PROPERTIES AND FUNCTIONS OF THREE BACTERIAL KINASES
PART I: A HEXOKINASE SPECIFIC FOR D-MANNOSE AND D-FRUCTOSE FROM LEUCONOSTOC MESENTEROIDES
PART II: 1-PHOSPHOFRUCTOKINASE AND 6-PHOSPHOFRUCTOKINASE FROM AEROBACTER AEROGENES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
VIRGINIA L. SAPICO
1969

This is to certify that the

thesis entitled

Properties and Functions of Three Bacterial Kinases
Part I: A Hexokinase Specific for D-Mannose and D-Fructose from Leuconostoc mesenteroides
Part II: 1-Phosphofructokinase and 6-Phosphofructokinase from Aerobacter aerogenes

presented by

Virginia L. Sapico

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

R. L. Anderson
Major professor

Date June 26, 1969

O-169

ABSTRACT

PROPERTIES AND FUNCTIONS OF THREE BACTERIAL KINASES

PART I: A HEXOKINASE SPECIFIC FOR D-MANNOSE AND D-FRUCTOSE FROM LEUCONOSTOC MESENTEROIDES

PART II: 1-PHOSPHOFRUCTOKINASE AND 6-PHOSPHOFRUCTOKINASE FROM AEROBACTER AEROGENES

By

Virginia L. Sapico

Part I describes a hexokinase (adenosine 5'-triphosphate:hexose 6-phosphotransferase) specific for D-mannose and D-fructose. The enzyme was purified to apparent homogeneity from extracts of Leuconostoc mesenteroides. D-Mannose and D-fructose were phosphorylated at equal rates, whereas D-glucose and 29 other sugars and sugar derivatives tested were not phosphorylated and did not inhibit the enzyme. The apparent K_m value for either hexose or adenosine 5'-triphosphate (ATP) varied with pH, but was independent of the concentration of the other. D-Mannose was a competitive inhibitor of D-fructose. Product inhibition occurred with adenosine 5'-diphosphate (ADP) (competitive with ATP) but not with D-fructose 6-phosphate. The pH-activity curves were different for the two hexoses, with the D-mannokinase to D-fructokinase ratios being about 1.0 at pH 6.9, 0.5 at pH 8.5, and 0.3 at pH 8.9. The enzyme

had a molecular weight estimated at 47,000. The products of the phosphorylation of D-mannose and D-fructose were identified as D-mannose 6-phosphate and D-fructose 6-phosphate, respectively. The basis for the unusual specificity of the enzyme can be rationalized from an inspection of molecular models of the preferred conformations of α-D-mannopyranose and β-D-fructofuranose. Although one of the models consists of a six-membered ring and the other a five-membered ring, the positions of equivalent atoms on the two models are superimposable. Such topological similarity is not mimicked by D-glucose or any of the other sugars tested as possible substrates.

Part II describes the functions, properties, and control mechanisms of 1-phosphofructokinase and 6-phosphofructokinase from Aerobacter aerogenes. Analysis of mutants lacking 6-phosphofructokinase and fructose 1,6-diphosphatase indicated that D-fructose metabolism in this organism is primarily through D-fructose 1-phosphate rather than D-fructose 6-phosphate.

6-Phosphofructokinase was purified six-fold from extracts of A. aerogenes PRL-R3. 1-Phosphofructokinase was purified 315-fold from extracts of a 6-phosphofructokinaseless mutant. Comparative studies on the two enzymes indicated that they are governed by different control mechanisms. 6-Phosphofructokinase exhibited a sigmoidal dependence of rate on D-fructose-6-P concentration, whereas 1-phosphofructokinase exhibited hyperbolic

dependence of rate on D-fructose-1-P concentration. ATP inhibited both enzymes under conditions of Mg^{++} to ATP ratios below 2:1. Inhibition of 6-phosphofructokinase by ATP was relieved by Mg^{++}, D-fructose-6-P, ADP, and various other nucleoside diphosphates. In contrast, only Mg^{++} was found to relieve inhibition of 1-phosphofructokinase activity by ATP. Both enzymes showed a sigmoidal dependence of rate on Mg^{++} concentration. Increased levels of D-fructose-6-P shifted the 6-phosphofructokinase curve from sigmoidal to hyperbolic, whereas D-fructose-1-P had no effect on a similar plot for 1-phosphofructokinase. Other nucleoside triphosphates were used as phosphoryl donors by both enzymes, and inhibited activity under conditions of Mg^{++} to nucleotide ratios below 2:1. In contrast with the result with ATP, the inhibition of 6-phosphofructokinase by other nucleoside triphosphates could not be relieved by D-fructose-6-P. Citrate, D-fructose 1,6-diphosphate, and D-fructose-6-P inhibited the 1-phosphofructokinase reaction competitively with D-fructose-1-P, suggesting possible <u>in vivo</u> control of activity. The data indicated that whereas 6-phosphofructokinase exhibits allosteric properties and a regulatory pattern typical of 6-phosphofructokinases from a variety of organisms, 1-phosphofructokinase behaves more like a non-allosteric kinase. The molecular weight of 6-phosphofructokinase was estimated as 100,000, and that of 1-phosphofructokinase as

75,000. The apparent K_m of 1-phosphofructokinase for either substrate did not vary with the concentration of the other. This finding is consistent with a sequential mechanism of substrate binding to the enzyme.

PROPERTIES AND FUNCTIONS OF THREE BACTERIAL KINASES

PART I: A HEXOKINASE SPECIFIC FOR D-MANNOSE AND D-FRUCTOSE FROM *LEUCONOSTOC MESENTEROIDES*

PART II: 1-PHOSPHOFRUCTOKINASE AND 6-PHOSPHOFRUCTOKINASE FROM *AEROBACTER AEROGENES*

By

Virginia L. Sapico

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1969

ACKNOWLEDGMENTS

The author wishes to express her gratitude to Dr. Richard L. Anderson for his patient guidance and encouragement throughout the course of this work. Thanks are also due to T. E. Hanson for providing some of the mutants used in these studies, to R. W. Walter for doing some of the enzyme assays described in Part II, Section A, and to all fellow researchers for their helpful suggestions. The help of Mrs. Shirley Randall in the preparation of this manuscript is also appreciated.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	xi
PART I. A HEXOKINASE SPECIFIC FOR D-MANNOSE AND D-FRUCTOSE FROM *LEUCONOSTOC MESENTEROIDES*	1
Introduction	1
Materials and Methods	3
Source and confirmation of identity of *Leuconostoc mesenteroides*	3
Growth of cells and preparation of extracts	3
Chemicals	4
Mannofructokinase assays	6
Other enzyme assays	8
Protein measurements	9
Disc gel electrophoresis	9
Sucrose density gradient centrifugation	9
Cellulose acetate electrophoresis	10
Results	11
Phosphorylation of hexoses with ATP by *L. mesenteroides* cell extracts	11
Requirements for D-mannose and D-fructose phosphorylation by cell extracts stored for 24 hr at 0-2°C	11
Purification of mannofructokinase	14
$MnCl_2$ fractionation	14
First ammonium sulfate fractionation	14
Heat treatment	18
Second ammonium sulfate fractionation	18
Calcium phosphate gel treatment	18
DEAE-cellulose chromatography	18
Sephadex chromatography	19

Page

Further attempts to separate the two kinase activities 22
Disc gel electrophoresis 22
Cellulose acetate electrohporesis . 23
Sucrose density gradient centrifugation 24
Thermal inactivation at 60°C . . . 24
Properties of mannofructokinase . . . 24
Stability 24
Phosphoryl acceptor specificity . . 24
Phosphoryl donor specificity . . . 28
Effect of pH 28
Determination of K_m for D-mannose and D-fructose 33
Determination of K_m for ATP 33
Inhibition of D-fructokinase activity by D-mannose 42
Product inhibition 42
Estimation of molecular weight . . 42
Product identification 51
Discussion 53
Summary 59

PART II. 1-PHOSPHOFRUCTOKINASE AND 6-PHOSPHOFRUCTOKINASE FROM AEROBACTER AEROGENES . 61

Introduction 61
Section A. Determination of the Relative Significance of the D-Fructose-1-P and D-Fructose-6-P Pathways in A. aerogenes by Analysis of Mutants Lacking 6-Phosphofructokinase and D-Fructose 1,6-Diphosphatase 64

Materials and methods 64
Bacteria 64
Culture media 64
Growth of cells and preparation of extracts 65
Chemicals 65
Enzyme assays 65
Protein determination 67
Results 68
Growth pattern 68
Enzyme activities in cell extracts 68
Discussion 76

Section B. Purification, Properties, and Regulation of 6-Phosphofructokinase and 1-Phosphofructokinase . . . 80

Page

Review of literature 80
Materials and methods 100
Bacteria 100
Growth of cells and preparation of extracts 100
Chemicals 101
Enzyme assays 102
Protein determination 103
Results 104
Purification of 6-Phosphofructokinase 104
Protamine sulfate precipitation 104
Ammonium sulfate fractionation . 104
Sephadex G-200 chromatography . 104
Purification of 1-phosphofructokinase 106
Protamine sulfate precipitation 106
Ammonium sulfate fractionation . 110
Sephadex G-200 chromatography . 110
Calcium phosphate gel adsorption and elution 110
Ammonium sulfate precipitation . 113
pH fractionation 113
Properties and regulation of 6-phosphofructokinase and 1-phosphofructokinase 114
Stability 114
ATP inhibition 114
Interaction of hexose phosphate substrate with ATP and Mg^{++} . 117
Effect of Mg^{++} 122
Effect of other nucleoside triphosphates 129
Effect of nucleoside diphosphates 135
Effect of nucleoside monophosphates 138
Substrate specificity 139
Test for inhibition of 6-PFK by D-fructose-1-P 140
Inhibition of 1-PFK by D-fructose-6-P 140
FDP inhibition 140
Effect of P_i 149
Effect of citrate 149
Effect of pH 149
Molecular weight determination . 158
Discussion 163
Summary of Part II 169

REFERENCES 171

LIST OF TABLES

Table		Page
I.	Phosphorylation of D-glucose, D-mannose, and D-fructose by cell extracts of *L. mesenteroides*	12
II.	Requirements for D-mannose and D-fructose phosphorylation in *L. mesenteroides* cell extracts after 24-hr storage at 0-2°C	13
III.	Purification of mannofructokinase from *L. mesenteroides*	15
IV.	Thermal inactivation of mannofructokinase at 60°C	27
V.	Phosphoryl donor specificity of mannofructokinase	29
VI.	Enzyme activities in crude cell extracts of *A. aerogenes*	71
VII.	Comparison of the properties of *A. aerogenes* 1-PFK and 6-PFK with those of 6-PFKs from other sources . . .	81
VIII.	Purification of 6-PFK from *A. aerogenes* PRL-R3	105
IX.	Purification of 1-PFK from mutant A9-1 .	109
X.	Phosphoryl donor specificity of 6-PFK and 1-PFK	130

LIST OF FIGURES

Figure		Page
1.	Disc gel electrophoretic patterns of fractions obtained from the latter stages of purification of mannofructokinase	17
2.	Elution profile of mannofructokinase on Sephadex G-100 and G-75	21
3.	Sedimentation pattern of mannofructokinase and peroxidase standard in a sucrose density gradient	26
4.	pH optima of mannofructokinase	31
5.	Lineweaver-Burk plots for determining the K_m values of mannofructokinase for D-mannose and D-fructose	35
6.	Lineweaver-Burk plots showing the relationship of mannofructokinase reaction velocity to D-mannose and D-fructose concentrations in the presence of varying ATP concentrations at pH 6.9 and 8.9	37
7.	Lineweaver-Burk plots for determining the K_m values of mannofructokinase for ATP	39
8.	Lineweaver-Burk plots showing the relationship of mannofructokinase reaction velocity to ATP concentration in the presence of varying concentrations of D-mannose and D-fructose	41
9.	Lineweaver-Burk plot showing the relationship of mannofructokinase reaction velocity to D-fructose concentration in the presence and absence of D-mannose	44
10.	Kinetic plot for determining the K_i for D-mannose	46

Figure		Page
11.	Lineweaver-Burk plot showing the relationship of mannofructokinase reaction velocity to ATP concentration in the presence of various concentrations of ADP	48
12.	Kinetic plot for determining the K_i for ADP	50
13.	Structural models showing the topological similarity between α-D-mannopyranose and β-D-fructofuranose	55
14.	Growth characteristics of A. aerogenes PRL-R3 and mutants 012 and A9-1 on D-glucose, D-fructose, and glycerol . .	70
15.	pH-activity profile of FDPase	75
16.	Pathways for the metabolism of D-glucose, D-fructose, and glycerol in A. aerogenes	78
17.	Elution pattern of 6-PFK on a Sephadex G-200 column	108
18.	Elution pattern of 1-PFK on a Sephadex G-200 column	112
19.	Inhibition of 6-PFK by ATP in the presence of various concentrations of Mg^{++} and D-fructose-6-P	116
20.	Inhibition of 1-PFK by ATP in the presence of various concentrations of Mg^{++} and D-fructose-1-P	119
21.	Dependence of initial velocity of 6-PFK and 1-PFK on the hexose phosphate concentration under conditions of varying Mg^{++} to ATP ratios	121
22.	Lineweaver-Burk plot for determining the K_m of 1-PFK for D-fructose-1-P in the presence of various ATP concentrations	124
23.	Lineweaver-Burk plot for determining the K_m of 1-PFK for ATP in the presence of various D-fructose-1-P concentrations	126

Figure		Page
24.	Dependence of 6-PFK and 1-PFK reaction velocity on Mg^{++} concentration in the presence of various concentrations of the hexose phosphate substrate	128
25.	Dependence of 6-PFK and 1-PFK reaction velocity on hexose phosphate concentration with other nucleoside triphosphates as phosphoryl donors	132
26.	Inhibition of 6-PFK by CTP in the presence of various concentrations of D-fructose-6-P and Mg^{++}.	134
27.	Dependence of 6-PFK reaction velocity on D-fructose-6-P concentration in the presence of various nucleotides	137
28.	Lineweaver-Burk plot showing the relationship of 1-PFK reaction velocity to D-fructose-1-P concentration in the presence of various concentrations of D-fructose-6-P	142
29.	Kinetic plot for determining the K_i of 1-PFK for D-fructose-6-P	144
30.	Lineweaver-Burk plot showing the relationship of 1-PFK reaction velocity to D-fructose-1-P concentration in the presence of various concentrations of FDP.	146
31.	Kinetic plot for determining the K_i of 1-PFK for FDP.	148
32.	Lineweaver-Burk plot showing the relationship of 1-PFK reaction velocity to D-fructose-1-P concentration in the presence of various concentrations of citrate.	151
33.	Kinetic plot for determining the K_i of 1-PFK for citrate	153
34.	pH-activity profiles of 6-PFK and 1-PFK	155
35.	Dependence of the rate of the 6-PFK reaction on D-fructose-6-P concentration at different pH values	157

Figure Page

36. Elution profiles of 1-PFK, 6-PFK, malic dehydrogenase, alcohol dehydrogenase, and cytochrome c on a Sephadex G-100 column 160

37. Plot of elution volume V_e vs log MW of the standards for the estimation of the molecular weights of 1-PFK and 6-PFK . . 162

LIST OF ABBREVIATIONS

D-Glucose-6-P, D-glucose 6-phosphate; D-glucose-1-P, D-glucose 1-phosphate; D-fructose-6-P, D-fructose 6-phosphate; D-fructose-1-P, D-fructose 1-phosphate; L-fructose-1-P, L-fructose 1-phosphate; D-mannose-6-P, D-mannose 6-phosphate; D-mannose-1-P, D-mannose 1-phosphate; L-ribulose-5-P, L-ribulose 5-phosphate; mannitol-1-P, mannitol 1-phosphate; FDP, D-fructose 1,6-diphosphate; FDPase, D-fructose 1,6-diphosphatase; 1-PFK, D-fructose 1-phosphate kinase; 6-PFK, D-fructose 6-phosphate kinase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; EDTA, ethylenediaminetetraacetate; triose-P, triose phosphate, PEP, phosphoenolpyruvate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; ATP, adenosine 5'-triphosphate; ATPase, adenosine 5'-triphosphatase; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; cyclic AMP, adenosine 3',5'-cyclic monophosphate; ITP, inosine 5'-triphosphate; IDP, inosine 5'-diphosphate; IMP, inosine 5'-monophosphate; UTP, uridine 5'-triphosphate; UDP, uridine 5'-diphosphate;

UMP, uridine 5'-monophosphate; GTP, guanosine 5'-triphosphate; GDP, guanosine 5'-diphosphate; GMP, guanosine 5'-monophosphate; CTP, cytidine 5'-triphosphate; CDP, cytidine 5'-diphosphate; CMP, cytidine 5'-monophosphate; TTP, thymidine 5'-triphosphate; TDP, thymidine 5'-diphosphate.

