NEW REACTIONS INSTRUMENTAL IN THE METABOLISM OF COMMON HEXOSES

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THESIS

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R. L. Anderson
Major professor

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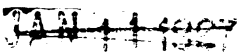

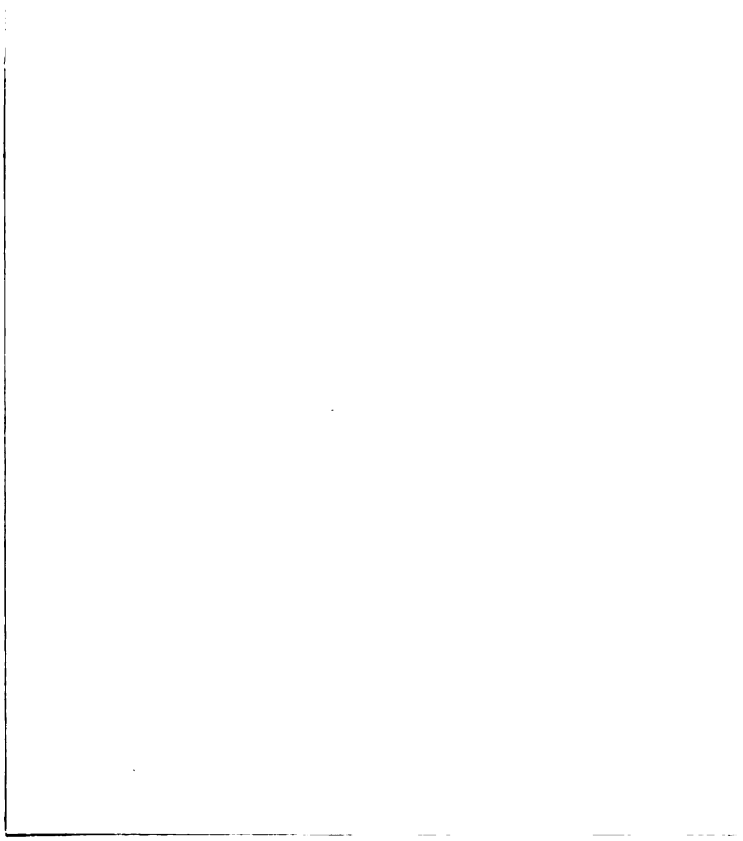

ABSTRACT

NEW REACTIONS INSTRUMENTAL IN THE METABOLISM OF COMMON HEXOSES

by Mamdouh Yehia Kamel

This thesis defines the enzymic basis for the initiation of the metabolism of common hexoses in Aerobacter aerogenes PRL-R3. This organism could not be shown to possess kinases for D-mannose, D-fructose, or D-mannitol even though it could utilize these compounds constitutively as sole carbon sources. Its constitutive hexokinase was purified over 1000-fold from extracts and shown to be highly stereospecific for D-glucose. The kinase was characterized with respect to pH optimum, substrate specificity, metal ion specificity, Michaelis constants, inhibition constants, and stability. The product of the D-glucokinase-catalyzed reaction was identified as D-glucose 6-phosphate.

An apparent D-mannokinase activity was detected in crude cell extracts, but was shown actually to involve an apparent 2-epimerization of D-mannose to D-glucose, the latter of which could be phosphorylated with ATP by the stereospecific D-glucokinase. The apparent 2-epimerization

was resolved into a cyclic process involving D-mannose 6-phosphate isomerase, D-glucose 6-phosphate isomerase D-glucokinase, and a new constitutive phosphotransferase which could phosphorylate D-mannose with D-glucose 6-phosphate, acetyl phosphate, or carbamyl phosphate, but not with adenosine triphosphate.

The new phosphotransferase was purified several hundred fold and characterized with respect to pH optimum, phosphoryl donor specificity and kinetic constants, phosphoryl acceptor specificity and kinetic constants, inhibition constants, stability, and reversibility of the catalyzed reactions. The reaction products were prepared and identified. The significance of the enzyme in metabolism was discussed.

NEW REACTIONS INSTRUMENTAL IN THE METABOLISM OF COMMON HEXOSES

By

Mamdouh Yehia Kamel

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I wish to express my sincere appreciation to Dr. R. L. Anderson for his encouragement, guidance and constructive criticism throughout the course of this work. I am also grateful to Mrs. Verachtert for technical assistance during a portion of this research and to Mr. D. P. Allison for his co-operation and help in the preparation of the coupling enzymes. Thanks go to Mr. T. Hanson for his help in developing the sugar phosphate separation method, to Mr. J. W. Mayo for the preparation of L-glucose and L-fructose, and to Mr. R. R. Hart for the preparation of L-galactose. I greatly appreciate the financial support from the Egyptian government.

Words cannot express the depth of my gratitude to my wife Samira for her patience, understanding and encouragement during the course of my graduate work.

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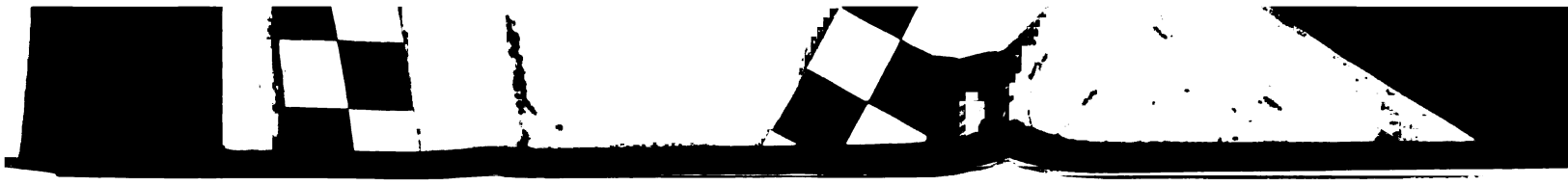

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VITA

Mamdouh Yehia Kamel was born in Cairo, Egypt on June 29, 1933. He graduated from Beni-Suef High School in June 1950 and received the B.S. degree in Chemistry and Botany from Cairo University in June, 1954. He accepted a scholarship from the National Research Center in Cairo, Egypt in 1954 and received the M.S. degree from Cairo University in 1957-1958. In 1958 he accepted a predoctoral fellowship from the Egyptian Government for further graduate work in Moscow University in Russia. In 1960 he changed his place of study to the United States of America and was accepted as a doctoral candidate in the Department of Biochemistry at Michigan State University in the summer of 1961. He will receive the Ph.D. degree in the fall of 1965.

Mr. Kamel is married and has a daughter, Hebba.

INTRODUCTION

In organisms in which carbohydrate metabolism has been thoroughly investigated, the degradation of D-mannose is initiated by phosphorylation at carbon atom 6 with ATP in a reaction mediated by a nonspecific hexokinase. D-Mannose 6-phosphate is then isomerized by a specific enzyme to yield D-fructose 6-phosphate, which may be metabolized further via the Embden-Meyerhof pathway or, after isomerization to D-glucose 6-phosphate, via one of the hexose monophosphate pathways.

Hexose and pentose utilization in Aerobacter aerogenes PRL-R3 has been shown to proceed through reactions of both the Embden-Meyerhof pathway and a hexose monophosphate (transketolase-transaldolase) pathway (1-4). Observations in this laboratory, however, had indicated that the initiation of the metabolism of hexoses in this organism did not conform to the established patterns. Its constitutive hexokinase appeared to be stereospecific for D-glucose; attempts to demonstrate unequivocally the existence of D-mannokinase in this organism had consistently yielded negative results in spite of the fact that both D-mannose and D-glucose could be utilized constitutively as sole carbon sources. Consequently, an investigation

of the enzymic mechanisms involved in the initiation of the metabolism of D-mannose and D-glucose in A. aerogenes PRL-R3 became the subject of this thesis. In addition, some observations on the metabolism of D-mannitol and D-fructose are reported.

This thesis consists of three parts. Part I describes the purification and properties of D-glucokinase from A. aerogenes PRL-R3 and establishes its unique stereospecificity. Part II describes a novel cyclic pathway for the metabolism of D-mannose which is independent of the involvement of D-mannokinase. Part III describes the purification and properties of a unique phosphotransferase, which is a key enzyme in the pathway described in Part II. Two abstracts and a preliminary communication on aspects of this work have been published (5-7).

PART I

Purification and Properties of the Stereospecific D-Glucokinase of Aerobacter aerogenes PRL-R3.

As noted in the general introduction, there was an indication that D-glucose but not D-mannose could be phosphorylated with ATP in A. aerogenes PRL-R3, although either of these hexoses could be metabolized constitutively as a sole carbon source. This implied (i) that the constitutive hexokinase of this organism had a unique specificity, and (ii) that D-mannose was metabolized by an unknown mechanism. To establish these points, the enzyme which catalyzed the phosphorylation of D-glucose with ATP was purified and its properties investigated. This section of the thesis describes the stereospecific D-glucokinase (ATP:D-glucose 6-phosphotoransferase, EC 2.7.1.2) of A. aerogenes PRL-R3, presents a procedure for its purification over 1,000-fold, and establishes its reaction product as D-glucose 6-phosphate.

EXPERIMENTAL PROCEDURE

Growth of Cells- A. aerogenes PRL-R3 was grown in 100-liter volumes in a New Brunswick Model 130 Fermacell fermentor at 30° with an aeration rate of 6 to 8 cubic feet per minute and an agitation speed of 300 rpm. The medium

consisted of 1.35% $Na_2HPO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.02% Dow Corning Antifoam B, and 0.5% D-glucose (autoclaved separately). The inoculum was 2.5 liters of an overnight culture in the same medium minus the antifoam. The cells were harvested with a Sharples AS-12 centrifuge 8 to 9 hours after inoculation. The yield was about 10 g (wet weight) of cells per liter.

Chemicals- L-Galactose was prepared by R.R. Hart by borohydride and sodium amalgam reduction of D-galacturonic acid (8). L-mannose was prepared by nitromethane addition to L-arabinose (9). L-glucose was prepared by J.W. Mayo by modifications of the procedures described by Hudson (10) and Frush and Isbell (11). L-Ribulose and D- and L-xylulose were prepared by refluxing L-arabinose and D- and L-xylose, respectively, with pyridine (12) and were purified by chromatography on Dowex 1-borate (13) after removing excess aldopentose by crystallization. L-Fructose was prepared enzymically by J.W. Mayo by an unpublished procedure. D-Allose and D-altrose were gifts of Dr. F. J. Simpson. D-Mannose 6-phosphate isomerase and D-glucose 6-phosphate isomerase were purified from extracts of A. aerogenes PRL-R3 by an unpublished procedure developed in this laboratory. Phosphoglucomutase, glucose 6-phosphate

dehydrogenase, lactic dehydrogenase (containing pyruvate kinase), and all other chemicals were obtained from commercial sources. D-Mannose and D-galactose were recrystallized (14,15) before use to remove interfering amounts of D-glucose.

D-Glucose 6-phosphate was determined spectrophotometrically by measuring the 340 mμ absorbance in the presence of NADP and D-glucose 6-phosphate dehydrogenase. D-Mannose 6-phosphate was determined spectrophotometrically with these same reagents with the addition of D-mannose 6-phosphate isomerase and D-glucose 6-phosphate isomerase.

D-Glucokinase Assay- D-Glucokinase was routinely assayed by measuring NADP reduction at 340 mμ with a Gilford absorbance-recording spectrophotometer thermostated at 25° using microcuvettes with a 1-cm light path. The reaction mixture contained in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.1 μmole of NADP, 5.0 μmoles of D-glucose, excess glucose 6-phosphate dehydrogenase, and D-glucokinase at concentrations which gave a linear response. The reaction was initiated by the addition of D-glucokinase. The activity of 6-phosphogluconate dehydrogenase (measured by replacing D-glucose plus ATP with 6-phosphogluconate in the assay mixture) in the crude cell extract was always less than 20%

of the D-glucokinase activity and, therefore, was not considered to contribute significantly to the observed D-glucokinase rate. Protein was determined spectrophotometrically with the aid of a nomograph (courtesy of Calbiochem) based on the data of Warburg and Christian (16). A unit of enzyme was defined as the amount which catalyzed the phosphorylation of 1 μmole of D-glucose per hour under the conditions described. Unless stated otherwise, the reported experiments were performed with the most highly purified fraction of D-glucokinase.

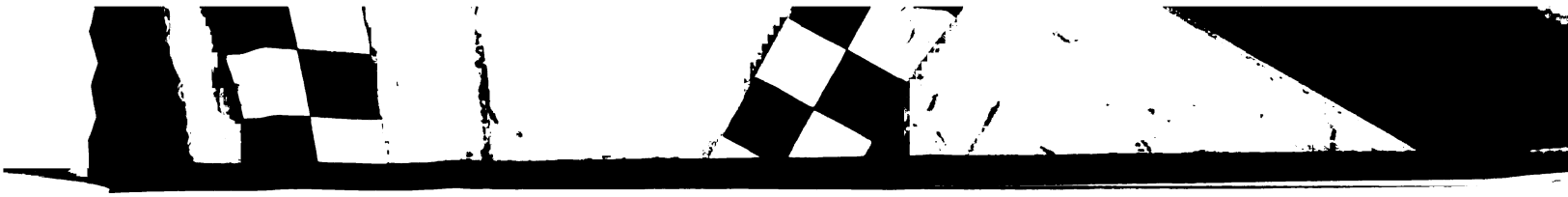
An alternate method for measuring kinase activity (as in the specificity experiment described in Fig. 7) was a pyruvic kinase-lactic dehydrogenase-linked assay based on the continuous spectrophotometric measurement of ADP (13).

RESULTS

Purification of D-Glucokinase

All operations were performed at 0 to 4°. Extracts were prepared by disrupting cells of A. aerogenes PRL-R3 suspended in water in a Raytheon 10-kc sonic oscillator. The broken-cell suspension was centrifuged at 13,200 x g, and the resulting supernatant solution was used as the cell extract. The extract used in the purification described below was obtained from 700 g (wet weight) of cells.

Bentonite Fractionation- Powdered bentonite, 223 g,



was suspended in 3,350 ml of cell extract containing 54 mg of protein per ml with a 280:260 mμ ratio of 0.69. Removal of the bentonite by centrifugation yielded a supernatant (2,300 ml) of 7-fold purified D-glucokinase containing 6.6 mg of protein per ml with a 280:260 mμ ratio of 0.61.

First Ammonium Sulfate Fractionation- Ammonium sulfate, 30.3 g, was dissolved in the above fraction, followed by 100 ml of 7.6% protamine sulfate. The precipitate that formed was removed by centrifugation and discarded. To the supernatant solution (2,375 ml) was added 1,169 g of ammonium sulfate (80% of saturation), and the resulting precipitate was dissolved in water to give 142 ml of 22-fold purified D-glucokinase containing 22 mg of protein per ml with a 280:260 mμ ratio of 1.15.

Acid Precipitation- The above fraction was diluted to 600 ml with water and the pH was lowered to 4.4 by the addition of acetic acid. The precipitated protein was removed by centrifugation and discarded. The pH of the supernatant solution was immediately raised to 7.0 with ammonium hydroxide. This yielded 600 ml of 33-fold purified D-glucokinase containing 3.8 mg of protein per ml with a 280:260 mμ ratio of 1.12.

Second Ammonium Sulfate Fractionation- To the above fraction was added 550 ml of saturated ammonium sulfate

(pH 7.0). The precipitate of crystalline and amorphous protein which appeared was removed by centrifugation and and discarded. To the supernatant solution was added 400 ml of saturated ammonium sulfate (pH 7.0). The resulting precipitate was collected by centrifugation and dissolved in water to yield 16 ml of 78-fold purified D-glucokinase containing 28 mg of protein per ml with a 280:260 mμ ratio of 1.18.

Sephadex G-100 Chromatography- The above fraction was placed on a column (5 x 153 cm) of Sephadex G-100 equilibrated with 0.01 M sodium phosphate buffer (pH 6.5) and eluted with the same buffer. Twenty-ml fractions were collected, and those which contained most of the activity were pooled. This yielded 120 ml of 590-fold purified D-glucokinase with a protein concentration of 0.33 mg per ml and a 280:260 mμ ratio of 1.55.

DEAE-Cellulose Chromatography- DEAE-Cellulose (Bio-Rad Cellex D, exchange capacity = 0.95 meq per g) was pretreated as recommended by Peterson and Sober (17) and equilibrated with 0.01 M sodium phosphate buffer (pH 6.5) in a column 1.5 x 12 cm. The above fraction was added to the column and eluted with 500 ml (5 ml fractions) of the same buffer containing NaCl in a linear gradient from 0 to 0.8M. The five fractions containing most of the

activity (6.6% of the D-glucokinase activity of the cell extract) were 1,530- to 1,980-fold purified, contained 0.20 to 0.35 mg of protein per ml, and had 280:260 mμ ratios ranging from 1.61 to 1.71. A summary of the purification procedure is given in Table I.

Properties of D-Glucokinase

pH Optima- D-Glucokinase activity as a function of pH was maximal at pH 7.5 in glycylglycine buffer and at about pH 8.9 in glycine buffer (Fig. 1).

Phosphoryl Donor Specificity- The relative rates of D-glucose phosphorylation in the presence of various phosphoryl donors (3.3mM) is given in Table II. ATP was the most effective phosphoryl donor. The observed phosphorylation with ITP was competitive with ATP, the rate with 3.3 mM ATP being 34% inhibited in the presence of 13.2 mM ITP. With saturating (33.3mM) D-glucose, the K_m for ATP was determined to be 0.8 mM (Fig 2).

Metal Ion Specificity- After treatment of purified D-glucokinase with 0.01 M EDTA (pH 7.0) and removal of excess EDTA by passage through a Sephadex column, activity was nil in the absence of added divalent cations. The relative rates of D-glucose phosphorylation by the Sephadex-treated enzyme in the presence of various metal salts is given in Table III. Mg^{++} was the most effective activator,

TABLE I

Purification of D-Glucokinase

Fraction	Total Activity	Recovery	Specific Activity
	units*	%	units/mg protein
Cell extract	401,000	100	2.2
Bentonite supernatant	229,000	57	15.1
Ammonium sulfate I	146,000	37	46.7
pH 4.4 supernatant	158,000	39	69.2
Ammonium sulfate II	73,400	18	164
Sephadex G-100	49,100	12	1,230
DEAE-cellulose, fraction 24	7,000		4,000
" " " 25	7,000		4,360
" " " 26	4,420	6.6	3,360
" " " 27	4,310		4,100
" " " 28	3,580		3,500

*µMoles of D-glucose phosphorylated per hour.

Fig. 1. pH Optima of D-glucokinase. The routine assay was used except that the buffer composition and pH were varied as indicated, with the D-glucokinase (DEAE-cellulose fraction) concentration constant. The pH measurements were made on duplicate reaction mixtures with a Sargent DR pH meter equipped with a Jenaer combination microelectrode. The pH did not change during the 5-minute assay period.

Figure 1.

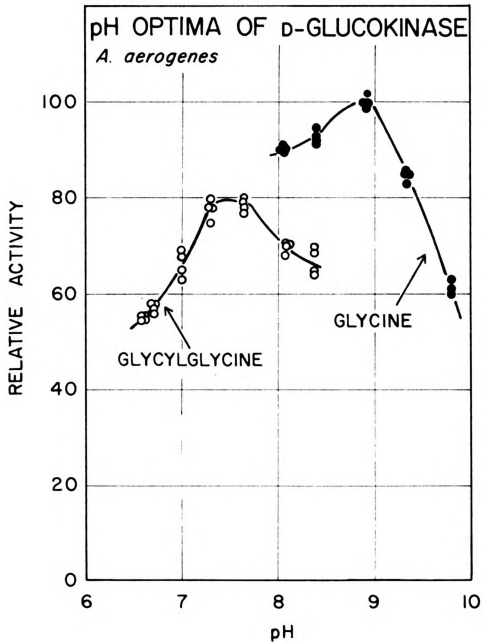

pH OPTIMA OF D-GLUCOKINASE
A. aerogenes
RELATIVE ACTIVITY
100
80
60
40
20
0
6
7
8
9
10
pH
GLYCYLGLYCINE
GLYCINE

TABLE II

Phosphoryl donor specificity of D-glucokinase

The reaction mixture contained in a volume of 0.15 ml: 5 µmoles of D-glucose, 1 µmole of $MgCl_2$, 0.1 µmole of NADP, 0.5 µmole of phosphoryl compound, 8 µmoles of glycylglycine buffer (pH 7.5), purified D-glucokinase, and excess glucose 6-phosphate dehydrogenase.

Phosphoryl donor	Relative phosphorylation rate
ATP	100
ITP	13
GTP	3
UTP	3
CTP	0
ADP	0
acetyl phosphate	0
carbamyl phosphate	0
creatine phosphate	0

Fig. 2. Lineweaver-Burk plot relating D-glucokinase reaction velocity to ATP concentration. The routine assay was used except that the ATP concentration was varied as indicated, with the D-glucokinase (DEAE-cellulose fraction) concentration constant. The $MgCl_2$ concentration was maintained at twice the ATP concentration.

Figure 2.

