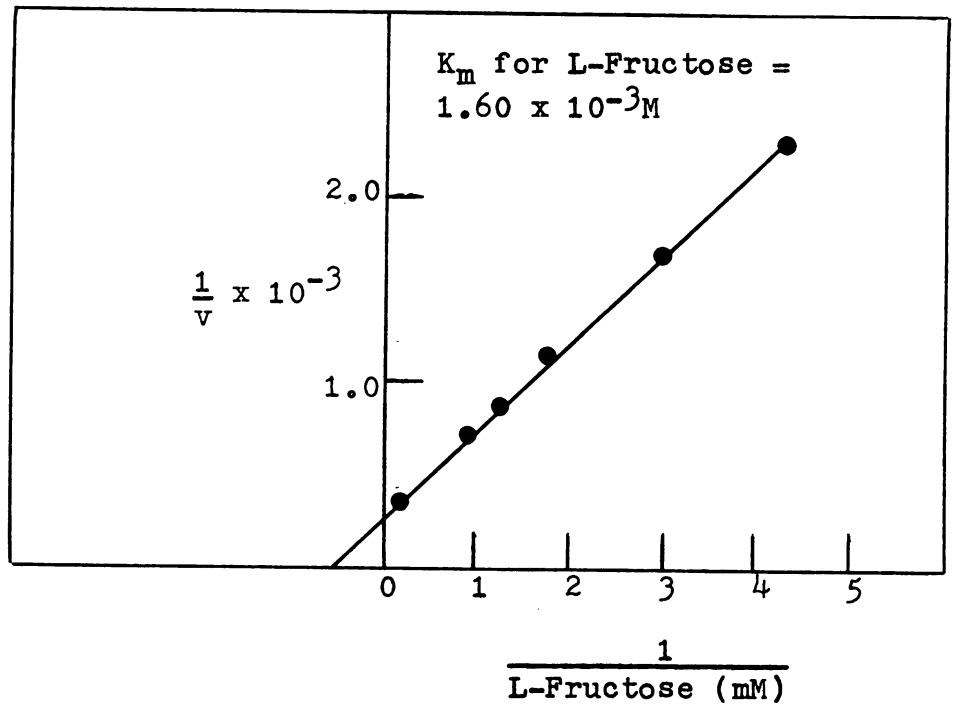

MUTANT
O- Protein
●- L-Fructose
⊙- L-Rhamulose
WILD TYPE
mg PROTEIN/FRACTION
nmoles ADP/5 MIN
FRACTION NO.
0
10
20
30
40
60
2
4
6
8

Fig. 28. Lineweaver-Burk plot of L-fructose (L-rhamnulose) kinase from L-mannose-positive cells. The standard kinase assay was used except that the substrate concentration was varied as indicated with the kinase (Sephadex G-100 fraction) concentration constant.

Figure 28.


K_m for L-Fructose =
1.60×10^{-3}M
2.0
1.0
$\frac{1}{v} \times 10^{-3}$
0
1
2
3
4
5
$\frac{1}{\text{L-Fructose (mM)}}$

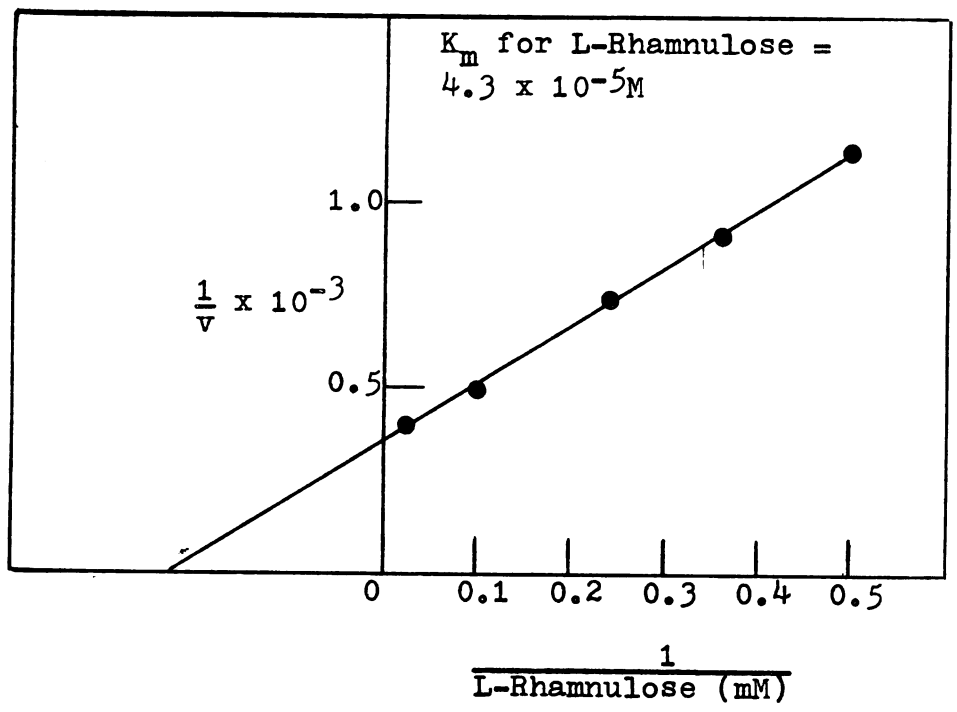
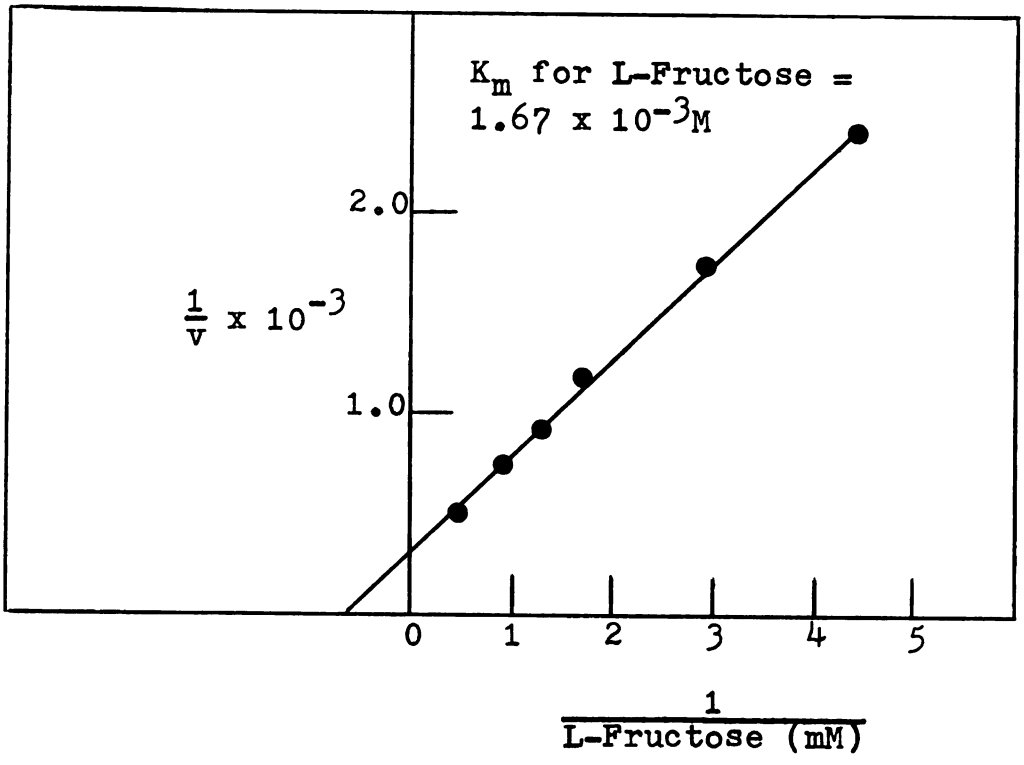

K_m for L-Rhamnulose =
4.3×10^{-5}M
1.0
0.5
$\frac{1}{v} \times 10^{-3}$
0
0.1
0.2
0.3
0.4
0.5
$\frac{1}{\text{L-Rhamnulose (mM)}}$

Fig. 29. Lineweaver-Burk plot of L-fructose (L-rhamnulose) kinase from wild-type cells.

Figure 29.


K_m for L-Fructose =
1.67×10^{-3}M
2.0
1.0
$\frac{1}{v} \times 10^{-3}$
0
1
2
3
4
5
$\frac{1}{\text{L-Fructose (mM)}}$

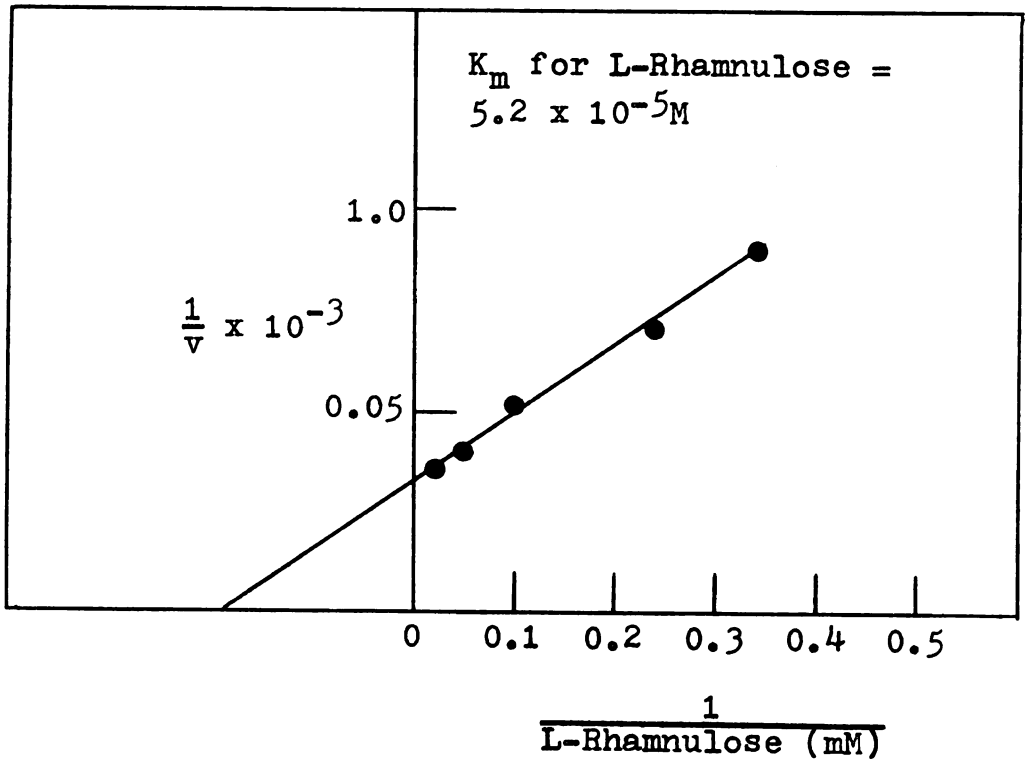

K_m for L-Rhamnulose =
5.2×10^{-5}M
1.0
0.05
$\frac{1}{v} \times 10^{-3}$
0
0.1
0.2
0.3
0.4
0.5
$\frac{1}{\text{L-Rhamnulose (mM)}}$

TABLE XI

Substrate specificity of the kinase

The routine kinase assay was employed. With the exception of L-rhamnulose the assay contained 1.5 μmoles of each substrate. The enzyme was fractionated on Sephadex G-100 before use.

Substrate	L-Rhamnose-Grown Cells Comparative Rate (%)	L-Mannose-Grown Cells Comparative Rate (%)
L-Rhamulose	100	100
L-Fructose	110	110
D-Fructose	1.8	2.5
L-Sorbose	0	0
L-Mannose	0	0

TABLE XII

Effect of mixing substrates on kinase activity

The routine kinase assay was used. When both substrates were mixed the concentration of each substrate was the same as that used in the routine assay. The kinase was fractionated on Sephadex G-100 before use.

	Growth Substrate	
Substrate	L-Rhamnose	L-Mannose
	Specific Activity (μmoles/min/mg)	
L-Rhamnulose	1.76	1.43
L-Fructose	1.95	1.60
L-Rhamnulose + L-Fructose	1.83	1.35

when assayed from cells grown on either L-mannose or L-rhamnose.

(f) pH Optimum- The pH optimum of the kinase ranged from pH 7.0-8.0 in both wild-type and mutant cells when either L-fructose or L-rhamnulose was the substrate (Figures 30 and 31).

Properties of L-Fructose-1-P (L-Rhamnulose-1-P) Aldolase:

(a) Activity in Crude Extracts- The rates of cleavage of L-fructose-1-P and L-rhamnulose-1-P were compared as a function of aldolase concentration in crude extracts from both wild-type and mutant cells (Figure 32).

(b) Partial Purification of the Aldolase- The aldolase was purified about 6-fold with a 20% recovery of total units (Table XIII). Fractionation with ammonium sulfate and Sephadex G-100 failed to separate the L-fructose-1-P from the L-rhamnulose-1-P aldolase activity (Figure 33).

(c) Km of the Aldolase- The aldolase bound L-rhamnulose-1-P 10 times more strongly than L-fructose-1-P. The Km value for L-rhamnulose-1-P was 0.5 mM compared to 5 mM for L-fructose-1-P (Figure 34).