PART I

A HEXOKINASE SPECIFIC FOR D-MANNOSE AND D-FRUCTOSE FROM *LEUCONOSTOC MESENTEROIDES*

INTRODUCTION

Heterofermentative lactic acid bacteria of the genera *Leuconostoc* and *Lactobacillus* ferment hexoses through a hexose monophosphate (phosphoketolase) pathway (1, 2). Although a constitutive hexokinase (ATP: hexose phosphotransferase) has been implicated in initiating the pathway (3, 4), other investigators have reported D-glucokinase activity in extracts to be weak or undetectable (5, 6). Preliminary investigations in this laboratory of possible alternative phosphorylating mechanisms involving phosphoryl donors other than ATP revealed instead high levels of kinase activity for D-glucose, D-mannose, and D-fructose in fresh extracts of *Leuconostoc mesenteroides*. Storage of the extracts at 0-2°C for 24 hours, however, invariably caused a disappearance of D-glucokinase activity without affecting the kinase activity for D-mannose or D-fructose. This observation substantiated previous indications (6) that more than one enzyme was involved in the phosphorylation of the three hexoses, and suggested that

previous failures to detect D-glucokinase activity were due to the lability of the enzyme rather than to its absence from the organism. Cifferi, Blakley, and Simpson (6) have pointed out that it was unknown whether the apparent phosphorylation of D-mannose and D-fructose in L. mesenteroides was mediated by a single kinase, by two specific kinases, or by a kinase specific for either D-mannose or D-fructose in conjunction with an isomerase which interconverted the two hexoses. Because D-mannose isomerase (7, 8) and specific kinases which phosphorylate either D-mannose (9-11) or D-fructose (9-12) at carbon atom 6 have been found in a variety of organisms, the latter two possibilities seemed likely. However, evidence presented in this part of the thesis indicates that the phosphorylation of D-mannose and D-fructose in L. mesenteroides is effected by a single enzyme with a unique specificity, and that an isomerase which interconverts the two hexoses is not involved. These conclusions are based on an investigation of the properties of the mannofructokinase (ATP:hexose 6-phosphotransferase specific for D-mannose and D-fructose) after its purification to apparent homogeneity.

The findings described in this part of the thesis have been published (13). The common identity of the D-mannokinase and D-fructokinase activities and the instability of the D-glucokinase activity of L. mesenteroides have recently been confirmed by DeMoss (14).

MATERIALS AND METHODS

Source and Confirmation of Identity of L. Mesenteroides

The strain of L. mesenteroides (designated LM) used in this investigation was obtained from Dr. W. A. Wood. Records denoting the original source of this strain were not available. Its identity was confirmed by the following observations: colonies on sucrose agar plates were large and mucoid, indicating the synthesis and deposition of dextran around the cells; growth in glucose broth was accompanied by gas production; and cells viewed by phase contrast microscopy appeared as chains of spheres or short rods.

Growth of Cells and Preparation of Extracts

The organism was grown and maintained in LBS medium (15) as modified by Costilow, Etchells, and Anderson (16). The medium contained per liter of broth: 10 g of trypticase, 5 g of yeast extract, 6 g of KH_2PO_4, 2 g of ammonium citrate, 1 g of monosorbitan oleate complex (trade name, Tween 80; purchased from E. H. Sargent Co.), 20.5 g of anhydrous sodium acetate, 0.6 g of $MgSO_4$, 0.1 g of $MnSO_4$, 0.03 g of $FeSO_4$, and 20 g of D-glucose (autoclaved separately). The cells were grown without

agitation in 20-liter carboys at 30°C. The inoculum was 1 liter of a 24-hr culture in the same medium. The cells were harvested with a Sharples centrifuge approximately 30 hr after inoculation and were washed once with distilled water. The yield was about 2 g (wet weight) of cells per liter.

Extracts were prepared from washed cell suspensions in distilled water by treatment for 12 to 15 minutes in a Raytheon 250-watt, 10-kc sonic oscillator circulated with ice water. The supernatant obtained after centrifugation of the broken cell suspension at 16,300 x g was used as the crude extract.

Chemicals

Horseradish peroxidase (Grade C; $A_{403}/A_{275} > 1.5$) and twice-recrystallized rabbit muscle lactic dehydrogenase (containing pyruvic kinase) were from Worthington Biochemical Corp., Freehold, N. J. Glucose-6-P isomerase (A grade, Boehringer) had <0.01% of contaminating activities of 6-phosphogluconate dehydrogenase, phosphoglucomutase, 6-phosphofructokinase, and glucose reductase. Glucose-6-P dehydrogenase (Boehringer) contained the following nominal impurities: glucose reductase, <0.05%; hexokinase, <0.2%; and 6-phosphogluconate dehydrogenase, <0.01%. Crystalline yeast hexokinase (substantially free of ATPase, adenylate kinase, glucose-6-P dehydrogenase, and 6-phosphogluconate dehydrogenase) was from Sigma

Chemical Co., St. Louis, Mo.

D-Mannose-6-P isomerase was purified from D-glucose-grown Aerobacter aerogenes PRL-R3 (17). An extract was prepared by treatment of washed cells in 0.05 M sodium phosphate buffer (pH 7.0) for 5 to 7 min in a sonic oscillator, followed by centrifugation of the resulting broken cell suspension. Solid $(NH_4)_2SO_4$ was added to the crude extract to a final concentration of 0.1 M, and nucleic acids were precipitated by the slow addition of 20% by volume of a 2% aqueous solution of protamine sulfate. Solid $(NH_4)_2SO_4$ was added to 50% saturation to the supernatant from the protamine sulfate step. The precipitate was dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.0) and the solution obtained was passed through a Sephadex G-100 column (40 x 2.9 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) and eluted with the same buffer. Fractions of 4 ml each were collected. Fraction 22 was 10-fold purified over the extract and was free ($< 0.1\%$ of D-mannose-6-P isomerase) from 6-phosphogluconate dehydrogenase, glucose-6-P isomerase, and glucose-6-P dehydrogenase.

D-Fructose was from Pfanstiehl Lab., Waukegan, Ill., D-fructose-6-P from Schwarz Bioresearch, D-fructose-1-P and PEP from Calbiochem, and D-mannose-1-P from Sigma. L-Mannose, L-glucose, L-ribulose, D-allose, D-altrose,

D- and L-galactose, and D- and L-xylulose were obtained as described by Kamel, Allison, and Anderson (17). L-Fructose was the preparation described by Mayo and Anderson (18). D-Mannose (c.p. grade) was recrystallized twice (19) to reduce the contaminating D-glucose to 0.07%, as determined with a stereospecific D-glucokinase (20). D-Mannose-6-P free from D-fructose-6-P and D-glucose-6-P was prepared enzymically in a reaction mixture containing 3 mmoles of ATP, 3 mmoles of $MgCl_2$, 3 mmoles of twice-recrystallized D-mannose, and crystalline yeast hexokinase. The pH of the mixture was maintained at pH 7.5 by titration with 4.1 N NH_4OH with the use of a Sargent recording pH stat. The D-mannose-6-P formed was isolated as described for L-ribulose-5-P (21).

Pyridine nucleotides and nucleoside di- and triphosphates were from P-L Biochemicals, Milwaukee, Wis. The nominal impurities of the latter were as follows: ITP, <4% IDP; UTP <4% UDP; GTP <4% GDP; ATP, <4% ADP; CTP, <4% CDP; ADP, <4% each of ATP and AMP; IDP, <4% ITP; and TTP, <4% TDP.

All other chemicals were from standard chemical sources.

Mannofructokinase Assays

Two types of assay were employed: a non-specific pyruvate kinase-lactate dehydrogenase-linked assay and a

specific glucose-6-P dehydrogenase-linked assay. Both were done in microcuvettes with a 1-cm light path and were monitored at 340 nm with a Gilford multiple sample absorbance-recording spectrophotometer thermostated at 25°C. The units of the coupling enzymes used in the assays refer to the number of μmoles of product formed per minute at pH 6.9 and 25°C. The pyruvate kinase-lactate dehydrogenase-linked assay contained in a volume of 0.15 ml: 8 μmoles of glycylglycine buffer (pH 6.9), 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.4 μmole of PEP, 0.05 μmole of NADH, 0.26 unit of lactate dehydrogenase, 0.03 unit of pyruvate kinase, 1.0 μmole of hexose substrate, and limiting amounts of mannofructokinase. Controls to correct for NADH oxidase and ATPase activities contained all components of the reaction mixture except the hexose substrate. This procedure was used for routine assays during purification of the enzyme and for determination of K_m values, pH optimum, sugar specificity, and inhibition by D-fructose-6-P.

The glucose-6-P dehydrogenase-linked assay contained in a volume of 0.15 ml: 8 μmoles of glycylglycine buffer (pH 6.9), 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.1 μmole of NADP, 0.08 unit of glucose-6-P dehydrogenase, 0.31 unit of glucose-6-P isomerase, 1.0 μmole of D-fructose or 1.0 μmole of D-mannose plus 0.005 unit of mannose-6-P isomerase, and limiting amounts of mannofruc-

tokinase. This assay was used for determining nucleotide specificity, for testing inhibition by compounds other than D-fructose-6-P, for determining the K_i for ADP, and for identifying the product.

Identical rates were obtained with both assays. The rates were linear with time for 12 to 15 min and were directly proportional to the amount of mannofructokinase present. A unit was defined as the amount of enzyme that catalyzed the phosphorylation of 1 μmole of D-mannose or D-fructose per minute at pH 6.9 and 25°C. Specific activity was defined as the number of units per milligram of protein.

Other Enzyme Assays

Peroxidase was determined by measuring 460 nm absorbance increase of a reaction mixture consisting of 0.15 ml of 0.003% H_2O_2 in 0.01 M sodium phosphate buffer (pH 6.0), 2 μl of 1.0% o-dianisidine in methanol, and 1 μl of peroxidase solution of an appropriate dilution.

Several enzyme activities were measured by adaptations of the glucose-6-P dehydrogenase-linked assay for mannofructokinase. The assay for 6-phosphogluconate dehydrogenase contained 1.0 μmole of 6-phosphogluconate in place of hexose and ATP. Assays for phosphofructomutase and phosphomannomutase contained 1.0 μmole of D-fructose-1-P or D-mannose-1-P, respectively, in place

of D-fructose plus ATP or D-mannose plus ATP. The assay for adenylate kinase contained ADP in place of ATP.

6-Phosphofructokinase was measured in the pyruvate kinase-lactate dehydrogenase-linked assay with D-fructose-6-P as the substrate.

Protein Measurements

Protein was estimated spectrophotometrically at 280 and 260 nm with the aid of a nomograph based on the data of Warburg and Christian (22).

Disc Gel Electrophoresis

This was performed in polyacrylamide gels at pH 8.6 according to directions supplied by Canalco (Rockville, Md.). The lower gel contained 7.5% acrylamide, one-half the standard amount of N,N,N',N'-tetramethylethylenediamine, and no $K_3Fe(CN)_6$. Ferricyanide was omitted because it inactivated mannofructokinase. When assays were to be made on the electrophoresed protein, the lower gel was sliced lengthwise prior to staining.

Sucrose Density Gradient Centrifugation

This employed a 3-ml linear gradient of 5 to 20% sucrose in 0.2 M $(NH_4)_2SO_4$ (pH 7.0). The gradient was carefully layered with a mixture of 25 µl (50 µg of protein) of the concentrated Sephadex G-75 fraction and 20 µl (10 µg of protein) of peroxidase. The centrifugation

was run for 14 hr at 39,000 rpm in a Spinco model L centrifuge. At the end of the run, the bottom of the tube was punctured and 5-drop fractions were collected.

Cellulose Acetate Electrophoresis

The cellulose acetate paper strips (2.5 x 12 cm, trade name: Sepraphore III) were from the Gelman Instrument Co. The barbital-acetate buffer contained 5 g of sodium barbital and 2.3 g of anhydrous sodium acetate per liter of solution. The pH of the buffer was adjusted with HCl to pH 6.0, 7.4, or 8.6. Seven µl (40 µg of protein) of the kinase preparation were applied on each paper strip. The runs were conducted at 4°C for 2 hours with a current of 1 ma per strip. Protein bands were developed by dipping the strips in 0.2% nigrosine (bacteriological stain, Allied Chemical) solution for 1 min, and immersing in 5% trichloroacetic acid. The proteins from the unstained strips corresponding to the protein bands were eluted with 0.2 M $(NH_4)_2SO_4$.

RESULTS

Phosphorylation of Hexoses With ATP by L. mesenteroides Cell Extracts

As shown in Table I, fresh extracts of L. mesenteroides exhibited kinase activity on D-glucose, D-fructose, and D-mannose at nearly equal rates. Storage of the crude extracts for 24 hr at 0-2°C, however, resulted in a complete loss of the D-glucokinase activity but had no effect on the kinase activity for either D-mannose or D-fructose. The D-glucokinase activity could not be protected from inactivation by 4 mM reduced glutathione or 2-mercaptoethanol.

Sonic oscillation of cell suspensions for varying time periods (5 to 20 minutes) or freezing of the cells for 48 hr prior to preparation of the extracts had no significant effect on the phosphorylating capacity for any of the three hexoses.

Requirements for D-Mannose and D-Fructose Phosphorylation by Crude Extracts Stored for 24 Hours at 0-2°C

Table II shows an agreement of results obtained from the pyruvate kinase-lactate dehydrogenase-linked and the glucose-6-P dehydrogenase-linked assays for kinase activity. The specificity for the corresponding

TABLE I

[redacted]oxylation of D-glucose, D-mannose, and D-fructose by cell extracts of L. mesenteroides

TABLE I

Phosphorylation of D-glucose, D-mannose, and D-fructose by cell extracts of L. mesenteroides

	Specific activity* on D-glucose	Specific activity* on D-mannose	Specific activity* on D-fructose
a) Fresh crude extract	0.66	0.77	0.68
b) Crude extract stored for 24 hrs at 0-2°C	0	0.77	0.68

*Specific activity: μmoles of substrate phosphorylated per minute per milligram protein at 25°C and pH 6.9.