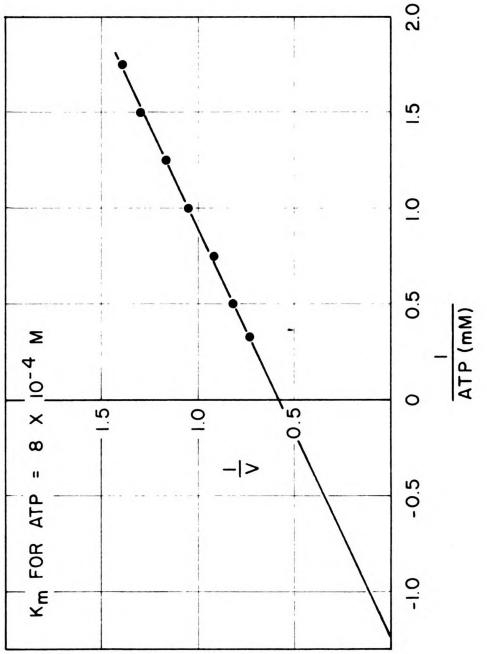

K_m FOR ATP = 8 X 10^{-4} M
1.5
1.0
0.5
$\frac{1}{V}$
-1.0
-0.5
0
0.5
1.0
1.5
2.0
$\frac{1}{\text{ATP (mM)}}$

TABLE III

Metal ion specificity of D-glucokinase

The reaction mixture contained in a volume of 0.15 ml. 5 μmoles of D-glucose, 0.1 μmole of NADP, 0.5 μmole of ATP 8 μmoles of glycylglycine buffer (pH 7.5), 1 μmole of the metal salt, purified D-glucokinase (treated with 0.01 M EDTA, pH 7.0, and dialyzed by passage through Sephadex), and excess glucose 6-phosphate dehydrogenase. The reaction was initiated by the addition of D-glucokinase.

Metal salt	Relative phosphorylation rate
$MgCl_2$	100
$MgSO_4$	100
$MnCl_2$	43
$CoCl_2$	24
$NiSO_4$	3
$CaCl_2$	0
$ZnSO_4$	0
None	0

with Mn^{++} and Co^{++} being partially effective.

Substrate Specificity- The glucose 6-phosphate dehydrogenase-linked assay was used to obtain an indication of specificity by measuring the inhibition of phosphorylation of 1 mM D-glucose in the presence of 100 mM concentrations of other sugars. Inhibition was detected only with D-glucosamine and D-xylose. Compounds which caused no inhibition were: 2-deoxy-D-glucose, α-methyl-D-glucoside, L-glucose, D- and L-mannose, D-allose, D-altrose, D- and L-galactose, D- and L-fucose, L-rhamnose, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, D-sorbitol, D-mannitol, D- and L-arabitol, ribitol, xylitol, L-sorbose, sucrose, L-xylose, D-lyxose, D-ribose, D- and L- arabinose, D- and L- ribulose, and D- and L-xylulose. The observed inhibition with D-glucosamine and D-xylose was competetive with D-glucose (Figs. 3 and 4), with the K_i being 0.4 mM for D-glucosamine (Fig.5) and 3 mM for D-xylose (Fig. 6).

The nonspecific pyruvic kinase-lactic dehydrogenase-linked assay (8) was used to measure possible phosphorylation. Fig. 7 shows that D-fructose, D-mannose, and 2-deoxy-D-glucose were not phosphorylated in an assay which was sufficiently sensitive to detect phosphorylation at 0.2% of the rate of phosphorylation of D-glucose. D-Glucosamine was phosphorylated at about 26% of the rate on D-glucose

Fig. 3. Lineweaver-Burk plot showing the relationship of D-glucose concentration to D-glucokinase reaction velocity in the presence of various concentrations of D-glucosamine. The routine assay was used except that the D-glucose and D-glucosamine concentrations were varied as indicated, with the D-glucokinase (DEAE-cellulose fraction) concentration constant.

Figure 3.

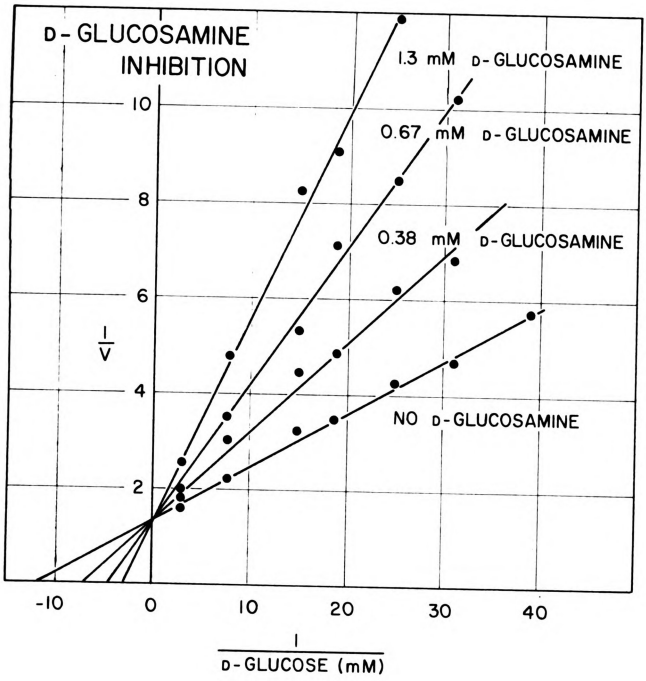

D-GLUCOSAMINE INHIBITION
1.3 mM D-GLUCOSAMINE
0.67 mM D-GLUCOSAMINE
0.38 mM D-GLUCOSAMINE
NO D-GLUCOSAMINE
10
8
6
4
2
1/V
-10
0
10
20
30
40
1/D-GLUCOSE (mM)

Fig. 4. Lineweaver-Burk plot showing the relationship of D-glucose concentration to D-glucokinase reaction velocity in the presence of various concentrations of D-xylose. The routine assay was used except that the D-glucose and D-xylose concentrations were varied as indicated, with the D-glucokinase (DEAE-cellulose fraction) concentration constant.

Figure 4.

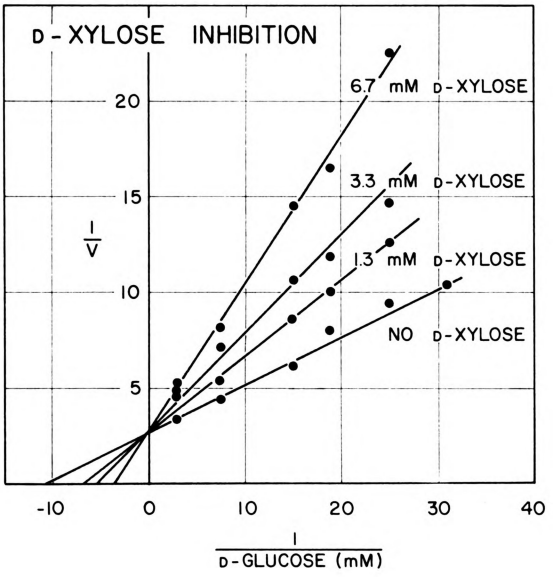

D - XYLOSE INHIBITION
6.7 mM D-XYLOSE
3.3 mM D-XYLOSE
1.3 mM D-XYLOSE
NO D-XYLOSE
1/V
20
15
10
5
-10
0
10
20
30
40
1/D-GLUCOSE (mM)

Fig. 5. Kinetic plot for obtaining the K_1 for D-glucosamine. The data are from the experiment described in Fig. 3.

Figure 5.

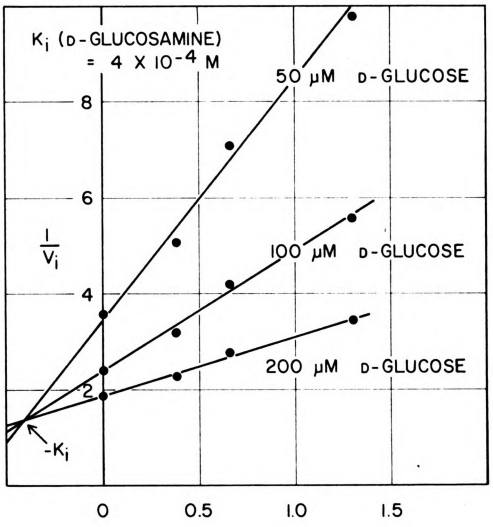

K_i (D-GLUCOSAMINE)
= 4 X 10^{-4} M
50 μM D-GLUCOSE
100 μM D-GLUCOSE
200 μM D-GLUCOSE
8
6
4
2
$\frac{1}{V_i}$
$-K_i$
0
0.5
1.0
1.5
D-GLUCOSAMINE CONCENTRATION (mM)

Fig. 6. Kinetic plot for obtaining the K_1 for D-xylose. The data are from the experiment described in Fig. 4.

Figure 6.

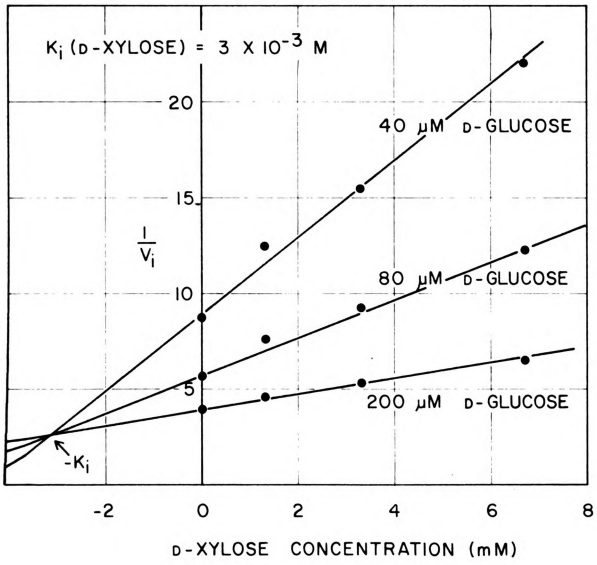
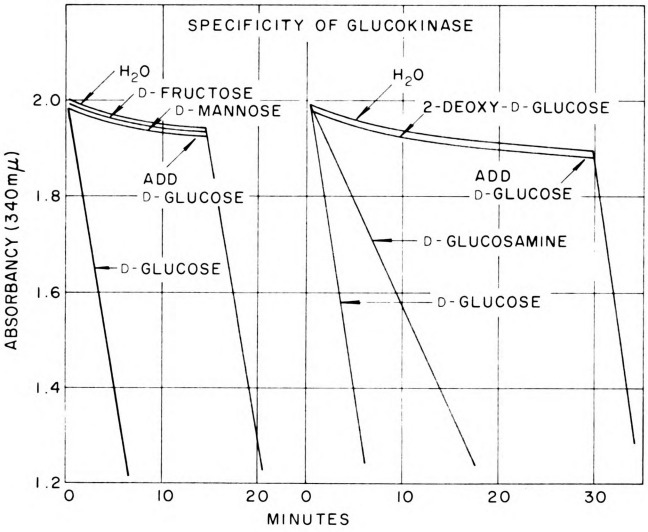

K_i (D-XYLOSE) = 3 X 10^{-3} M
20
15
10
5
$\frac{1}{V_i}$
40 μM D-GLUCOSE
80 μM D-GLUCOSE
200 μM D-GLUCOSE
$-K_i$
-2
0
2
4
6
8
D-XYLOSE CONCENTRATION (mM)

Fig. 7. Specificity of D-glucokinase. Each cuvette contained in a volume of 0.15 ml: 10 µmoles of glycylglycine buffer (pH 7.5), 0.5 µmole of ATP, 1.0 µmole of $MgCl_2$, 0.5 µmole of phosphoenolpyruvate, 0.05 µmole of NADH, 0.15 µmole of sugar, excess crystalline lactate dehydrogenase (containing pryuvate kinase), and purified D-glucokinase. The cuvette compartment was thermostated at 25°. The reaction rate was proportional with D-glucokinase concentration. Controls without ATP were negative.

Figure 7.


SPECIFICITY OF GLUCOKINASE
H2O
D-FRUCTOSE
D-MANNOSE
ADD
D-GLUCOSE
D-GLUCOSE
H2O
2-DEOXY-D-GLUCOSE
ADD
D-GLUCOSE
D-GLUCOSAMINE
D-GLUCOSE
ABSORBANCY (340mμ)
2.0
1.8
1.6
1.4
1.2
0
10
20
0
10
20
30
MINUTES

The addition of D-glucose to the negative cuvettes after 15 to 30 minutes resulted in a rapid decrease in absorbance, verifying that D-glucokinase and the coupling enzymes were not inactivated or inhibited by the other sugars. Similar experiments with higher levels of D-glucokinase for increased sensitivity indicated that the following compounds were also not phosphorylated: D- and L-xylose, α-methyl-D-glucoside, L-glucose, L-mannose, L-fructose, D-allose, D-altrose, D- and L-galactose, D- and L-fucose, L-rhamnose, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, D-sorbitol, D-mannitol, D- and L-arabitol, ribitol, xylitol, L-sorbose, D-lyxose, D-ribose, D- and L-arabinose, D- and L-ribulose, and D- and L-xylulose.

From the data depicted in Fig. 8, the K_m for D-glucose was determined to be 80 μM.

Product Inhibition- With the pyruvic kinase-lactic dehydrogenase-linked assay (see Fig. 7), no inhibition of the phosphorylation of 1 mM D-glucose was observed in the presence of 10 mM D-glucose 6-phosphate. D-Mannose 6-phosphate, although not a product, was also tested and found to give no inhibition at these concentrations. ADP inhibition was competitive with ATP (Fig. 9), with the K_i being 0.4 mM (Fig. 10).

Fig. 8. Lineweaver-Burk plot relating D-glucokinase reaction velocity to D-glucose concentration. The routine assay was used except that the D-glucose concentration was varied as indicated, with the D-glucokinase (DEAE-cellulose fraction) concentration constant.

Figure 8.

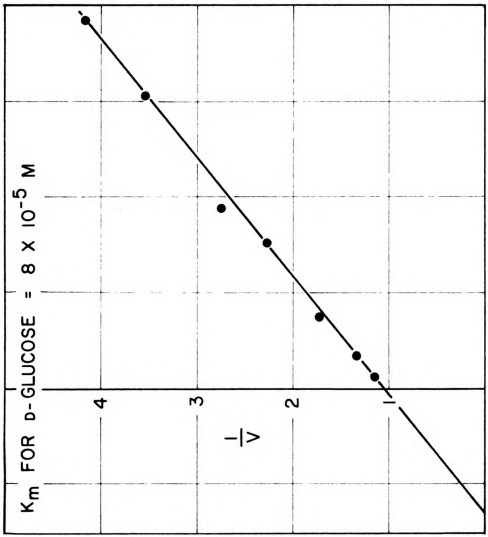

K_m FOR D-GLUCOSE = 8 X 10^{-5} M
$\frac{1}{V}$
4
3
2
1
-10
0
10
20
30
$\frac{1}{\text{D-GLUCOSE (mM)}}$

Fig. 9. Lineweaver-Burk plot showing the relationship of ATP concentration to D-glucokinase reaction velocity in the presence of various concentrations of ADP. The routine assay was used except that the ATP and ADP concentrations were varied as indicated with the D-glucokinase (DEAE-cellulose fraction) concentration constant. The $MgCl_2$ concentration was maintained at twice the concentration of ATP plus ADP.

Figure 9.

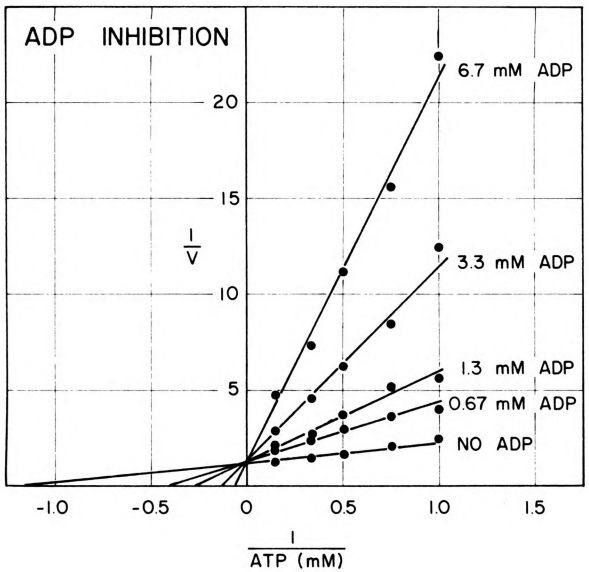
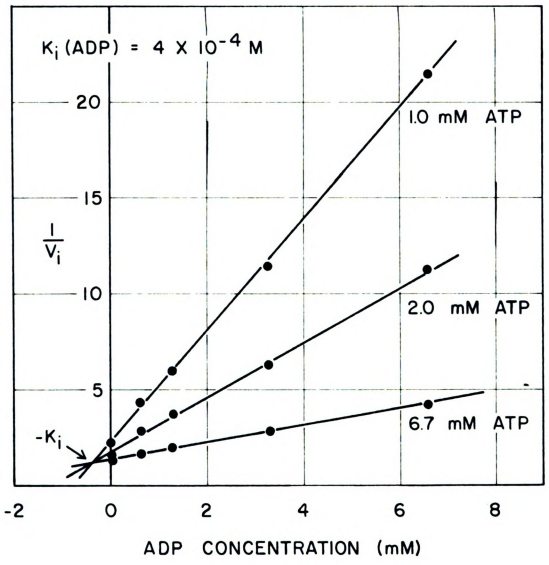

ADP INHIBITION
6.7 mM ADP
3.3 mM ADP
1.3 mM ADP
0.67 mM ADP
NO ADP
20
15
10
5
1/V
-1.0
-0.5
0
0.5
1.0
1.5
1/ATP (mM)

Fig. 10. Kinetic plot for obtaining the K_i for ADP. The data are from the experiment described in Fig. 9.

Figure 10.


K_i (ADP) = 4 X 10^{-4} M
20
15
10
5
$\frac{1}{V_i}$
-K_i
1.0 mM ATP
2.0 mM ATP
6.7 mM ATP
-2
0
2
4
6
8
ADP CONCENTRATION (mM)

Stability- Purified D-glucokinase was kept at room temperature for several days or at 0° (unfrozen) for several weeks without a significant loss of activity. It was unstable to storage in the frozen state at -20°.

Product Identification

The product of the D-glucokinase-catalyzed reaction was prepared by incubating in a microcuvette: 2.5 units of D-glucokinase (DEAE-cellulose fraction), 0.030 µmole of D-glucose, 0.5 µmole of ATP, 1.0 µmole of $MgCl_2$, 0.2 µmole of NADP, and 10.0 µmoles of glycylglycine buffer (pH 7.5), in a volume of 0.15 ml. After incubation at 25° for 25 minutes, excess glucose 6-phosphate dehydrogenase was added. This resulted in an increase in absorbance at 340 mµ equivalent to the oxidation of 0.029 µmole of D-glucose 6-phosphate. The further addition of excess phosphoglucomutase did not result in a change in absorbance after correcting for dilution. Other experiments indicated that D-glucokinase and glucose 6-phosphate dehydrogenase were free from phosphoglucomutase and 6-phosphogluconate dehydrogenase. Thus, it was established that the product of the D-glucokinase-catalyzed reaction is D-glucose 6-phosphate and not D-glucose 1-phosphate.

Effect of Growth Substrate on D-Glucokinase Level

The specific activity of D-glucokinase in extracts of cells grown on D-glucose-free (<0.0001%) nutrient broth

(0.5% Difco peptone, 0.3% Difco beef extract, pH 7.0) or on the mineral medium with 0.5% glycerol in place of D-glucose was the same as in extracts of cells grown on the D-glucose-mineral medium. Therefore, D-glucokinase may be considered to be constitutive in this organism.

DISCUSSION

Although several other kinases presumably stereospecific for D-glucose have been reported (18-24), their existence and specificity had not been established after extensive purification. After this work was completed, however, a report by Saito (25) described a D-glucokinase purified 113-fold from Brevibacterium fuscum. In addition, a kinase which phosphorylates D-glucose and D-mannose but not most other sugars has recently been purified 200-fold from rabbit liver (26).

Specificity studies on the A. aerogenes D-glucokinase indicate that for a compound to bind at the D-glucose site, it must possess an aldehydo group at carbon atom 1 and the D-gluco configuration at carbon atoms 2,3, and 4. The -OH at carbon atom 2 may be replaced by $-NH_2$ (as in D-glucosamine) but not by -H (as in 2-deoxy-D-glucose). D-Xylose satisfies these criteria and competitively inhibits the phosphorylation of D-glucose, but is not itself phosphorylated. To be phosphorylated, the compound binding at the

D-glucose site must also contain a hydroxmethyl group attached to carbon atom 5. Thus, the only compounds that have been demonstrated to be phosphorylated by this D-glucokinase are D-glucose and D-glucosamine.