(d) Effect of Mixing Substrates- When L-fructose-1-P and L-rhamnulose-1-P were mixed together, their combined

Fig. 30. pH optimum of the kinase in wild-type cells. The routine kinase assay was used. The kinase was fractionated on Sephadex G-100 before use. Details of the buffers and pH ranges used are given in Figure 24. The pH of the reaction was recorded at the end of the incubation period. The amount of protein per assay was 0.08 mg.

Figure 30.

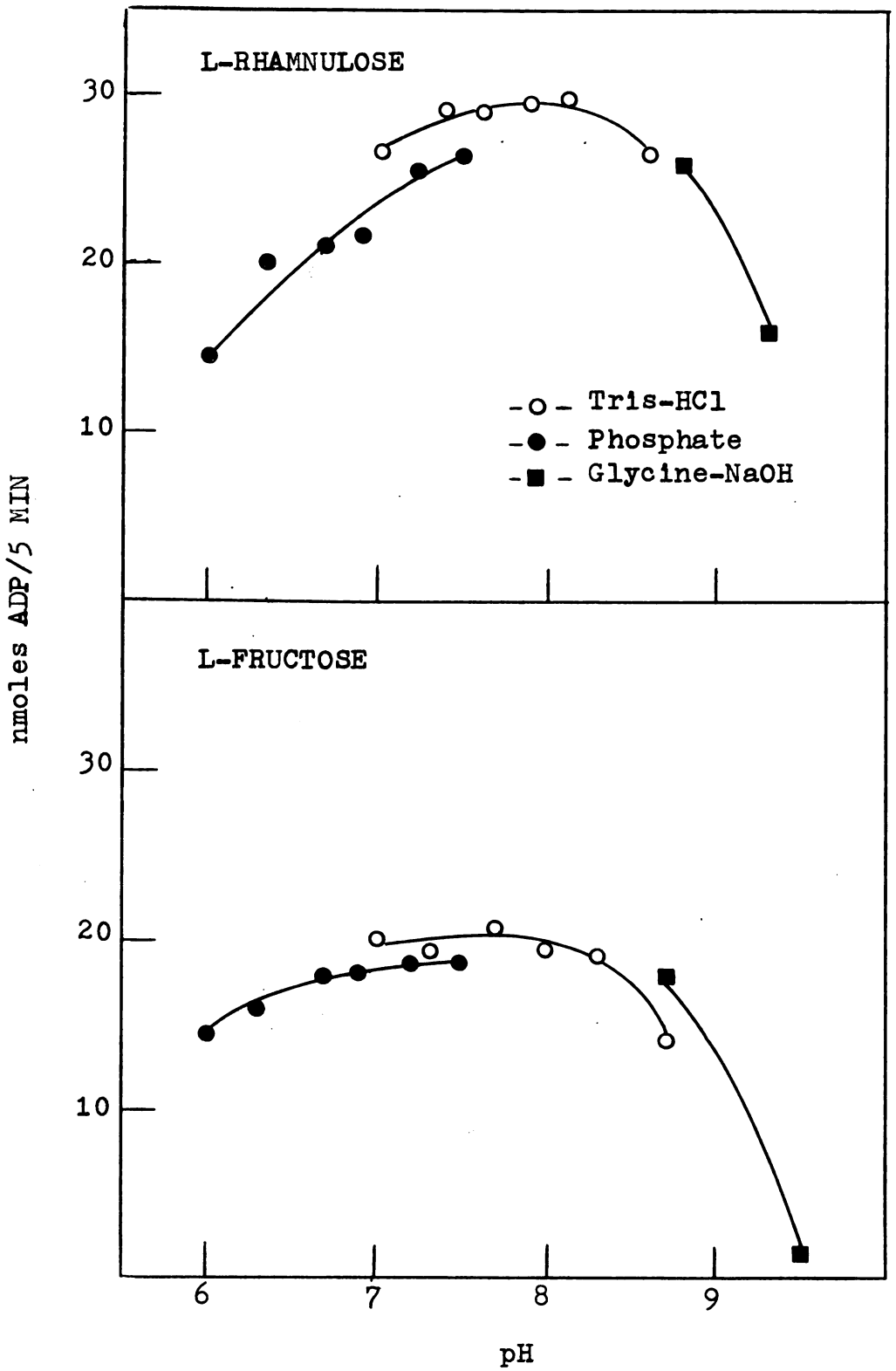

L-RHAMNULOSE
30
20
10
-○- Tris-HCl
-●- Phosphate
-■- Glycine-NaOH
L-FRUCTOSE
30
20
10
nmoles ADP/5 MIN
6
7
8
9
pH

Fig. 31. pH optimum of the kinase in the L-mannose-positive cells. Refer to Figure 30 for details. The amount of protein per assay was 0.04 mg.

Figure 31.

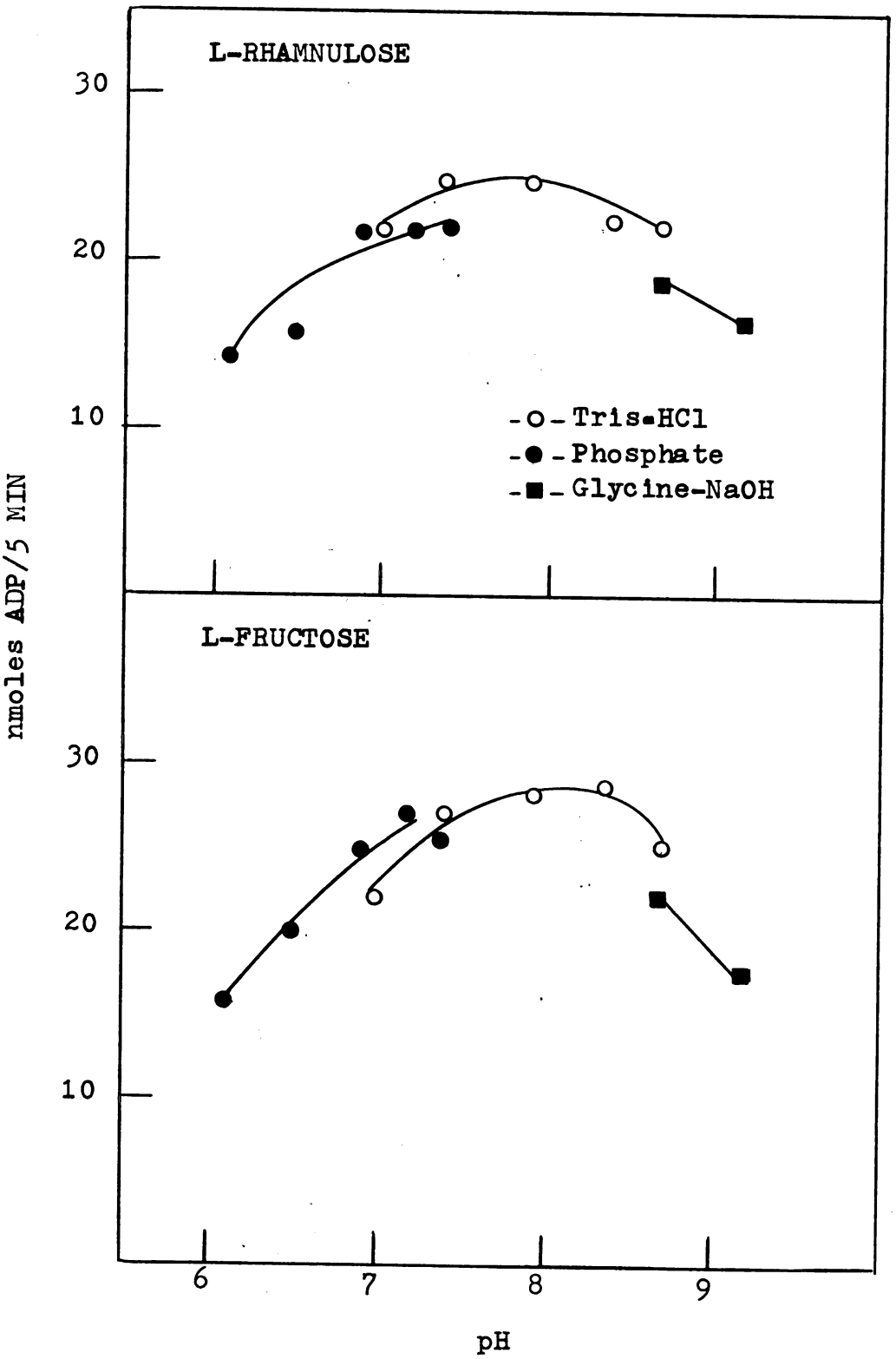

L-RHAMNULOSE
30
20
10
-○- Tris-HCl
-●- Phosphate
-■- Glycine-NaOH
L-FRUCTOSE
30
20
10
nmoles ADP/5 MIN
6
7
8
9
pH

Fig. 32. Activity of L-fructose-1-P (L-rhamnulose-1-P) in crude extracts. The routine aldolase assay was used except that the protein was varied.

Figure 32.

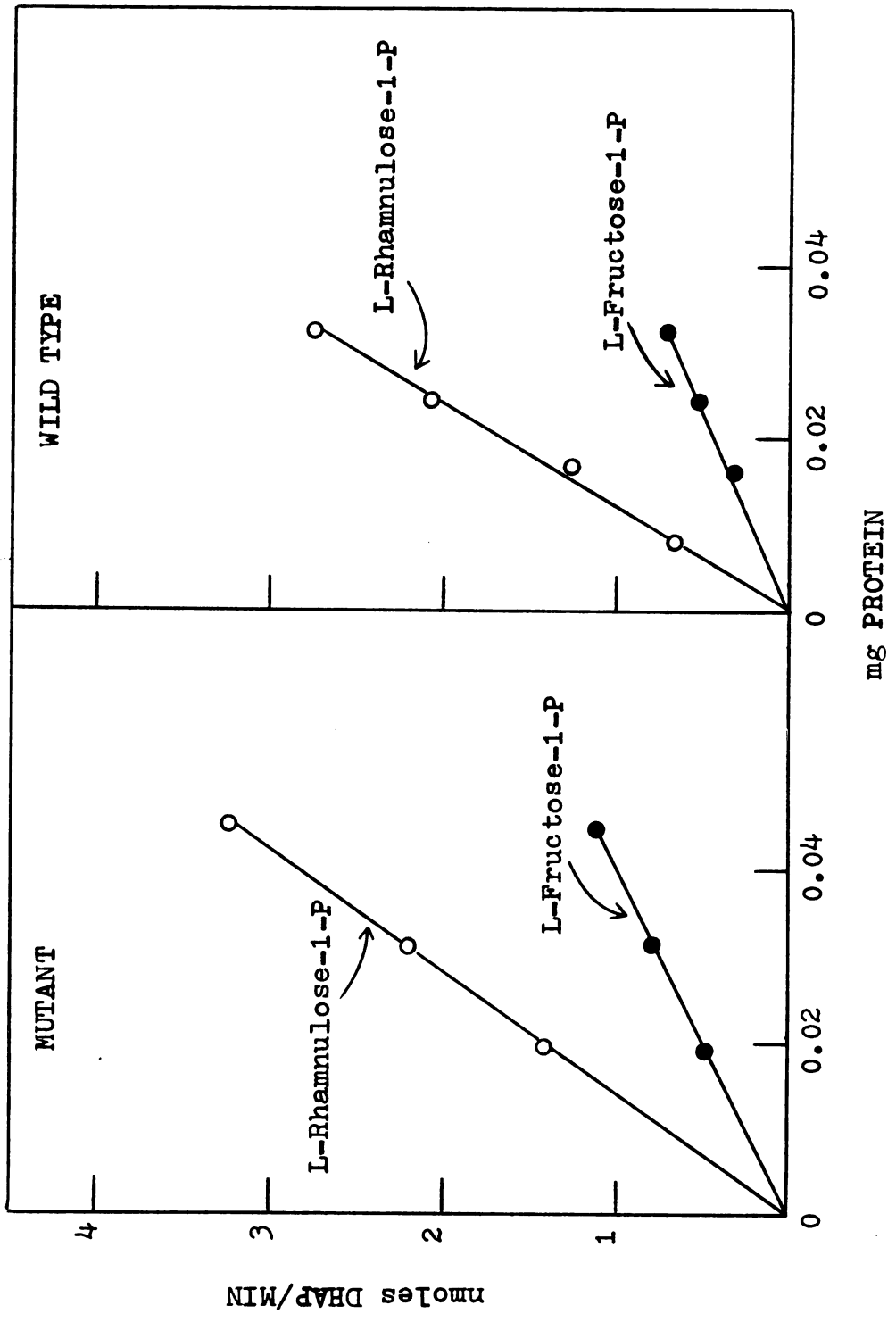

WILD TYPE
MUTANT
L-Rhamnulose-1-P
L-Fructose-1-P
L-Rhamnulose-1-P
L-Fructose-1-P
nmoles DHAP/MIN
mg PROTEIN
0
1
2
3
4
0.02
0.04

TABLE XIII

Partial purification of L-fructose-1-P (L-rhamnulose-1-P)

aldolase from the L-mannose-positive mutant

Fraction	Total Activity		Recovery	Specific Activity	
	Units*		%	Units/mg protein	
	L-fructose-1-P	L-rhamnulose-1-P		L-fructose-1-P	L-rhamnulose-1-P
Crude extract	4.0	11.0	100	0.03	0.07
Ammonium sulfate	3.4	9.5	86	0.10	0.25
Sephadex G-100	0.9	2.3	21	0.15	0.37

* Micromoles of NADH oxidized per minute at 25° in the standard assay.