D-glucose phosphorylation was measured by use of the glucose-6-P dehydrogenase-linked assay. D-Mannose and D-fructose phosphorylations were measured by use of the pyruvate kinase-lactate dehydrogenase-linked assay.

Requirements for
in cell ex

Complete pyruvate
lactate dehydrogena
assay mixture

minus ATP

minus hexose

minus PEP

Complete glucose-6-
dehydrogenase-linke
mixture

minus hexose

minus mannose-6-
glucose-6-P isom
glucose-6-P dehy

minus mannose-6-

Units: micromoles
at 340 and pH

TABLE II

Requirements for D-mannose and D-fructose phosphorylation in cell extracts after 24-hr storage at 0-2°C

	Activity on D-mannose	Activity on D-fructose
	units*/mg	units*/mg
Complete pyruvate kinase-lactate dehydrogenase-linked assay mixture	0.36	0.45
minus ATP	0.006	0.006
minus hexose	0.01	0.01
minus PEP	0.006	0.008
Complete glucose-6-P dehydrogenase-linked assay mixture	0.36	0.45
minus hexose	0	0
minus mannose-6-P isomerase, glucose-6-P isomerase, and glucose-6-P dehydrogenase	0	0
minus mannose-6-P isomerase	0	0.45

*Units: micromoles of substrate phosphorylated per minute at 25°C and pH 6.9.

hexose-6-P of th

dehydrogenase-1

products of D-ma

were D-mannose-6

The lack

mannose-6-P isom

linked assay for

both mannose iso

crude extracts u

Puri

Unless ot

formed at 0-4°C.

in Table III. D

from the latter

$MnCl_2$ Fractionat

$MnCl_2$ (1

extract (13 mg p

tion of 0.05 M.

centrifugation a

First Ammonium S

Solid amm

ring to the abov

between 40 and 7

gation and disso

hexose-6-P of the coupling enzymes in the glucose-6-P dehydrogenase-linked assay suggested that the principal products of D-mannose and D-fructose phosphorylation were D-mannose-6-P and D-fructose-6-P, respectively.

The lack of NADP reduction in the absence of mannose-6-P isomerase in the glucose-6-P dehydrogenase-linked assay for mannokinase indicated the absence of both mannose isomerase and mannose-6-P isomerase in the crude extracts under the assay conditions employed.

Purification of Mannofructokinase

Unless otherwise stated, all operations were performed at 0-4°C. A summary of the purification is given in Table III. Disc electrophoretic patterns of fractions from the latter stages of purification are shown in Fig. 1.

$MnCl_2$ Fractionation

$MnCl_2$ (1 M) was added with stirring to the crude extract (13 mg protein per ml) to give a final concentration of 0.05 M. The resulting precipitate was removed by centrifugation and discarded.

First Ammonium Sulfate Fractionation

Solid ammonium sulfate was added slowly with stirring to the above fraction. The protein precipitating between 40 and 70% saturation was collected by centrifugation and dissolved in water. This solution contained

TABLE III

Purification of mannofructokinase from Leuconostoc mesenteroides

	Total	Total activity (D-fructo-	Specific activity (D-fructo-	Recovery (D-fructo-	Specific activity (D-manno-	D-Mannokinase / D-fructokinase

TABLE III

Purification of mannofructokinase from Leuconostoc mesenteroides

Fraction	Total protein	$A_{280}:A_{260}$	Total activity (D-fructo-kinase)	Specific activity (D-fructo-kinase)	Recovery (D-fructo-kinase)	Specific activity (D-manno-kinase)	D-Mannokinase / D-fructokinase
	mg		units*	units/mg protein	%	units/mg protein	
Cell extract	4316	0.64	7110	1.65	100	1.37	0.83
$MnCl_2$	1670	0.62	6760	4.04	95	4.13	1.02
First $(NH_4)_2SO_4$ (40-70%)	756	1.10	5540	7.32	78	7.84	1.07
Heat	147	0.61	2560	17.4	(36)	14.8	0.85
Second $(NH_4)_2SO_4$ (pH 7.0)	124	1.16	2930	23.4	(41)	23.6	1.01
$Ca_3(PO_4)_2$ gel	126	1.10	3900	31.0	55	31.0	1.00
DEAE-cellulose fractions 36-56	47	1.6	1140	24.3	16	24.3	1.00
Sephadex G-100 fractions 46-55	4.8	1.7	365	76.0	5.1	76.0	1.00

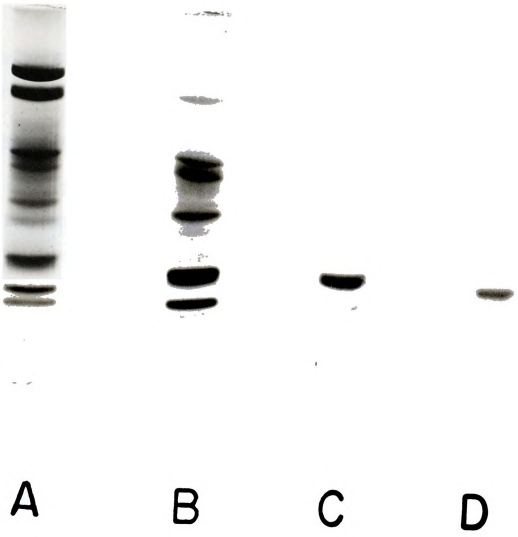
*Units: μmoles of hexose phosphorylated per minute at 25°C and pH 6.9.

Figure 1

A

Figure 1: Disc gel electrophoretic patterns of fractions obtained from the latter stages of purification. A, calcium phosphate gel supernatant; B, DEAE-cellulose Fractions 36-56; C, Sephadex G-75 Fractions 47 to 49; D, enzyme obtained by eluting the region corresponding to the darker band in C. The direction of migration was down.

Figure 1



ns of
ter stages
osphate
ose Frac-
ractions
eluting
darker
gration

considerable sa
fate in the pre

Heat Treatment

The abov
in an 80°C wate
precipitate was

Second Ammonium

Three vol
tion (pH 7) were
the above fracti
and dissolved in

Calcium Phosphat

Calcium p
added to the abo
per ml of enzyme
and discarded.
Sephadex G-25 co
phosphate buffer
eluted with the
est mannofructok

DEAE-Cellulose C

The Sephad
(1.5 x 23 cm) of

considerable salt because of the occluded ammonium sulfate in the precipitated protein.

Heat Treatment

The above fraction was held at 50°C for 3 minutes in an 80°C water bath and cooled rapidly on ice. The precipitate was removed by centrifugation and discarded.

Second Ammonium Sulfate Fractionation

Three volumes of saturated ammonium sulfate solution (pH 7) were added with stirring to two volumes of the above fraction. The precipitate was centrifuged down and dissolved in 20 ml of water.

Calcium Phosphate Gel Treatment

Calcium phosphate gel (Sigma, 11% solids) was added to the above fraction at a ratio of 0.04 ml of gel per ml of enzyme solution. The gel was centrifuged down and discarded. The supernatant was passed through a Sephadex G-25 column equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). Fractions (5 ml each) were eluted with the same buffer, and those that had the highest mannofructokinase activity were combined.

DEAE-Cellulose Chromatography

The Sephadex fraction was passed through a column (1.5 x 23 cm) of DEAE-cellulose (Sigma, exchange capacity

= 0.9 meq per g
phate buffer (p
with 225 ml of
fate in a linea
36 to 56, which
combined. As s
cant loss in sp
Disc gel electr
contaminating p
DEAE step incre

Sephadex Chroma

The abov
ing with solid
centrifuged pre
tion was then p
Sephadex G-100
(pH 7.0). Frac
0.2 M ammonium
ammonium sulfat
glycylglycine b
in enzyme activ
a relatively hig
It was not estab
were actually re
purified, 5% rec
ammonium sulfate

= 0.9 meq per g) equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). Fractions (3 ml) were eluted with 225 ml of the same buffer containing ammonium sulfate in a linear gradient from 0 to 0.4 M. Fractions 36 to 56, which had the highest specific activity, were combined. As shown in Table III, an apparent significant loss in specific activity resulted from this step. Disc gel electrophoresis (Fig. 1B), however, showed that contaminating proteins had been removed. Moreover, the DEAE step increased the A_{280}:A_{260} ratio from 1.1 to 1.6.

Sephadex Chromatography

The above fraction was concentrated by precipitating with solid ammonium sulfate and dissolving the centrifuged precipitate in 3.5 ml of water. This solution was then passed through a column (2.7 x 38 cm) of Sephadex G-100 equilibrated with 0.2 M ammonium sulfate (pH 7.0). Fractions (1.5 ml) were eluted with neutral 0.2 M ammonium sulfate (Fig. 2). Substitution of the ammonium sulfate as eluent with either water or 0.05 M glycylglycine buffer (pH 7.0) led to a considerable loss in enzyme activity, indicating that the enzyme requires a relatively high ionic strength for maximum stability. It was not established whether ammonium or sulfate ions were actually required. Fractions 46 to 55 (50-fold purified, 5% recovery) were combined, concentrated by ammonium sulfate precipitation, and passed through a

Figure 2: Top graph, chromatography of DEAE-cellulose Fractions 36 to 56 on Sephadex G-100; bottom graph, chromatography of Sephadex G-100 Fractions 46 to 55 on Sephadex G-75. Details are given in the text.

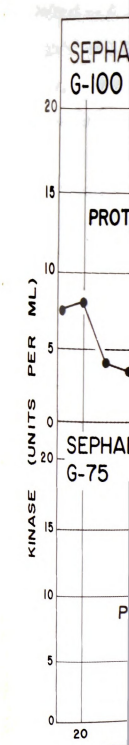
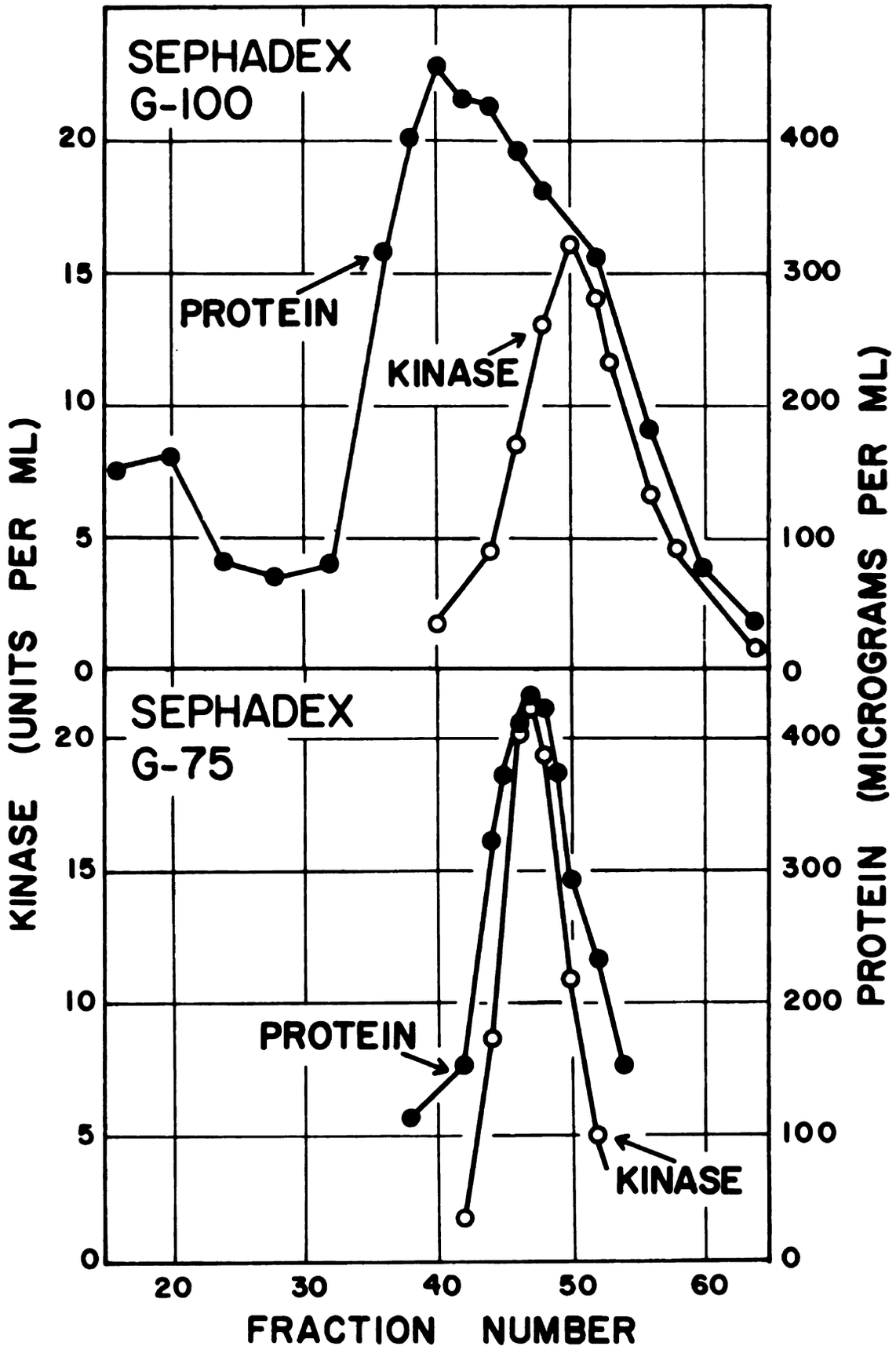


Figure 2


SEPHADEX
G-100
PROTEIN
KINASE
SEPHADEX
G-75
PROTEIN
KINASE
KINASE (UNITS PER ML)
PROTEIN (MICROGRAMS PER ML)
FRACTION NUMBER
0
5
10
15
20
100
200
300
400
20
30
40
50
60

Sephadex G-50 c
sulfate (pH 7.0
activity was at
concentrated, a
Again, there wa
2). Fractions
by ammonium sul
phoresis at thi
one minor prote

The kina
were consistent
1:1 throughout

Further Attem

Disc Gel Electro

A sample
(2 mg protein pe
disc gel electro
from Canalco. T
ponding to the p
eluted with 0.2
No mannofructoki
eluted solutions
ponent on the en
the enzyme. Low
$K_3Fe(CN)_6$ and, t

Sephadex G-50 column equilibrated with 0.2 M ammonium sulfate (pH 7.0). No further increase in specific activity was attained. The peak fractions were combined, concentrated, and passed through a Sephadex G-75 column. Again, there was no increase in specific activity (Fig. 2). Fractions 47 to 49 were combined and concentrated by ammonium sulfate precipitation. Disc gel electrophoresis at this stage showed that the enzyme contained one minor protein impurity (Fig. 1C).

The kinase activities on D-mannose and D-fructose were consistently present at a ratio of approximately 1:1 throughout the purification described above.