D-Glucokinase is constitutive in A. aerogenes PRL-R3 and presumably functions in initiating the metabolism of D-glucose and D-glucosamine. Hexokinases (ATP:hexose phosphotransferases) for other common hexoses such as D-mannose and D-fructose, however, have escaped detection in extracts of A. aerogenes PRL-R3, even though these compounds can be metabolized constitutively by this organism. A similar situation presumably exists in Escherichia coli. Fraenkel, Falcoz-Kelly, and Horecker (27) have described a mutant (FR-1) of E. coli which lacks the specific D-glucokinase, and another mutant (MM-6) which, unlike the wild type or mutant FR-1, is unable to grow on D-fructose. The genetic defect in mutant MM-6 has been postulated to be due to either (i) altered permeability (28), or (ii) lack of a "nonspecific hexokinase...which, for some reason, is difficult to measure in extracts" (27). Since several enzymes have now been described which phosphorylate hexoses with phosphoryl donors other than ATP (6,7, 29-31), it is possible that not one, but several, nonspecific phosphotransferases act in concert to phosphorylate hexoses in any one

organism. Because some of these enzymes can use D-glucose phosphate as the phosphoryl donor (6, 30, 31), it is attractive to speculate that the stereospecific D-glucokinase described here functions not only in the initiation of the metabolism of D-glucose, but also indirectly in the metabolism of other hexoses for which ATP:hexose phosphotransferase activity has not been demonstrated.

SUMMARY OF PART I

A constitutive, stereospecific D-glucokinase was purified over 1,000-fold from extracts of A. aerogenes PRL-R3. Only D-glucose (K_m= 8 x 10^{-5}M) and D-glucosamine (K_i= 4 x 10^{-4}M) were phosphorylated. The enzyme was inhibited by D-xylose (competitive with D-glucose, K_i=3 x 10^{-3}M) but not by 34 other sugars and related compounds tested. It was inhibited by ADP (competitive with ATP, K_i= 4 x 10^{-4}M) but not by D-glucose 6-phosphate or D-mannose 6-phosphate. The pH optimum was 7.5 in glycylglycine buffer and about 8.9 in glycine buffer. Other properties studied were phosphoryl donor specificity, metal ion specificity, and stability. The product of D-glucose phosphorylation was identified as D-glucose 6-phosphate.

PART II

A Cyclic Pathway

for the Metabolism of D-Mannose

Because of the widespread occurrence of hexokinase and D-mannose 6-phosphate isomerase in yeast, animal tissues, and bacteria, it is generally believed that D-mannose is metabolized by phosphorylation with ATP to yield D-mannose 6-phosphate, followed by isomerization to D-fructose 6-phosphate. The constitutive hexokinase of A. aerogenes PRL-R3, however, has been purified over 1,000-fold and shown to be highly stereospecific for D-glucose (see Part I). Attempts to demonstrate unequivocally the existence of D-mannokinase in this organism have consistently yielded negative results in spite of the fact that D-mannose is metabolized constitutively. Rather, an apparent 2-epimerization of D-mannose to D-glucose was detected in extracts. In an effort to reconcile these observations, we have identified a sequence of reactions which lead us to propose a cyclic pathway for the metabolism of D-mannose which is independent of the involvement of D-mannokinase. In addition to D-glucokinase, D-mannose 6-phosphate isomerase, and D-glucose 6-phosphate isomerase, the

pathway involves the participation of acyl phosphate:hexose phosphotransferase (5), which has now been purified several hundred fold and shown also to possess hexose phosphate: hexose phosphotransferase activity (7) (see Part III). This section of the thesis outlines the pathway and describes the experiments which led to its proposal.

EXPERIMENTAL PROCEDURE

Growth of Organism- A. aerogenes PRL-R3 was grown aerobically at 30° for 18 hours and harvested by centrifugation. Unless otherwise specified, the D-glucose-mineral medium described in Part I was used. The peptone-beef extract medium used in one experiment consisted of 0.5% Difco peptone and 0.3% Difco beef extract, pH 7.0.

Preparation of Cell Extracts- Cell extracts were prepared by treatment of cell suspensions for 5 to 10 minutes in a Raytheon Model DF-101, 250 watt, 10-kc sonic oscillator circulated with ice water. The supernatant fluid, after removal of the cellular debris by centrifugation at 31,000 x g, was the crude extract.

Reagents- Intestinal alkaline phosphatase and glucose oxidase (Glucostat) were obtained from the Worthington Biochemical Corporation. Glucose 6-phosphate dehydrogenase of suitable purity for the enzyme-coupled assays was obtained from a variety of commercial sources.

D-Glucokinase was the preparation described in Section I, and purified acyl phosphate:hexose phosphotransferase was the preparation described in Section III. D-Mannose (C.P. grade) was twice recrystallized (14) to remove interfering amounts of D-glucose. All other chemicals were used as obtained from commercial sources.

Analytical Procedures- Spectrophotometric measurements of reduced pyridine nucleotides were made at 340 mμ with a Gilford absorbance-recording spectrophotometer thermostated at 25°, using microcuvettes with a 1-cm light path. Manometric measurements were made using conventional techniques (32). Descending paper chromatography of sugars employed Whatman No. 1 paper (washed with 1N HCl and water) with 80% phenol as the solvent. The sugars on the chromatograms were visualized with silver nitrate (33) or with N,N-dimethy-p-phenylenediamine monohydrochloride (34). Radioactivity scans of paper chromatograms were made with a Nuclear-Chicago Model 1036 4-pi Actigraph II scanner, Nuclear-Chicago Model 1620CS analytical count ratemeter, and Sargent Model SRL recorder. D-Glucose was measured with glucose oxidase or by measuring NADP reduction in the presence of excess purified stereospecific D-glucokinase, glucose 6-phosphate dehydrogenase, and ATP. Other procedures were as described in Section I.

Enzyme Assays- D-Glucokinase and acyl phosphate:hexose phosphotransferase were assayed as described in Parts I and III, respectively. D-Glucose 6-phosphate isomerase was assayed spectrophotometrically by measuring NADP reduction in the presence of D-fructose 6-phosphate (containing limited D-glucose 6-phosphate), and glucose 6-phosphate dehydrogenase. D-Mannose 6-phosphate isomerase was assayed spectrophometrically by measuring NADP reduction in the presence of D-mannose 6-phosphate, D-glucose 6-phosphate isomerase, and glucose 6-phosphate dehydrogenase.

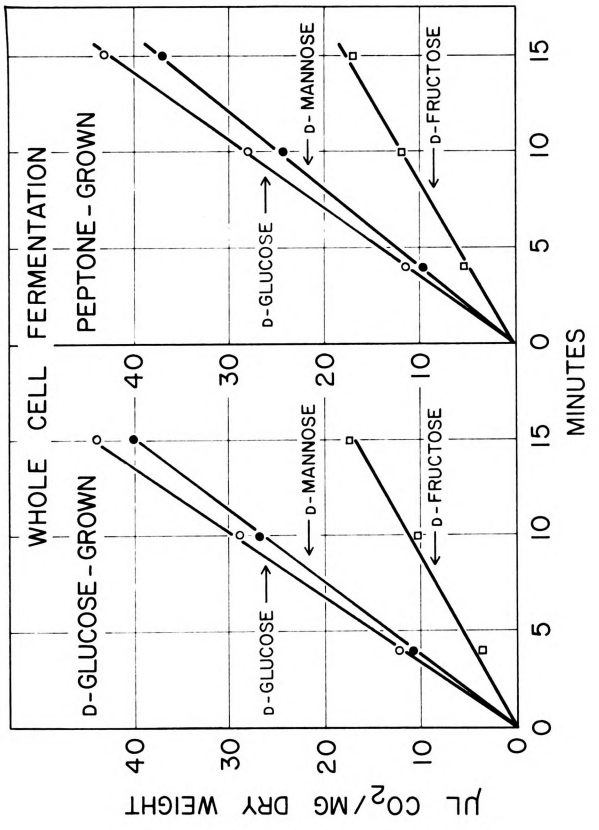
RESULTS AND DISCUSSION

Whole Cell Fermentation- Cells of A. aerogenes PRL-R3 grown on either the D-glucose-mineral medium or on a peptone-beef medium fermented D-glucose and D-mannose at equivalent rates (Fig. 11), indicating that the metabolism of these two hexoses is constitutive in this organism.

Absence of D-Mannokinase- Several different assay procedures were used in attempts to detect the possible phosphorylation of D-mannose with ATP. These included measurement of the disappearance of reducing sugars (35) after removal of the phosphate esters with barium (36); measurement of acid production manometrically (37), spectrophotometrically (38), and by titration with NaOH to maintain a constant pH; and the measurement of ADP

Fig. 11. Fermentation of D-glucose, D-mannose, and D-fructose by cells of A. aerogenes PRL-R3 grown on D-glucose-mineral salts and on peptone-beef extract. Each Warburg vessel contained in a volume of 0.5 ml: 10 μmoles of $NaHCO_3$, 10 μmoles of hexose, and washed cells (1.86 mg dry weight). The gas phase was 5% CO_2 in nitrogen, and the temperature was 30°. An endogeneous rate of about 6 μl of CO_2 per hr per mg dry weight has been subtracted from the rates shown.

Figure 11.


WHOLE CELL FERMENTATION
D-GLUCOSE-GROWN
PEPTONE-GROWN
D-GLUCOSE
D-MANNOSE
D-FRUCTOSE
D-GLUCOSE
D-MANNOSE
D-FRUCTOSE
μL CO2/MG DRY WEIGHT
MINUTES
0
10
20
30
40
0
5
10
15

formation in a pyruvate kinase-lactate dehydrogenase-linked assay (13). Although the results obtained with the different methods varied, an apparent activity could always be detected in crude extracts by manometric and titrimetric assays. Attempts to purify the apparent D-mannokinase by various fractionation procedures, however, invariably led to a loss of activity. Typical data are shown in Fig. 12. The titrimetric assay for kinases showed activity on both D-glucose and D-mannose in the crude extract, but only on D-glucose in the ammonium sulfate fraction. Other fractions were also devoid of D-mannokinase activity. The use of EDTA, reduced glutathione, or mercaptoethanol during fractionation and assay had no effect on preserving D-mannokinase activity. D-Mannokinase activity was also not detected in particulate fractions of broken-cell suspensions. Assays with CTP, GTP, ITP, and UTP in place of ATP were also negative. Cell extracts prepared by procedures other than sonic vibration, such as with a French pressure cell, also contained no detectable D-mannokinase activity after fractionation.

Although it is possible that a very labile or otherwise peculiar D-mannokinase does exist in A. aerogenes PRL-R3, the apparent activity that was detected in crude extracts can also be explained on a basis other than the direct phosphorylation of D-mannose with ATP. In the titrimetric

Fig. 12. Titrimetric assay for D-glucokinase and D-mannokinase in a crude extract and an ammonium sulfate fraction. Each reaction mixture contained in a volume of 1.64 ml: 40 μmoles of ATP, 80 μmoles of $MgCl_2$, 80 μmoles of the indicated hexose, and extract (25 mg of protein for the crude extract and 20 mg of protein for the ammonium sulfate fraction). The pH was maintained between 7.2 and 7.4 with the periodic addition of NaOH. The temperature was 25°.

Figure 12.

μEQ. OH⁻ TO MAINTAIN pH 7.4
CRUDE EXTRACT
D-GLUCOSE
D-MANNOSE
ENDOGENOUS
40-60% $(NH_4)_2SO_4$ FRACTION
D-GLUCOSE
ADD D-GLUCOSE
D-MANNOSE
ENDOGENOUS
MINUTES
0
5
10
15
20
25
30
40

assay shown in Fig. 13, D-mannose was preincubated with the crude extract before the addition of ATP. With the addition of ATP to the preincubated mixture, a rapid initial rate was observed, followed by a slow rate approximating that which was obtained without preincubation. This suggested that D-mannose was not phosphorylated directly, but was first converted to another compound, which was phosphorylated. Since an ATP:hexose phosphotransferase was known for D-glucose, but could not be demonstrated for D-fructose, a likely candidate for the unknown compound was considered to be D-glucose.

Apparent 2-Epimerization of D-Mannose to D-Glucose- The experiments described above suggested that D-mannose was converted to D-glucose, which then served as the phosphoryl accepter for ATP by the action of D-glucokinase. To test this hypothesis, crude extracts of A. aerogenes PRL-R3 were incubated with D-mannose at 25° and at pH 7.5. The accumulation of D-glucose was then determined with glucose oxidase and with the stereospecific D-glucokinase. D-Glucose was found to be formed at rates up to about 0.3 μmole per hr per mg of protein. The rate of D-glucose formation was half maximal at a D-mannose concentration of about 20 mM (Fig. 14).

The identification of the product as D-glucose by the

Fig. 13. Titrimetric assay for D-mannokinase-type activity in a crude extract. Each reaction mixture contained in a volume of 1.44 ml: 10 μmoles of ATP, 20 μmoles of $MgCl_2$, 200 μmoles of D-mannose, and extract (18 mg of protein). The endogeneous control was minus D-mannose. The reaction mixtures were pre-incubated with D-mannose for the times indicated before the addition of ATP, and adjusted to pH 7.4 with NaOH before the clock was started for 0 time. The pH was then maintained between 7.2 and 7.4 with the periodic addition of NaOH. The temperature was 25°. Controls in which the extract was preincubated without D-mannose before the addition of ATP gave the same rates as the indicated endogeneous rate.

Figure 13.

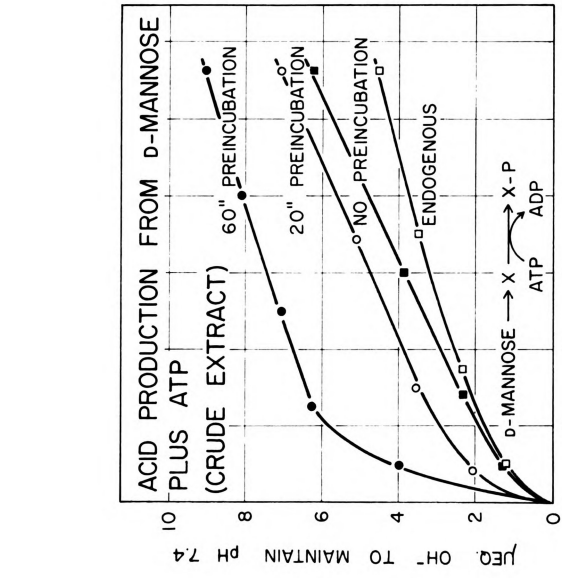
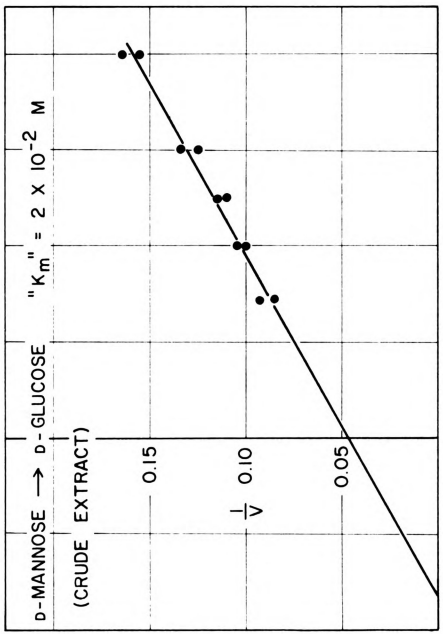

ACID PRODUCTION FROM D-MANNOSE
PLUS ATP
(CRUDE EXTRACT)
60" PREINCUBATION
20" PREINCUBATION
NO PREINCUBATION
ENDOGENOUS
D-MANNOSE → X → X-P
ATP ADP
μEQ. OH⁻ TO MAINTAIN pH 7.4
MINUTES
0
2
4
6
8
10
12

Fig. 14. Double reciprocal plot of the rate of D-glucose formation as a function of D-mannose concentration in a crude extract. Each reaction mixture contained in a volume of 1.0 ml:100 μmoles of glycylglycine buffer (pH 7.5), crude extract (12 mg of protein), and varying concentrations of D-mannose. Controls were minus D-mannose. After incubation at 25° for 0 and 90 minutes, the tubes were heated in a boiling water bath for 5 minutes, cooled, and centrifuged. Aliquots of the supernatant solutions were then assayed for D-glucose with D-glucokinase-glucose 6-phosphate dehydrogenase. The difference in D-glucose at 0 and 90 minutes, minus the endogeneous controls, was a measure of the rate of D-glucose formation.

Figure 14.

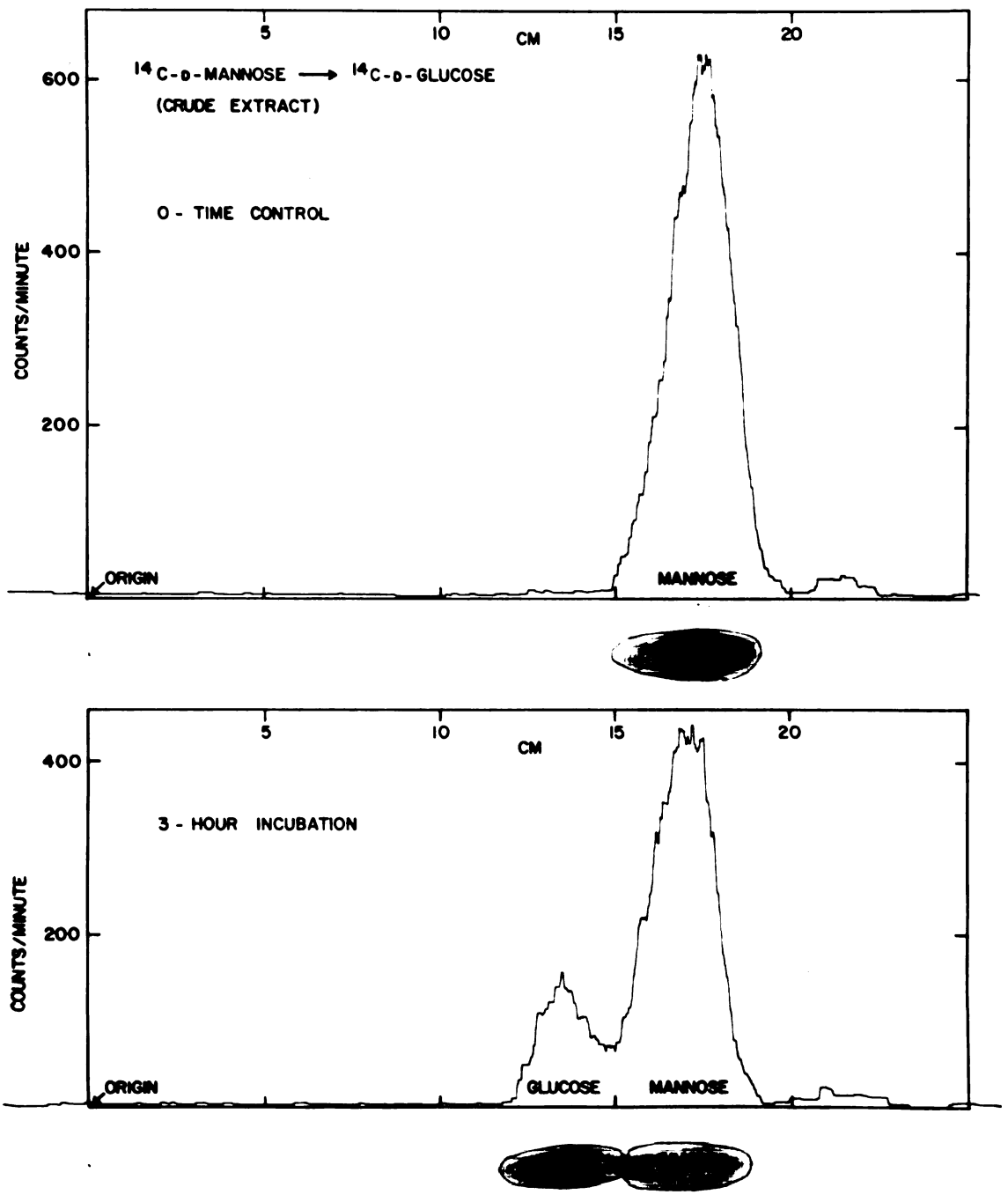

D-MANNOSE → D-GLUCOSE "K_m" = 2 X 10^{-2} M
(CRUDE EXTRACT)
0.15
0.10
0.05
$\frac{1}{V}$
-25
0
25
50
75
100
$\frac{1}{\text{D-MANNOSE (M)}}$

use of glucose oxidase and D-glucokinase as specific reagents was corroborated by paper chromatography. With 80% phenol as the solvent, a spot which migrated at a rate corresponding to D-glucose was detected with $AgNO_3$ and with N,N- dimethyl-p-phenylenediamine monohydrochloride. The size of the glucose spot increased with increasing times of incubation of D-mannose with the crude extract. An endogenous formation of D-glucose was slight, and was not detected at the shorter incubation times. To show that the D-glucose was derived from added D-mannose rather than from an endogenous compound by glycosyl exchange reactions, ^{14}C-D-mannose was used. Fig. 15 shows that at 0 time essentially all of the radioactivity on the chromatogram migrated with D-mannose, whereas after 3 hours of incubation, the spots corresponding to both D-glucose and D-mannose were radioactive.