Fig. 33. Fractionation of the aldolase on Sephadex G-100. The routine aldolase assay was employed. Details of the procedure are outlined in the text. Each fraction was assayed for activity on L-fructose-1-P and L-rhamnulose-1-P.

Figure 33.

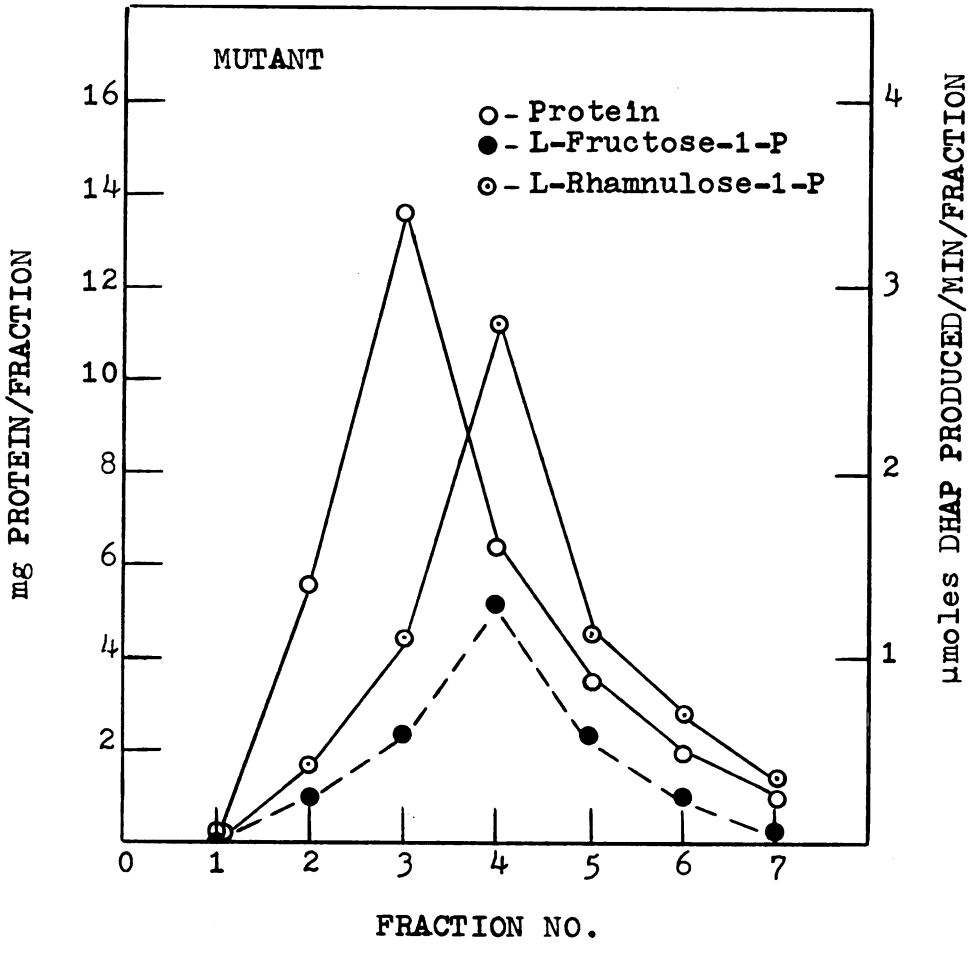
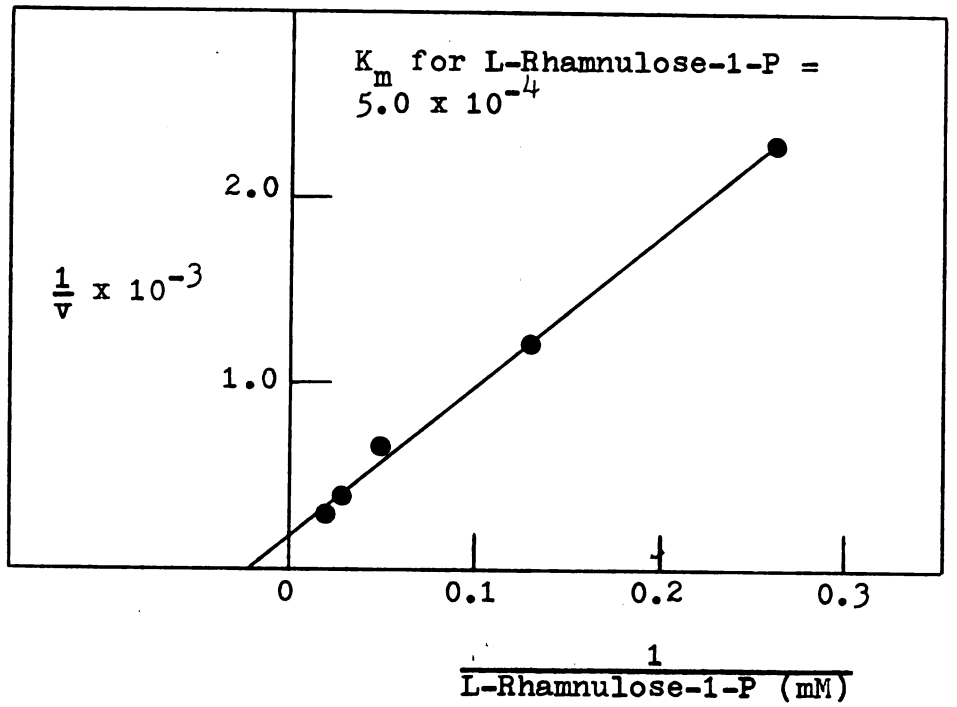
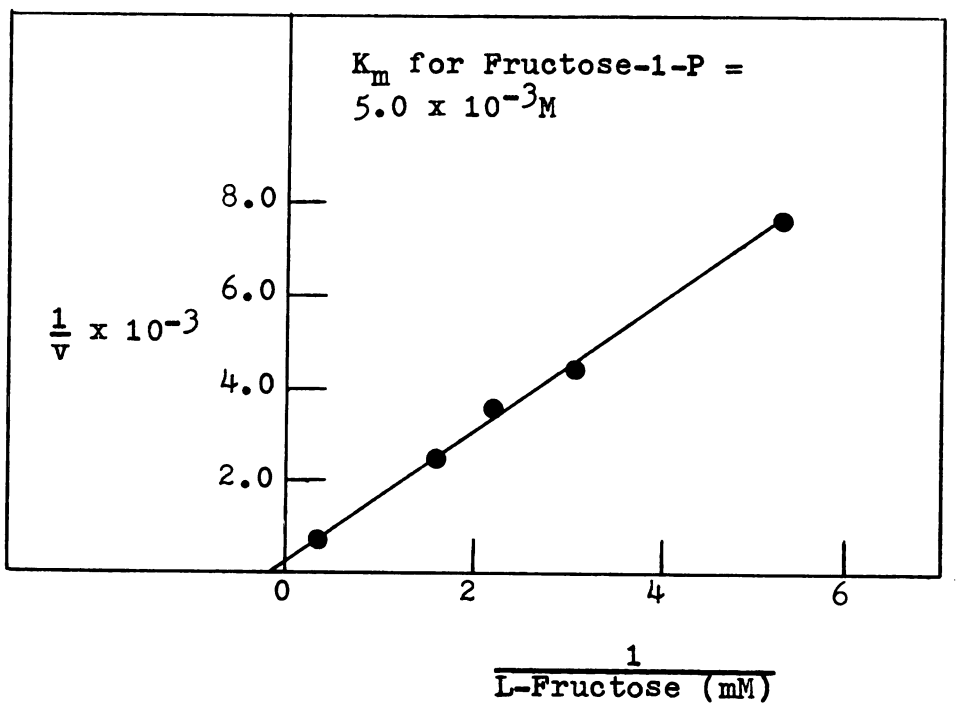

MUTANT
O - Protein
● - L-Fructose-1-P
⊙ - L-Rhamnulose-1-P
mg PROTEIN/FRACTION
µmoles DHAP PRODUCED/MIN/FRACTION
FRACTION NO.
0
1
2
3
4
5
6
7
2
4
6
8
10
12
14
16
1
2
3
4

Fig. 34. Lineweaver-Burk plot relating aldolase reaction velocity to substrate concentration. The routine assay was used except that the substrate was varied as indicated with the aldolase (Sephadex G-100 fraction) concentration constant. The enzyme was obtained from cells previously grown on L-mannose.

Figure 34.


K_m for L-Rhamnulose-1-P = 5.0×10^{-4}
$\frac{1}{v} \times 10^{-3}$
2.0
1.0
0
0.1
0.2
0.3
$\frac{1}{\text{L-Rhamnulose-1-P (mM)}}$


K_m for Fructose-1-P = 5.0×10^{-3}M
$\frac{1}{v} \times 10^{-3}$
8.0
6.0
4.0
2.0
0
2
4
6
$\frac{1}{\text{L-Fructose (mM)}}$

aldolase activities were not additive (Table XIV).

(e) Substrate Specificity- The aldolase when purified from both wild-type and mutant cells had the same substrate specificity (Table XV). It cleaved only L-rhamnulose-1-P and L-fructose-1-P, and did not cleave D-fructose-1-P or D-glucose-1-P. Activity on the other substrates was due to contaminating enzymes in the preparation and are listed in the table.

DISCUSSION

The evidence obtained in Part II of this thesis indicates that the same enzymes degrade L-mannose and L-rhamnose (Figure 35). They were induced by L-mannose and L-rhamnose in both the wild-type and mutant cells and the ratios of the specific activities of the individual enzymes for L-mannose, L-rhamnose, and their metabolic intermediates were the same in each strain. In addition, whether the enzymes were isolated from the wild-type or mutant cells, the activities on L-mannose or L-rhamnose could not be separated when subjected to partial purification. The enzymes showed the same substrate specificity when induced by either L-hexose and their individual specific activities were not additive when measured in the presence of both substrates. Finally, neither L-mannose nor L-rhamnose was able to induce the isomerase

TABLE XIV

Effect of mixing substrates on aldolase activity

The routine aldolase assay was employed. When both substrates were mixed, the concentration of each substrate was the same as that used in the routine assay. The aldolase was fractionated previously on Sephadex G-100 before use.

Substrate	Specific Activity (μmoles DHAP produced/ min/mg protein)
L-Fructose-1-PO_4	0.20
L-Rhamnulose-1-PO_4	0.33
L-Fructose-1-PO_4 + L-Rhamnulose-1-PO_4	0.24

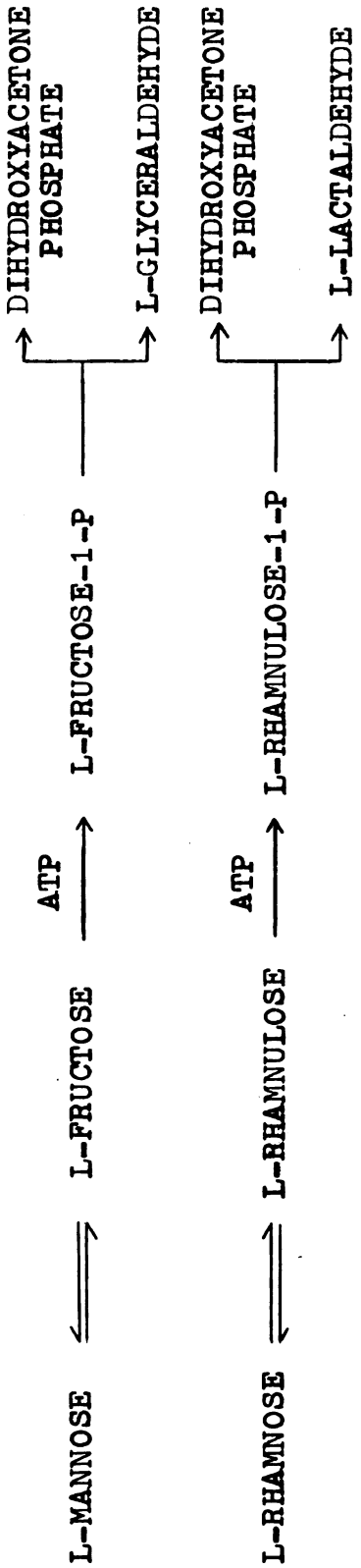
TABLE XV

Substrate specificity of the aldolase

The routine aldolase assay was used. With the exception of L-rhamnulose-1-P, the assay contained 1.5 μmoles of each substrate when tested. Contaminating activities are listed in cases where activity was expressed but was not due to cleavage by the aldolase.

Substrate	Comparative Rate (%)
L-Rhamnulose-1-P	100
L-Fructose-1-P	40-60
D-Fructose-1-P	0
D-Glucose-1-P	0
D-Fructose-6-P	mannitol-1-P dehydrogenase activity
D-Glucose-6-P	D-Fructose-6-P isomerase activity
D-Fructose-1,6-diP	glycolytic aldolase (trace)

Figure 35.


L-MANNOSE
L-FRUCTOSE
ATP
L-FRUCTOSE-1-P
DIHYDROXYACETONE PHOSPHATE
L-GLYCERALDEHYDE
L-RHAMNOSE
L-RHAMNULOSE
ATP
L-RHAMNULOSE-1-P
DIHYDROXYACETONE PHOSPHATE
L-LACTALDEHYDE

in a mutant deficient in this enzyme.