Further Attempts to Separate the Two Kinase Activities

Disc Gel Electrophoresis

A sample (0.1 ml) of the Sephadex G-75 fraction (2 mg protein per ml) was examined for homogeneity by disc gel electrophoresis using premixed gel solutions from Canalco. The portions of the unstained gel corresponding to the protein bands of the stained half were eluted with 0.2 M ammonium sulfate (pH 7.0) and assayed. No mannofructokinase activity was detected in any of the eluted solutions. Tests on the effect of each gel component on the enzyme showed that $K_3Fe(CN)_6$ inactivated the enzyme. Lower gel A was therefore prepared without $K_3Fe(CN)_6$ and, to prevent a too rapid polymerization of

the acrylamide
of the standard
the gel minus
the bands in th
from the premix
ing to the dark
D-mannose and D

Homogene
tokinase band (
ium sulfate (pH
a second disc e
the second run
on D-mannose an
activity of the
amount of elute
determination.

Cellulose Acetat

No sharp
any of the runs
at pH 8.6, manno
only a thin port
The kinase activ
approximately eq
fructokinase act

the acrylamide, the volume of TEMED was reduced to half of the standard amount. The enzyme remained active in the gel minus $K_3Fe(CN)_6$ and the number and position of the bands in the gel remained identical to those obtained from the premixed gel solutions. The section corresponding to the darker band (Fig. 1C) exhibited activity on D-mannose and D-fructose in a 1:1 ratio at pH 6.9.

Homogeneity was attained by eluting the mannofructokinase band (about 680 µg of protein) with 0.2 M ammonium sulfate (pH 7.0), and using the solution obtained for a second disc electrophoresis run. The single band from the second run (Fig. 1D) also exhibited equal activities on D-mannose and D-fructose at pH 6.9. The specific activity of the band could not be ascertained because the amount of eluted protein was too small for an accurate determination.

Cellulose Acetate Electrophoresis

No sharp resolution of proteins was obtained in any of the runs made at pH 8.6, 7.4, and 6.0. However, at pH 8.6, mannofructokinase activity was eluted from only a thin portion (about 5 mm) of the protein band. The kinase activities on D-mannose and D-fructose were approximately equal in all the slices that had mannofructokinase activity.

Sucrose Densit

Fig. 3 s
fructokinase ar
density gradien
activity had ar

Thermal Inactiv

Table IV
of the two kina
tivation of D-m
fructokinase ac

Pr

Stability

The enzym
ing the peak frs
stable for over
presence of 0.2
pH 6.9, 8.5, and

Phosphoryl Accep

Of 32 sug
concentration of
were phosphoryla
fructokinase in

Sucrose Density Gradient Centrifugation

Fig. 3 shows the sedimentation pattern of mannofructokinase and horseradish peroxidase on a sucrose density gradient. Each fraction which had D-mannokinase activity had an equal D-fructokinase activity at pH 6.9.

Thermal Inactivation at 60°C

Table IV shows the rates of thermal inactivation of the two kinase activities at 60°C. The rate of inactivation of D-mannokinase activity paralleled that of D-fructokinase activity at pH 6.9, 8.5, and 8.9.

Properties of Mannofructokinase

Stability

The enzyme preparation obtained after concentrating the peak fractions from the Sephadex G-75 step was stable for over 6 months when stored at 0-2°C in the presence of 0.2 M $(NH_4)_2SO_4$. The ratios of activity at pH 6.9, 8.5, and 8.9 remained constant throughout storage.

Phosphoryl Acceptor Specificity

Of 32 sugars and sugar derivatives tested at a concentration of 70 mM, only D-mannose and D-fructose were phosphorylated. With 0.016 unit of purified mannofructokinase in the pyruvate kinase-lactate dehydrogenase-

Figure 3: Sedimentation pattern of mannofructokinase and peroxidase standard (molecular weight = 40,000) in a sucrose density gradient. The kinase fractions had equal activities on D-mannose and D-fructose. Details are given in the text.

Figure 3

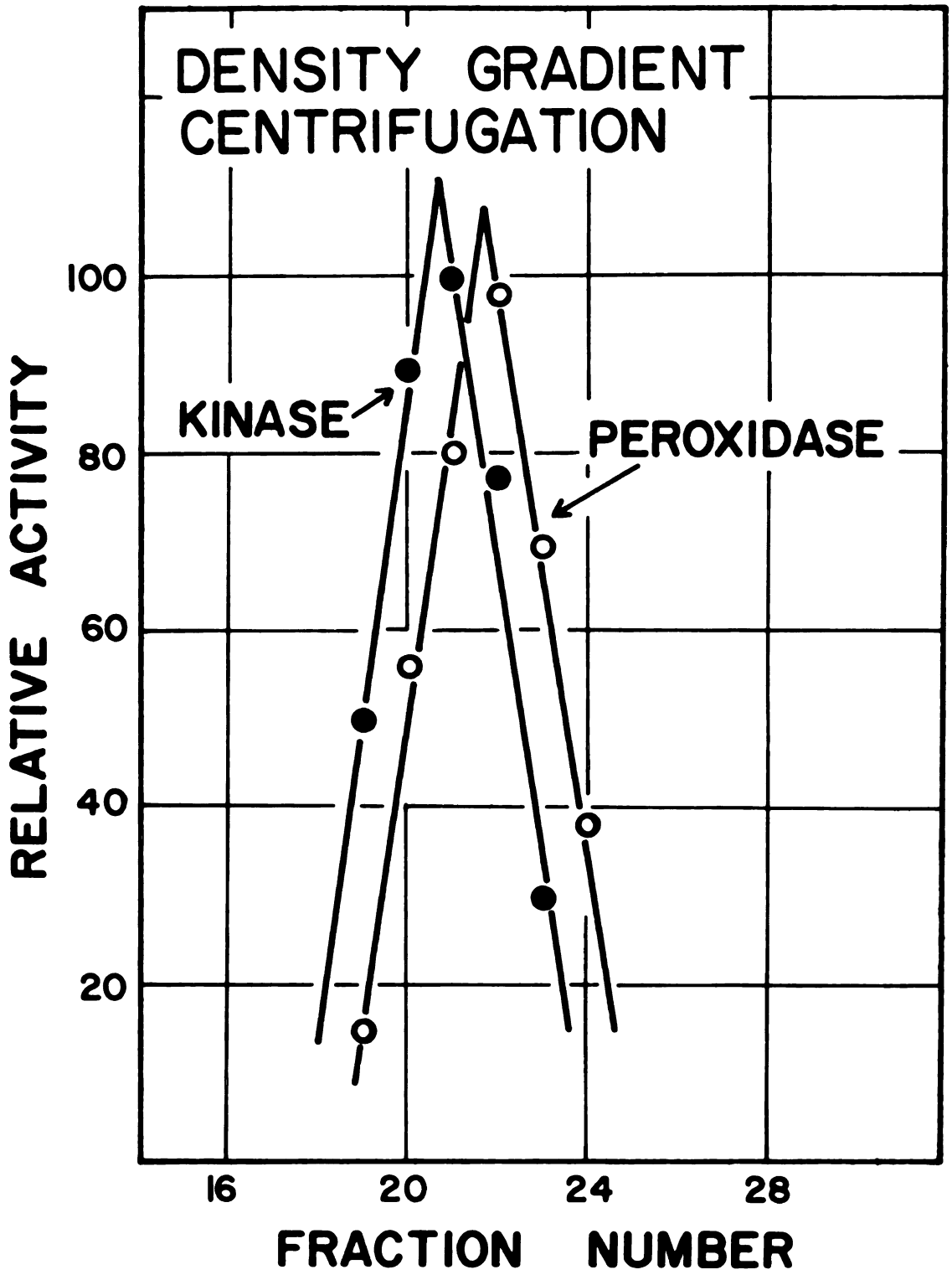

DENSITY GRADIENT
CENTRIFUGATION
KINASE
PEROXIDASE
RELATIVE ACTIVITY
100
80
60
40
20
16
20
24
28
FRACTION NUMBER

TABLE IV

Thermal inactivation of mannofructokinase at 60°C

Mannofructokinase was purified through the second $(NH_4)_2SO_4$ step (specific activity = 9.3). The enzyme (0.8 mg per ml) was adjusted to pH 7.0 and was heated in polypropylene centrifuge tubes in a 60°C

TABLE IV

Thermal inactivation of mannofructokinase at 60°C

Mannofructokinase was purified through the second $(NH_4)_2SO_4$ step (specific activity = 9.3). The enzyme (0.8 mg per ml) was adjusted to pH 7.0 and was heated in polypropylene centrifuge tubes in a 60°C water bath. At the indicated times, the tubes were quickly cooled on ice, the precipitates were centrifuged off, and samples were assayed for D-mannokinase and D-fructokinase activities at the indicated pH values.

Heating time	At pH 6.9		At pH 8.5		At pH 8.9	
	% of initial activity	Ratio of D-mannokinase to D-fructokinase activities	% of initial activity	Ratio of D-mannokinase to D-fructokinase activities	% of initial activity	Ratio of D-mannokinase to D-fructokinase activities
min						
0	100	0.90	100	0.55	100	0.36
3	91	0.94	92	0.56	100	0.40
5	72	1.02	75	0.54	74	0.31
10	36	0.93	36	0.54	36	0.31
15	20	0.87	20	0.51	17	0.32
20	3.5	0.90	3.7	0.55	3.6	0.35
25	0		0		0	

linked assay,
could not be d
D- and L-gluco
tose, D-altros
D-lyxose, D- a
D- and L-ribul
conate, D-gluc
D-mannitol, D-
using a larger
was determined
at a level of
the glucose-6-P
above compounds
0.56 mM D-fruct
lation was cons
lished with the

Phosphoryl Donc

The rela
tose in the pre
given in Table

Effect of pH

Kinase a
Fig. 4. In dif
ity at pH 6.9 v
activity for th

linked assay, phosphorylation of the following compounds could not be detected (< 3% of the rate on D-fructose): D- and L-glucose, 2-deoxy-D-mannose, L-mannose, L-fructose, D-altrose, D-allose, D- and L-galactose, L-sorbose, D-lyxose, D- and L-xylose, D- and L-arabinose, D-ribose, D- and L-ribulose, D- and L-xylulose, L-rhamnose, D-gluconate, D-glucuronate, D-galacturonate, D-glucitol, D-mannitol, D- and L-arabitol, ribitol, and xylitol. By using a larger amount of the kinase in this assay, it was determined that D-glucose was not phosphorylated even at a level of 0.04% of the rate with D-fructose. With the glucose-6-P dehydrogenase-linked assay none of the above compounds (70 mM) inhibited the phosphorylation of 0.56 mM D-fructose, indicating that possible phosphorylation was considerably less than the 3% maximum established with the other assay.

Phosphoryl Donor Specificity

The relative rates of phosphorylation of D-fructose in the presence of various nucleotides (3.3 mM) are given in Table V.

Effect of pH

Kinase activity as a function of pH is shown in Fig. 4. In different experiments, D-fructokinase activity at pH 6.9 varied from 90 to 100% of D-mannokinase activity for the same enzyme preparation. The activity

The nucleo
3.3 mM. The gluc
D-fructokinase ac

Nucleotide
ATP
ITP
TTP
GTP
UTP
CTP
IDP
ADP

TABLE V

Phosphoryl donor specificity
of mannofructokinase

The nucleotides were tested at a concentration of 3.3 mM. The glucose-6-P dehydrogenase-linked assay for D-fructokinase activity was used.

Nucleotide	Percent relative activity
ATP	100
ITP	75
TTP	62
GTP	9
UTP	9
CTP	<4
IDP	<4
ADP	<4

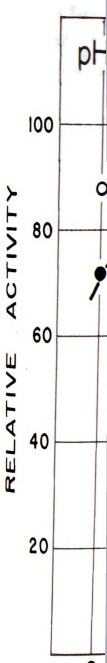
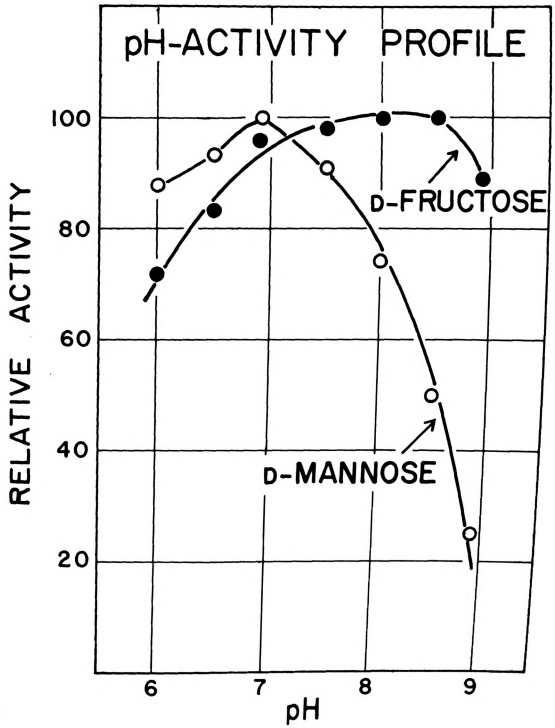


Figure 4: pH optima of mannofructokinase. Buffers (0.053 M) used were: cacodylate, pH 6.0 to 6.5; glycylglycine, pH 6.9 to 8.0; and glycine, pH 8.5 to 8.9. The pyruvate kinase-lactate dehydrogenase-linked assay was used for both hexoses throughout the pH range shown. Activity on D-fructose through pH 6.9 to 8.9 was verified by the glucose-6-P dehydrogenase-linked assay. pH measurements were made on duplicate reaction mixtures. The pH did not vary with time during the assay period.

Figure 4


pH-ACTIVITY PROFILE
RELATIVE ACTIVITY
100
80
60
40
20
D-FRUCTOSE
D-MANNOSE
6
7
8
9
pH

on D-mannose d
on D-fructose
around pH 8 to
mannose was ab
and about 32%
hexose and ATP
obtained for D
with the pyruva
and the glucos
late kinase an
not be detecte
either the kina
oxidation of NA
with D-fructose
6-P as substrat
genase-linked a
pH values. Lik
of NADP could b
glucose-6-P deh
at any pH. Thi
profiles were a
that the differ
the result of c
contribute to o
tides.

The D-ma
at pH 6.9, 8.5,

on D-mannose dropped considerably around pH 8, while that on D-fructose increased slightly and reached a peak around pH 8 to 8.5. The rate of phosphorylation of D-mannose was about 54% of that of D-fructose at pH 8.5 and about 32% at pH 8.9. The enzyme was saturated with hexose and ATP at all pH values. Identical curves were obtained for D-fructokinase activity from pH 6.9 to 8.9 with the pyruvate kinase-lactate dehydrogenase-linked and the glucose-6-P dehydrogenase-linked assays. Adenylate kinase and 6-phosphofructokinase activities could not be detected at pH values of 6.9, 7.5, and 8.5 in either the kinase fraction or the coupling enzymes. No oxidation of NADH or reduction of NAD could be detected with D-fructose, D-mannose, D-mannose-6-P, or D-fructose-6-P as substrates in the pyruvate kinase-lactate dehydrogenase-linked assay mixture minus ATP at the same three pH values. Likewise, no oxidation of NADPH or reduction of NADP could be detected with these substrates in the glucose-6-P dehydrogenase-linked assay mixture minus ATP at any pH. This indicates that the observed pH-activity profiles were an expression of the kinase activities and that the differences on D-mannose and D-fructose were not the result of contamination with other enzymes that might contribute to oxidation or reduction of pyridine nucleotides.