The observed conversion of D-mannose to D-glucose did not involve aldose-ketose isomerization with D-frutose as a free intermediate. The isomerization of D-mannose to D-fructose has been detected in extracts of this organism only after growth on D-lyxose (39); the isomerization of D-glucose to D-fructose, as determined by the Roe (40) procedure, has never been detected in extracts of this organism. Thus, an apparent 2-epimerization of D-mannose

Fig. 15. Formation of ^{14}C-D-glucose from ^{14}C-D-mannose in a crude extract. The reaction mixture contained 200 μmoles (2 μcuries) of D-mannose-1-^{14}C and crude cell extract (432 mg of protein, adjusted to pH 7.5) in a volume of 15 ml. Samples (7-ml) were removed at 0 time and after incubation at 25° for 3 hours, heated in a boiling water bath for 5 minutes, cooled, and centrifuged. Absolute ethanol (28 ml) was added to each supernatant solution, and after cooling to 0°, the precipitates were removed by centrifugation. The samples were then evaporated to dryness under reduced pressure and re-dissolved in 3 ml of water. They were then treated three times with Dowex 50W-X8 (H^+), after which the pH was 3, followed by three treatments with Amberlite CG-4B, after which the pH was 6.4. The volumes were then reduced to 0.1 ml with a Buchler Rotary Evapo-Mix. One μl (2,500 cpm) of the 3-hr sample and 1.3 μl (2,800 cpm) of the 0-time sample were spotted on paper and developed as described in Experimental Procedure. The spots were visualized with silver nitrate. The chromatogram scans employed a 0.25-inch slit width, a 0.2-inch per minute scan speed, and a 20-second time constant.

Figure 15.


5
10
CM
15
20
600
400
200
COUNTS/MINUTE
^{14}C-D-MANNOSE ⟶ ^{14}C-D-GLUCOSE
(CRUDE EXTRACT)
0 - TIME CONTROL
ORIGIN
MANNOSE
5
10
CM
15
20
400
200
COUNTS/MINUTE
3 - HOUR INCUBATION
ORIGIN
GLUCOSE
MANNOSE

to D-glucose was indicated.

Inactivation of the D-Mannose to D-Glucose Conversion by Charcoal- Treatment of a crude extract with 10% and 20% charcoal (Darco G-60) caused a loss of the ability to convert D-mannose to D-glucose 53% and 100%, respectively, suggesting the involvement of a cofactor. This was not unexpected since other epimerases are known which require charcoal-adsorbable cofactors. For example, N-acyl-D-glucosamine 2-epimerase requires ATP (41), and UDP-galactose 4-epimerase requires NAD (42). However, attempts to re-activate the charcoal-inactivated extract with ATP, ITP, UTP, GTP, CTP, TTP, or NAD were unsuccessful. Furthermore, attempts to elute from the charcoal a cofactor which would reactivate the extract were unsuccessful. The possibility existed that a protein was adsorbed by the charcoal, although analysis of the charcoal-treated extract for enzymes such as D-glucokinase, D-glucose 6-phosphate isomerase, and D-mannose 6-phosphate isomerase indicated that they were not adsorbed. The possibility also existed that the suspected 2-epimerase acted on nucleoside diphosphate derivatives of D-mannose and D-glucose rather than on the free sugars (43), and that the necessary sugar nucleotides were adsorbed on the charcoal. This scheme would require another enzyme, a transferase which would catalyze a glycosyl exchange of

D-mannose with a nucleoside diphosphate-D-glucose to yield a nucleoside diphosphate-D-mannose and D-glucose. Experiments which are described below, however, indicate that the conversion of D-mannose to D-glucose can be explained on a basis other than one involving two hypothetical enzymes.

Inhibition of the D-Mannose to D-Glucose Conversion by Alkaline Phosphatase- When a crude extract was supplemented with alkaline phosphatase and incubated with D-mannose, no D-glucose was formed (Table IV). This suggested the participation of phosphomonoesters in the D-mannose to D-glucose conversion.

ATP-Dependence of the D-Mannose to D-Glucose Conversion- Molecular sieving of a crude extract by passage through Sephadex G-25 abolished its ability to convert D-mannose to D-glucose. The activity could be restored, however, by the addition of ATP to the reaction mixture (Fig. 16). The rate was maximal at an ATP concentration of about 0.5 mM. At higher ATP concentrations, the D-glucose formed was phosphorylated to D-glucose 6-phosphate. Preliminary experiments on the stoichiometry of the reaction indicated that more than two moles of D-glucose were formed per mole of ATP, suggesting that the ATP was acting catalytically. Thus, the reaction had a superficial resemblance to the ATP-dependent 2-epimerization of N-acyl-D-glucosamine

TABLE IV

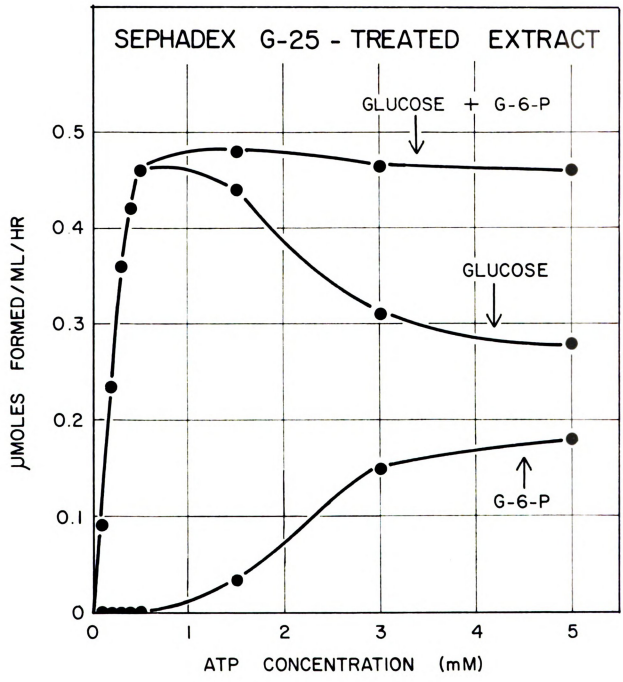
Effect of added alkaline phosphatase on the formation of D-glucose from D-mannose by a crude cell extract

The complete reaction mixture contained in a volume of 1.0 ml: 60 μmoles of glycylglycine buffer (pH 7.5), 50 μmoles of D-mannose, 12 μmoles of $MgCl_2$, crude cell extract (11.4 mg of protein), and 2 mg of alkaline phosphatase. Controls were minus phosphatase or D-mannose as indicated. After incubation for the times indicated, the reaction mixtures were heated in a boiling water bath for 2 minutes, cooled, and centrifuged. Aliquots of the supernatant solutions were then assayed for D-glucose with D-glucokinase-glucose 6-phosphate dehydrogenase.

Incubation Time	D-Glucose Formed			
	Minus Phosphatase		Plus Phosphatase	
	+ D-Mannose	- D-Mannose	+ D-Mannose	- D-Mannose
Minutes	mμmoles/mg protein	mμmoles/mg protein	mμmoles/mg protein	mμmoles/mg protein
0	0	0	7	7
20	56	0	17	20
40	86	0	23	20
60	126	0	23	23

Fig. 16. Inactivation of the ability of a crude extract to convert D-mannose to D-glucose by chromatography on Sephadex G-25 and reactivation with ATP. Crude extract (10 ml) was passed through a column (20 x 3.5 cm) of Sephadex G-25. The activity of the resulting protein fraction was then tested in reaction mixtures consisting of 60 μmoles of glycylglycine buffer (pH 7.5), 12 μmoles of $MgCl_2$, 50 μmoles of D-mannose, crude extract (2.4 mg of protein), and ATP in amounts varying from 0 to 5 μmoles, in a volume of 1.0 ml. After incubation at 25° for 60 minutes, the tubes were heated in a boiling water bath for 2 minutes, cooled, and centrifuged. Aliquots of the supernatant solutions were then assayed for D-glucose and D-glucose 6-phosphate by the use of D-glucokinase and glucose 6-phosphate dehydrogenase. G-6-P, D-glucose 6-phosphate.

Figure 16.


SEPHADEX G-25 - TREATED EXTRACT
GLUCOSE + G-6-P
GLUCOSE
G-6-P
μMOLES FORMED/ML/HR
0
0.1
0.2
0.3
0.4
0.5
0
1
2
3
4
5
ATP CONCENTRATION (mM)

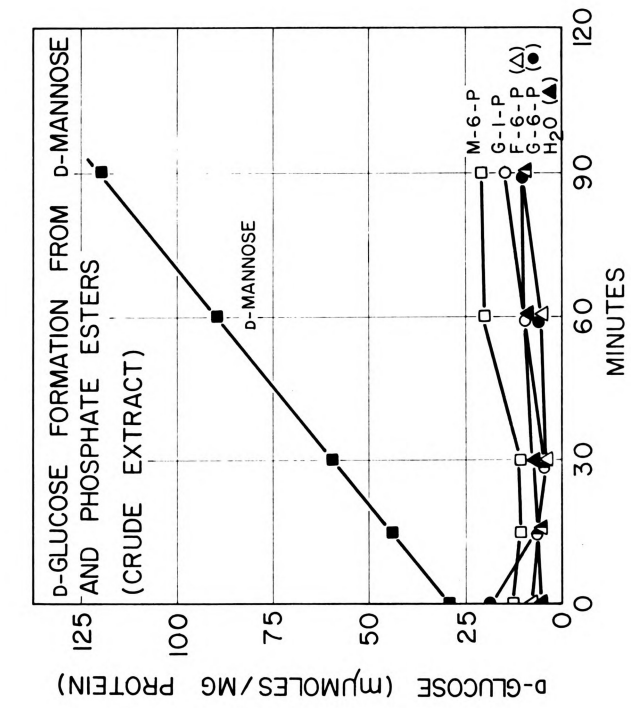
recently described by Ghosh and Roseman (41). However, experiments which are described below indicate that the D-mannose to D-glucose conversion observed in extracts of A. aerogenes PRL-R3 was mediated by the concerted action of several enzymes rather than a single ATP-dependent enzyme.

Evidence that D-Glucose was not Derived from D-Glucose Phosphate by the Hydrolytic Action of a Phosphatase- In view of the ATP-dependence and alkaline phosphatase-sensitivity of the D-mannose to D-glucose conversion, the possibility existed that D-mannose was somehow phosphorylated to D-mannose 6-phosphate and converted to D-glucose 6-phosphate and D-glucose 1-phosphate by isomerase- and mutase-catalyzed reactions. The D-glucose might then arise from the hydrolysis of D-glucose 6-phosphate or D-glucose 1-phosphate. Evidence which militates against this is illustrated in Fig. 17; a crude extract formed D-glucose from D-mannose but not from the phosphate esters of D-mannose, D-glucose, or D-fructose.

Measurement of the Conversion of D-Mannose to D-Glucose 6-Phosphate in a Continuous Spectrophotometric Assay; Discovery of Acyl Phosphate:Hexose Phosphotransferase- Because crude extracts contained an active D-glucokinase with a high affinity for D-glucose ($K_m = 8 \times 10^{-5}$ M), the ATP-dependent conversion of D-mannose to D-glucose could conveniently be

Fig. 17. Measurement of the D-glucose formed from D-mannose and from hexose phosphates in a crude extract. The reaction mixtures contained in a volume of 7 ml: 100 μmoles of hexose phosphate or 500 μmoles of D-mannose, and crude extract (252 mg of protein), adjusted to pH 7.5. One-ml samples were removed at time intervals, heated in a boiling water bath for 2 minutes, cooled, and centrifuged. Aliquots of the supernatant solutions were then assayed for D-glucose with glucose oxidase. M-6-P, D-mannose 6-phosphate; G-1-P D-glucose 1-phosphate; F-6-P, D-fructose 6-phosphate; G-6-P, D-glucose 6-phosphate.

Figure 17.


D-GLUCOSE FORMATION FROM D-MANNOSE AND PHOSPHATE ESTERS (CRUDE EXTRACT)
D-MANNOSE
M-6-P
G-1-P
F-6-P (△)
G-6-P (●)
H2O (▲)
D-GLUCOSE (μMOLES/MG PROTEIN)
MINUTES
0
25
50
75
100
125
30
60
90
120

measured spectrophotometrically in a continuous assay consisting of D-mannose, ATP, NADP, glucose 6-phosphate dehydrogenase, and crude or Sephadex G-25-treated extract. Subsequently, it was found that acetyl phosphate could replace ATP in this assay. This led to the discovery of a phosphotransferase which phosphorylated D-glucose (5) and D-mannose (7) with acetyl phosphate to yield the respective 6-phosphates (see Part III.) This enzyme, which was found to be inactivated by or adsorbed by charcoal, could account for the acetyl phosphate-dependent conversion of D-mannose to D-glucose 6-phosphate, assuming the presence of D-mannose 6-phosphate isomerase and D-glucose 6-phosphate isomerase. It could not, however, account for the ATP-dependent conversion of D-mannose to D-glucose.

Fractionation of the Enzymes Involved in the Acetyl Phosphate-Dependent and ATP-Dependent Conversions of D-mannose to D-glucose 6-phosphate- Chromatography of a crude extract on Sephadex G-75 and analysis of the fractions for the ability to convert D-mannose to D-glucose 6-phosphate in the continuous spectrophotometric assay revealed a loss of most of the activity. The activity could be restored, however, by the recombination of certain fractions, indicating that two or more proteins were involved in the acetyl phosphate-

dependent conversion of D-mannose to D-glucose 6-phosphate. A typical experiment is shown in Fig. 18. Fraction 14, which contained almost no activity, activated fractions 6 through 9. Fraction 6, which contained no activity alone, greatly stimulated the activity in fractions 10 through 14. Essentially identical results were obtained when ATP replaced acetyl phosphate in the reaction mixture. Fractions 6 through 9 were subsequently found to contain acyl phosphate:hexose phosphotransferase, whereas fractions 10 through 14 were found to contain D-glucokinase, D-glucose 6-phosphate isomerase, and D-mannose 6-phosphate isomerase. To explain the ATP-dependent conversion of D-mannose to D-glucose, however, it seemed necessary to postulate an additional reaction wherein the phosphoryl group of D-glucose 6-phosphate, which could be formed from a D-glucokinase-catalyzed phosphorylation of D-glucose contamination (0.08%) in the D-mannose, could be transferred to the 6-position of D-mannose.

Hexose 6-Phosphate:Hexose 6-Phosphotransferase- An experiment which demonstrates the phosphorylation of D-mannose with D-glucose 6-phosphate to yield D-glucose plus D-mannose 6-phosphate, as postulated above, is shown in Table V. The reaction was measured in reverse; i.e., the D-glucose-dependent conversion of D-mannose 6-phosphate

Fig. 18. Chromatography of a crude extract on Sephadex G-75 and assay of the fractions (singly and in combination) for the ability to catalyze an acetyl phosphate-dependent conversion of D-mannose to D-glucose 6-phosphate. A crude extract was passed through a column (30 x 2.5 cm) of Sephadex G-75 at 4° and collected in 4.5-ml fractions. Aliquots (0.03 ml) of the fractions were then assayed by measuring the rate of absorbance increase at 340 mμ in reaction mixtures consisting of 10 μmoles of glycylglycine buffer (pH 7.5), 5 μmoles of D-mannose, 0.5 μmole of acetyl phosphate, 1 μmole of $MgCl_2$, 0.2 μmole of NADP, and excess glucose 6-phosphate dehydrogenase in a volume of 0.15 ml. Where combinations of fractions are indicated, 0.03 ml of each fraction was used.

Figure 18.

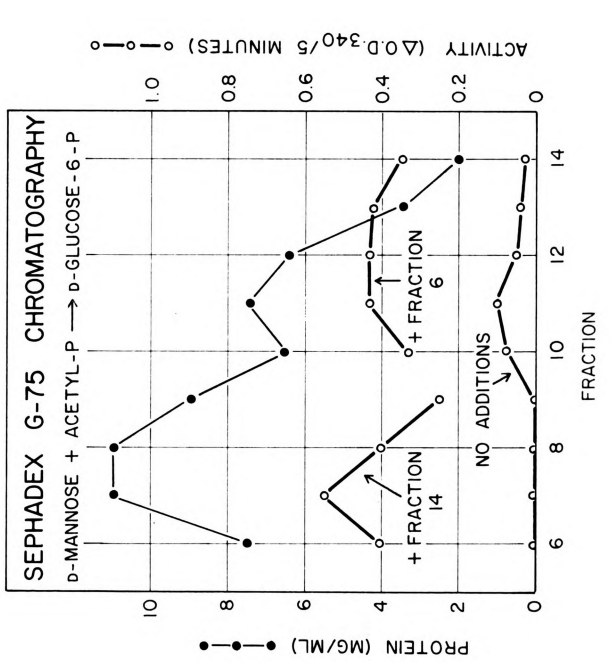

SEPHADEX G-75 CHROMATOGRAPHY
D-MANNOSE + ACETYL-P ⟶ D-GLUCOSE-6-P
PROTEIN (MG/ML) ●—●—●
ACTIVITY (ΔO.D.340/5 MINUTES) o—o—o
FRACTION
+ FRACTION 14
+ FRACTION 6
NO ADDITIONS

TABLE V

Demonstration of hexose phosphate:hexose phosphotransferase activity: formation of D-glucose 6-phosphate from D-glucose plus D-mannose 6-phosphate

The complete reaction mixture contained in a volume of 0.15 ml: 10 µmoles of glycylglycine buffer (pH 7.5), 0.1 µmole of NADP, 1 µmole of $MgCl_2$, 1 µmole of D-glucose, 1 µmole of D-mannose 6-phosphate, a Sephadex G-75 fraction of crude extract (200 µg of protein), and excess glucose 6-phosphate dehydrogenase. The reaction rates were measured by observing the increase in absorbance at 340 mµ.

Reaction Mixture	Rate of D-glucose 6-phosphate formation
	mµmoles/hr/mg of protein
Complete.....	196
Minus D-glucose.....	0
Minus D-mannose-6-P..	0

to D-glucose 6-phosphate was measured by coupling the reaction to glucose 6-phosphate dehydrogenase. With a Sephadex G-75 fraction, D-glucose 6-phosphate was formed from a mixture of D-mannose 6-phosphate and D-glucose, but not from either one alone. This reaction is difficult to detect in crude extracts because the conversion of D-mannose 6-phosphate to D-glucose 6-phosphate is not then D-glucose-dependent due to the action of D-mannose 6-phosphate isomerase and D-glucose 6-phosphate isomerase.

The phosphorylation of D-mannose with D-glucose 6-phosphate has also been measured in the forward direction by enzymic determinations of D-glucose, D-glucose 6-phosphate and D-mannose 6-phosphate, and by the demonstration that ^{14}C-D-mannose 6-phosphate is formed from D-glucose 6-phosphate and ^{14}C-D-mannose (see Part III). The enzyme which catalyzes this reaction is believed to be the same enzyme as that which phosphorylates hexoses with acetyl phosphate and carbamyl phosphate (Part III).

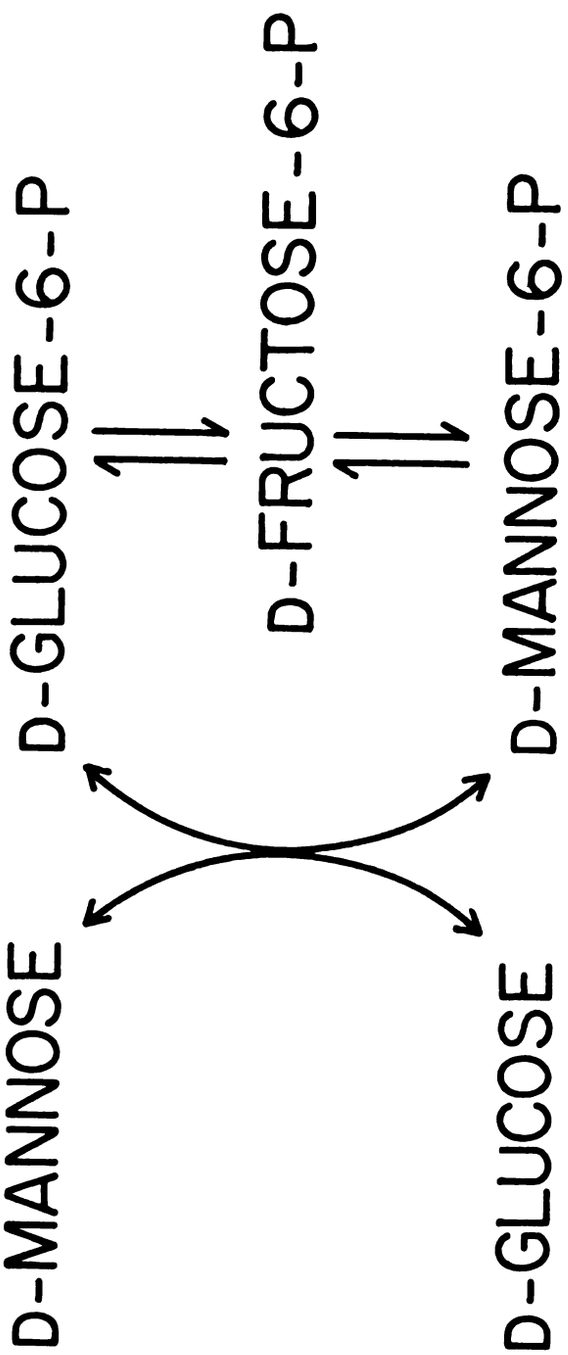
The dependence of the conversion of D-mannose to D-glucose 6-phosphate on either acetyl phosphate or ATP and on two sephadex G-75 fractions may now be rationalized. With acetyl phosphate, D-mannose was phosphorylated to D-mannose 6-phosphate by the acyl phosphate:hexose phosphotransferase, and converted to D-glucose 6-phosphate

by the action of D-mannose 6-phosphate isomerase and D-glucose 6-phosphate isomerase. With ATP, the D-glucose which contaminated even the recrystallized D-mannose at a level of 0.08% was phosphorylated by the action of D-glucokinase. The D-glucose 6-phosphate formed then served as a phosphoryl donor for D-mannose by the action of hexose phosphate:hexose phosphotransferase, yielding D-mannose 6-phosphate and free D-glucose which could be phosphorylated again with ATP. The D-mannose 6-phosphate was then converted to D-glucose 6-phosphate by isomerization reactions. Consistent with this explanation are the K_m values. The K_m of D-glucokinase for D-glucose is 8×10^{-5}M, and the K_m of acyl phosphate:hexose phosphotransferase is 4×10^{-4}M for D-mannose 6-phosphate and 1.2×10^{-2}M for D-mannose. The relatively large K_m for D-mannose presents no difficulty because D-mannose was supplied in excess.