The metabolism of L-rhamnose has been described previously (36-41) and also found to undergo isomerization to L-rhamnulose, phosphorylation with ATP to L-rhamnulose-1-P, and cleavage to dihydroxyacetone phosphate and L-lactaldehyde. Domagk and Zech (37) purified L-rhamnose isomerase from Lactobacillus plantarum and showed that it isomerized L-mannose at a rate of <1% that of L-rhamnose. Chiu and Feingold (67) reported that L-rhamnulose-1-P aldolase from E. coli would not condense dihydroxyacetone phosphate and L-glyceraldehyde but Ghalambor and Heath (43) showed that L-fuculose-1-P aldolase would condense them to yield L-sorbose-1-P. In most cases, however, L-mannose or its metabolic intermediates have not been employed as test substrates.

The mutual antagonistic effect of Mn^{++} and Co^{++} on L-mannose and L-rhamnose is an interesting characteristic of the isomerase. Swada (39) showed that L-rhamnose isomerase from E. coli was stimulated by Mn^{++} and inhibited by Co^{++}, but L-mannose was not tested as a substrate. Similar cases of changes in substrate specificity due to changes in cations have been reported. Folk and Gladmer (68) showed that Co^{++} and Cd^{++} produced changes in the

peptidase and esterase activities of carboxypeptidase B. Co^{++} increased the peptidase activity and inhibited the esterase activity whereas Cd^{++} reversed the phenomenon. DNA polymerase also shows a similar effect (69); normally, the reaction proceeds in the presence of Mg^{++}, and only deoxyribonucleotide triphosphates serve as substrates. If Mn^{++} replaces Mg^{++}, however, ribonucleotides also may serve as substrates.

D-Mannose isomerase from Pseudomonas saccharophilia (70) has been purified about 6-fold and shown to be highly specific for D-mannose and D-rhamnose but inactive on L-mannose and L-rhamnose. D-Mannose isomerase, unlike L-mannose isomerase, is not affected by Mn^{++} or Co^{++}.

PART III

Comparison of the Utilization of L-Mannose by Wild-Type A. Aerogenes and the L-Mannose-Positive Mutant

The first two parts of this thesis defined the biodegradative pathway of L-mannose in A. aerogenes and confirmed that L-mannose and L-rhamnose were metabolized by the same enzymes. A mutant strain of A. aerogenes has been isolated which, unlike the wild type, readily grows on L-mannose as the sole carbon source. The third part of this thesis investigates the basis for the gain in the ability of this mutant to grow on L-mannose.

EXPERIMENTAL PROCEDURE

Growth Curves- Growth curves were performed in test tubes (150 mm x 18 mm) containing 7.0 ml of mineral medium or nutrient broth (71). The mineral medium was supplemented with 0.28% hexose and inoculated with 0.05 ml of actively growing cells to give an initial $O.D._{540}$= 0.01. For growth recorded on limiting amounts of hexose, the mineral medium or nutrient broth was supplemented with 0.08% hexose solution. The tubes were shaken at 30° on a reciprocating shaker. All growth measurements were recorded at 540 nm with a Coleman Junior Spectrophotometer.

Analytical Procedures- L-Rhamnose and L-mannose were determined by the method of Folin and Malmrose (11).

When the L-hexoses were mixed together, L-rhamnose was determined by the cysteine-sulfuric acid test (72) and L-mannose determined by the difference in the total reducing sugar present.

Reagents- L-Galactose was prepared by the method of Frush and Isbell (73) (courtesy of R. Hart). DL-Glyceraldehyde was purchased from Calbiochem., Los Angeles, California. Chloramphenicol was purchased from the Parke Davis and Company, Detroit, Michigan, and puromycin was a gift from Dr. A. Morris.

Determination of the Number of Generations- The total number of cells during growth was determined with a Petroff-Hauser Counter and viable cells determined by plating on nutrient agar.

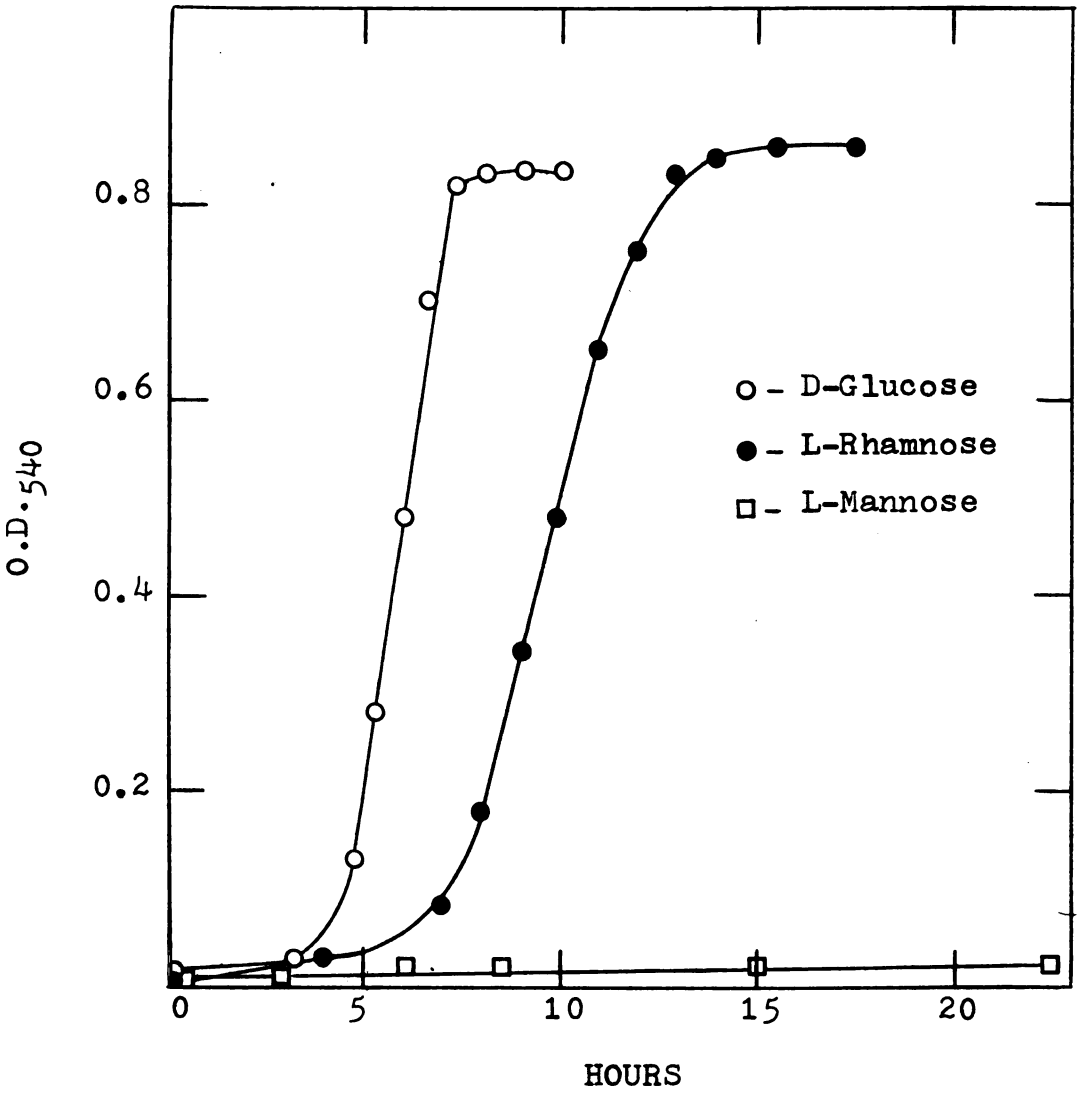
RESULTS

Growth of Wild-Type Cells on D-Glucose, L-Rhamnose, and L-Mannose- Wild-type A. aerogenes readily grew on L-rhamnose but no significant growth occurred on L-mannose during the same incubation period (Figure 36).

Selection of the L-Mannose-Positive Cells- If the wild-type cells were incubated with agitation for an additional period of time with L-mannose, growth on L-mannose eventually occurred but at a very slow rate. If then these cells were inoculated into fresh mineral

Fig. 36. Growth of wild-type cells on D-glucose, L-rhamnose, and L-mannose.

Figure 36.


0.8
0.6
0.4
0.2
O.D.540
0
5
10
15
20
HOURS
O - D-Glucose
● - L-Rhamnose
□ - L-Mannose

medium supplemented with L-mannose, growth occurred at a faster rate and by subsequent transfer a strain was eventually selected which readily grew on L-mannose. Figure 37 shows the relation between generation time and transfer number. There was a gradual decrease in the generation time to a minimum of 2.5 hours.

Stability of the L-Mannose-Positive Mutant- The L-mannose-positive strain retained its ability to grow on L-mannose. The cells could be stored for several weeks on nutrient agar and when inoculated into medium supplemented with L-mannose, the cells grew readily. Furthermore, if the mutant cells were grown on other carbon sources, such as D-glucose or L-rhamnose, they retained their ability to grow on L-mannose (Figure 38), indicating that growth on L-mannose was due to a mutation rather than an adaptation.

Response of the Wild Type and Mutant to Imvic Tests and a Specific Phage- Both wild-type and mutant cells responded identically to Imvic tests (71) (Table XVI) and to a phage which lysed A. aerogenes (Figure 39), indicating that the L-mannose positive cells selected were derived from A. aerogenes and not a contaminant.

Diauxie Curves--Effect of L-Mannose on the Growth of the Wild Type and Mutant- Since the manometric studies

Fig. 37. Selection of the L-mannose-positive mutant. Each transfer was performed after the growth culture reached an $O.D._{540}$ = 0.40. A 0.05-ml volume of cells was transferred to fresh medium supplemented with L-mannose. The generation time was defined as the time in which the culture grew from an $O.D._{540}$ = 0.15 to 0.30.

Figure 37.

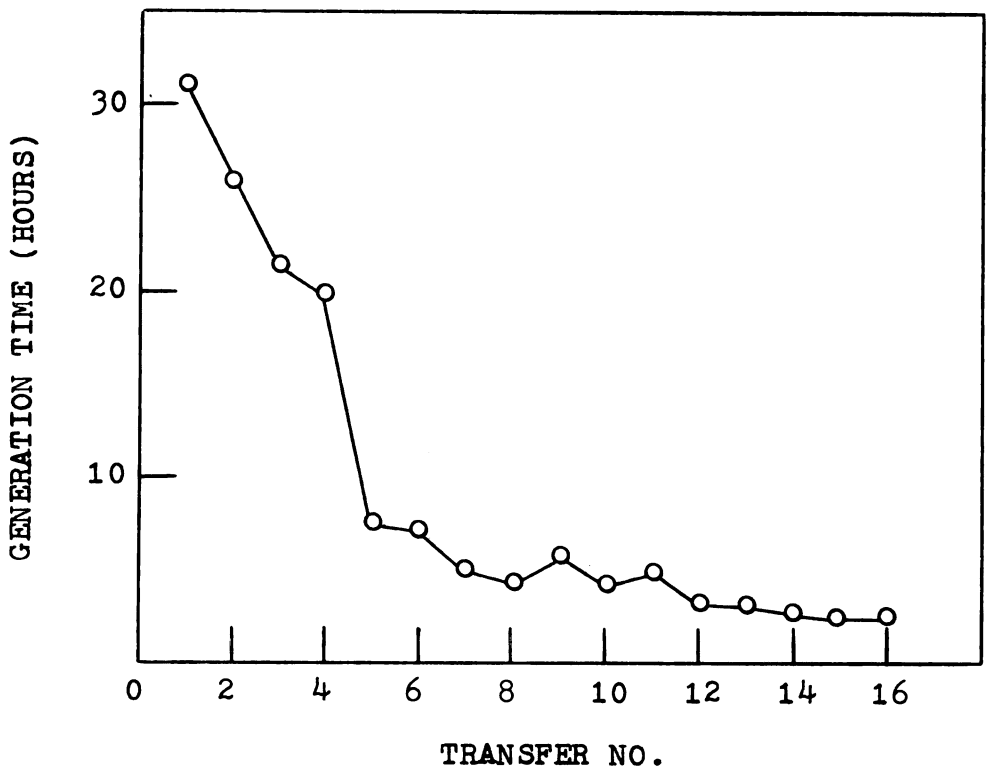


Fig. 38. Stability of the L-mannose-positive mutant.