The D-mannokinase to D-fructokinase activity ratios at pH 6.9, 8.5, and 8.9 remained constant at various stages

of purificatio

eous on disc e

Determination
and D-Fructose

Because
mal velocity w
interest to de
affect the K_m
D-mannose (0.4
that for D-fru
pH 8.9 (Fig. 5
to 4 mM did no
at pH 6.9 and
Burk plot all
strate axis (F

Determination o

With D-m
was 0.4 mM at p
D-fructose as t
at pH 6.9 and 1
of D-mannose an
had no effect o
8.9 (Fig. 8).

of purification, including the fraction that was homogeneous on disc electrophoresis.

Determination of K_m for D-Mannose and D-Fructose

Because of the different effects of pH on the maximal velocity with the two hexose substrates, it became of interest to determine whether pH would significantly affect the K_m for D-mannose or D-fructose. The K_m for D-mannose (0.4 mM) was the same at pH 6.9 and 8.9, while that for D-fructose was 0.4 mM at pH 6.9 and 0.7 mM at pH 8.9 (Fig. 5). Concentrations of ATP ranging from 0.2 to 4 mM did not affect the apparent K_m for either hexose at pH 6.9 and 8.9. The curves obtained in a Lineweaver-Burk plot all converged at a common point on the 1/substrate axis (Fig. 6).

Determination of K_m for ATP

With D-mannose as the substrate, the K_m for ATP was 0.4 mM at pH 6.9 and 2 mM at pH 8.9 (Fig. 7). With D-fructose as the substrate, the K_m for ATP was 0.1 mM at pH 6.9 and 1 mM at pH 8.9 (Fig. 7). Concentrations of D-mannose and D-fructose ranging from 0.2 to 3.3 mM had no effect on the apparent K_m for ATP at pH 6.9 and 8.9 (Fig. 8).

Figure 5: Lineweaver-Burk plots for determining the K_m values for D-mannose and D-fructose. The pyruvate kinase-lactate dehydrogenase-linked assay was used except that the pH and hexose concentrations were varied as indicated.

Figure 5

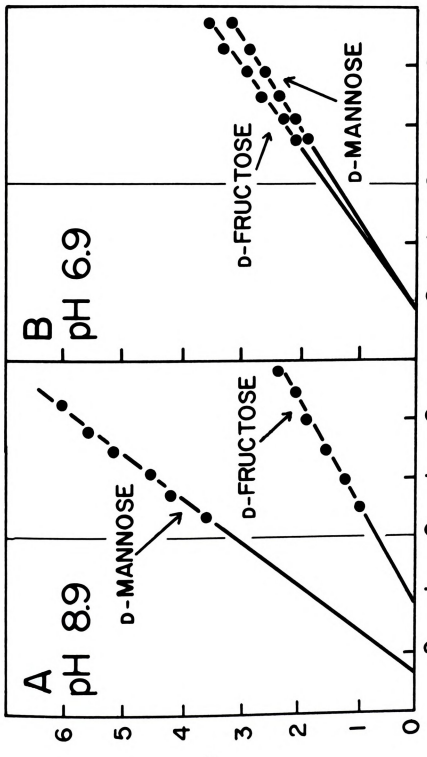

A
pH 8.9
D-MANNOSE
D-FRUCTOSE
B
pH 6.9
D-FRUCTOSE
D-MANNOSE
1/V
1/HEXOSE (mM)
0
1
2
3
4
5
6
-2
-1
0
1
2

Figure 6

Figure 6: Lineweaver-Burk plots showing the relationship of reaction velocity to D-mannose and D-fructose concentrations in the presence of varying concentrations of ATP at pH 6.9 and 8.9. The pyruvate kinase-lactate dehydrogenase-linked assay was used except that the pH and ATP and hexose concentrations were varied as indicated. The numbers along the curves represent the ATP concentration (in mM). The Mg^{++} to ATP ratio was maintained at 2:1. A, plot of 1/v vs 1/mM D-fructose at pH 8.9; B, plot of 1/v vs 1/mM D-fructose at pH 6.9; C, plot of 1/v vs 1/mM D-mannose at pH 8.9; D, plot of 1/v vs 1/mM D-mannose at pH 6.9.

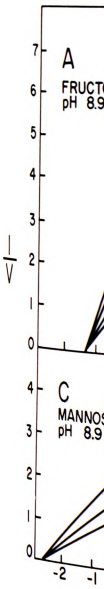


Figure 6

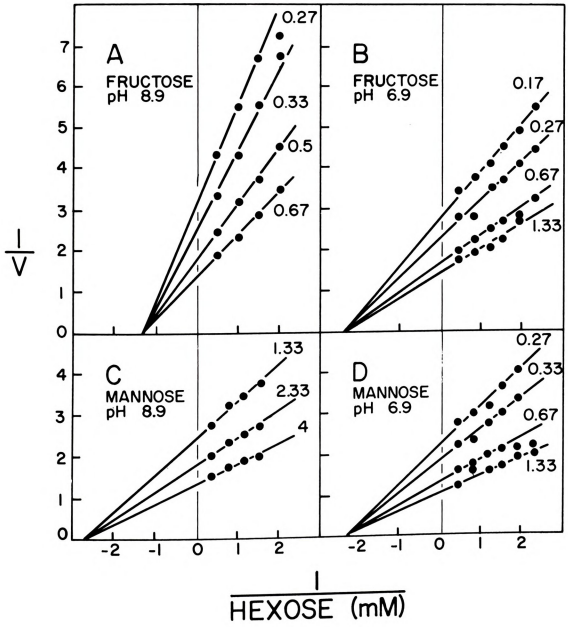

A
FRUCTOSE
pH 8.9
0.27
0.33
0.5
0.67
B
FRUCTOSE
pH 6.9
0.17
0.27
0.67
1.33
C
MANNOSE
pH 8.9
1.33
2.33
4
D
MANNOSE
pH 6.9
0.27
0.33
0.67
1.33
1/V
1/HEXOSE (mM)

Figure 7

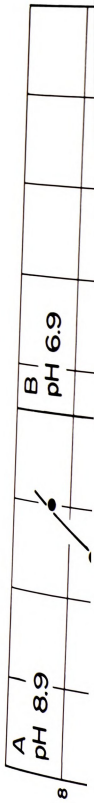


Figure 7: Lineweaver-Burk plots for determining the K_m values for ATP. The pyruvate kinase-lactate dehydrogenase-linked assay was used except that the pH and ATP concentration were varied as indicated. The Mg^{++}:ATP ratio was maintained at 2:1.

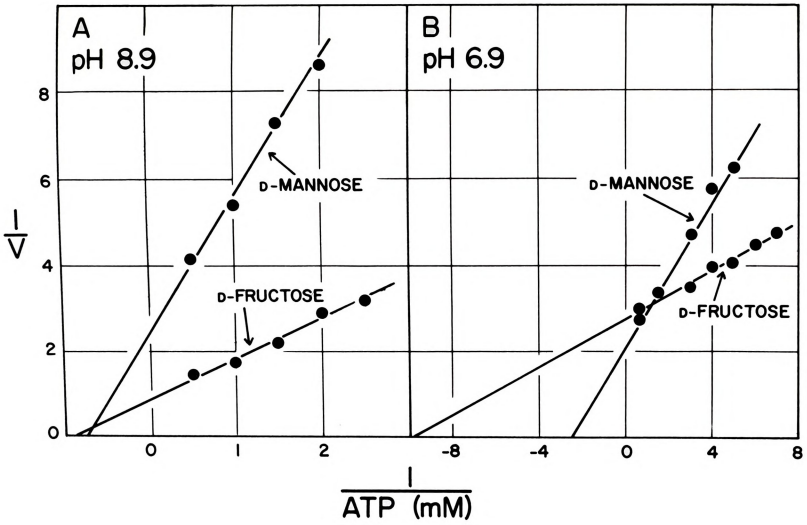
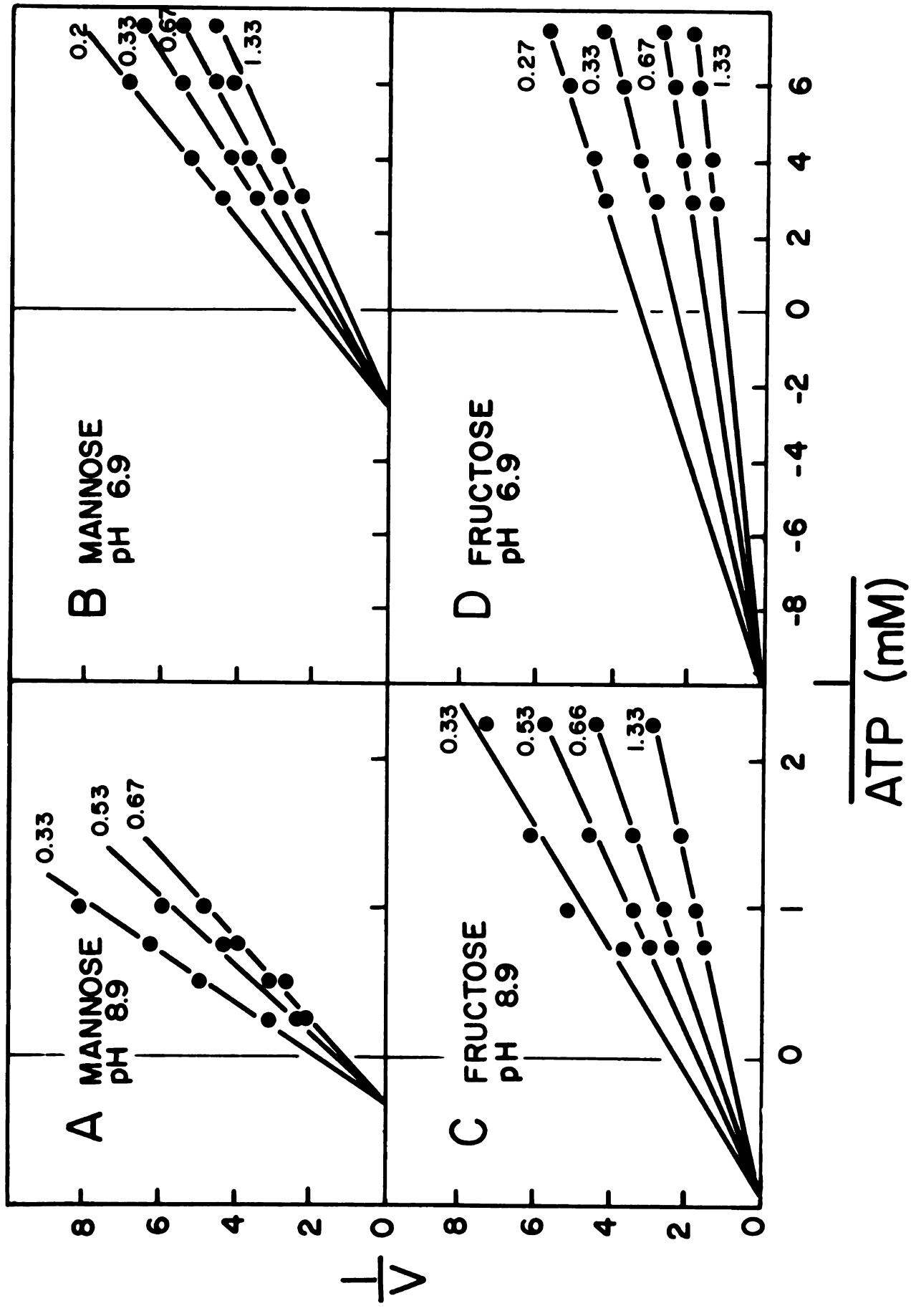

A
pH 8.9
B
pH 6.9
D-MANNOSE
D-FRUCTOSE
D-MANNOSE
D-FRUCTOSE
1/V
1/ATP (mM)
0
2
4
6
8
0
1
2
-8
-4
0
4
8

Figure 7

Figure 8: Lineweaver-Burk plots showing the relationship of reaction velocity to ATP concentration in the presence of varying concentrations of D-mannose and D-fructose at pH 6.9 and 8.9. The routine pyruvate kinase-lactate dehydrogenase-linked assay was used except that the pH and ATP and hexose concentrations were varied as indicated. The numbers along the curves represent the concentration (in mM) of the hexose substrate. The Mg^{++}:ATP ratio was maintained at 2:1. A, plot of 1/v vs 1/mM ATP at pH 8.9 with D-mannose as the substrate; B, plot of 1/v vs 1/mM ATP at pH 6.9 with D-mannose as the substrate; C, plot of 1/v vs 1/mM ATP at pH 8.9 with D-fructose as the substrate; D, plot of 1/v vs 1/mM ATP at pH 6.9 with D-fructose as the substrate.

Figure 8


A MANNOSE pH 8.9
0.33
0.53
0.67
B MANNOSE pH 6.9
0.2
0.33
0.67
1.33
C FRUCTOSE pH 8.9
0.33
0.53
0.66
1.33
D FRUCTOSE pH 6.9
0.27
0.33
0.67
1.33
1/V
ATP (mM)

Inhibition o
Activity by

The re
fructose were
tive, at pH 6
(Fig. 9) show
tion by D-man
shown in Fig.
to be 0.4 mM,
(Fig. 5).

Product Inhib

Inhibi
was tested at
with ATP (Fig
K_i for ADP wa
at concentrat
phorylation o

Estimation of

Data fo
in a sucrose g
ing 3.5 S as t
dase standard
mannofructokin
distance$_2$ = S$_2$

Inhibition of D-Fructokinase Activity by D-Mannose

The rates of phosphorylation of D-mannose and D-fructose were found to be competitive, rather than additive, at pH 6.9, 8.5, and 8.9. A Lineweaver-Burk plot (Fig. 9) shows the inhibition of D-fructose phosphorylation by D-mannose at pH 6.9. From the kinetic plot shown in Fig. 10, the K_i for D-mannose was determined to be 0.4 mM, which is the same as its K_m as a substrate (Fig. 5).

Product Inhibition

Inhibition of D-fructose phosphorylation by ADP was tested at pH 6.9 and was found to be competitive with ATP (Fig. 11). From a kinetic plot (Fig. 12), the K_i for ADP was estimated to be 0.3 mM. D-Fructose-6-P, at concentrations up to 15 mM, did not inhibit the phosphorylation of D-fructose at either pH 6.9 or pH 8.9.

Estimation of Molecular Weight

Data for the sedimentation of mannofructokinase in a sucrose density gradient are shown in Fig. 3. Taking 3.5 S as the sedimentation coefficient of the peroxidase standard (23), the sedimentation coefficient of mannofructokinase was calculated by the equation $S_1 \times \text{distance}_2 = S_2 \times \text{distance}_1$ (24) to be 4.1 S. Assuming

Figure 9: Lineweaver-Burk plot showing the relationship of reaction velocity to D-fructose concentration in the presence and absence of D-mannose. The glucose-6-P dehydrogenase-linked assay for D-fructokinase activity was used except that D-fructose and D-mannose were varied as indicated. The pH was 6.9.