Reconstitution of the Reactions Involved in the Apparent 2-Epimerization of D-Mannose to D-Glucose. The apparent 2-epimerization of D-mannose to D-glucose that was observed in crude extracts may be explained by the reactions shown in Fig. 19, involving hexose phosphate:hexose phosphotransferase, D-glucose 6-phosphate isomerase, and D-mannose 6-phosphate isomerase. This sequence was reconstituted by

Fig. 19. Proposed reaction sequence for the conversion of D-mannose to D-glucose in crude extracts.

Figure 19.



purified enzymes, as is demonstrated in Table VI. D-Glucose was formed from D-mannose in the presence of a catalytic amount of D-glucose 6-phosphate and the three enzymes, but was not formed to a significant extent when one of the components was omitted (Experiment 1). D-Glucose 6-phosphate could be replaced by D-glucokinase, ATP, and a catalytic amount of D-glucose (Experiment 2). Consistent with this explanation for the D-mannose to D-glucose conversion are the observations that (i) the K_m of purified hexose phosphate:hexose phosphotransferase for D-mannose (1.2×10^{-2}M) is about the same as the concentration which effects the half-maximal rate of D-glucose formation (2×10^{-2} M, see Fig. 14) in the crude extract, and (ii) the specific activity of hexose phosphate:hexose phosphotransferase in the crude extract (about 0.2 μmole of hexose phosphorylated per hour per mg of protein) is about the same as the rate of D-glucose formation by the crude extract, as described earlier in Part II of this thesis.

A Cyclic Pathway for the Metabolism of D-Mannose- Fig. 20 summarizes the reactions which we propose to be involved in the constitutive utilization of D-mannose by A. aerogenes PRL-R3. The essential features of this scheme are that although D-glucose can be phosphorylated with ATP, D-mannose cannot be. Instead, D-mannose is phosphorylated with

TABLE VI

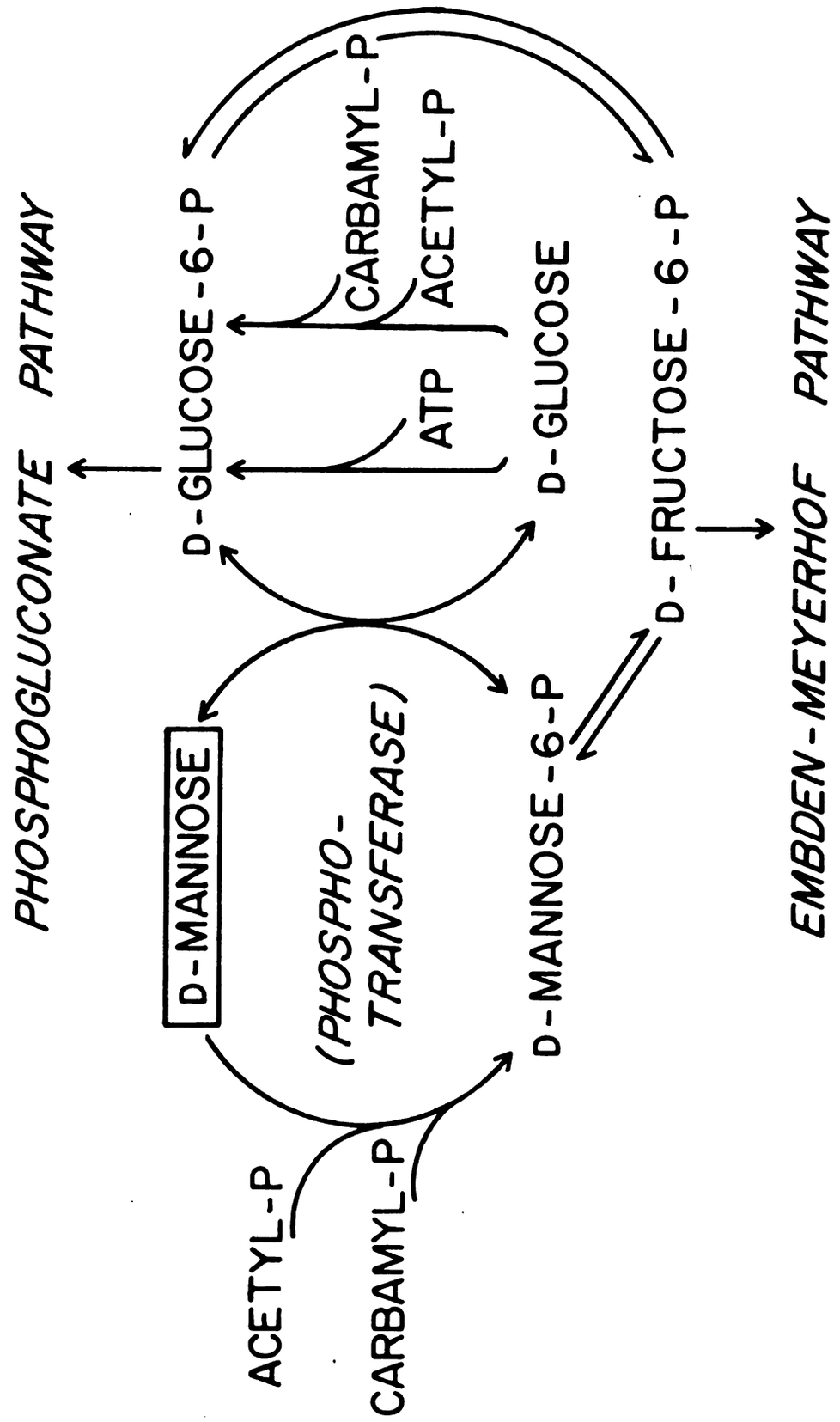
Reconstitution of the D-mannose to D-glucose conversion with purified enzymes

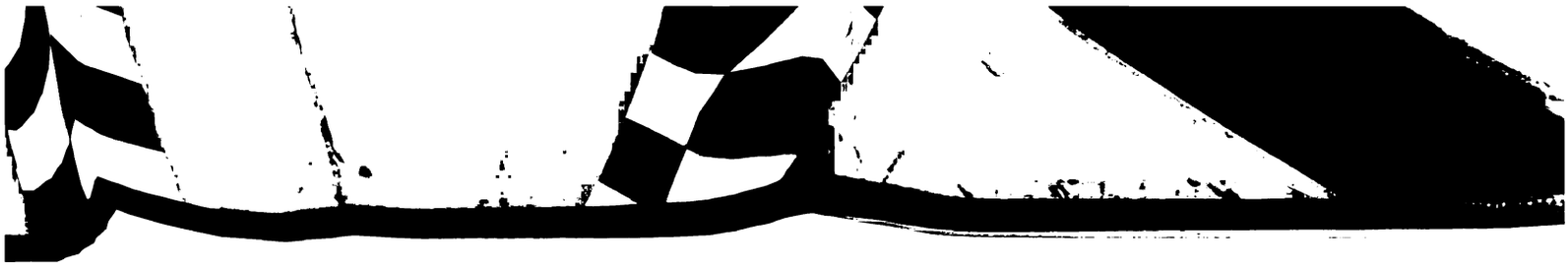
The complete reaction mixture in Experiment 1 contained in a volume of 1.8 ml: 200 μmoles of glycylglycine buffer (pH 7.5), 10 μmoles of $MgCl_2$, 200 μmoles of D-mannose, 0.5 μmole of D-glucose 6-phosphate, 11 units of acyl phosphate: hexose phosphotransferase, 5 units of D-mannose 6-phosphate isomerase, and 5 units of D-glucose 6-phosphate isomerase. The complete reaction mixture in Experiment 2 contained 1 μmole of ATP, 0.3 μmole of D-glucose, and 7 units of D-glucokinase in place of D-glucose 6-phosphate. The units of the enzymes used in this experiment refer to the μmoles of the substrate reacted per hour at 25°, pH 7.5, and with saturating levels of substrate. After incubation of the reaction mixtures at 25° for the times indicated, aliquots were removed, heated in a boiling water bath for 2 minutes, cooled, centrifuged, and the supernatants assayed for the increase in D-glucose (measured as D-glucose + D-glucose 6-phosphate with D-glucokinase and glucose 6-phosphate dehydrogenase) above 0 time controls.

Experiment No.	Reaction Mixture	D-glucose formed	
		1 hr	2 hrs
		mμmoles/ml	mμmoles/ml
1	Complete	498	847
	" minus phosphotransferase	-60	-12
	" minus isomerases	-12	-12
	" minus D-glucose 6-phosphate	0	40
	" minus D-mannose	-30	-30
2	Complete	435	1,037
	" minus ATP	-45	-71

Fig. 20. Summary of the reactions believed to be instrumental in the metabolism of D-mannose in A. aerogenes PRL-R3.

Figure 20.


PHOSPHOGLUCONATE PATHWAY
D-GLUCOSE-6-P
CARBAMYL-P
ACETYL-P
ATP
D-GLUCOSE
D-FRUCTOSE-6-P
EMBDEN-MEYERHOF PATHWAY
D-MANNOSE
(PHOSPHO-TRANSFERASE)
D-MANNOSE-6-P
ACETYL-P
CARBAMYL-P



D-glucose 6-phosphate to yield D-mannose 6-phosphate and D-glucose. D-Glucose 6-phosphate may be regenerated by isomerization of D-mannose 6-phosphate through D-fructose 6-phosphate, or by phosphorylation of D-glucose with ATP. Hexose phosphates that are depleted by further metabolism may be replenished by phosphorylation of D-mannose with acetyl phosphate or carbamyl phosphate. The apparent 2-epimerization of D-mannose to D-glucose that was observed in crude extracts may be explained by a cyclic process involving D-mannose 6-phosphate isomerase, D-glucose 6-phosphate isomerase, acyl phosphate:hexose phosphotransferase (hexose phosphate:hexose phosphotransferase), and possibly D-glucokinase to provide sparking amounts of D-glucose 6-phosphate. Observations in support of the pathway depicted in Fig. 20 are as follows: (i) D-mannose is utilized constitutively, (ii) the constitutive hexokinase is stereospecific for D-glucose, (iii) D-mannokinase activity cannot be detected, (iv) crude extracts convert D-mannose to D-glucose as determined with glucose oxidase, a stereospecific D-glucokinase, and paper chromatography using ^{14}C-D-mannose, (v) D-fructose is not a free intermediate in the conversion of D-mannose to D-glucose, (vi) the D-mannose to D-glucose conversion is inactivated by alkaline phosphatase, charcoal, and chromatography on Sephadex G-25, (vii) the Sephadex

G-25-inactivated extract can be reactivated with ATP but the charcoal-inactivated extract cannot be, (viii) the accumulated D-glucose is not derived from D-glucose 1-phosphate or D-glucose 6-phosphate by the action of a phosphatase, (ix) acetyl phosphate can replace ATP in the conversion of D-mannose to D-glucose 6-phosphate, (x) an enzyme which phosphorylates D-mannose with D-glucose 6-phosphate, carbamyl phosphate, or acetyl phosphate has been identified and purified several hundred fold, (xi) D-mannose 6-phosphate isomerase, D-glucose 6-phosphate isomerase, D-glucokinase, and acyl phosphate:hexose phosphotransferase are constitutive enzymes, (xii) acyl phosphate:hexose phosphotransferase is adsorbed by or inactivated by charcoal, (xiii) the ATP-dependent and acetyl phosphate-dependent conversions of D-mannose to D-glucose 6-phosphate are inactivated by chromatography on Sephadex G-75, but can be restored by combining two of the fractions, one of which contains acyl phosphate:hexose phosphotransferase and the other of which contains D-mannose 6-phosphate isomerase, D-glucose 6-phosphate isomerase, and D-glucokinase, (xiv) the conversion of D-mannose to D-glucose can be reconstituted by D-mannose 6-phosphate isomerase, D-glucose 6-phosphate isomerase, acyl phosphate:hexose phosphotransferase, and a

catalytic amount of D-glucose 6-phosphate, (xv) the K_m value of the phosphotransferase for D-mannose is the same as the D-mannose concentration which effects a half maximal rate of conversion of D-mannose to D-glucose in crude extracts, and (xvi) the specific activity of acyl phosphate:hexose phosphotransferase in crude extracts is the same as the rate at which D-mannose is converted to D-glucose in crude extracts. Of interest is the fact that even though D-glucokinase is stereospecific for D-glucose, it can presumably function in D-mannose metabolism by supplying a suitable phosphoryl donor (D-glucose 6-phosphate).

Whether the observed rate of D-mannose metabolism in extracts (an average of about 0.2 μmole per hour per mg of protein at pH 7.5 and 25°) is adequate to account for the rate at which cells of A. aerogenes PRL-R3 metabolize D-mannose is difficult to ascertain because the degree of inactivation during extraction is not known, nor is the effect of disruption of cellular organization. Furthermore, the enzymes involved have widely divergent pH optima (e.g., pH 9 for acyl phosphate:hexose phosphotransferase and less than pH 6 for D-mannose 6-phosphate isomerase), and so the conditions used were not necessarily optimal. At higher pH values and higher temperatures, rates have been obtained

which were several fold greater and therefore approached the specific activities of other enzymes involved in monosaccharide metabolism in this organism (44). The possibility also exists that other phosphotransferases which use different phosphoryl donors (29,30) may be involved in hexose metabolism in this organism.

SUMMARY OF PART II

Evidence was presented for the operation of a unique cyclic pathway of D-mannose metabolism in A. aerogenes PRL-R3. The pathway involves reactions catalyzed by D-glucose 6-phosphate isomerase, D-mannose 6-phosphate isomerase, a stereospecific D-glucokinase, and a phosphotransferase which phosphorylates D-mannose with D-glucose 6 phosphate, acetyl phosphate, or carbamyl phosphate. A functional significance for the pathway is indicated by an apparent lack of D-mannokinase in this organism, even though it can metabolize D-mannose constitutively. The pathway also accounts for an apparent 2-epimerization of D-mannose to D-glucose that was observed in extracts.

PART III

Purification and Properties of Acyl Phosphate:Hexose Phosphotransferase (Hexose Phosphate:Hexose Phosphotransferase) from *Aerobacter aerogenes* PRL-R3

The preceding section of this thesis reported the participation of acyl phosphate:D-mannose 6-phosphotransferase activity and D-glucose 6-phosphate:D-mannose 6-phosphotransferase activity in a novel pathway of D-mannose metabolism in *A. aerogenes* PRL-R3. This demonstrated utilization of acetyl phosphate, carbamyl phosphate, and D-glucose 6-phosphate, rather than ATP, for the phosphorylation of hexoses in an energy-generating pathway is so far unique in metabolism. Consequently, the enzymes which catalyzed these reactions were purified and their properties investigated. This section of the thesis describes the purification and properties of acyl phosphate: hexose phosphotransferase from *A. aerogenes* PRL-R-3, and presents evidence for its common identity with hexose phosphate: hexose phosphotransferase. Kinetic and specificity studies indicate that the enzyme may also participate in the metabolism of D-fructose and D-mannitol.

EXPERIMENTAL PROCEDURE

Growth of Organism- A. aerogenes PRL-R3 was grown as described in Part I.

Chemicals- D-Mannose 6-phosphate and D-fructose 6-phosphate were prepared enzymically by the method of Slein (45). D-Mannitol 1-phosphate and D-sorbitol 6-phosphate were prepared by chemical reduction of D-mannose 6-phosphate and D-glucose 6-phosphate, respectively (46). α-D-Mannose 1-phosphate and α-D-galactose 1-phosphate were gifts from Dr. R.G. Hansen. Other chemicals were obtained as described in Parts I and II.

Enzymes- D-Mannitol 1-phosphate dehydrogenase was purified from A. aerogenes PRL-R3 grown on D-mannitol or D-sorbitol. A 10-fold increase in specific activity was achieved by protamine sulfate treatment, ammonium sulfate fractionation, and chromatography on Sephadex G-100. The other coupling enzymes used were obtained as reported in Parts I and II.

Analytical Procedures- The chromatographic separation of sugar phosphates was accomplished on Whatman No. 1 paper (washed with 1 N HCL and water) with the modified Hanes-Isherwood solvent (picric acid, t-butanol, H_2O, 2:80:20) (47). Inorganic orthophosphate was determined by the method of Fiske and SubbaRow (48). D-Ribose 5-phosphate

was determined by an orcinol method (49). Other methods used were as described in Parts I and II.

Acyl Phosphate:Hexose Phosphotransferase Assay--The enzyme was routinely assayed by measuring NADP reduction at 340 mμ with a Gilford absorbance-recording spectrophotometer thermostated at 25°, using microcuvettes with a 1-cm light path. The reaction mixture contained in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 0.2 μmole of NADP, 1.0 μmole of acetyl phosphate, 1.0 μmole of D-glucose, excess glucose 6-phosphate dehydrogenase, and acyl phosphate:hexose phosphotransferase at concentrations which gave a linear response. In early experiments a divalent metal ion such as Mg^{++} or Mn^{++} was included in the assay, but was later omitted because it was found to have no effect on the rate. A unit of enzyme was defined as the amount that catalyzed the phosphorylation of 1 μmole of D-glucose per hour under the conditions described.

When hexoses other than D-glucose were used as phosphoryl acceptors, the following additional coupling enzymes were included in excess: for D-fructose, D-glucose 6-phosphate isomerase; for D-mannose, D-mannose 6-phosphate isomerase and D-glucose 6-phosphate isomerase. With mannitol as the phosphoryl acceptor, 0.1 μmole of NAD and excess

mannitol 1-phosphate dehydrogenase replaced NADP and glucose 6-phosphate dehydrogenase.

The activity of 6-phosphogluconate dehydrogenase (measured by replacing D-glucose and acetyl phosphate with 6-phosphogluconate in the assay mixture) in the crude cell extract was about twice the activity of the acyl phosphate: hexose phosphotransferase. Therefore, its contribution to the observed acyl phosphate:hexose phosphotransferase rate was considered in the initial steps of purification by dividing the specific activity values for the crude extract, protamine sulfate fraction, and first ammonium sulfate fraction by two (no 6-phosphogluconic acid dehydrogenase activity was detected after the heat step).

RESULTS

Purification of Acyl Phosphate:Hexose Phosphotransferase

Preparation of Extracts- Seventy grams (wet weight) of cells were suspended in 80 ml of water. Extracts were prepared by treating the cell suspension for 15 minutes in a Raytheon 10-kc sonic oscillator circulated with ice water. The broken-cell suspension was centrifuged at 13,200 x *g*, and the resulting supernatant fluid was used as the cell extract. Unless stated otherwise, the fractionation procedures described below were performed at 0 to 4°.

Protamine Treatment- The cell extract was diluted with

water to give 675 ml with a protein concentration of 21 mg per ml and a 280:260 mμ ratio of 0.73. Ammonium sulfate (8.91 g) was added to give a concentration of 0.1 M, followed by the slow addition of 136 ml of a 2% solution of protamine sulfate. The mixture was stirred for 10 minutes and the precipitate that formed was removed by centrifugation and discarded. The supernatant solution (780 ml) contained 9 mg of protein per ml and had a 280:260 mμ ratio of 0.90.

First Ammonium Sulfate Fractionation- Ammonium sulfate (210.8 g) was added to the protamine sulfate-treated extract, and the precipitate that formed was removed by centrifugation and discarded. To the supernatant solution was added 233.6 grams of ammonium sulfate (50 to 90% of saturation), and the resulting precipitate was collected by centrifugation and dissolved in water. This solution (85 ml) contained 29 mg of protein per ml and had a 280:260 mμ ratio of 1.11.

Heat Treatment- The temperature of the above fraction was raised quickly to 50°, held for 5 minutes, and quickly cooled. The precipitated protein was removed by centrifugation and discarded. The supernatant solution contained 13 mg of protein per ml and had a 280:260 mμ ratio of 1.10.