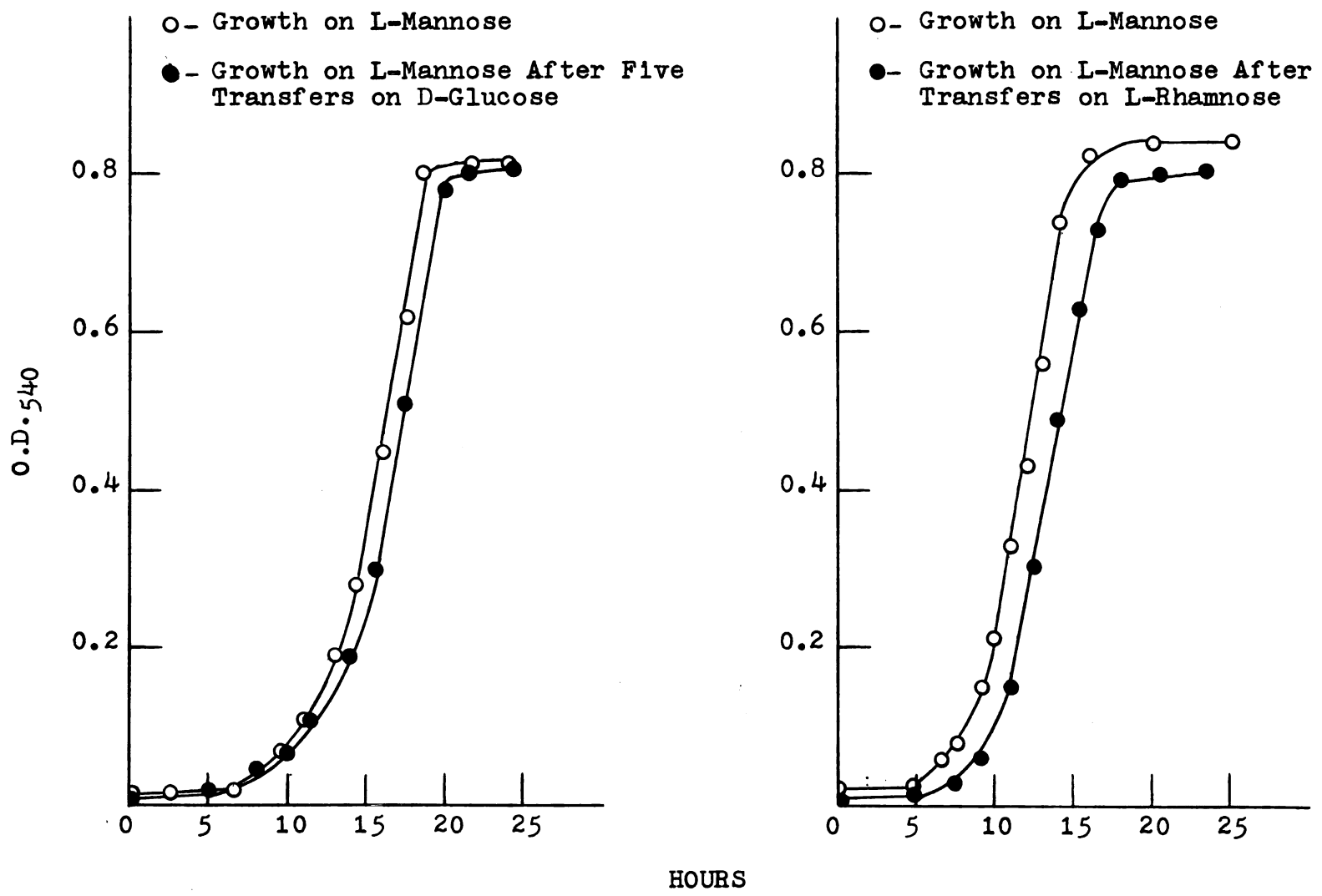

O- Growth on L-Mannose
●- Growth on L-Mannose After Five Transfers on D-Glucose
O.D.540
0.8
0.6
0.4
0.2
0
5
10
15
20
25
HOURS
O- Growth on L-Mannose
●- Growth on L-Mannose After Three Transfers on L-Rhamnose
0.8
0.6
0.4
0.2
0
5
10
15
20
25

Figure 38.

TABLE XVI

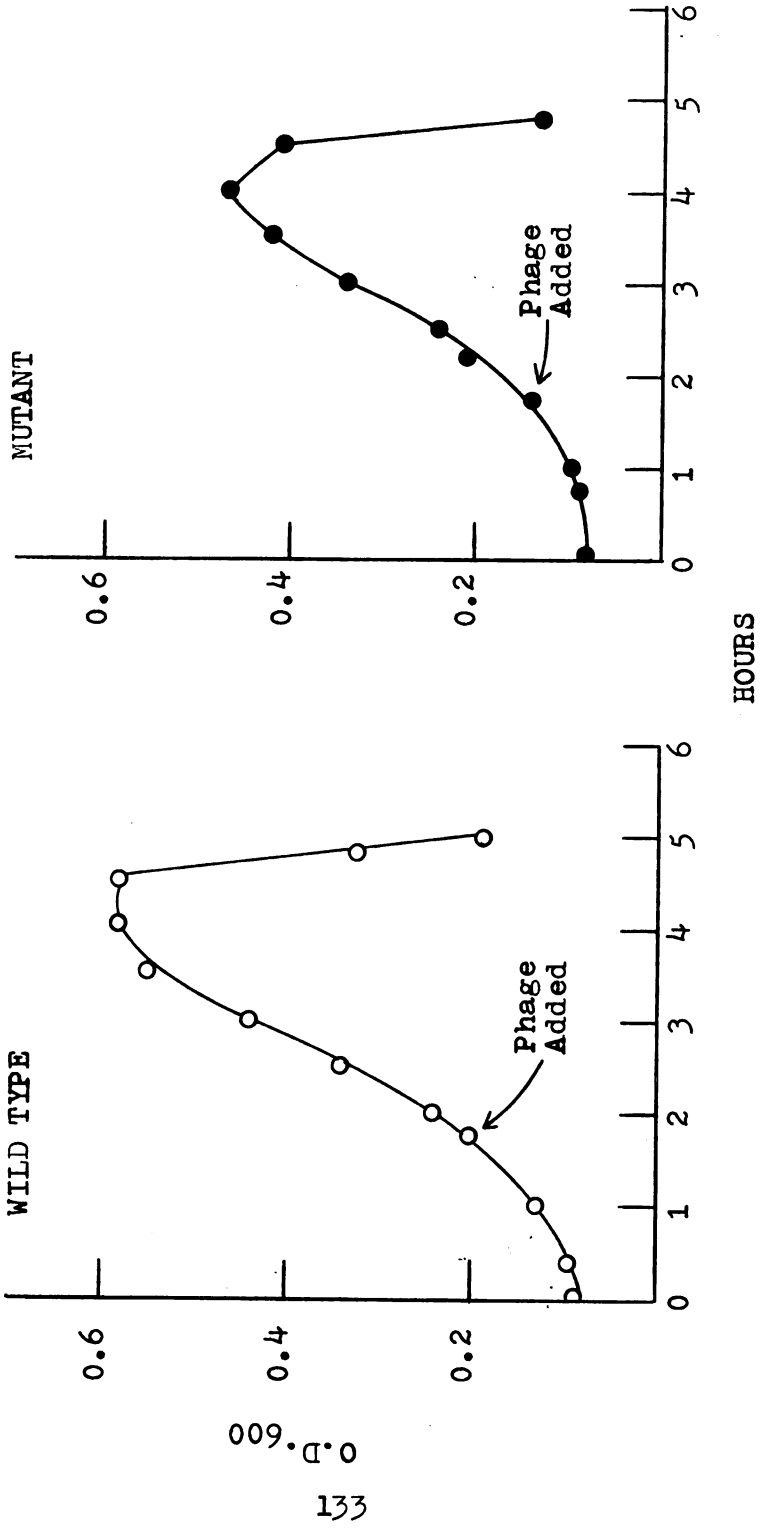
Response of the wild type and mutant to imvic tests

The tests were performed according to the procedures outlined in the Difco manual (71).

Cell Type	Indole	Methyl Red	Voges-Proskauer	Citrate
Wild	+	+	-	+
L-Mannose Positive	+	+	-	+

Fig. 39. Phage specificity of A. aerogenes.

Figure 39.


WILD TYPE
MUTANT
Phage Added
Phage Added
0.6
0.4
0.2
0
1
2
3
4
5
6
HOURS
O.D.600

presented in Part II showed that the wild-type cells could utilize L-mannose if the biodegradative enzymes were previously induced by L-rhamnose, investigations were carried out to determine what effect L-mannose had on actively growing wild-type cells. Since the wild-type cells grew very slowly on L-mannose as a sole carbon source, the growth medium was supplemented with a limiting amount of L-rhamnose to induce the degradative enzymes, and, after the L-rhamnose was completely metabolized, L-mannose utilization would begin. Figure 40 compares the utilization of L-rhamnose and L-mannose by wild-type cells. The cells grew preferentially on D-glucose, and when the D-glucose was completely degraded, they grew on L-rhamnose but not on L-mannose. L-Mannose-positive cells produced a different result (Figure 41). D-Glucose and L-rhamnose were readily utilized but, unlike the wild type, the mutant cells also grew on L-mannose.

When the wild-type cells were allowed to grow on a mixture of L-mannose and L-rhamnose, a change in the growth rate occurred; growth on L-rhamnose was rapid and when completely metabolized growth on L-mannose began but with a decrease in the growth rate (Figure 42). In contrast, the mutant readily grew on L-rhamnose and L-mannose, and in the mixture of these hexoses the cells grew to a greater

Fig. 40. Growth of wild-type A. aerogenes on D-glucose, and mixtures of D-glucose and L-rhamnose, and D-glucose and L-mannose.

Figure 40.

D-GLUCOSE
D-GLUCOSE +
L-RHAMNOSE
D-GLUCOSE +
L-MANNOSE
0.6
0.4
0.2
0
5
10
HOURS
O.D. 540

Fig. 41. Growth of the L-mannose-positive strain on D-glucose and mixtures of D-glucose and L-rhamnose, and D-glucose and L-mannose.

Figure 41.

D-GLUCOSE
D-GLUCOSE +
L-RHAMNOSE
D-GLUCOSE +
L-MANNOSE
0.6
0.4
0.2
0
10
20
HOURS
O.D.540

Fig. 42. Growth of the wild-type cells on L-rhamnose, L-mannose, and a mixture of these L-hexoses.

Figure 42.

L-RHAMNOSE
L-MANNOSE
L-RHAMNOSE +
L-MANNOSE
0.6
0.4
0.2
0
10
20
HOURS
O.D.540

extent than the wild type with no change in the growth rate when L-mannose utilization had begun (Figure 43). Thus, when L-mannose entered the wild-type cell, L-mannose itself, or its metabolic intermediates, exerted a toxic or inhibitory effect on the organism. This effect was overcome by the mutant resulting in active growth on L-mannose.

Hexose Utilization as a Function of Growth- To demonstrate a more direct effect of L-mannose on the growth of wild-type and mutant cells, growth and hexose utilization were measured simultaneously. Figure 44 compares the utilization of L-rhamnose and L-mannose as a function of growth. L-Rhamnose, but not L-mannose, was readily utilized by the wild type. When both hexoses were mixed, the wild-type cells showed a preferential growth on L-rhamnose which, after completely degrading it, began to metabolize L-mannose. However, the growth rate on L-mannose decreased, eventually leveling off but with continuous utilization of L-mannose. Unlike the wild type, the mutant readily grew on L-mannose after the L-rhamnose was metabolized (Figure 45).

Although the diauxie studies indicated that L-mannose exerted an inhibitory effect on the growth of the wild-type cells, these investigations were conducted under conditions

Fig. 43. Growth of the L-mannose-positive strain on L-rhamnose, L-mannose, and a mixture of these L-hexoses.

Figure 43.

L-RHAMNOSE
L-MANNOSE
L-RHAMNOSE +
L-MANNOSE
0.6
0.4
0.2
0
10
20
HOURS
O.D.540

Fig. 44. Determination of L-mannose and L-rhamnose utilization by actively growing wild-type cells. Hexose utilization was stopped by removing 100 µl aliquots from the growth medium and adding it to 100 µl of 0.2 N H_2SO_4. Aliquots of this solution then were tested for hexoses by the following procedures:

a. When either L-rhamnose or L-mannose was the sole carbon source, it was measured by the method of Folin and Malmrose (11).

b. When L-rhamnose and L-mannose were mixed, L-rhamnose was measured by the cysteine-sulfuric acid test (62). The difference in optical density at 396 nm and 429 nm gave the amount of L-rhamnose in the presence of L-mannose.