Figure 9

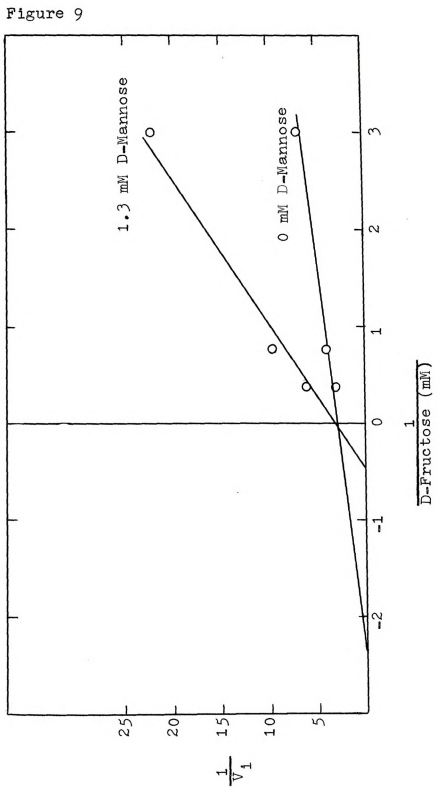

1.3 mM D-Mannose
0 mM D-Mannose
25
20
15
10
5
-2
-1
0
1
2
3
1/V1
1/D-Fructose (mM)

Figure 10: Kinetic plot for determining the K_i for D-mannose. The data are taken from the experiment described in Figure 9.

Figure 10

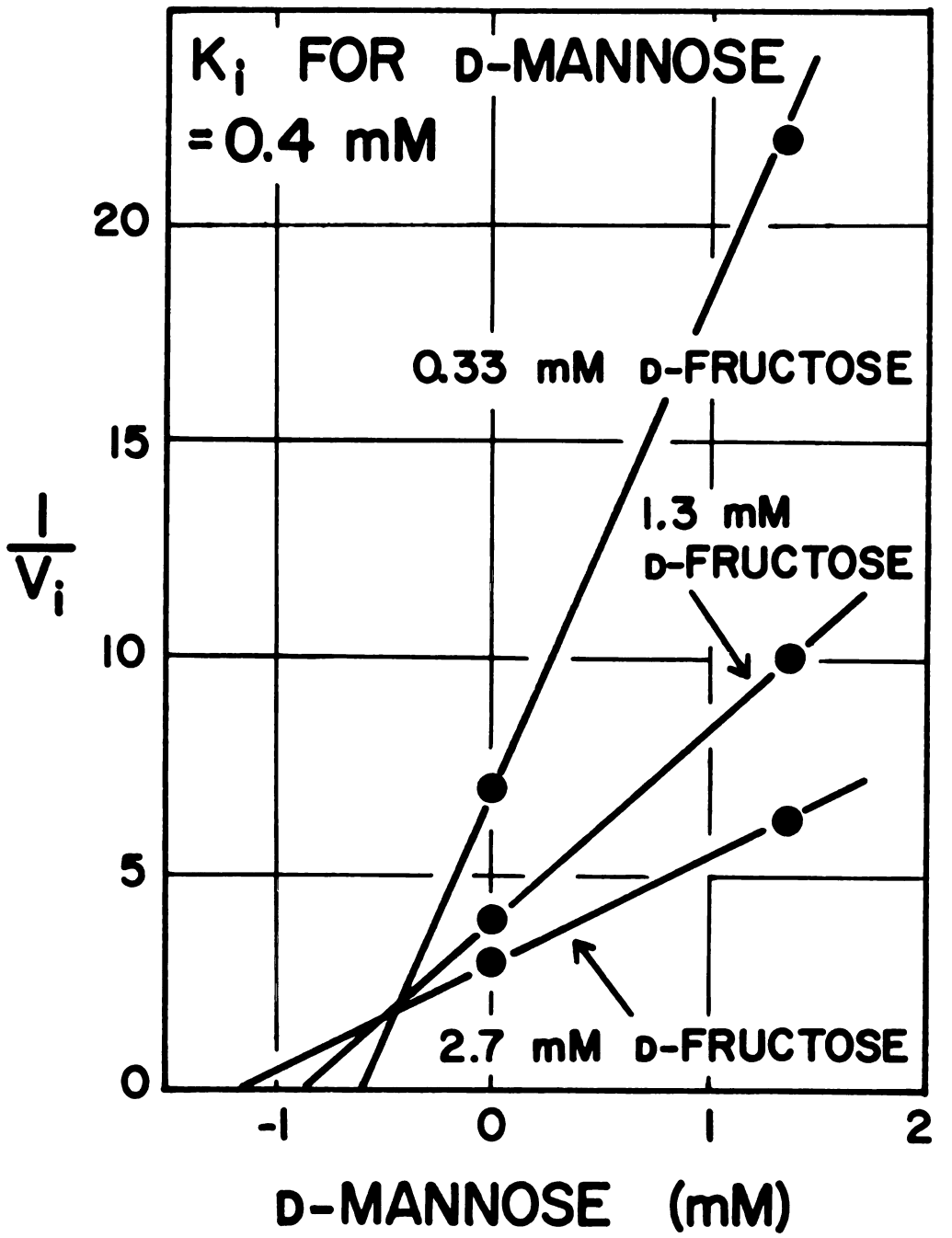

K_i FOR D-MANNOSE
=0.4 mM
0.33 mM D-FRUCTOSE
1.3 mM
D-FRUCTOSE
2.7 mM D-FRUCTOSE
$\frac{1}{V_i}$
20
15
10
5
0
-1
0
1
2
D-MANNOSE (mM)

Figure 11: Lineweaver-Burk plots showing the relationship of reaction velocity to ATP concentration in the presence of various concentrations of ADP. The glucose-6-P dehydrogenase-linked assay was used except that ATP and ADP were varied as indicated. The Mg^{++} concentration was maintained at twice the total concentration of ATP plus ADP. The pH was 6.9.

Figure 11

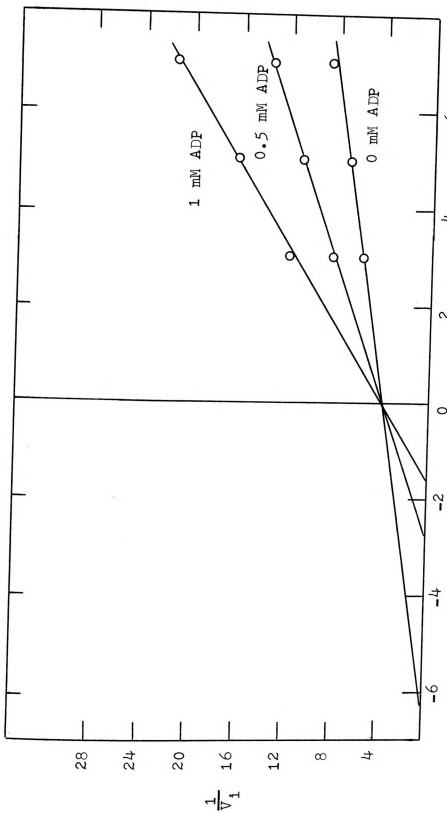
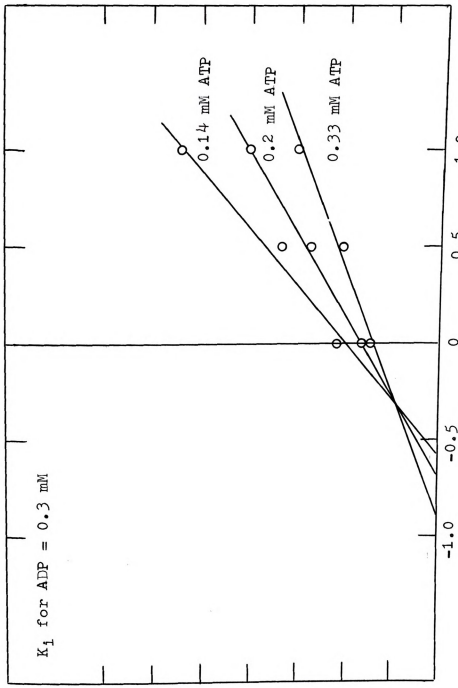

1 mM ADP
0.5 mM ADP
0 mM ADP
$\frac{1}{V_1}$
$\frac{1}{ATP}$ (mM)
28
24
20
16
12
8
4
-6
-4
-2
0
2
4
6

Figure 12: Kinetic plot for determining the K_i for ADP. The data are taken from the experiment described in Figure 11.

Figure 12


K_i for ADP = 0.3 mM
0.14 mM ATP
0.2 mM ATP
0.33 mM ATP
$\frac{1}{V_i}$
28
24
20
16
12
8
4
-1.0
-0.5
0
0.5
1.0
mM ADP

that the man
its molecula
of log S ver
Tanford (25)
approximatel
sedimentatio

The p
reactions we
5 µl (0.06 u
obtained fro
strate (D-fr
µmole of MgC
glycine buff
0.15 ml. Af
addition of
caused no in
tose as the
of glucose-6
increase at
µmole of D-g
with D-manno
0.0048 unit
the cuvette
absorbance i
lent to the

that the mannofructokinase molecule is roughly spherical, its molecular weight was estimated with the aid of a plot of log S versus log molecular weight, using the data of Tanford (25). This method gives a molecular weight of approximately 47,000 for the experimentally determined sedimentation coefficient of 4.1 S.

Product Identification

The products of the mannofructokinase-catalyzed reactions were prepared by incubating in a microcuvette: 5 µl (0.06 unit) of the homogeneous enzyme preparation obtained from disc electrophoresis, 0.02 µmole of substrate (D-fructose or D-mannose), 0.5 µmole of ATP, 1.0 µmole of $MgCl_2$, 0.1 µmole of NADP, 8.0 µmoles of glycylglycine buffer (pH 6.9), and water to a final volume of 0.15 ml. After incubation at 25°C for one hour, the addition of excess glucose-6-P dehydrogenase (0.078 unit) caused no increase in absorbance at 340 nm. With D-fructose as the substrate, the further addition of 0.31 unit of glucose-6-P isomerase resulted in an absorbance increase at 340 nm equivalent to the oxidation of 0.022 µmole of D-glucose-6-P; no change was noted in the cuvette with D-mannose as the substrate. The further addition of 0.0048 unit of mannose-6-P isomerase caused no change in the cuvette with D-fructose but resulted in a 340 nm absorbance increase in the cuvette with D-mannose equivalent to the oxidation of 0.021 µmole of D-glucose-6-P.

Assay

dehydrogenas

in the kinas

negative. Th

phosphorylat

tose-6-P and

Assays for possible contaminating 6-phosphogluconate dehydrogenase, phosphofructomutase, or phosphomannomutase in the kinase preparation and in the coupling enzymes were negative. These results indicate that the products of the phosphorylation of D-fructose and D-mannose were D-fructose-6-P and D-mannose-6-P, respectively.

Previ

nose or D-fr

D-glucose an

efficiencies

hexose (9-12

ent in that

which is an

(D-fructose)

other sugars

basis for thi

tural formuls

tion of molec

31.6% in the

sidered to be

tion that the

in their opti

α-D-mannopyra

ferred confor

tofuranose ar

the positions

models are su

model are ske

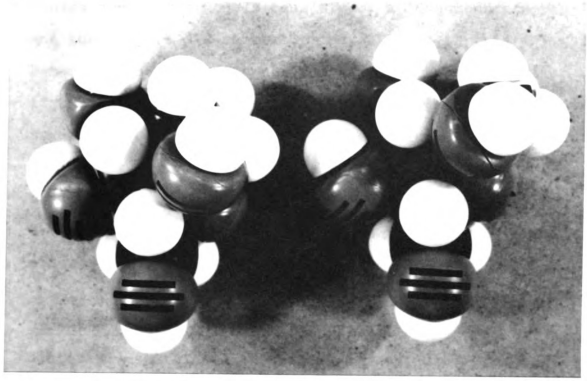
positional co

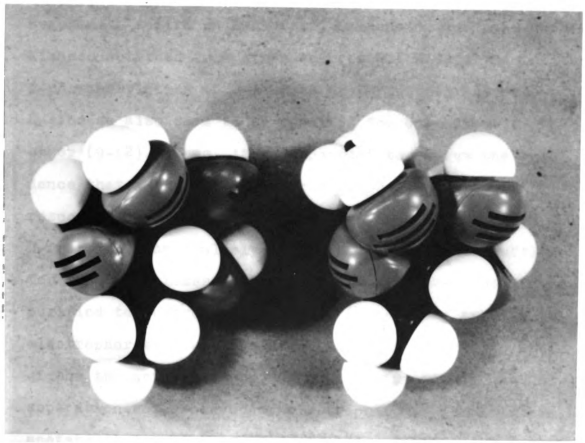
DISCUSSION

Previously reported hexokinases active on D-mannose or D-fructose at carbon atom 6 also phosphorylate D-glucose and often several other sugars with varying efficiencies (26-35), or are specific for a single hexose (9-12). The hexokinase described here is different in that it is equally active on two hexoses, one of which is an aldose (D-mannose) and the other a ketose (D-fructose), but has no detectable activity on many other sugars (<0.04% in the case of D-glucose). The basis for this specificity is not apparent from structural formulas but can be rationalized from an inspection of molecular models. D-Fructose in solution occurs 31.6% in the furanose form (36) and is generally considered to be the β anomer (37), presumably on the assumption that the α and β anomers would differ significantly in their optical rotations. D-Mannose in solution is 69% α-D-mannopyranose (38). Molecular models of the preferred conformations of α-D-mannopyranose and β-D-fructofuranose are depicted in Fig. 13. It can be seen that the positions of the oxygen and hydrogen atoms on the two models are superimposable, although the positions on one model are skewed somewhat relative to the other. This positional correspondence of equivalent atoms holds even

Figure 13: Structural models showing the topological similarity between α-D-mannopyranose (left side of each photograph) and β-D-fructofuranose. Upper, bottom view of the models; lower, top view of the models. Carbon atom 6 is located at the bottom of each photograph.

Figure 13





though one
and the oth
D-mannose a
ity which i
sugars test
enzyme has
strate site
met by both
other sugar

Moor

Streptococc
bacterium,
specific D-f
hexokinase a
Kinases obta
6-phosphoryl
D-glucose al
other (9-12)
dence that a
responsible
D-fructose,
for the two
purified to
electrophore
of the two a
separation w
acetate strip

though one of the models consists of a five-membered ring and the other a six-membered ring. Viewed in this way, D-mannose and D-fructose bear a close structural similarity which is not mimicked by D-glucose or any of the other sugars tested as possible substrates. Thus, although the enzyme has strict requirements for binding at the substrate site, it can be seen how these conditions may be met by both D-mannose and D-fructose to the exclusion of other sugars.

Moore and O'Kane (11) presented evidence that Streptococcus faecalis, a homofermentative lactic acid bacterium, contains a specific D-mannokinase and a specific D-fructokinase in addition to a nonspecific hexokinase active on D-glucose, D-mannose, and D-fructose. Kinases obtained from other sources which catalyze the 6-phosphorylation of D-mannose or D-fructose but not D-glucose also seem to be specific for one hexose or the other (9-12). Thus, it is important to review the evidence that a single enzyme from L. mesenteroides is responsible for the phosphorylation of both D-mannose and D-fructose, particularly since the pH-activity profiles for the two hexoses are different. The enzyme has been purified to apparent homogeneity (determined by disc gel electrophoresis) with no significant change in the ratio of the two activities throughout the purification. No separation was achieved by electrophoresis on cellulose acetate strips or by sucrose density gradient centrifuga-

tion.
value
ally,
tive
the s

and D.
pect
be no
bit di
exampl
genase
(28).

fructo
kinase
data p
ity of
streng
in 0.1
for mo

toklna
of one
K_m of
of subs

tion. Thermal inactivation rates assayed at three pH values were identical for the two activities. And finally, the phosphorylation of the two hexoses was competitive rather than additive, with the K_1 for D-mannose being the same as its K_m as a substrate.