Second Ammonium Sulfate Fractionation- Ammonium sulfate,

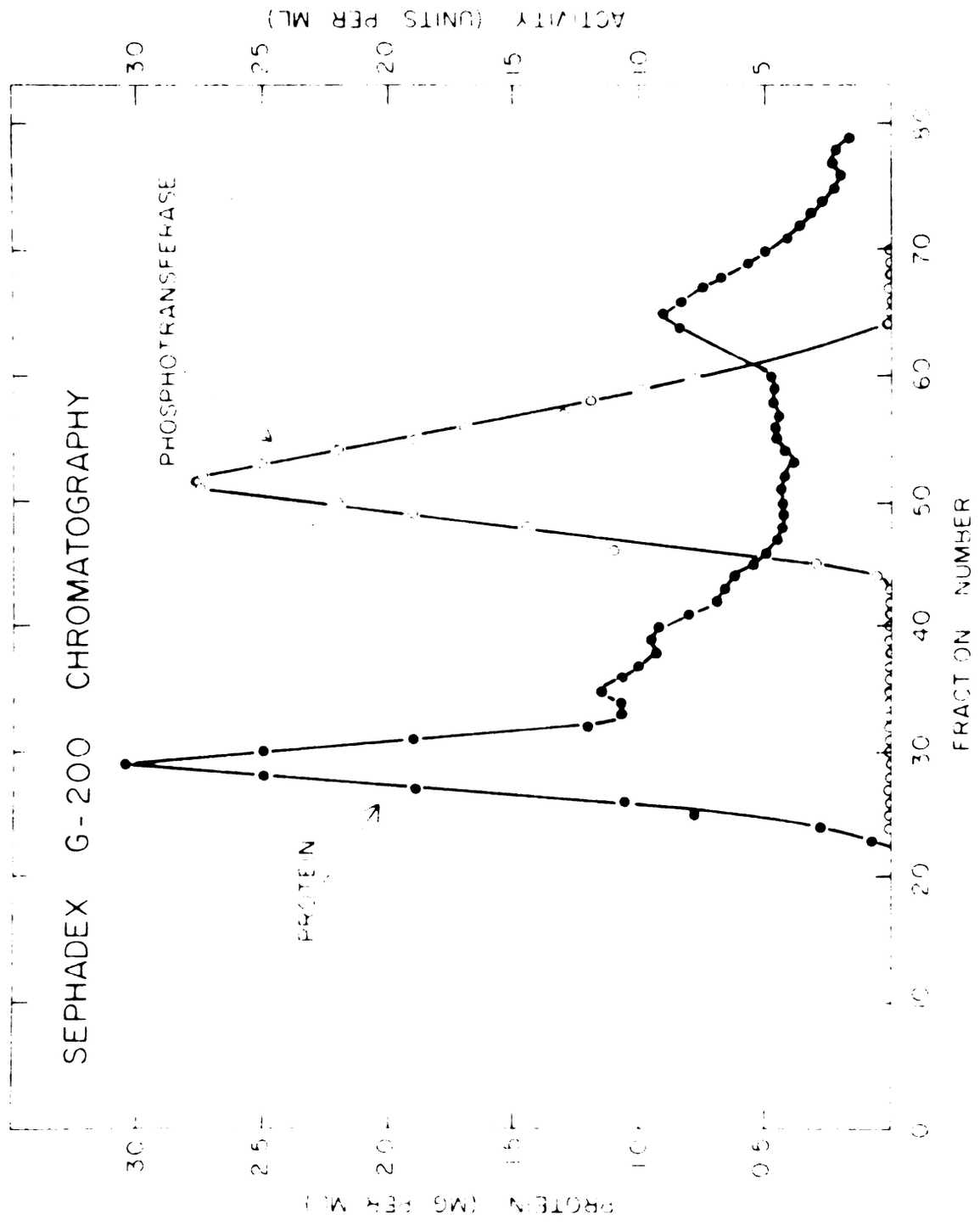
19.7 g (0 to 50% of saturation), was added to 70 ml of the above fraction, and the resulting precipitate was collected by centrifugation and dissolved in water. This solution (30 ml) contained 14 mg of protein per ml and had a 280:260 mμ ratio of 1.20.

Chromatography on Sephadex G-200- The above fraction was placed on a column (5 x 50 cm) of Sephadex G-200 and eluted with water at a flow rate of 80 ml per hour. Five-ml fractions were collected, and those which contained the highest specific activities (tubes 60 to 70) were pooled. Fig. 21 represents a typical elution profile. Turbidity of the pooled fractions was removed by centrifugation. The supernatant solution (46 ml) contained 0.81 mg of protein per ml and had a 280:260 mμ ratio of 1.52.

Chromatography on DEAE-Cellulose- The above fraction was concentrated by lyophilization and redissolved in 5.5 ml of water. Turbidity was removed by centrifugation. The solution was kept on ice overnight, and a precipitate of crystalline and amorphous protein which appeared was removed by centrifugation and discarded. All of the phosphotransferase activity remained in the supernatant. DEAE-cellulose (Bio-Rad Cellex D, exchange capacity = 0.95 meq per g) was treated with 0.2 M glycylglycine buffer (pH 6.5) and equilibrated with 0.02 M glycylglycine buffer (pH 6.5).

Fig. 21. Typical elution profile of phosphotransferase on Sephadex G-200. The units in this figure refer to an A_{340} increase of 1.0 per 5 minutes.

Figure 21.


SEPHADEX G-200 CHROMATOGRAPHY
PHOSPHOTRANSFERASE
PROTEIN
ACTIVITY (UNITS PER ML)
PROTEIN (MG PER ML)
FRACTION NUMBER
0
5
10
15
20
25
30
40
50
60
70

A column (1.5 x 10 cm) was prepared and 4.4 ml of the lyophilized Sephadex fraction was added. The protein was eluted (4-ml fractions) with 100 ml of 0.02 M glycyl-glycine buffer (pH 6.5) containing NaCl in a linear gradient from 0 to 0.3 M. From the elution profile (Fig. 22), it can be seen that the phosphotransferase was associated with a major proetin peak. Disc electrophoresis of the peak fraction revealed only one major band and one very faint minor band, indicating that the purified phosphotransferase was essentially homogenous. The two fractions with the highest specific activities were pooled to yield 8 ml of 710-fold purified phosphotransferase with a protein concentration of 0.16 mg per ml and a 280:260 mμ ratio of 1.7. Correcting for the portion of fractions not used for further purification, the yield was 8.5% of the activity in the crude extract. A summary of the purification procedure is given in Table VII.

Properties of Phosphotransferase and Product Identification

pH Optimum- Phosphotransferase activity as a function pH was maximal at a pH of 9 and a half maximal at a pH of about 7.5 (Fig. 23).

Phosphoryl Donor Specificity- The relative rates for the conversion of D-glucose to D-glucose 6 phosphate in

Fig. 22. Elution profile of phosphotransferase on DEAE-cellulose. The details are described in the text. The units in this figure refer to an A_{340} increase of 1.0 per 5 minutes.

Figure 22.

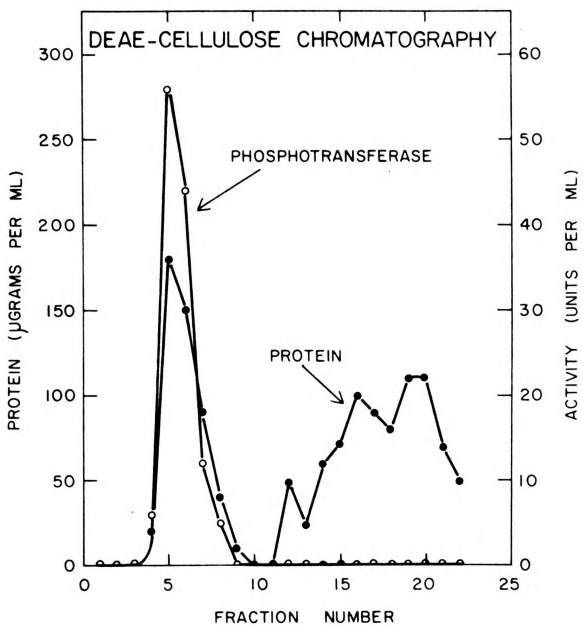

DEAE-CELLULOSE CHROMATOGRAPHY
PHOSPHOTRANSFERASE
PROTEIN
PROTEIN (μGRAMS PER ML)
ACTIVITY (UNITS PER ML)
FRACTION NUMBER
0
50
100
150
200
250
300
10
20
30
40
50
60
5
15
25

TABLE VII

Purification of acyl phosphate:hexose phosphotransferase

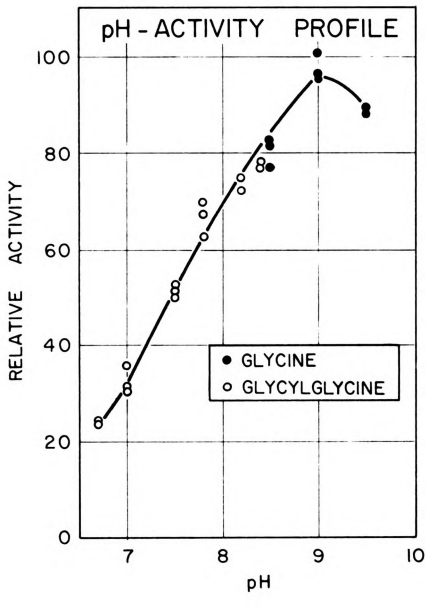
Fraction	Total Activity[a]	Yield	Specific Activity
	Units	%	Units/mg
Cell extract	1,570[b]	(76)	0.11
Protamine sulfate	2,060[b]	100	0.29
Ammonium sulfate I	1,240[b]	60	0.50
Heat	1,250	61	1.1
Ammonium sulfate II	1,300	63	2.5
Sephadex G-200	294	14	6.5
DEAE-cellulose	174	8.5	78

[a] μMoles of D-glucose phosphorylated per hour; corrected for the portion of fractions not used for further purification.

[b] Corrected for the 6-phosphogluconate dehydrogenase contribution by dividing the observed rates by 2.

Fig. 23. pH optimum of acyl phosphate:hexose phosphotransferase. The routine assay was used except that the buffer composition and pH were varied as indicated, with the enzyme concentration constant at 0.03 unit per cuvette. The pH measurements were made on duplicate reaction mixtures. the pH did not vary with time during the 5-minute assay period.

Figure 23.


pH - ACTIVITY PROFILE
RELATIVE ACTIVITY
100
80
60
40
20
0
7
8
9
10
pH
GLYCINE
GLYCYLGLYCINE

the presence of various phosphoryl donors are given in Table VIII. The Km and Vmax values were determined for four of the more active phosphoryl donors. The Km values for acetyl phosphate, carbamyl phosphate, and D-mannose 6-phosphate were essentially equal, at 4 x 10^{-4}M, whereas the Km value for D-ribose 5-phosphate was 5-fold larger, at 2 x 10^{-3}M (Fig. 24). The Vmax values were equal for acetyl phosphate and carbamyl phosphate, whereas the values for D-mannose 6-phosphate and D-ribose 5-phosphate were about 75% and 31% (Fig. 24), respectively, of the Vmax value for acetyl phosphate and carbamyl phosphate.

Phosphoryl Acceptor Specificity- Using specific enzyme-coupled assays with acetyl phosphate as the phosphoryl donor, the phosphotransferase was demonstrated to phosphorylate D-glucose, D-mannose, and D-fructose at carbon atom 6, and D-mannitol at carbon atom 1. The apparent Km values were determined to be 1.6 x 10^{-4}M for D-glucose, 1.2 x 10^{-2}M for D-mannose, about 0.3 M for D-fructose, and 6.7 x 10^{-2}M for D-mannitol (Figs. 25 and 26). More recent experiments described below indicated that in the case of D-fructose, D-fructose 1-phosphate rather than D-fructose 6-phosphate was the predominant product. The Vmax values for the phosphorylation of D-glucose, D-mannose, D-fructose and D-mannitol with acetyl phosphate, within the limitations of

TABLE VIII

Phosphoryl donor specificity of phosphotransferase

The standard assay (with D-glucose as the phosphoryl acceptor) was used except that the phosphoryl donor (1 μmole) was varied. Phosphotransferase (DEAE-cellulose fraction) was 0.156 unit (1.8 μg of protein.)

General Type	Specific Example	Relative phosphorylation rate
Acyl phosphate	Acetyl phosphate	100
	Carbamyl phosphate	100
Enol phosphate	Phosphoenolpyruvate	0
Hydroxyalkyl phosphate	D-Mannose 6-phosphate	71
	D-Fructose 6-phosphate	25
	D-Ribose 5-phosphate	18
	D-Fructose 1-phosphate	15
	α-D-Glucose 1-phosphate	5
	D-Gluconate 6-phosphate	2.5
	D-Sorbitol 6-phosphate	2.5
	D-Mannitol 1-phosphate	0
	α-D-Mannose 1-phosphate	0
	α-D-Galactose 1-phosphate	0
	α-Glycerol phosphate	0
	D-Glycerate 3-phosphate	0
Alkyl triphosphate	ATP	0
Alkyl pyrophosphate	ADP	0
Phosphoramidate	Creatine phosphate	0
Inorganic pyrophosphate		0
Inorganic orthophosphate		0

Fig. 24. Phosphoryl donor kinetic constants for phosphotransferase. The routine assay was used except that the phosphoryl donor was varied as indicated, with the acyl phosphate: hexose phosphotransferase (DFAF-cellulose fraction) concentration constant at 0.092 unit.

Figure 24.

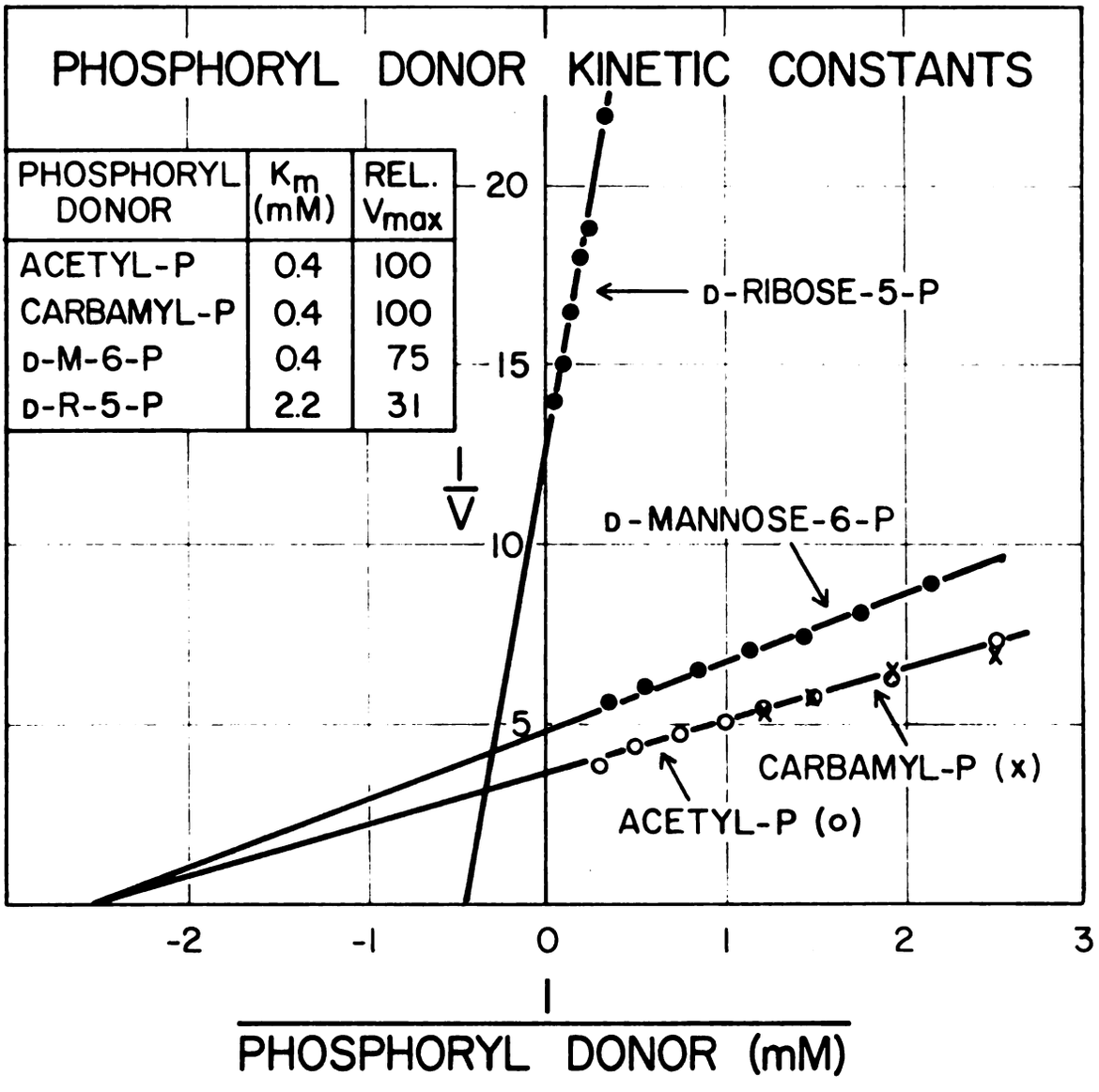

PHOSPHORYL DONOR KINETIC CONSTANTS
PHOSPHORYL DONOR
Km (mM)
REL. Vmax
ACETYL-P 0.4 100
CARBAMYL-P 0.4 100
D-M-6-P 0.4 75
D-R-5-P 2.2 31
D-RIBOSE-5-P
D-MANNOSE-6-P
CARBAMYL-P (x)
ACETYL-P (o)
1/V
20
15
10
5
-2
-1
0
1
2
3
1
PHOSPHORYL DONOR (mM)

Fig. 25. Lineweaver-Burk plot relating phosphotransferase reaction velocity to substrate concentration. The routine assays were used except that the substrate was varied as indicated with the phosphotransferase (DEAE-cellulose fraction) concentration constant at 0.108 unit per cuvette.

Figure 25.