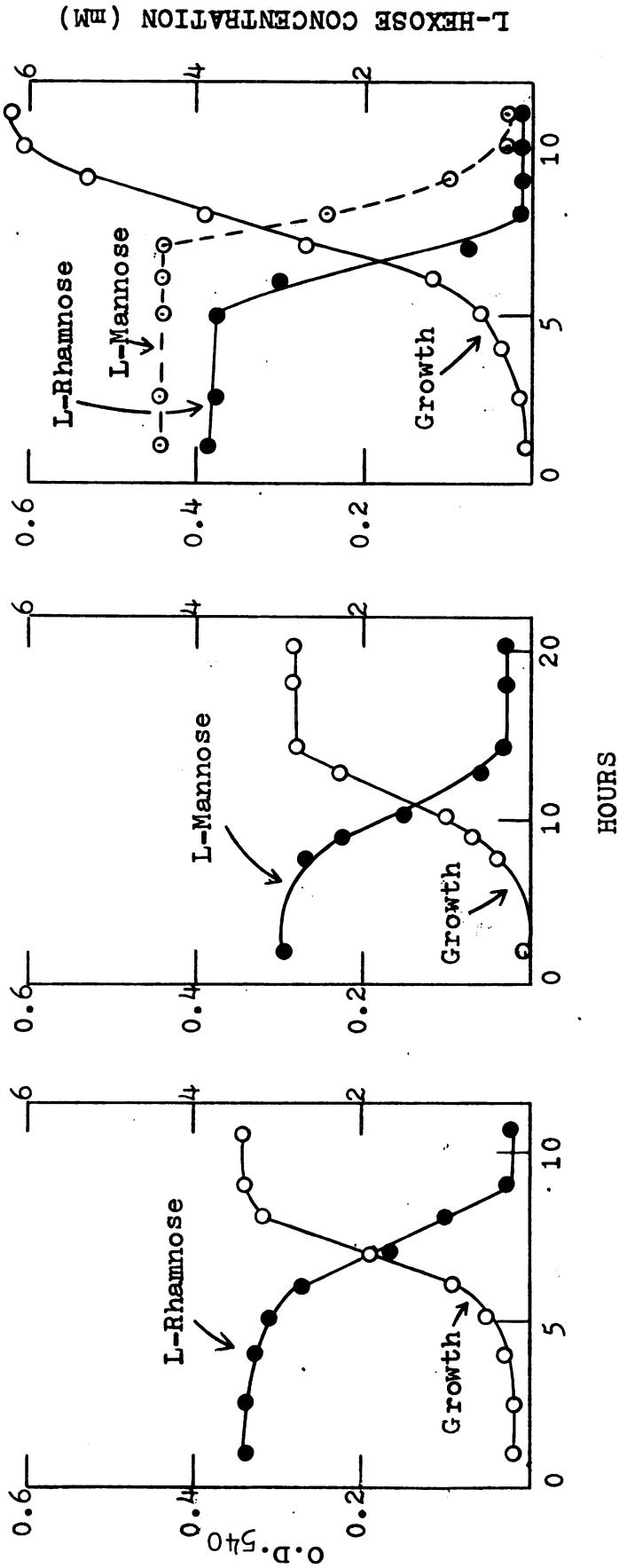
c. To determine L-mannose in the presence of L-rhamnose, the total optical density of the mixture was determined by the procedure of Folin and Malmrose. With L-rhamnose determined by the procedure in part b, its contribution to the optical density measured at 520 nm in the mixture was calculated. Thus, the difference in the optical density of the mixture and the calculated value for L-rhamnose gave the amount of L-mannose.

Figure 44.

L-HEXOSE CONCENTRATION (mM)
O.D.540
HOURS
L-Rhamnose
L-Mannose
Growth
0.6
0.4
0.2
0
5
10
2
4
6
20

Fig. 45. Utilization of L-rhamnose, L-mannose, and a mixture of these L-hexoses by actively growing L-mannose-positive cells. Refer to Figure 44 for details.

Figure 45.


L-HEXOSE CONCENTRATION (mM)
O.D.540
HOURS
L-Rhamnose
L-Mannose
Growth

in which the biodegradative enzymes were induced by L-rhamnose. Both cell types responded differently to growth on nutrient broth supplemented with L-mannose (Figure 46). The growth of the parent cells was inhibited when utilization of L-mannose had begun but with continued uptake of L-mannose. Likewise, the mutant cells were inhibited when L-mannose initially was utilized, but unlike the wild type, the inhibition was overcome and growth on L-mannose continued to completion.

Induction of L-Mannose Degradative Enzymes in the Wild Type- The isomerase, kinase, and aldolase activities were measured in the wild-type cells after induction by L-mannose and L-rhamnose (Table XVII). Both L-hexoses induced all three enzymes which explains the utilization of L-mannose by the wild-type organism.

Measurement of Enzymatic Activity upon Removal of the Inducer- To demonstrate if any or all of the first three degradative enzymes were constitutive in the L-mannose-positive cells, the inducer, L-mannose, was removed and the cells were grown for four and seven generations (Table XVIII) after which individual enzyme activities were measured (Table XIX). The simultaneous

Fig. 46. Growth of wild-type and L-mannose-positive cells in nutrient broth supplemented with L-mannose.

Figure 46.

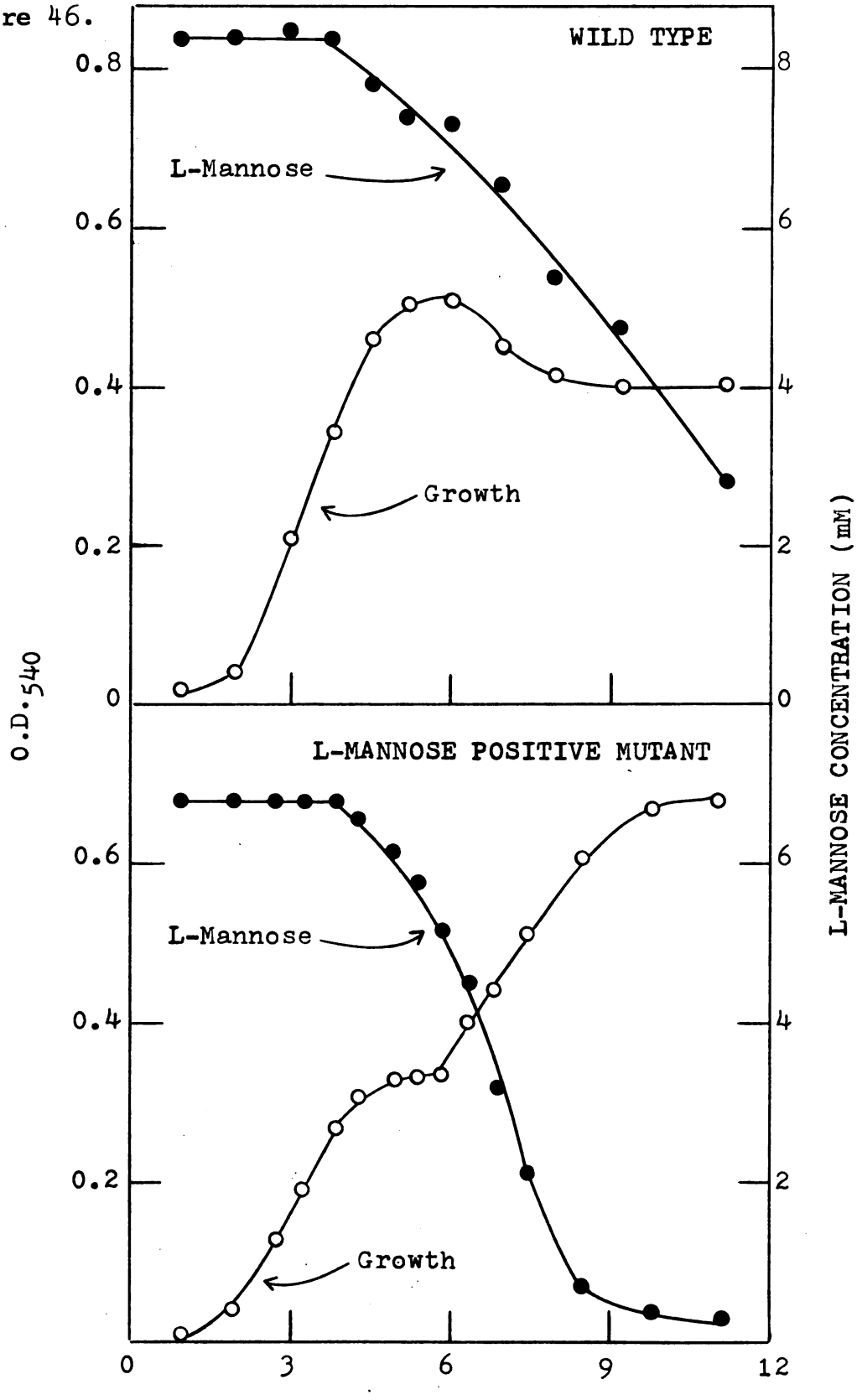

WILD TYPE
L-Mannose
Growth
L-MANNOSE POSITIVE MUTANT
L-Mannose
Growth
0.8
0.6
0.4
0.2
0
8
6
4
2
0
0.6
0.4
0.2
6
4
2
0
3
6
9
12
HOURS
O.D.540
L-MANNOSE CONCENTRATION (mM)

TABLE XVII

Induction of L-mannose enzymes in wild-type cells

a. Induction by L-Mannose- The cells were grown aerobically in nutrient broth supplemented with 350 mg of L-mannose. The cells were harvested when inhibition of growth by L-mannose was recorded.

b. Induction by L-Rhamnose- The cells were grown aerobically in nutrient broth for 6-8 hours, after which they were supplemented with 350 mg of L-rhamnose and allowed to shake an additional 70 minutes before harvest.

Inducer	Isomerase		Kinase*		Aldolase*	
	L-Rhamnose	L-Mannose	L-Rhamnulose	L-Fructose	L-Rhamnulose -1-P	L-Fructose -1-P
L-Rhamnose	0.24	0.07	1.25	1.40	0.11	0.03
L-Mannose	0.11	0.03	1.10	1.30	0.09	0.02

* The kinase and aldolase activities were determined after fractionating with ammonium sulfate. Activities are expressed as μmoles product/min/mg protein.

TABLE XVIII

Determining the number of generations upon removal of the inducer

A culture of the mutant cells previously grown on L-mannose was diluted to a final $O.D._{540}$ = 0.50 and 10 ml of this solution inoculated into 490 ml of nutrient broth and allowed to grow. The total number of cells at a specific time interval was determined with a Petroff-Hauser bacteria counter and the viable count determined by plating the cells on nutrient agar. This procedure was used to determine when the cells had grown to four generations. For seven generations of growth, 2 ml of the diluted mutant culture was inoculated into 498 ml of nutrient broth and the above procedure repeated.

Four Generations			
Hours of Growth	$O.D._{540}$	Viable Count (cells/ml) x 10^8	Total Count (cells/ml) x 108
0	0.010	0.21	0.20
1.25	0.016	0.23	0.25
2.00	0.038	0.30	0.40
2.75	0.080	0.50	0.56
3.50	0.168	1.35	1.40
4.75	0.300	3.30	4.60
Seven Generations			
0	0.001	0.05	0.05
1.25	-	0.05	0.08
2.00	-	0.07	0.10
2.75	0.025	0.15	0.20
3.50	0.055	0.28	0.38
4.25	0.140	0.65	0.80
5.00	0.290	3.30	1.94
5.75	0.400	5.70	4.00

TABLE XIX

Measurement of enzyme activity upon removal of the inducer

After growing for four and seven generations the cells were harvested and enzyme activity in the crude extracts measured as described in the text.

Growth Substrate	Generations	Isomerase		Kinase		Aldolase	
		L-Rhamnose	L-Mannose	L-Rhamnulose	L-Fructose	L-Rhamnulose -1-P	L-Fructose -1-P
L-Mannose	-	0.78	0.20	0.27	0.31	0.16	0.06
Nutrient Broth	4	0.008	0.003	0.005	0.005	0*	0*
Nutrient Broth	7	0*	0*	0*	0*	0*	0*

Results are expressed as μmoles of product/min/mg of protein.

* < 0.002

decrease in the level of all three enzymes indicated that the mutant was not constitutive for them.

Effect of Protein Inhibitors on the Utilization of L-Mannose and L-Rhamnose- To show further that the first three enzymes were not constitutive, the inducers L-mannose and L-rhamnose were added back to the mutant cells previously grown in the absence of inducers and the rate of their utilization measured manometrically. To distinguish between constitutive and newly induced enzymes, either chloramphenicol or puromycin was added to the reaction. As Figures 47 and 48 indicate, in the absence of these inhibitors L-rhamnose was readily utilized and reached a maximal rate comparable to D-glucose. L-Mannose also was oxidized but at a slower rate than L-rhamnose. In the presence of either chloramphenicol or puromycin neither L-mannose nor L-rhamnose was utilized. The results thus showed that the oxidation of L-mannose and L-rhamnose by the mutant cells was due to the biosynthesis of new enzymes and not to a constitutive mutation.

The results indicated that another mechanism was operating within the cell to regulate growth on L-mannose. Since dihydroxyacetone phosphate was readily metabolized, L-glyceraldehyde was restudied to determine if it exerted

Fig. 47. Effect of chloramphenicol and puromycin on enzyme induction and hexose utilization in the mutant after four generations of growth in the absence of inducer. Each Warburg vessel contained in a volume of 0.55 ml, 10 μmoles of either L-rhamnose or L-mannose, 180 μmoles of KOH (center well), 1000 μg of chloramphenicol or 500 μg of puromycin, and about 2 mg dry weight of cells. As a control water was substituted for the inhibiter of protein biosynthesis and for endogenous activity water was substituted for the hexose.

Figure 47.