The differential pH-activity profiles for D-mannose and D-fructose are of interest and at first led me to suspect that two enzymes were involved. However, it should be noted that several other enzymes are known which exhibit different pH optima for different substrates, for example, fructose diphosphatase (39), glutamate dehydrogenase (40), and hexokinase from Aspergillus parasiticus (28).

The common identity of the D-mannokinase and D-fructokinase activities and the instability of D-glucokinase of L. mesenteroides were recently corroborated by data presented by DeMoss (14). He attributed the lability of D-glucokinase observed by us to the low ionic strength of our cell extracts. His enzyme preparation in 0.1 M potassium phosphate buffer (pH 7.5) was stable for months at -20°C and for days at 0°C (14).

Although the reaction mechanism of this mannofructokinase has not been studied in detail, the inability of one substrate (hexose or ATP) to affect the apparent K_m of the other is consistent with a sequential mechanism of substrate binding (41), as for yeast hexokinase (42-44),

E. coli galactokinase (45), and rat muscle hexokinase type II (46), rather than with the "ping-pong" type (41), as is characteristic of nucleoside diphosphate kinase (47, 48) and rat muscle hexokinase type I (49). Earlier studies on particulate (50) and solubilized (51) brain hexokinase suggested that these enzymes exhibit a "ping-pong" mechanism of action. Recent data, however, indicate that the above mechanism is incorrect, and that the mechanism appears to be sequential (52, 53).

SUMMARY

An adenosine 5'-triphosphate:hexose 6-phosphotransferase specific for D-mannose and D-fructose (mannofructokinase) was purified to apparent homogeneity from extracts of Leuconostoc mesenteroides. D-mannose and D-fructose were phosphorylated by the enzyme at equal rates, whereas D-glucose, 2-deoxy-D-mannose, and 28 other sugars and sugar derivatives were not phosphorylated and did not inhibit the enzyme. The pH-activity curves were different for D-mannose and D-fructose, with the D-mannokinase activity to D-fructokinase activity ratios being about 1.0 at pH 6.9, 0.5 at pH 8.5, and 0.3 at pH 8.9. The enzyme was further characterized with regard to phosphoryl donor specificity, kinetic constants, inhibition constants, and molecular weight. The products of the phosphorylation of D-mannose and D-fructose were identified as D-mannose-6-P and D-fructose-6-P, respectively.

To explain the unique specificity of this kinase, it was postulated that α-D-mannopyranose and β-D-fructofuranose are the molecular species that serve as substrates. It was shown with molecular models that equivalent functional groups of the preferred conformations of the two species occupy nearly identical spatial positions,

even though one of the molecules consists of a five-membered ring and the other a six-membered ring. Viewed in this way, D-mannose and D-fructose bear a close structural similarity which is not mimicked by D-glucose or any of the other sugars tested as possible substrates. Thus, although the enzyme has strict requirements for binding at the substrate site, it can be seen how these conditions may be met by both D-mannose and D-fructose to the exclusion of other sugars.

PART II

1-PHOSPHOFRUCTOKINASE AND 6-PHOSPHOFRUCTOKINASE FROM *AEROBACTER AEROGENES*

INTRODUCTION

The discovery in 1966 of an inducible kinase specific for D-fructose 1-phosphate in *Aerobacter aerogenes* PRL-R3 (54) suggested a previously unrecognized pathway of D-fructose metabolism. More recently, a four-component PEP:fructose 1-phosphotransferase system from this organism was characterized and genetic evidence was presented for the requirement of the enzyme system for normal growth on D-fructose (55). This work thus established the pathway for D-fructose metabolism to be the following:

$$\text{D-Fructose} \xrightarrow{\text{PEP system}} \text{D-fructose-1-P} \xrightarrow{\text{1-PFK}} \text{FDP}$$

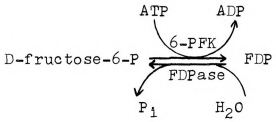
In addition to the PEP system and 1-PFK, *A. aerogenes* PRL-R3 has also an inducible D-fructokinase (ATP:D-fructose 6-phosphotransferase) and a constitutive 6-PFK. An alternate route,

$$\text{D-fructose} \xrightarrow{\text{D-fructokinase}} \text{D-fructose-6-P} \xrightarrow{\text{6-PFK}} \text{FDP},$$

may therefore be operative in this organism. Section A of this part of the thesis assesses the relative impor-

tance of these two pathways during growth on D-fructose. Analysis of mutants lacking 6-PFK and FDPase corroborate the view that D-fructose is metabolized via the D-fructose-1-P pathway and establishes that 6-PFK is functional in the metabolism of D-glucose but not D-fructose. This work has been published recently (56).

The presence in A. aerogenes of two phosphofructokinases with different roles makes it of interest to study the two enzymes and compare their control mechanisms. FDP, the product of the 6-PFK reaction, can be converted back to D-fructose-6-P by FDPase:



The D-fructose-6-P-FDP cycle functions as a net ATPase if not controlled; hence it has been termed a "futile" cycle (57). 1-PFK, on the other hand, is not known to participate in such a cycle. It is therefore to be expected that 6-PFK would be subject to more complex control mechanisms than would 1-PFK. 6-PFK from a variety of organisms is being intensively studied with respect to its regulation (see Review of Literature in Part II, Section B). 1-PFK, on the other hand, which is now known also to occur in Bacteroides symbiosus (58) and Escherichia coli (59), has not previously been sub-

jected to kinetic analysis. The last portion of this thesis (Part II, Section B) presents evidence that the 6-PFK from A. aerogenes, like those from most other organisms, displays sigmoidal kinetics and is modified by several effectors, whereas the 1-PFK exhibits regular Michaelis-Menten kinetics and more closely resembles other non-allosteric kinases.

SECTION A

DETERMINATION OF THE RELATIVE SIGNIFICANCE OF D-FRUCTOSE-1-P AND D-FRUCTOSE-6-P PATHWAYS IN *A. AEROGENES* BY ANALYSIS OF MUTANTS LACKING 6-PHOSPHOFRUCTOKINASE AND D-FRUCTOSE 1,6-DIPHOSPHATASE

MATERIALS AND METHODS

Bacteria

The parental strains used in this investigation were *Aerobacter aerogenes* PRL-R3 and a uracil auxotroph, PRL-R3(U^-), derived from it. The uracil auxotroph was given to us by Dr. Robert P. Mortlock of the University of Massachussetts. Mutant 012, derived from strain PRL-R3, was isolated by T. E. Hanson (56); mutant A9-1, derived from strain PRL-R3(U^-), was isolated by Dr. R. L. Anderson (56).

Culture Media

The basal mineral medium used for strain PRL-R3 and mutant 012 consisted of 0.71% Na_2HPO_4, 0.15% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.009% $MgSO_4$, and 0.0005% $FeSO_4 \cdot 7H_2O$. This medium was supplemented with 0.005% uracil for the growth of strain PRL-R3(U^-) and mutant A9-1. Sugars were autoclaved separately and added to the basal mineral medium at a concentration of 0.5%.

Growth of Cells and Preparation of Extracts

The growth curves were done in 18 x 50 mm culture tubes containing 7.0 ml of medium. The inoculum was 0.1 ml of an overnight culture on D-glucose (except for mutant A9-1, which was on D-fructose). The tubes were slanted at an angle of 55° and were agitated on a water bath reciprocal shaker at 148 cycles per min at 30°C.

For enzyme studies, the cells were grown in 500 ml of medium in Fernbach flasks on a rotary shaker at 32°C. The cells were harvested by centrifugation during the late log phase of growth, suspended in distilled water, and broken by sonication for 10 minutes as described in Part I.

Chemicals

FDP and crystalline α-glycerophosphate dehydrogenase-triose phosphate isomerase were from Sigma. D-fructose-6-P was from Boehringer. D-fructose-1-P, yeast glucose-6-P dehydrogenase (A grade), rabbit muscle FDP aldolase (A grade), and crystalline rabbit muscle glucose-6-P isomerase (A grade) were from Calbiochem. All other chemicals were obtained as described in Part I.

Enzyme Assays

All assays involved the oxidation or reduction of pyridine nucleotide coenzymes and were monitored at 340 nm

with a Gilford automatic absorbance-recording spectrophotometer thermostated at 25°C. The reactions were carried out in 0.15-ml volumes in microcuvettes with a 1-cm light path. In all cases, the amount of extract assayed was limiting, so that the rates were proportional to the enzyme concentration. Specific activity was defined as the number of μmoles of substrate utilized per minute per milligram of protein.

The assays for 1-PFK and 6-PFK contained 1.0 μmole of ATP; 2.0 μmoles of $MgCl_2$; 0.05 μmole of NADH; 1.0 μmole of D-fructose-1-P or D-fructose-6-P; excess FDP aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase; and 10.0 μmoles of buffer [glycylglycine (pH 7.5) for 1-PFK, and glycine (pH 8.2) for 6-PFK]. The control assays contained all components of the reaction mixture except ATP.

The assay for FDPase contained 1.0 μmole of FDP, 1.0 μmole of $MgCl_2$, 0.2 μmole of EDTA, 0.1 μmole of NADP, excess glucose-6-P isomerase and glucose-6-P dehydrogenase, and 10.0 μmoles of glycylglycine buffer (pH 7.5).

The assay for D-fructokinase activity contained 1.0 μmole of D-fructose, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.1 μmole of NADP, excess phosphoglucose isomerase and glucose-6-P dehydrogenase, and 10.0 μmoles of glycylglycine buffer (pH 7.5).

The assay for D-glucokinase, as described by Kamel, Allison, and Anderson (17), contained 1.0 μmole

of D-glucose, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.1 μmole of NADP, excess glucose-6-P dehydrogenase, and 10.0 μmoles of glycylglycine buffer (pH 7.5).

Protein Determination

Protein was estimated as described in Part I.

RESULTS

Growth Pattern

Growth characteristics of the parental strain (PRL-R3) and the two mutants (A9-1 and 012) on D-glucose, D-fructose, and glycerol are shown in Fig. 14. Strain PRL-R3 grew well on all three substrates. Mutant A9-1 mimicked the parent on D-fructose and glycerol, but grew only slowly on D-glucose. Mutant 012 grew well on D-glucose but failed to grow on D-fructose or glycerol; after 24 hr, slight growth occurred occasionally on D-fructose but not on glycerol.

Enzyme Activities in Cell Extracts

The data in Table VI show that all strains contained similar levels of D-glucokinase, whereas mutant 012 was missing FDPase and mutant A9-1 was missing 6-PFK. D-Fructokinase activity was low in all extracts, but was consistently higher in cells grown on D-fructose than on D-glucose. This apparent D-fructokinase activity has not been purified, so it has not been established that the observed activity in crude cell extracts is the result of a single enzyme possessing ATP:D-fructose 6-phosphotransferase activity. The presence, however, of a phosphofructomutase in the extract and the coupling

Figure 14: Growth characteristics of strain PRL-R3 and mutants 012 and A9-1 on D-glucose, D-fructose, and glycerol. The growth pattern of PRL-R3(U^-) (not shown) was the same as that for PRL-R3. An optical density of 0.35 was equivalent to a viable count of 8.2×10^8 cells per ml.

Figure 14

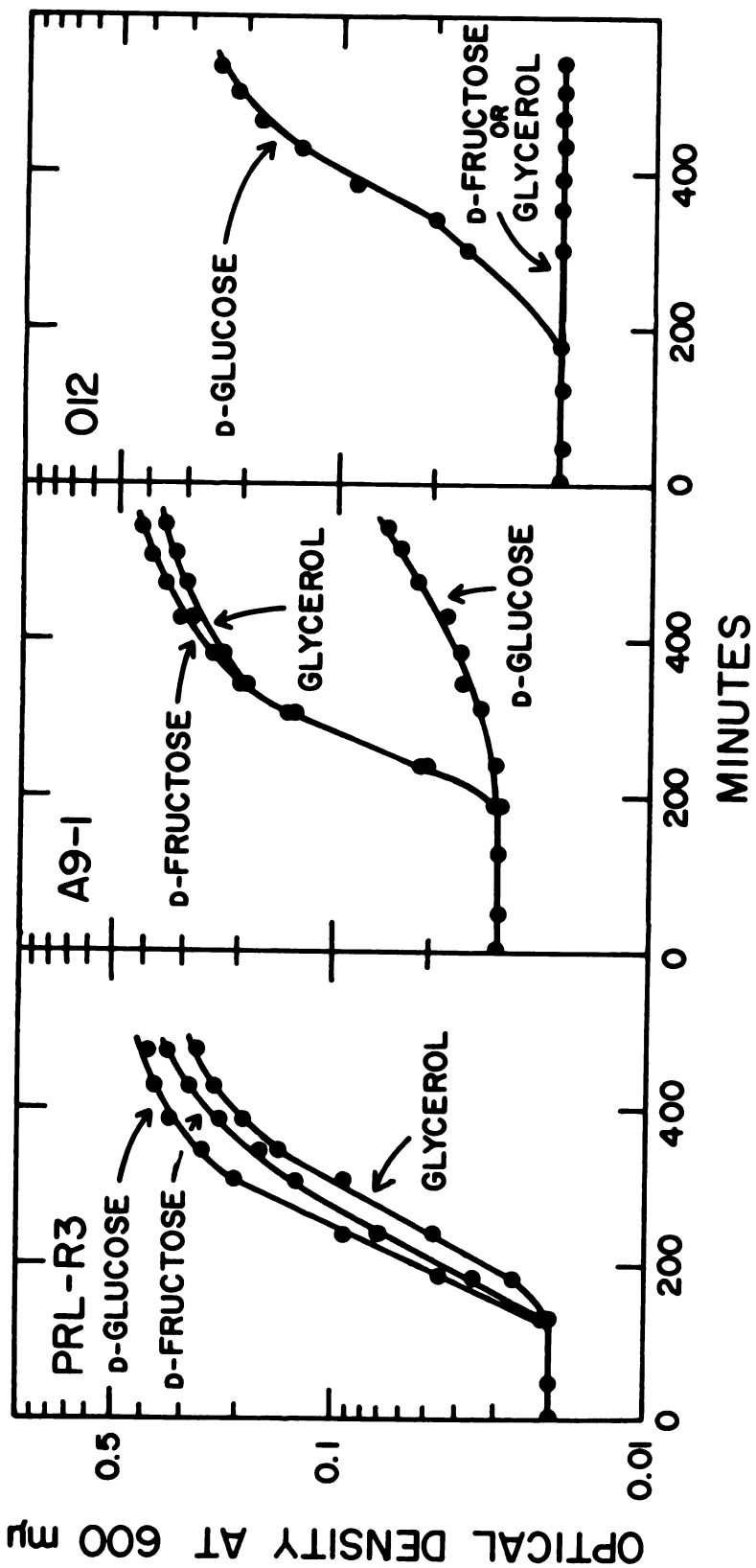

PRL-R3
A9-1
012
D-GLUCOSE
D-FRUCTOSE
GLYCEROL
D-FRUCTOSE OR GLYCEROL
0.5
0.1
0.01
0
200
400
MINUTES
OPTICAL DENSITY AT 600 mμ

TABLE VI

Enzyme activities* in crude cell extracts of Aerobacter aerogenes

Strain	Growth substrate	D-Fructose 1,6-diphosphatase	6-Phospho-fructokinase	1-Phospho-fructokinase	D-Glucokinase	D-Fructokinase
PRL-R3	D-Glucose	0.032	0.060	< 0.002	0.099	0.002
	D-Fructose	0.032	0.065	0.095	0.111	0.009
PRL-R3(U^-)	D-Glucose	0.034	0.070	< 0.002	0.120	0.003
	D-Fructose	0.033	0.084	0.111	0.117	0.013
012	D-Glucose	< 0.0002	0.060	< 0.002	0.110	0.003
A9-1	D-Fructose	0.028	< 0.002	0.096	0.112	0.011

*Results are expressed as micromoles of substrate utilized per minute per milligram of protein in the standard assays.

enzymes under the assay conditions was ruled out since the replacement of D-fructose plus ATP with 1.0 μmole of D-fructose-1-P in the assay gave no detectable reaction; thus the product of the D-fructokinase reaction was shown to be D-fructose-6-P.