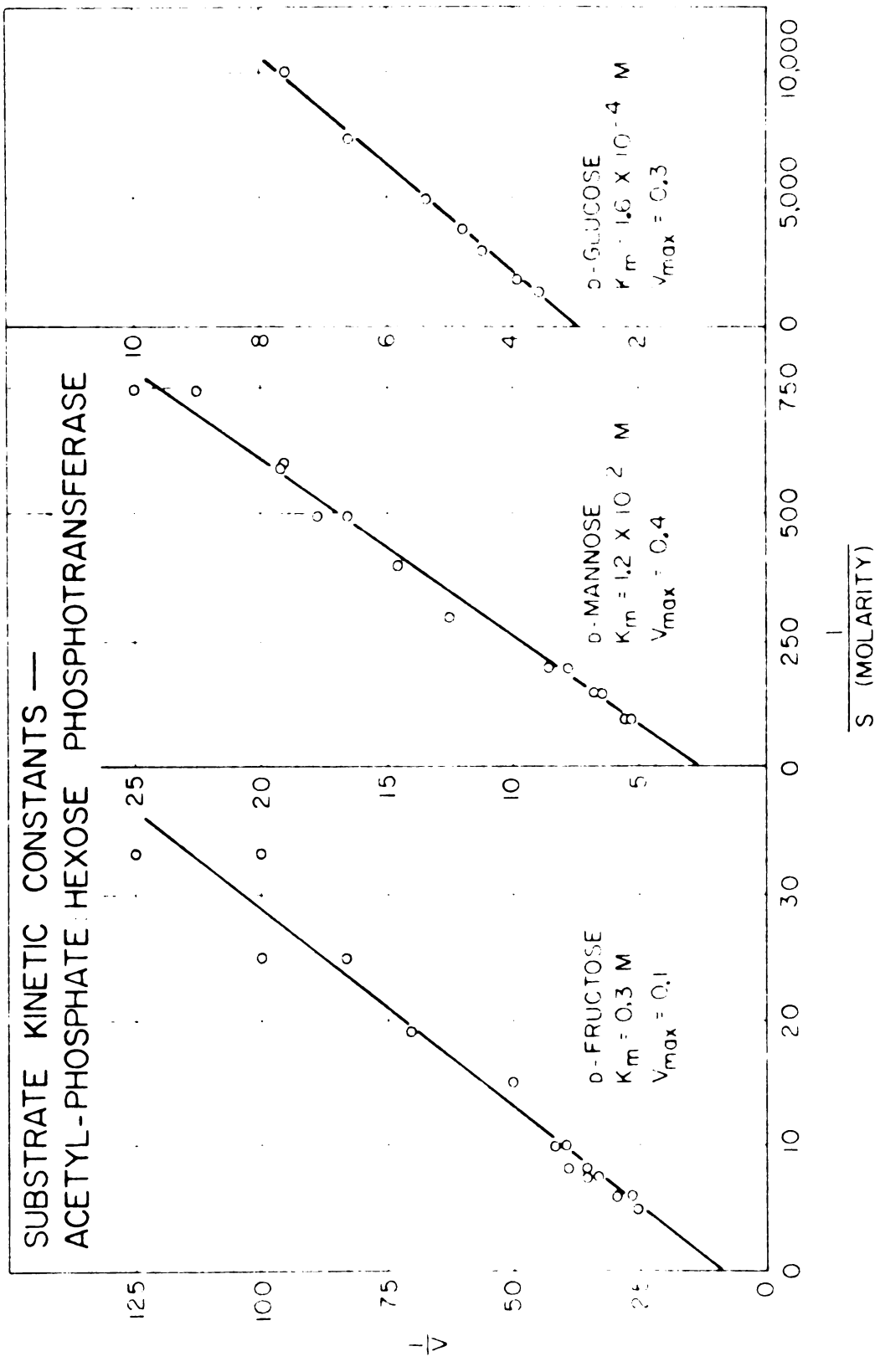
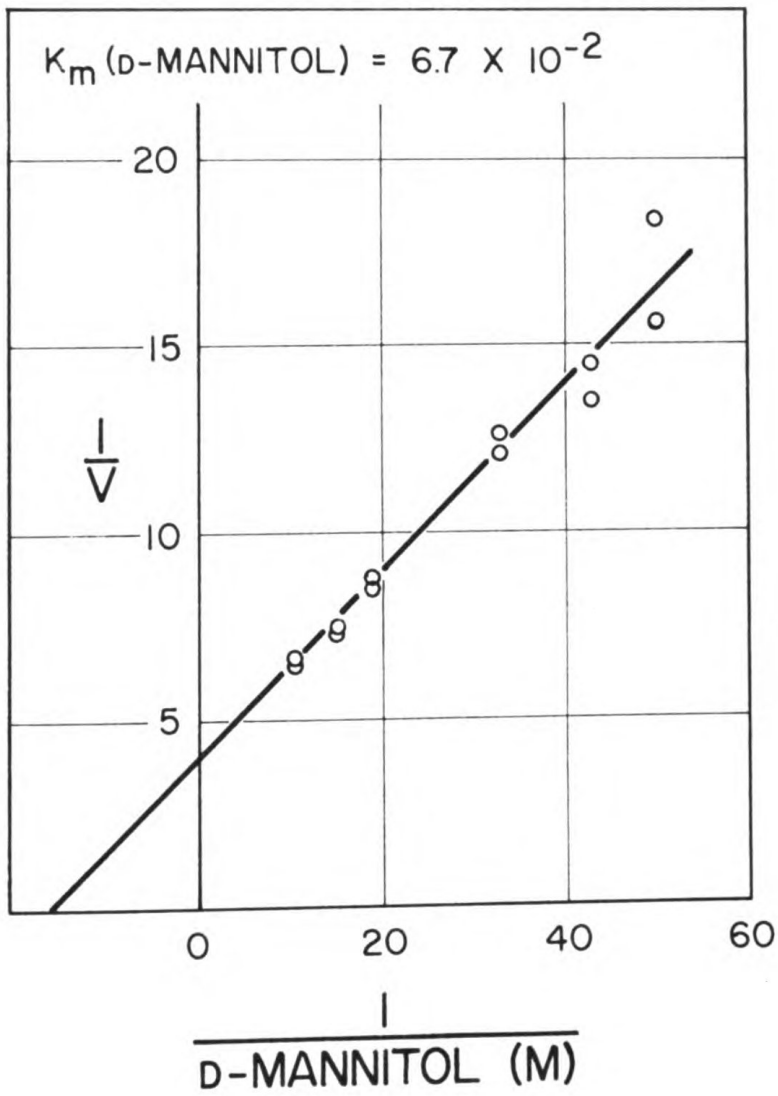

SUBSTRATE KINETIC CONSTANTS —
ACETYL-PHOSPHATE HEXOSE PHOSPHOTRANSFERASE
D-FRUCTOSE
K_m = 0.3 M
V_{max} = 0.1
D-MANNOSE
K_m = 1.2 X 10^{-2} M
V_{max} = 0.4
D-GLUCOSE
K_m = 1.6 X 10^{-4} M
V_{max} = 0.3
$\frac{1}{V}$
$\frac{1}{S}$ (MOLARITY)

Fig. 26. Lineweaver-Burk plot relating phosphotransferase reaction velocity to D-mannitol concentration. The routine assay employing the mannitol 1-phosphate coupling enzymes were used, with the phosphotransferase (DEAE-cellulose fraction) constant at 0.108 unit per cuvette.

Figure 26.


K_m (D-MANNITOL) = 6.7 X 10^{-2}
20
15
10
5
$\frac{1}{V}$
0
20
40
60
$\frac{1}{\text{D-MANNITOL (M)}}$

determinations by kinetic plots, were about equal (Figs. 25 and 26).

Identification of the Products of Phosphorylation of D-Glucose, D-Mannose, D-Fructose, and D-Mannitol with Acetyl Phosphate- The product of the phosphorylation of D-glucose with acetyl phosphate was prepared on a micro scale by incubating in a cuvette: 0.32 unit of acyl phosphate:hexose phosphotransferase (DEAE-cellulose fraction), 15.0 mμmoles of D-glucose, 1.0 μmole of acetyl phosphate, 0.2 μmole of NADP, and 10 μmoles of glycylglycine buffer (pH 7.5), in a volume of 0.15 ml. After incubation at 25° for 37 minutes, excess glucose 6-phosphate dehydrogenase was added. This resulted in an increase in absorbance at 340 mμ equivalent to the oxidation of 14.9 mμmoles of D-glucose 6-phosphate. The further addition of excess phosphoglucomutase did not result in a change in absorbance after correcting for dilution, indicating an absence (<0.2 mμmole) of D-glucose 1-phosphate. Other experiments indicated that the glucose 6-phosphate dehydrogenase and acyl phosphate:hexose phosphotransferase were devoid of phosphoglucomutase activity. Thus, the product of phosphorylation of D-glucose with acetyl phosphate was identified as D-glucose 6-phosphate and not D-glucose 1-phosphate.

The products of the phosphorylation of D-glucose,

D-mannose, D-fructose, and D-mannitol with acetyl phosphate were prepared on a larger scale as follows. The reaction mixtures contained in a volume of 10.0 ml: 1 mmole of glycylglycine buffer (pH 8.5), 600 μmoles of acetyl phosphate, 11.4 units of phosphotransferase (0.39 mg protein of a DEAE cellulose fraction), and substrate (100 μmoles of D-glucose, 1 mmole of D-mannose, 2 mmoles of D-fructose, or 1 mmole of D-mannitol). After 2 hours of incubation at 25° the amounts of product were determined enzymically to be as follows: 26.8 μmoles of D-glucose 6-phosphate, 13.0 μmoles of D-mannose 6-phosphate, 9.6 μmoles of D-fructose 6-phosphate, and 9.0 μmoles of D-mannitol 1-phosphate. The reactions were stopped at 2 hours by the addition of 1.2 mmoles of barium acetate to each. After chilling on ice for 30 minutes, the three samples were treated separately as follows: the pH was adjusted to 8-8.2 with sodium hydroxide, and 4 volumes of 95% alcohol were added. The solution was kept in the refrigerator overnight, and the precipitate that formed was collected by centrifugation and washed with 80%, 90%, 95% and finally absolute ethyl alcohol. The residue (the barium salt of the sugar phosphate) was dried under a vacuum. The weight yields of the barium salts of the products were as follows: D-glucose, 315 mg; D-mannose,

300 mg; D-fructose, 480 mg; and D-mannitol, 540 mg. For enzymic assay and chromatography, the barium salt was treated with Dowex-50 (H^+) to remove the barium. The complete removal of barium ions from the solution was checked by the addition of ammonium sulfate. The recoveries of the products, as determined by enzymic assays, were 18.3 μmoles of D-glucose 6-phosphate, 9.1 μmoles of D-mannose 6-phosphate, 2.1 μmoles of D-fructose 6-phosphate, and 3.4 μmoles of D-mannitol 1-phosphate.

Paper chromatography of the products of phosphorylation of D-glucose, D-mannose, and D-fructose are shown in Fig. 27. The phosphorylation products of D-glucose and D-mannose each showed one spot on the chromatogram, corresponding to authentic samples of D-glucose 6-phosphate and D-mannose 6-phosphate, respectively. The product of D-fructose phosphorylation showed two spots, a minor spot corresponding to authentic D-fructose 6-phosphate and a major spot which did not correspond to any of the standards. However, in another chromatogram (Fig. 28) the major spot corresponded to authentic D-fructose 1-phosphate. When the enzymically prepared fructose phosphate ester was dephosphorylated with alkaline phosphatase and chromatographed (after deionization) on Whatman No. 1 paper with 80% phenol as a solvent (as described in Part II), only one

Fig. 27. Chromatography of the products of phosphorylation of D-glucose, D-mannose, and D-fructose with acetyl phosphate. The details are given in the text. The standards used in this chromatogram were commercial preparations.

Figure 27.

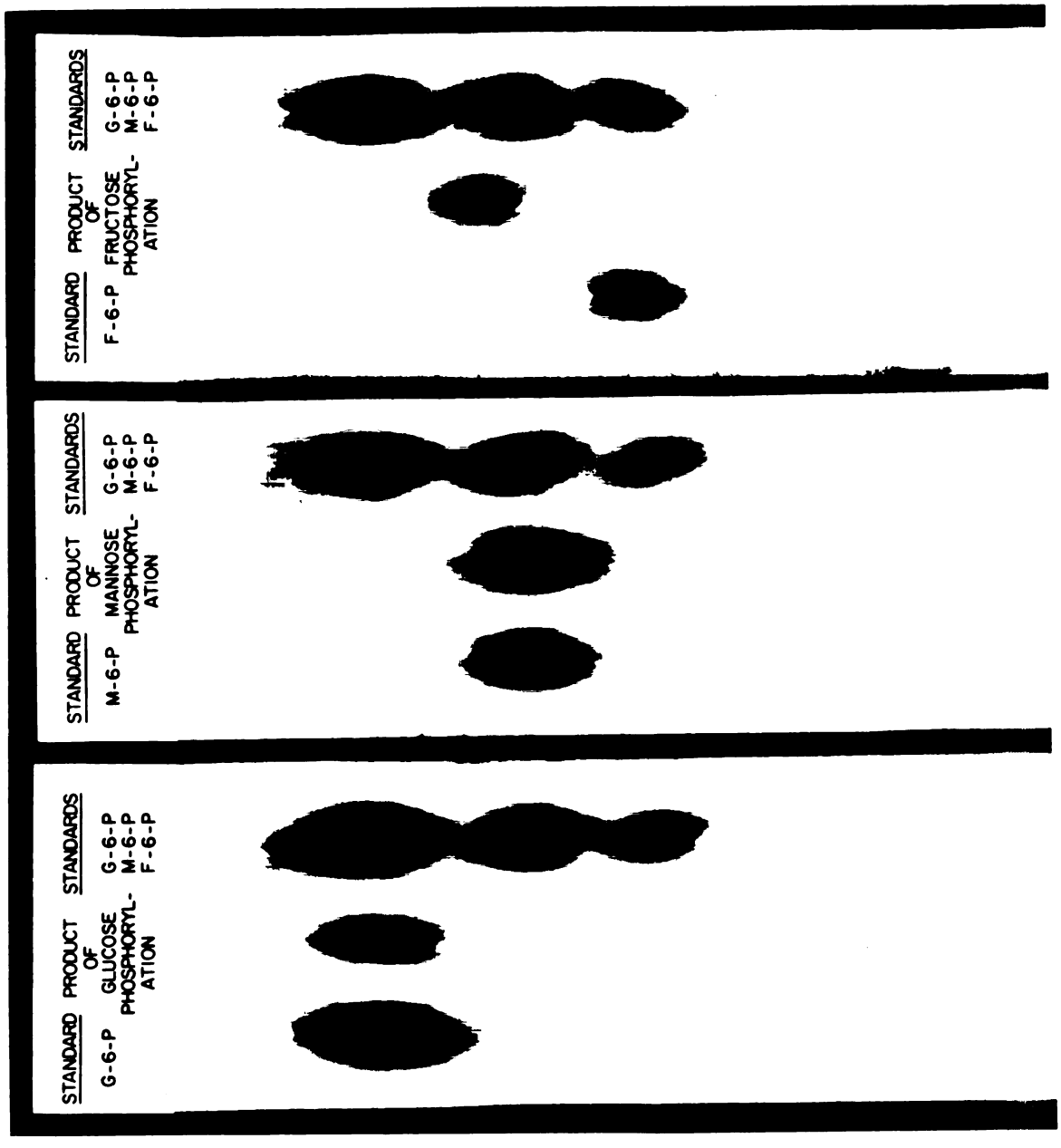
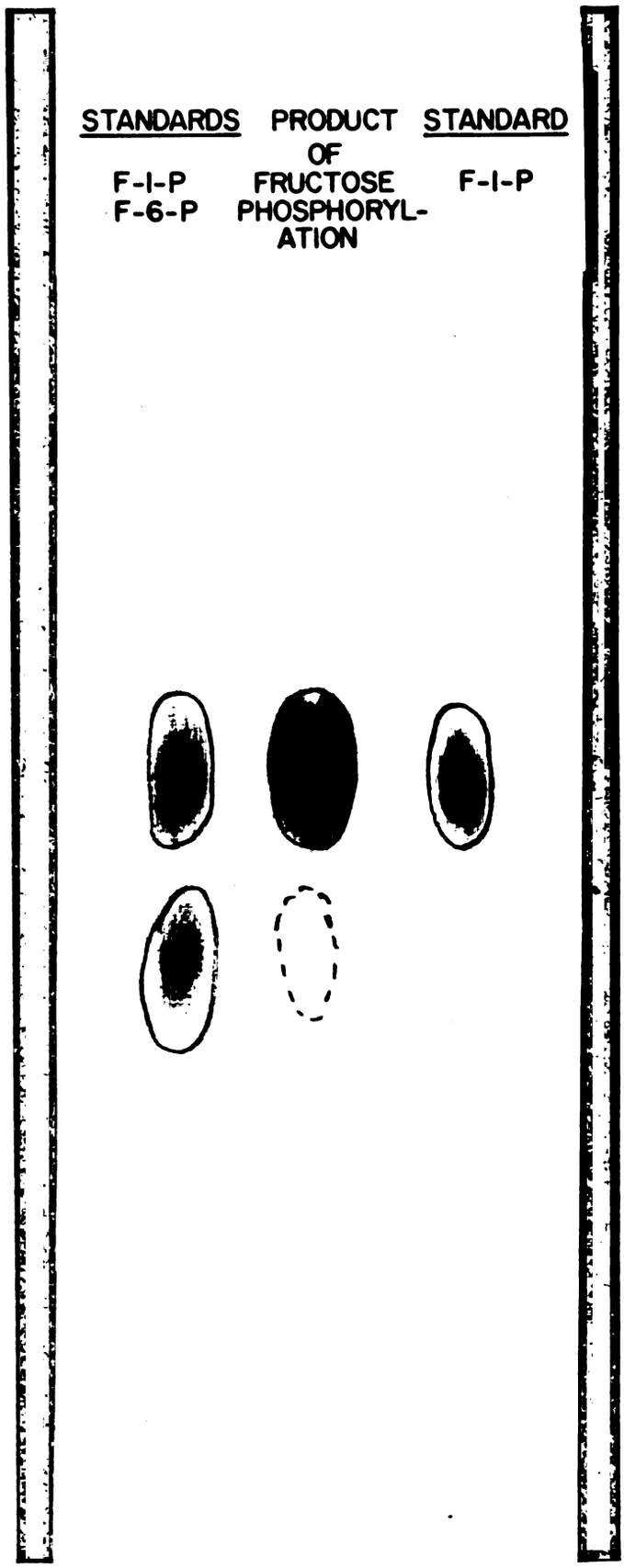

STANDARD
G-6-P
PRODUCT OF GLUCOSE PHOSPHORYL-ATION
STANDARDS
G-6-P
M-6-P
F-6-P
STANDARD
M-6-P
PRODUCT OF MANNOSE PHOSPHORYL-ATION
STANDARDS
G-6-P
M-6-P
F-6-P
STANDARD
F-6-P
PRODUCT OF FRUCTOSE PHOSPHORYL-ATION
STANDARDS
G-6-P
M-6-P
F-6-P

Fig. 28. Chromatography of the product of phosphorylation of D-fructose with acetyl phosphate. The details are given in the text.

Figure 28.


STANDARDS
F-1-P
F-6-P
PRODUCT OF FRUCTOSE PHOSPHORYL-ATION
STANDARD
F-1-P

spot was observed, which corresponded to D-fructose. Analysis of the product of D-fructose phosphorylation by the use of a specific D-fructose 1-phosphate kinase coupled with rabbit muscle aldolase indicated that it contained at least twice as much D-fructose 1-phosphate as D-fructose 6-phosphate.* Chromatography of the product of D-mannitol phosphorylation (not shown) produced only one spot, which corresponded to authentic D-mannitol 1-phosphate.

Evidence for the Common Identity of Acyl Phosphate: Hexose Phosphotransferase and Hexose phosphate:Hexose Phosphotransferase- Data reported above indicated that both acyl phosphates and hydroxyalkylphosphates (especially D-mannose 6-phosphate and D-glucose 6-phosphate) could function as phosphoryl donors for hexose phosphorylation. The relative rates of phosphorylation of D-glucose with acetyl phosphate and D-mannose 6-phosphate remained constant during various stages of purification of the enzyme, even when purified to apparent homogeneity, suggesting that the same enzyme phosphorylated D-glucose with either phosphoryl donor. Further support for the common identity of the enzymes responsible for phosphorylating

*T.F. Hanson and R.L. Anderson, personal communication.

D-glucose with acyl phosphates and hydroxyalkylphosphates is provided by the following three experiments:

a] D-mannose inhibition- D-Mannose inhibited the phosphorylation of D-glucose competitively when either acetyl phosphate or D-mannose 6-phosphate was used as the phosphoryl donor (Fig. 29). The K_i for D-mannose was the same, at 1.2×10^{-2}M, with either phosphoryl donor (Fig. 30). This K_i value was the same as the K_m for D-mannose when D-mannose was the phosphoryl acceptor (Fig. 25).

b] Comparison of acetyl phosphate:D-glucose 6-phosphotransferase and D-mannose 6-phosphate:D-glucose 6-phosphotransferase activities with respect to K_m for D-glucose- The K_m for D-glucose was determined to be 1.6×10^{-4} with either acetyl phosphate or D-mannose 6-phosphate as the phosphoryl donor (Fig. 31).

c] Competitive phosphorylation of D-glucose with acetyl phosphate and D-mannose 6-phosphate- The results in Fig. 32 show that the rate of phosphorylation of D-glucose with a mixture of acetyl phosphate and D-mannose 6-phosphate was intermediate between the rates obtained with either one alone, indicating that phosphorylation of D-glucose with these two phosphoryl donors was competitive rather than additive.

Phosphatase Activity of Phosphotransferase and Reversi-

Fig. 29. Lineweaver-Burk plot showing the relationship of D-glucose concentration to phosphotransferase reaction velocity in the presence of various concentrations of D-mannose. The routine assay was used with acetyl phosphate (1 µmole) as the phosphoryl donor in the left graph and D-mannose 6-phosphate (1 µmole) as the phosphoryl donor in the right graph. The phosphotransferase (DEAE-cellulose fraction) concentration was constant at 0.05 unit per cuvette.

Figure 29.