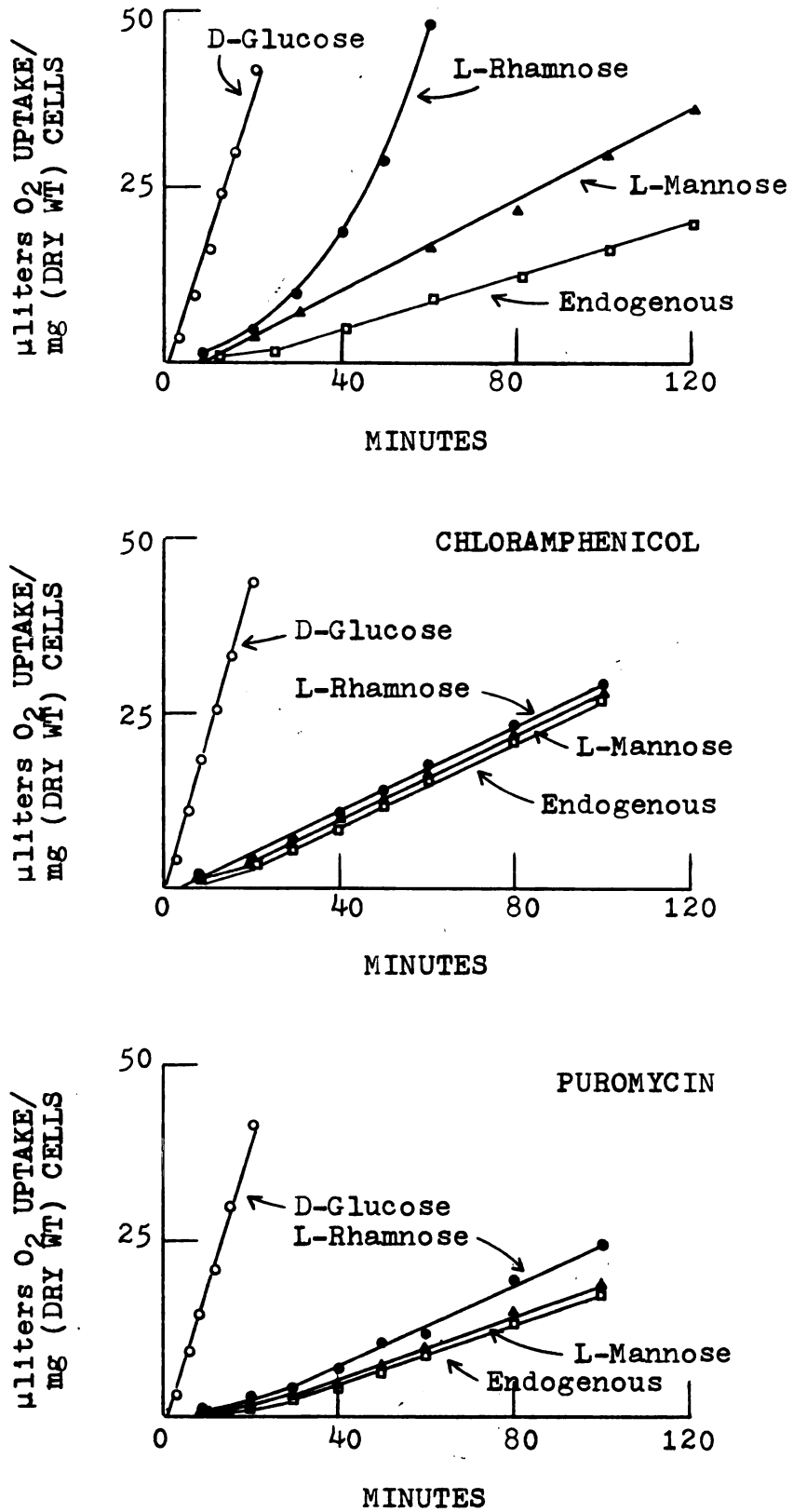
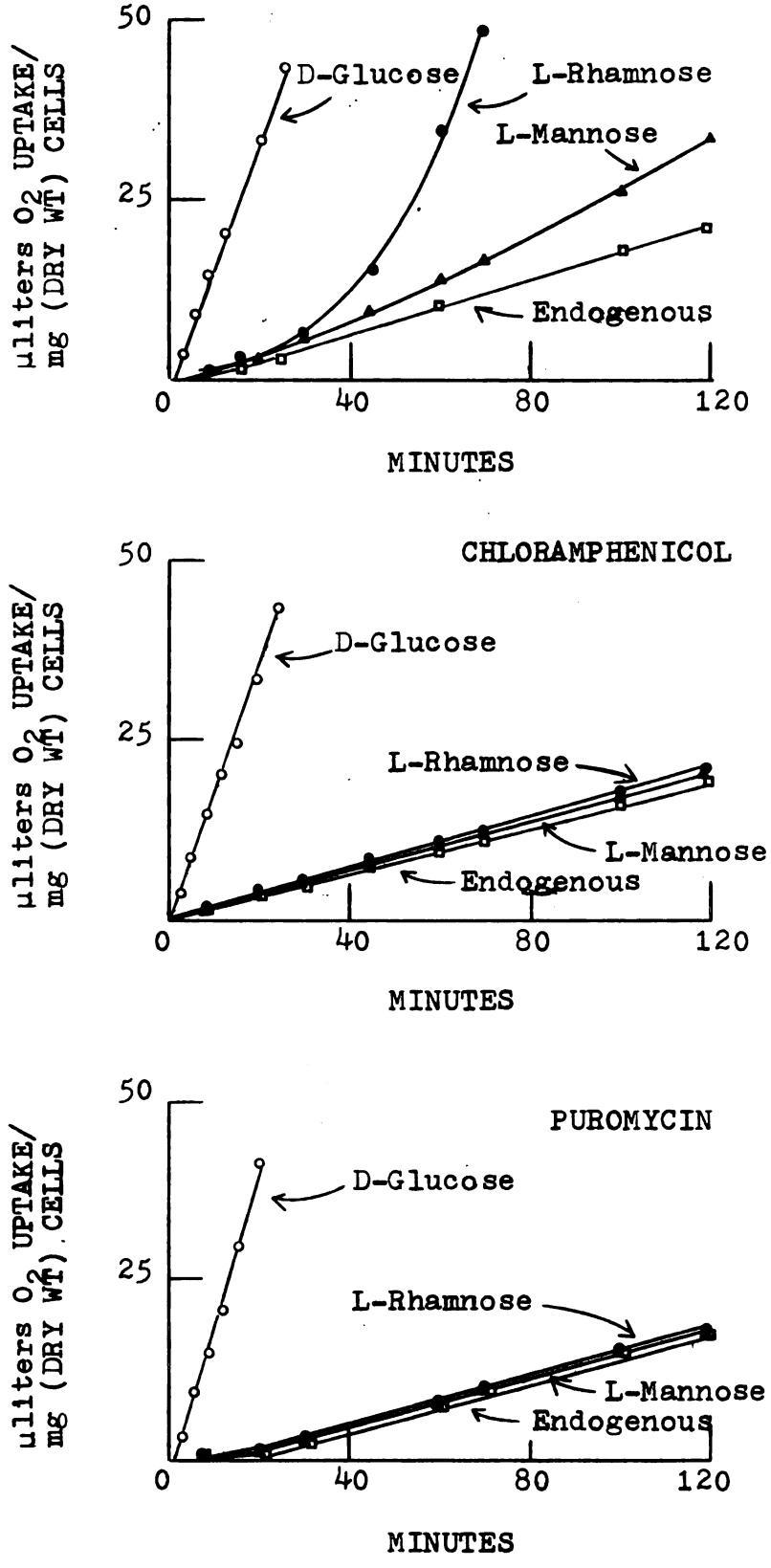

µliters O_2 UPTAKE/ mg (DRY WT) CELLS
50
25
0
40
80
120
MINUTES
D-Glucose
L-Rhamnose
L-Mannose
Endogenous
CHLORAMPHENICOL
PUROMYCIN

Fig. 48. Effect of chloramphenicol and puromycin on enzyme induction and hexose utilization in the mutant after seven generations of growth in the absence of the inducer. Refer to Figure 47 for details.

Figure 48.


µliters O_2 UPTAKE/ mg (DRY WT) CELLS
50
25
0
40
80
120
MINUTES
D-Glucose
L-Rhamnose
L-Mannose
Endogenous
CHLORAMPHENICOL
PUROMYCIN

any effect on the cells. Part I of this thesis showed that L-glyceraldehyde underwent oxidation to glyceric acid and reduction to glycerol. Thus, investigations were conducted to establish if these reactions were instrumental in regulating L-mannose metabolism. Since the wild-type cells completely utilized L-mannose without growth, the possibility existed that a degradative product of L-mannose which inhibited growth accumulated in the growth medium. Attempts to find such a product failed.

Contribution of Glyceric Acid to L-Glyceraldehyde Metabolism- The enzyme which converts L-glyceraldehyde to glyceric acid was detected only by identification of the reaction product (see Part I). Paper chromatography of the growth media obtained from both wild-type and mutant cells actively utilizing L-mannose showed the accumulation of a compound which co-chromatographed with DL-glyceric acid. Whether or not some of the glyceric acid was metabolized by these cells is not known. However, since it did accumulate as an excretory product of L-mannose degradation by both cell types, it was eliminated as a possible growth inhibitor.

Effect of Glycerol on the Growth of the Wild-Type and Mutant Cells- Both the mutant and the wild-type cells

grew equally well on glycerol (Figure 49). Although the reduction of L-glyceraldehyde to glycerol could not be assayed in crude extracts because of interfering reactions, partial fractionation with ammonium sulfate yielded a 45-60% fraction which showed reduction of L-glyceraldehyde. This enzyme was detected in fractionated extracts obtained from both wild-type and mutant cells previously grown on L-rhamnose or L-mannose respectively (Table XX). In addition, L-mannose also induced the enzyme in wild-type cells after they were previously grown on D-glucose. Since both L-hexoses induced the reductase in both cell types, the reduction of L-glyceraldehyde to glycerol was not considered to be an influential reaction in inhibiting the growth of the parent strain on L-mannose.

Effect of DL-Glyceraldehyde on the Growth of the Wild-Type and Mutant Cells- DL-Glyceraldehyde inhibited the growth of both wild-type and mutant cells above a concentration of 0.007 mM. Figure 50 compares the effect of DL-glyceraldehyde on the growth of the cells. In neither case was the inhibitory effect overcome at the higher concentration. No conclusion was drawn as to which enantiomorph caused the growth inhibition.

Growth of the Wild Type and Mutant on L-Galactose- A. aerogenes responded to growth on L-galactose, a rare

Fig. 49. Growth of A. aerogenes on glycerol.

Figure 49.

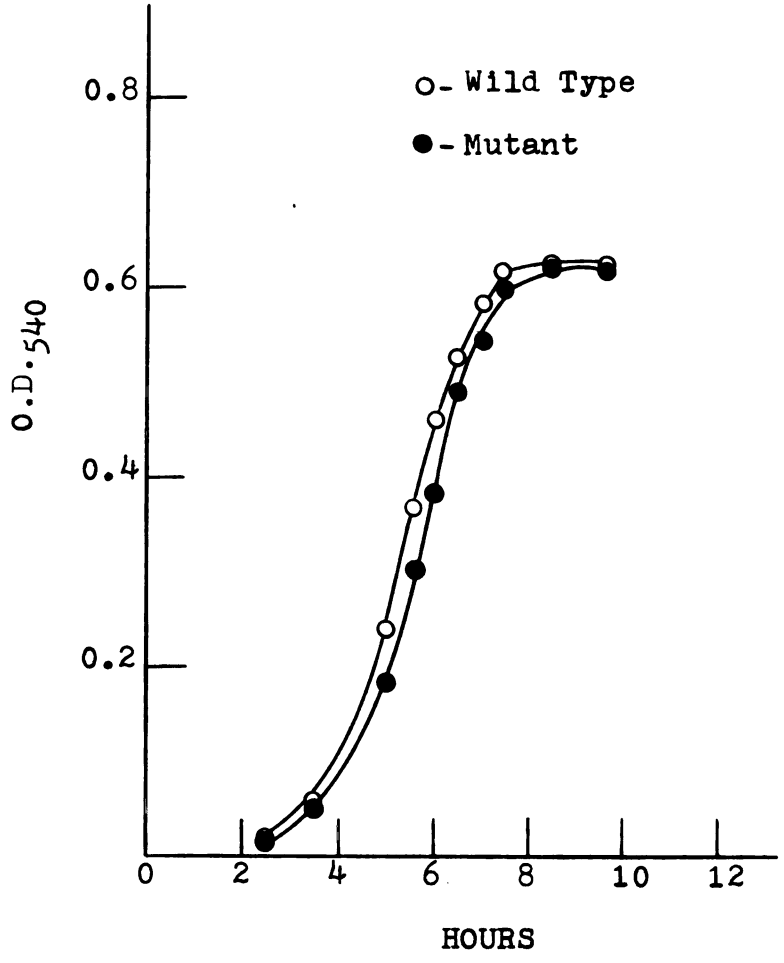


TABLE XX

Induction of L-glyceraldehyde reductase

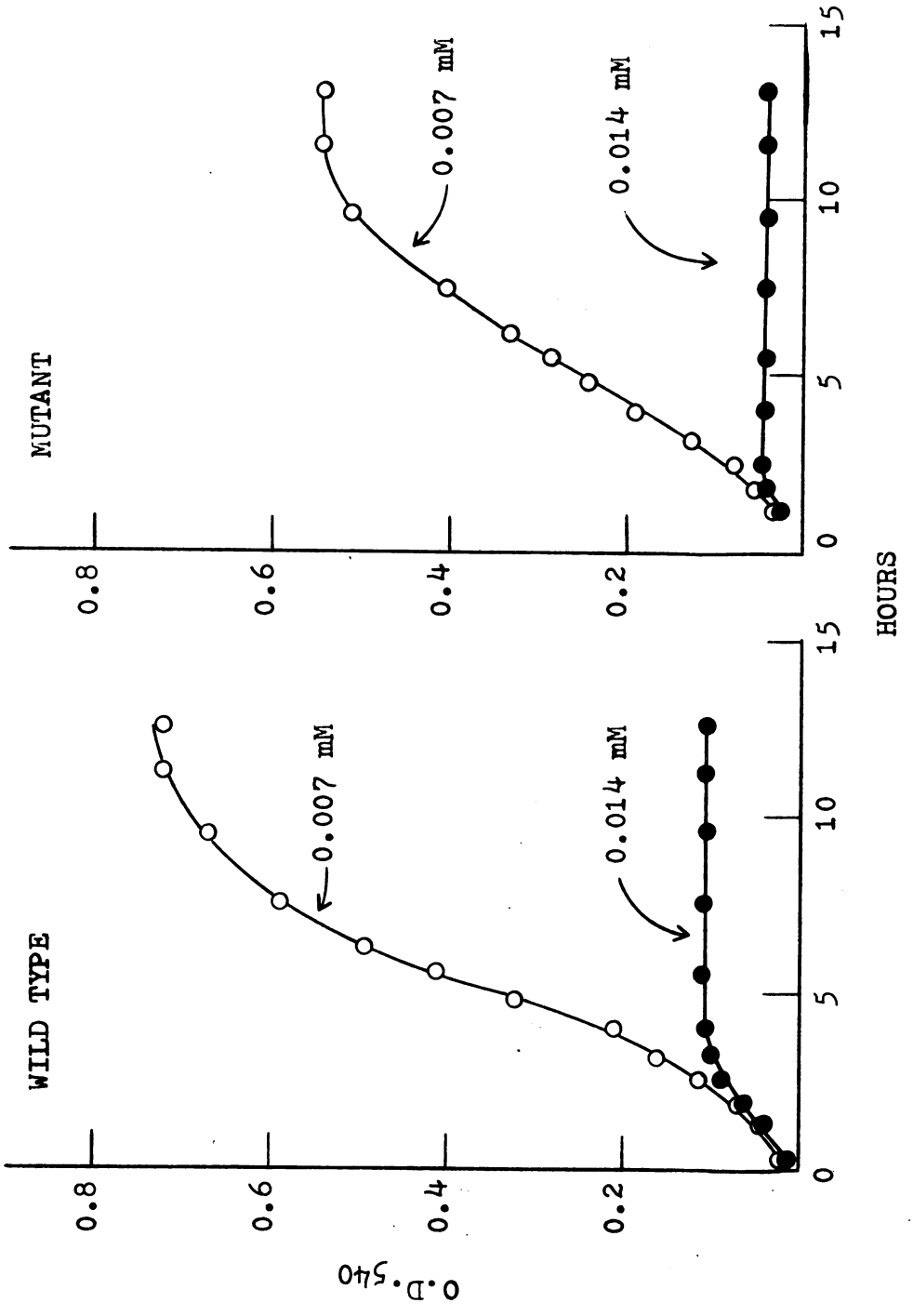
Reductase activity was measured using the routine assay. The enzyme was from a 45-60% ammonium sulfate fraction.

Cell Type	Growth Substrate	Reductase Activity*
Wild	L-Rhamnose	0.088
	D-Glucose and L-Mannose	0.020
	D-Glucose	0.000
Mutant	L-Mannose	0.021
	D-Glucose	0.000

* Activity is expressed as μmoles NADH oxidized/min/mg of protein.

Fig. 50. Growth of A. aerogenes in nutrient broth supplemented with varying amounts of DL-glyceraldehyde.

Figure 50.


WILD TYPE
MUTANT
0.007 mM
0.014 mM
0.007 mM
0.014 mM
0.2
0.4
0.6
0.8
0
5
10
15
HOURS
O.D.540

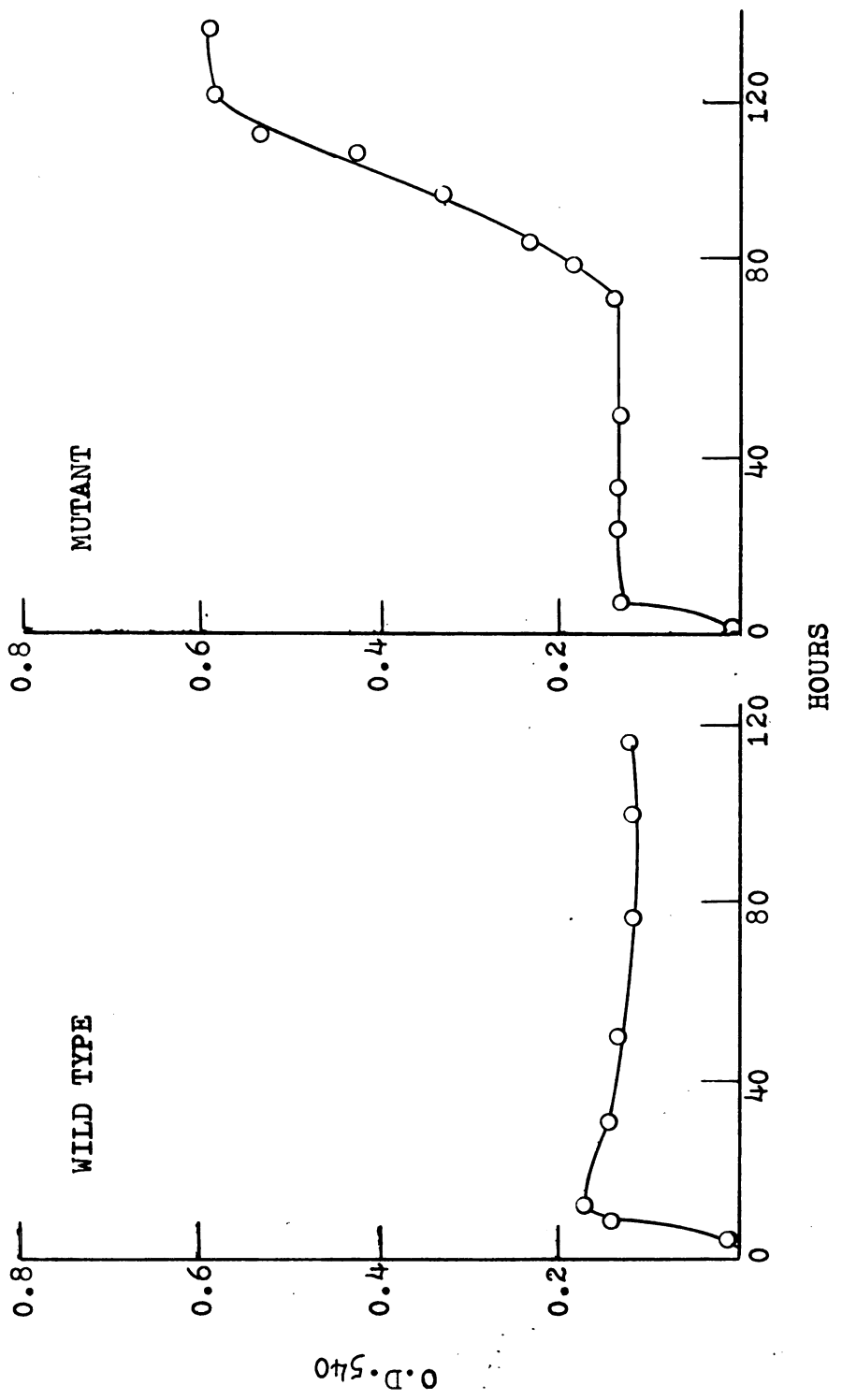
hexose, in the same way as it did to L-mannose. Although L-galactose and L-mannose differ structurally, they both undergo similar biodegradative patterns and produce L-glyceraldehyde as a major intermediate. L-Galactose metabolism has been established in A. aerogenes (Mayo and Anderson, unpublished results) and found to be isomerized to L-tagatose, phosphorylated with ATP to L-tagatose-1-P, and cleaved to dihydroxyacetone phosphate and L-glyceraldehyde. If the basis for growth on L-mannose involved L-glyceraldehyde or a subsequent metabolic intermediate, then the L-mannose positive mutant should also grow on L-galactose more readily than the wild type. As Figure 51 indicates, a 75-hour lag preceded growth of the mutant on L-galactose whereas the wild type showed no appreciable growth even after 120 hours. There was some initial growth by both cell types but this was attributed to an unknown contaminent rather than to growth on L-galactose. The mutant cells after growing on L-galactose also grew on L-mannose.

DISCUSSION

The growth of A. aerogenes on L-mannose involves a mechanism other than a constitutive mutation for the first three enzymes in the pathway. The evidence in this investigation suggests that the basis for the gain in the

Fig. 51. Growth of A. aerogenes on L-galactose.

Figure 51.



ability of the mutant to grow on L-mannose lies in its ability to metabolize L-glyceraldehyde or a subsequent metabolic intermediate. The growth of both the wild type and the mutant was inhibited when the cells began to utilize L-mannose, but only the mutant overcame the inhibition. This suggested that a compound accumulated within the cells which inhibited their growth rather than the selection of a mutant strain which resisted the inhibitory effect of this product. Since L-mannose induced the isomerase, kinase, and aldolase in both types of cells, the first three enzymes in the biodegradative pathway were not influential in regulating growth of the cells on L-mannose. In addition, none of the enzymes was constitutive when the inducer was removed.

L-Glyceraldehyde inhibits a variety of cellular processes but the exact site(s) of inhibition are still questionable. Mendel (74) was the first to report that L-glyceraldehyde inhibited the formation of lactic acid from glucose in tumor cells. Rudney (75) had shown that hexokinase in rat skeletal muscle, rat sarcoma, beef brain, and yeast also was inhibited by L-glyceraldehyde. In a later report Wenzel, Joel, and Oelkers (76) demonstrated that L-glyceraldehyde inhibited glycolysis in Ehrlich ascite tumor cells. On the other hand, D-glyceraldehyde

has been implicated by Rapkine et al (77) to inhibit D-glyceraldehyde-3-P dehydrogenase. Possibly DL-glyceraldehyde is working in a dual capacity to inhibit growth of A. aerogenes. Lardy and coworkers (78) have shown that rabbit muscle aldolase condenses L-glyceraldehyde and dihydroxyacetone phosphate to yield L-sorbose-1-P which strongly inhibits hexokinase. Whether or not a similar reaction occurs in bacteria is not known. Possibly the bacterial glycolytic aldolase is capable of condensing the products of the L-fructose-1-P cleavage producing L-sorbose-1-P which may be toxic to the cell. If this is the case, the mutant may overcome this toxicity by synthesizing an enzyme which converts L-sorbose-1-P to a common metabolite.

Although L-glyceraldehyde is simultaneously reduced to glycerol and oxidized to glyceric acid, the enzymes were present in both wild type and mutant. Both cell types grew readily on glycerol and when grown on L-mannose, glyceric acid accumulated in the growth medium. Thus, these reactions were not considered to be significant in regulating L-mannose metabolism. The mutant cells, however, were capable of growing on L-galactose more readily than the wild type and L-glyceraldehyde is a

common product of both L-hexoses.

Previous investigators have shown that the metabolism of unnatural sugars involved a constitutive mutation not for the synthesis of new enzymes but rather for the production of a non-specific enzyme capable of converting the unnatural substrate to a common metabolic intermediate. Mortlock et al (7) have shown that growth of A. aerogenes on xylitol and L-arabitol was explainable by the selection of a mutant constitutive for ribitol dehydrogenase, which converted xylitol and L-arabitol to D- and L-xylulose, respectively. Similarly, growth on L-xylose was due to selection of a mutant constitutive for L-fucose isomerase (2). In contrast, D-lyxose appeared to be an exception to this generalized phenomenon. Allison and Anderson (4) reported that D-lyxose was isomerized to D-xylulose but they were unable to isolate constitutive mutants for D-lyxose isomerase. The enzyme was induced only by D-lyxose, and although D-mannose also was isomerized by this same enzyme, it was unable to induce it. Lin et al (6) reported that two genetic events regulate the metabolism of mannitol in A. aerogenes. The suppression of the phosphotransferase pathway by which mannitol was normally metabolized gave rise to a constitutive mutation for the production of D-arabitol dehydrogenase which converted

mannitol to D-fructose. Other systems involving the utilization of an unnatural substrate by derepression of an enzyme include: altrose-galactoside via β-galactosidase (79), β-glycerolphosphate via alkaline phosphatase (80), and putrescine via diamine-α-keoglutarate transaminase (81). The enzymes of L-mannose metabolism also are non-specific in that they degrade the naturally occurring hexose, L-rhamnose. However, unlike the previously mentioned cases, the first three enzymes involved in L-mannose metabolism are not constitutive.

To conclude, both mutant and wild-type cells utilized L-mannose and their growth was inhibited by a product of L-fructose-1-P. This inhibitor may be L-glyceraldehyde or some product thereof. However, only the mutant was able to overcome the inhibition resulting in active growth on L-mannose.

SUMMARY

The biodegradative pathway of L-mannose by A. aerogenes PRL-R3 has been elucidated. L-Mannose is isomerized to L-fructose which is phosphorylated with ATP and a kinase to L-fructose-1-P. L-Fructose-1-P is cleaved by an aldolase to dihydroxyacetone phosphate and L-glyceraldehyde. L-Fructose and a new sugar phosphate,

L-fructose-1-P, were isolated and identified, and an alternative procedure was developed for the preparation of L-fructose. The enzymes in the pathway have been characterized. They were induced by either L-mannose or L-rhamnose and the biodegradation of these two L-hexoses has been shown to involve the same enzymes. A mutant strain of A. aerogenes has been isolated, which, unlike the wild type, grows readily on L-mannose as the sole carbon source. The mutant is not constitutive for the first three enzymes in the biodegradative pathway. Thus, the basis for growth of the mutant on L-mannose possibly lies in its ability to metabolize L-glyceraldehyde or a product thereof.

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