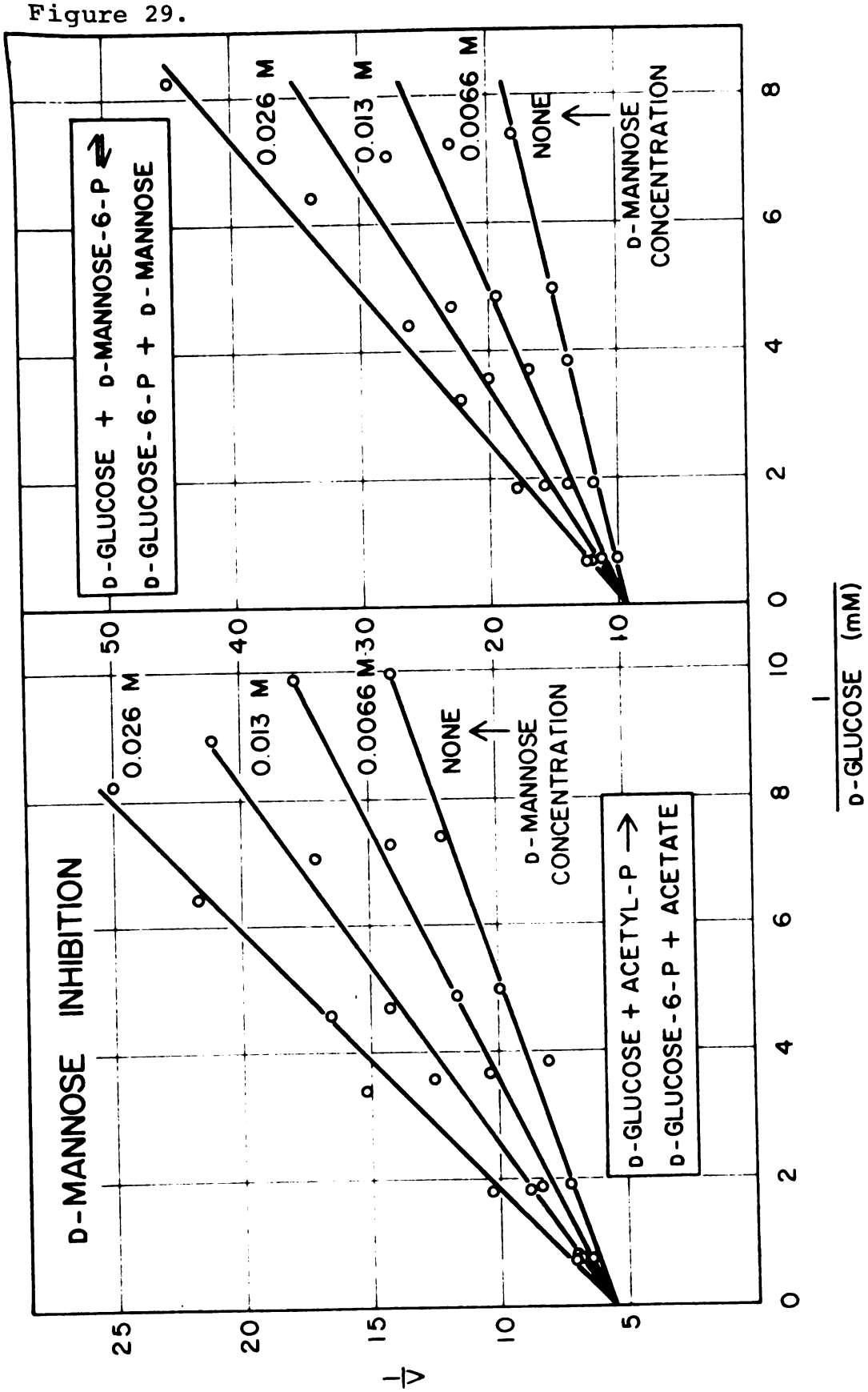

D-MANNOSE INHIBITION
D-GLUCOSE + ACETYL-P →
D-GLUCOSE-6-P + ACETATE
0.026 M
0.013 M
0.0066 M
NONE
D-MANNOSE CONCENTRATION
D-GLUCOSE + D-MANNOSE-6-P ⇌
D-GLUCOSE-6-P + D-MANNOSE
1/V
1/D-GLUCOSE (mM)

Fig. 30. Kinetic plot for obtaining the K_i for D-mannose using either D-mannose 6-phosphate or acetyl phosphate as the phosphoryl donor. The data are taken from the experiment described in Fig. 29.

Figure 30.

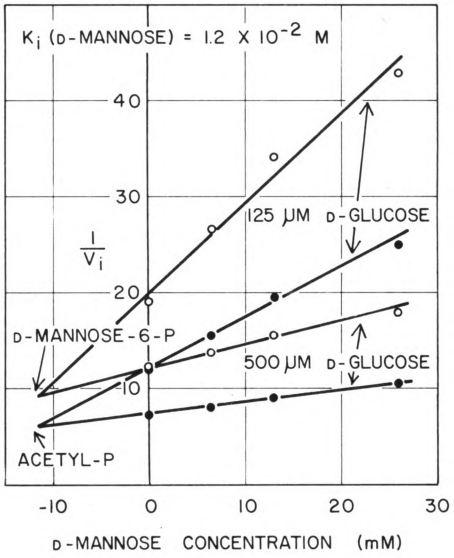

K_i (D-MANNOSE) = 1.2 X 10^{-2} M
40
30
20
10
$\frac{1}{V_i}$
125 μM D-GLUCOSE
500 μM D-GLUCOSE
D-MANNOSE-6-P
ACETYL-P
-10
0
10
20
30
D-MANNOSE CONCENTRATION (mM)

Fig. 31. Lineweaver-Burk plot showing the relationship of D-glucose concentration to reaction velocity at constant concentrations of acetyl phosphate and D-mannose 6-phosphate. The data are taken from the experiment described in Fig. 29.

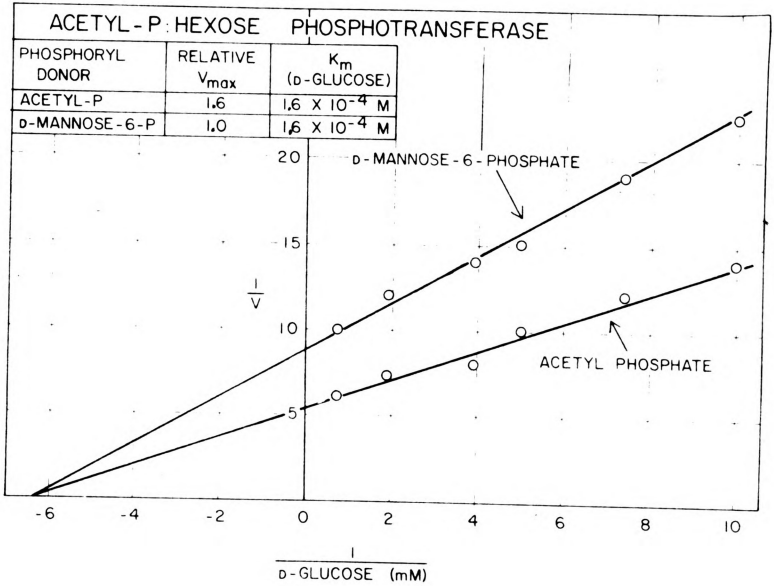
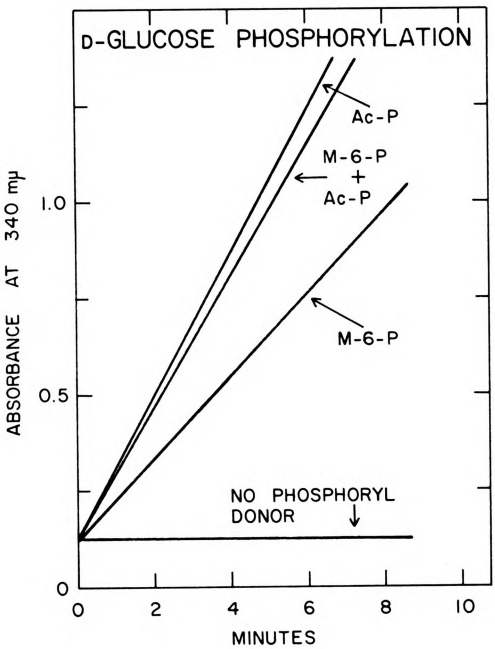

ACETYL - P : HEXOSE PHOSPHOTRANSFERASE
PHOSPHORYL DONOR
RELATIVE V_{max}
K_m (D-GLUCOSE)
ACETYL-P
1.6
1.6×10^{-4} M
D-MANNOSE-6-P
1.0
1.6×10^{-4} M
D-MANNOSE-6-PHOSPHATE
ACETYL PHOSPHATE
$\frac{1}{V}$
$\frac{1}{\text{D-GLUCOSE (mM)}}$
20
15
10
5
-6
-4
-2
0
2
4
6
8
10

Figure 31.

Fig. 32. Competitive phosphorylation of D-glucose with D-mannose 6-phosphate and acetyl phosphate. The routine phosphotransferase assay was used except that the phosphoryl donor (1.0 μmole each) was varied as indicated. M-6-P, D-mannose 6-phosphate; ac-P, acetyl phosphate.

Figure 32.

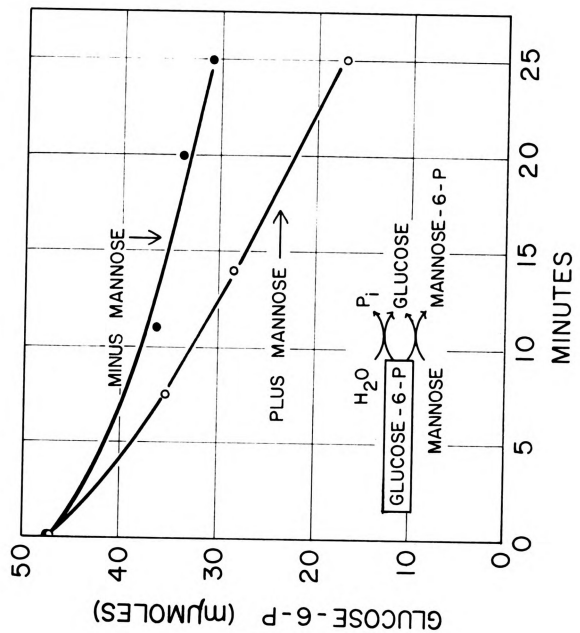

D-GLUCOSE PHOSPHORYLATION
Ac-P
M-6-P
+
Ac-P
M-6-P
NO PHOSPHORYL
DONOR
ABSORBANCE AT 340 mμ
1.0
0.5
0
0
2
4
6
8
10
MINUTES

bility of the D-Mannose 6-Phosphate:D-Glucose 6-Phosphotransferase Reaction- Hexose phosphate:hexose phosphotransferase activity was routinely determined by measuring D-glucose 6-phosphate formation in a glucose 6-phosphate dehydrogenase-linked assay. However, it is believed that the reaction is of physiological significance in the reverse direction, with D-glucose 6-phosphate, formed by action of the stereospecific D-glucokinase, serving as a phosphoryl donor for other substrates such as D-mannose and D-fructose. The rate of phosphotransferase-catalyzed disappearance of D-glucose 6-phosphate in the presence and absence of D-mannose is shown in Fig. 33. The D-glucose 6-phosphate that disappeared was accounted for as D-glucose, as determined with D-glucokinase-glucose 6-phosphate dehydrogenase. The rate of D-glucose 6-phosphate disappearance in the absence of D-mannose was a measure of the hydrolase activity of phosphotransferase.* The increased rate in the presence of D-mannose, minus the rate in the absence of D-mannose, was a minimal value for phosphotransferase activity because of an inhibition of hydrolysis by D-mannose, as will be described in the

* In preliminary experiments, a phosphotransferase-catalyzed hydrolysis of acetyl phosphate (50) which was inhibited by D-glucose was also demonstrated.

Fig. 33. Phosphotransferase-catalyzed disappearance of D-glucose 6-phosphate in the presence and absence of D-mannose. The reaction mixture contained in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 1 μmole of $MgCl_2$, 0.2 μmole of NADP, 5 μmoles of D-mannose, 0.048 μmole of D-glucose 6-phosphate and 0.076 unit of purified phosphotransferase. Excess glucose 6-phosphate dehydrogenase was added to the incubation mixture at the indicated time intervals to determine remaining D-glucose 6-phosphate. Excess purified D-glucokinase and ATP were then added to determine free D-glucose.

Figure 33.


GLUCOSE-6-P (μMOLES)
50
40
30
20
10
0
0
5
10
15
20
25
MINUTES
MINUS MANNOSE
PLUS MANNOSE
H_2O
P_i
GLUCOSE-6-P
GLUCOSE
MANNOSE
MANNOSE-6-P

following paragraph. To show that D-mannose 6-phosphate was a product in the phosphotransferase reaction, ^{14}C-D-mannose was used. Fig. 34 provides chromatographic evidence that ^{14}C-D-mannose 6-phosphate was formed from D-glucose 6-phosphate and ^{14}C-D-mannose.

The stoichiometry of the D-mannose 6-phosphate:D-glucose 6-phosphotransferase reaction, and the relative D-glucose 6-phosphatase activity, is shown in Table IX. Phosphatase activity in the absence of D-mannose (Experiment 2) was measured by D-glucose 6-phosphate disappearance and D-glucose appearance, which were equivalent. In the presence of D-mannose (Experiment 1), D-glucose 6-phosphate disappearance and D-glucose appearance increased about two-fold. Independent measurements of phosphatase and phosphotransferase activity indicated that phosphatase activity (Pi formation) was inhibited about 50% in the presence of D-mannose, while the remainder of the D-glucose 6-phosphate that disappeared was accounted for as D-mannose 6-phosphate formed from phosphotransferase activity. Thus, with D-glucose 6-phosphate as the phosphoryl donor and D-mannose as the phosphoryl acceptor, phosphotransferase activity was about three times as great as D-glucose 6-phosphatase activity.

Effect of Growth Substrate on Phosphotransferase Level- The specific activity of acetyl phosphate:D-glucose

Fig. 34. Formation of ^{14}C-D-mannose 6-phosphate from D-glucose 6-phosphate and ^{14}C-D-mannose. The reaction mixture contained in a volume of 5.0 ml: 450 µmoles of glycylglycine buffer (pH 7.5), 500 µmoles (5 µcuries) of D-mannose-U-^{14}C, 4.2 units of phosphotransferase (DEAE-cellulose fraction) and 90 µmoles of D-glucose 6-phosphate. After incubation for two hours at 25°, the reaction was stopped, and the sugar phosphate esters were isolated and chromatographed as described in the text in the section on product identification. The radioactivity scan was made as described in Fig. 15, except that the scan speed was 0.2 inch per minute and the time constant was 40 seconds.

Figure 34.

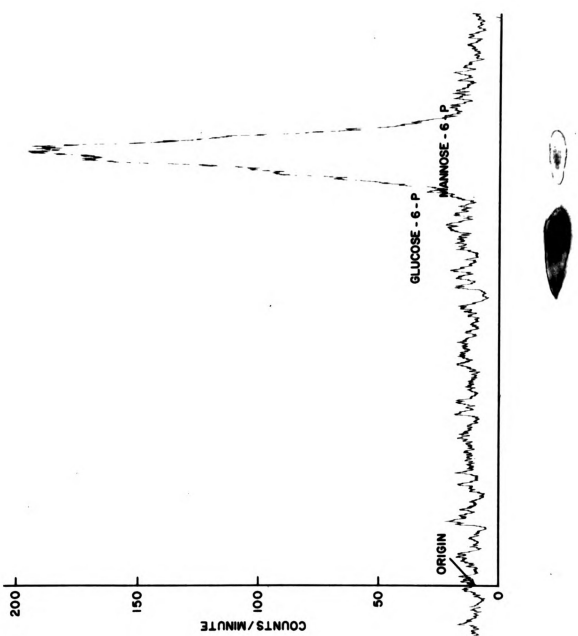


TABLE IX

Stoichiometry of the D-mannose 6-phosphate: D-glucose 6-phosphotransferase reaction, and D-glucose 6-phosphatase activity of phosphotransferase

The reaction mixture in Experiment 1 contained in a volume of 4.0 ml: 320 µmoles of glycylglycine buffer (ph 7.5), 16 µmoles of D-glucose 6-phosphate, 800 µmoles of D-mannose, and 11.3 units of phosphotransferase (DEAE-cellulose fraction). Samples (0.5 ml) were taken at 0, 15, and 30 minutes, centrifuged, and the supernatant used for analysis. The reaction mixture in Experiment 2 was minus D-mannose. Analysis of a control without enzyme indicated no changes from the 0-time values. The incubation temperature was 25°C.

	Time Minutes	D-Glucose -6-P	D-Glucose	D-Mannose -6-P	Pi	D-Mannose -6-P + Pi
		Δµmoles/ml				
Experiment 1						
D-Glucose-6-P	15	-1.58	1.79	1.18	0.35	1.53
+						
D-mannose	30	-2.01	2.06	1.47	0.50	1.97
Experiment 2	15	-0.72	0.69			
D-Glucose-6-P	30	-1.04	1.17			

6-phosphotransferase in extracts of cells grown on D-glucose-free (<0.0001%) nutrient broth (0.5% Difco peptone, 0.3% Difco beef extract, pH 7.0) or on the mineral medium with glycerol in place of D-glucose was the same as in extracts of cells grown on the D-glucose-mineral medium. Therefore, it may be concluded that the enzyme is constitutive.

Stability of Phosphotransferase- The most highly purified fractions of phosphotransferase have been stored at -20° for seven months with repeated thawing and freezing with no detectable loss of activity.

DISCUSSION

The reported data indicate that a single enzyme catalyzes all of the following reactions:

(i) acyl phosphate + hexose → organic acid + hexose phosphate

(ii) Hexose phosphate A + hexose B ⇌ hexose phosphate B + hexose A

(iii) hexose phosphate + H_2O → hexose + Pi

(iv) acyl phosphate + H_2O → organic acid + Pi

For the phosphotransferase reactions, the phosphoryl donor may be either an acyl phosphate (acetyl phosphate or carbamyl phosphate) or certain hydroxyalkylphosphates (e.g., D-mannose 6-phosphate or D-glucose 6-phosphate). Other hydroxyalkylphosphates (e.g., α-D-glucose 1-phosphate, D-sorbitol 6-phosphate, or α-glycerol phosphate), however, show little or no activity as phosphoryl donors. Other compounds (e.g., ATP, creatine phosphate, phosphoenolpyruvate and inorganic pyrophosphate) which have high phosphoryl transfer potential with certain enzymes also have no activity with this phosphotransferase. Although D-mannitol has been shown to serve as a phosphoryl acceptor, hexoses such as D-glucose and D-mannose have higher affinities (lower K_m values). Because of this fact, and because higher rates and higher affinities were observed with acyl phosphates and certain hexose phosphates than with other phosphoryl donors, the enzyme has

been named acyl phosphate (hexose phosphate):hexose phosphotransferase.

The reversibility of reaction (ii) was demonstrated with D-mannose and D-glucose and their respective 6-phosphates. The irreversibility of reaction (iii) was demonstrated by the inability of Pi to serve as a phosphoryl donor in the synthesis of D-glucose 6-phosphate. The irreversibility of reactions (i) and (iv) was not determined experimentally, but would be predicted from the reported values for the free energies of hydrolysis of the phosphoryl compounds (51).

The phosphotransferase-catalyzed hydrolysis of D-glucose 6-phosphate in the absence of an organic phosphoryl acceptor (D-mannose) was shown to be about half of the rate of D-glucose 6-phosphate disappearance in the presence of D-mannose. Since the hydrolysis of D-glucose 6-phosphate was inhibited about 50% in the presence of D-mannose, the hydrolase activity amounted to only about one-third of the phosphotransferase activity. Thus, the phosphotransferase described here differs from "phosphatases" in three important respects: (i) the hexose phosphotransferase activities of phosphatases are generally less than the hydrolase activities (31, 52, 53); (ii) the K_m values of phosphatases for organic phosphoryl acceptors are usually

large, often approaching 1 M (31, 54-58), whereas the K_m of this phosphotransferase for D-glucose is low (4×10^{-4} M); and (iii) the substrate specificities of phosphatases which can effect phosphotransferase reactions are usually broad (55, 57, 59, 60), whereas with this phosphotransferase it is relatively narrow. A possible exception to the last statement may be D-glucose 6-phosphatase, which possesses pyrophosphate:hexose phosphotransferase and hexose phosphate: hexose phosphotransferase activities (31).

As has already been discussed in Part II, a physiological role for the acyl phosphate (hexose phosphate):hexose phosphotransferase described here is readily apparent. A. aerogenes PRL-R3 can metabolize D-mannose constitutively yet apparently possesses no D-mannokinase (ATP:D-mannose 6-phosphotransferase). The described phosphotransferase, therefore, could substitute for D-mannokinase by phosphorylating D-mannose with D-glucose 6-phosphate, acetyl phosphate, or carbamyl phosphate.

A. aerogenes PRL-R3, like E. coli (27), also seems to lack D-fructokinase. Whether the phosphotransferase described here can phosphorylate D-fructose to D-fructose 6-phosphate at a suitable rate to be metabolically significant is doubtful, because of the apparent high K_m for D-fructose (about 0.3 M). However, more recent experiments revealed that D-fructose

1-phosphate rather than D-fructose 6-phosphate was the predominant product. It is possible that the K_m for D-fructose is lower than 0.3 M when it binds in a position suitable for phosphorylation at carbon atom 1.

Liss, Horwitz and Kaplan (61) have provided evidence that D-manitol is metabolized in A. aerogenes through D-mannitol 1-phosphate. However, a kinase for D-mannitol could not be demonstrated (61). Consequently, the phosphotransferase described here becomes a candidate for initiating the metabolism for D-mannitol in this organism.

Several other enzymes which phosphorylate hexoses with phosphoryl donors other than ATP have recently been described. These other phosphoryl donors include phosphoenolpyruvate (29), D-glucose 1-phosphate (30), phosphoramidate (62), inorganic pyrophosphate (31), and various nucleoside di- and triphosphates (31). It seems likely that in time still other physiologically significant phosphoryl donors (e.g., 1,3-diphosphoglycerate) for hexoses will be discovered.

SUMMARY OF PART III

A new enzyme, acyl phosphate (hexose phosphate):hexose phosphotransferase, was purified several hundred fold from extracts of A. aerogenes PRL-R3. It was characterized with respect to pH optimum, phosphoryl donor specificity and

kinetic constants, phosphoryl acceptor specificity and kinetic constants, inhibition constants, stability, and reversibility of the catalyzed reactions. The reaction products were prepared and identified. The significance of this constitutive enzyme in the metabolism of hexoses and D-mannitol was discussed.

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