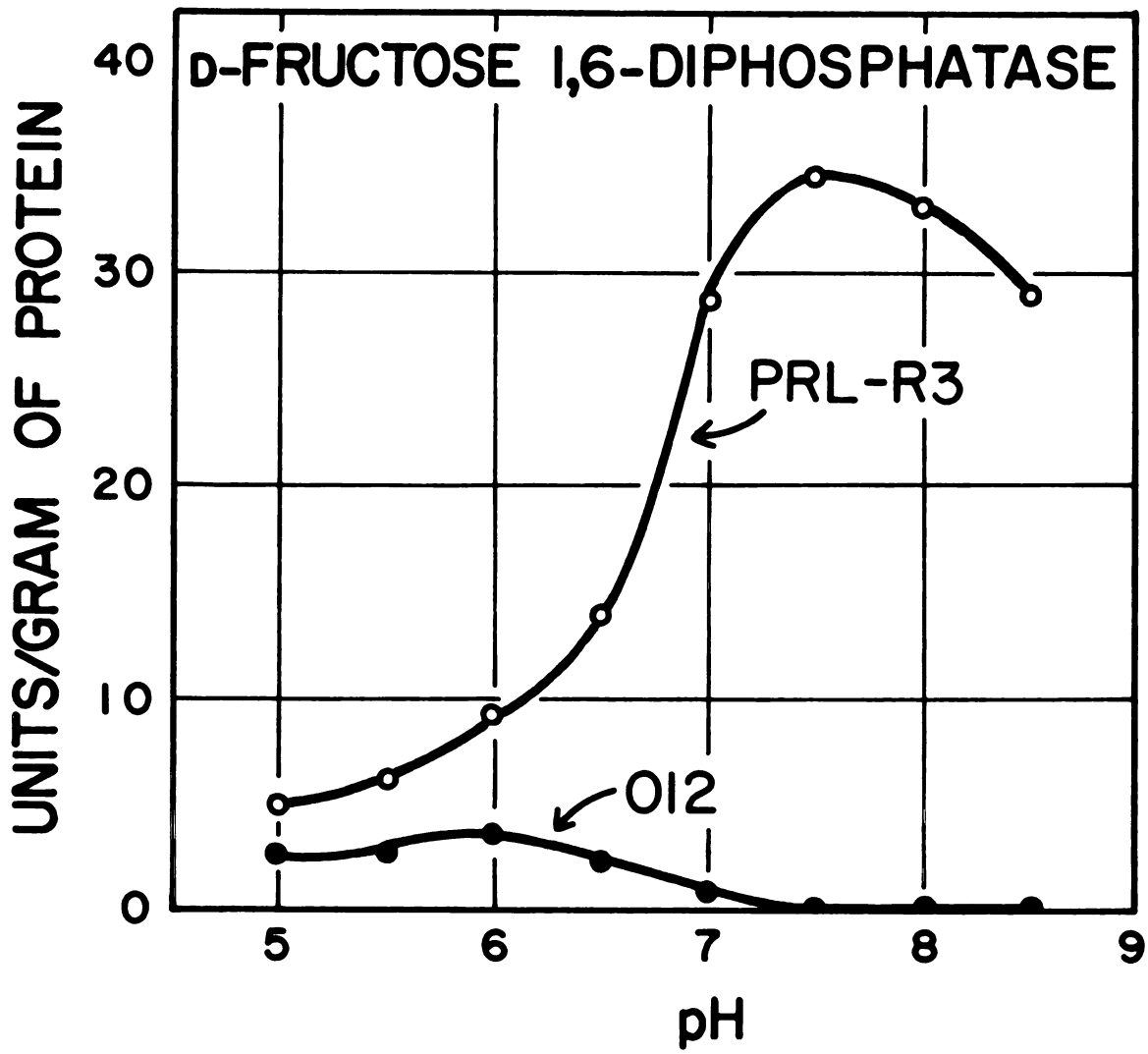
1-PFK was found only in extracts of cells grown on D-fructose (Table VI). The inability of mutant 012 to grow on D-fructose precluded a measurement of 1-PFK in this strain under conditions which induced the enzyme in the other strains. However, partial induction was achieved by incubating D-glucose-grown cells in 0.25% D-glucose plus 0.25% D-fructose in mineral medium for a period of time sufficient to allow complete utilization of the D-glucose. Under these conditions, the specific activities in extracts were 0.12 in strain PRL-R3 and 0.013 in mutant 012. When the cells were harvested and extracts prepared before D-glucose utilization was complete, 1-PFK activity remained undetectable, indicating repression in the presence of D-glucose. The partial induction observed in mutant 012 probably occurred in the short period of growth just before or immediately after the D-glucose was exhausted and D-glucose repression was relieved. A further attempt was made to induce 1-PFK in mutant 012 under conditions in which D-glucose repression would be absent by exposing D-glucose-grown cells to 0.25% D-fructose in nutrient broth (0.5% Difco peptone plus 0.3% Difco beef extract,

pH 7.0). 1-PFK activity remained undetectable in mutant 012, although a normal level of activity was induced in strain PRL-R3. This lack of induction in mutant 012 may be attributed to catabolite repression, which is known to be enhanced during catabolism under nongrowing conditions (60, 61); mutant 012 does not grow on nutrient broth, which is consistent with its lack of FDPase.

FDPase activity as a function of assay pH is shown in Figure 15. The extract from mutant 012 exhibited some activity at low pH values, but no activity at pH 7.5, which was the pH optimum for FDPase activity in extracts of strain PRL-R3 and mutant A9-1. The activity at low pH values is believed to be due to a nonspecific acid hexose phosphatase (62).

Figure 15: pH-Activity profile of FDPase. The standard assay was used except that the buffer composition and pH were varied. Buffers (10 μmoles) used were: cacodylate, pH 5.0 to 6.5; glycylglycine, pH 7.0 to 8.0; and glycine, pH 8.5. The profile for A9-1 (not shown) was the same as that shown for PRL-R3.

Figure 15


D-FRUCTOSE 1,6-DIPHOSPHATASE
UNITS/GRAM OF PROTEIN
40
30
20
10
0
5
6
7
8
9
pH
PRL-R3
012

DISCUSSION

The possible common pathways for the metabolism of D-glucose, D-fructose, and glycerol in A. aerogenes are as summarized in the scheme shown in Fig. 16. The metabolism of D-glucose through the Embden-Meyerhof pathway requires 6-PFK. If D-fructose were metabolized via D-fructose-6-P, then it follows that 6-PFK would also be required for normal growth on this substrate. On the other hand, if it were metabolized through D-fructose-1-P, then the 6-PFK-catalyzed reaction would be bypassed. In the latter case, normal growth on D-fructose would require FDPase to make D-fructose-6-P for biosynthetic reactions. Normal growth on glycerol would likewise require FDPase.

Mutant A9-1, missing 6-PFK, grows on D-fructose or glycerol as well as does the parental strain, PRL-R3 (U^-), but grows only slowly on D-glucose. This is consistent with the metabolism of D-fructose through D-fructose-1-P. If fructose were metabolized through D-fructose-6-P rather than D-fructose-1-P, this mutant would still be expected to grow well on glycerol, but no better on D-fructose than on D-glucose. The residual growth on D-glucose by this mutant could indicate that the defective 6-PFK is partially functioning in the

Figure 16: Pathways for the metabolism of D-glucose, D-fructose, and glycerol in A. aerogenes.

Figure 16

D-GLUCOSE
D-GLUCOSE-6-P
D-FRUCTOSE
D-FRUCTOSE-6-P
ATP
A9-1
ADP
D-FRUCTOSE-1-P
D-FRUCTOSE-1,6-P
P_i
012
H_2O
D-GLYCERALDEHYDE-3-P
DIHYDROXYACETONE-P
GLYCEROL

intact cell, but is more likely due to the metabolism of D-glucose through the hexose monophosphate shunt.

Mutant 012, missing FDPase, grows well on D-glucose but not on D-fructose or glycerol. This, too, is consistent with D-fructose being metabolized in the wild type primarily through D-fructose-1-P rather than D-fructose-6-P. If the pathway through D-fructose-6-P were of major significance, a FDPase-negative mutant would be expected to grow on both D-glucose and D-fructose.

Similar mutant analysis has recently been carried out on E. coli to assess the relative importance of the D-fructose-1-P and the D-fructose-6-P pathways in D-fructose metabolism of this organism (59, 62, 63). Although an earlier paper indicated that the metabolism goes through D-fructose-6-P (62), more recent results (59) indicate that the D-fructose-1-P pathway occurs also in E. coli. However, some mutants of this organism deficient in 6-PFK activity failed to grow normally on D-fructose, suggesting that 6-PFK may have some role in D-fructose metabolism (63). The identification of a PEP:D-fructose 1-phosphotransferase system and a 1-PFK in this organism provides support for the D-fructose-1-P pathway (59).

SECTION B

PURIFICATION, PROPERTIES, AND REGULATION OF 6-PHOSPHOFRUCTOKINASE AND 1-PHOSPHOFRUCTOKINASE

REVIEW OF LITERATURE

The central role of 6-PFK in the control of glycolysis is well recognized (57, 64-67). *In vivo* experiments determining metabolite flux during glycolysis have indicated that the main rate-controlling step is the 6-PFK-catalyzed production of FDP from D-fructose-6-P (68, 69). Studies of the kinetic properties of the enzyme provide important information on the possible mechanism for its control. Table VII gives a summary of most of these studies. Although some results varied with the system under investigation, it seems clear that the enzyme is subject to a number of complex control mechanisms. In all cases [except with the 6-PFK from *Dictyostelium discoideum* (91)], the enzyme is inhibited by high levels of ATP. In some studies, however, it is not certain whether the inhibition is a function of the amount of ATP *per se* or to the lack of sufficient Mg^{++} to bind all the ATP molecules in a MgATP complex (84, 89, 90), which is the real substrate of the reaction (113). With several mammalian 6-PFKs significant ATP inhibition was detected under conditions

TABLE VII

Comparison of the properties of A. aerogenes 1-PFK and 6-PFK with those of the 6-PFKs from other sources

Effector	Sheep brain	Sheep heart
ATP	Inhibits (greater at pH 7 than at pH 8) (71).	Inhibits at pH 6.9 but not at pH 8.2 (72).
D-Fructose-6-P	Relieves ATP inhibition (70,71); does not relieve Mg^{++} inhibition (71).	Relieves ATP inhibition (72).
FDP	Relieves ATP and citrate inhibition (70,71); activates (71).	Activates and relieves ATP inhibition (72).
Mg^{++}	Inhibits at pH 8 with K_i of 4-5 mM (71).	
Other NTPs	CTP, GTP, UTP, TTP and ITP are also substrates (71); GTP is less inhibitory than ATP (71).	UTP, ITP, and CTP are also substrates (72).
Cyclic AMP	Activates by lowering K_m for ATP and D-fructose-6-P and by increasing V_{max} (71).	Activates at non-inhibitory ATP levels; relieves ATP inhibition (72).
AMP	Relieves citrate inhibition (70); activates like cyclic AMP and relieves ATP inhibition (71).	Same effect as cyclic AMP (72).

ADP	Activates at 0.05 mM; inhibits at higher levels (71).	Same effect as cyclic AMP (72).
Other NMPs	GMP, IMP, CMP, UMP, TMP, and 3'-AMP have no effect (71).	
Other NDPs		
P_i	Relieves ATP and citrate inhibition (70,71).	
Citrate	Inhibits (more effective at pH 7 than at pH 8) (70).	
NH_4^+	Activates at non-inhibitory ATP levels (71); synergistic effect with P_i and AMP at pH 7 (71).	
pH	ATP inhibition greater at pH 7 than at pH 8 (71).	Hyperbolic kinetics at pH 8.2; sigmoidal kinetics at pH 6.9 (72).
Sedimentation behavior and MW		MW of native enzyme greater than 300,000 (72).

TABLE VII - Continued

Effector	Rabbit muscle	Guinea pig heart	Rat kidney cortex
ATP	Inhibits at pH 6.9 to 7.1 (73-75).	Inhibits at pH 6.9 but not at pH 8.2 (78).	Inhibits (80).
D-Fructose-6-P	Relieves ATP inhibition (74).	Relieves ATP inhibition (78).	Relieves ATP inhibition (80).
FDP	Activates in the presence of 2.5 mM ATP; no effect with 4 mM ATP (74).		Relieves ATP and citrate inhibition (80).
Mg^{++}			
Other NTPs	UTP inhibits like ATP; CTP is moderately inhibitory; ITP and GTP do not inhibit at 5 mM (73).	UTP is both substrate and inhibitor (78).	
Cyclic AMP	Relieves ATP inhibition (73-75).	Relieves ATP inhibition at pH 6.9; inhibits at pH 7.6 and 8.0; relieves UTP inhibition (78).	
AMP	Relieves ATP inhibition (73-75); relieves UTP inhibition (73).	Less effective than cyclic AMP (78).	Relieves ATP and citrate inhibition (80).

ADP	Little activation when used alone; becomes more effective with P_i (74).	Less effective than cyclic AMP (78).	Relieves ATP inhibition (80).
Other NMPs	CMP relieves ATP inhibition (73); UMP, IMP, and GMP have no effect (73).	CMP, GMP, IMP, UMP, and cyclic 2',3'-CMP have no effect (78).	
Other NDPs		CDP, IDP, and UDP have no effect (78).	
P_i	Relieves ATP inhibition (74,75).	Slight activation at 0.1 mM (78).	Relieves ATP inhibition (80).
Citrate			Inhibits synergistically with ATP (80).
NH_4^+	Activates; does not affect degree of ATP inhibition (75).		
pH	No ATP inhibition at pH 8 to 9; considerable ATP inhibition below pH 7 (73,75).	Cyclic AMP inhibits at pH 7.6 and 8.0 (78).	
Sedimentation behavior and MW	MW of active enzyme: 3.8×10^5 (76); MW of protomer: 93,200 (77).	S varies from 15.2 to 28.8 S (79).	

TABLE VII - Continued

Effector	Rat adipose tissue	Rat liver	Rat heart
ATP	Inhibits (81).	Inhibits (82).	Inhibits (83).
D-Fructose-6-P		Relieves ATP inhibition (82).	Relieves ATP inhibition (83).
FDP		Relieves ATP inhibition; effect less marked with citrate present (82).	
Mg^{++}			
Other NTPs			
Cyclic AMP	Relieves ATP inhibition (81).		
AMP	Relieves ATP inhibition (81).	Relieves ATP inhibition; effect less marked with citrate present (82).	

		effect less marked with citrate present (82).	
ADP	Below 1 mM; relieves ATP inhibition; higher levels inhibit (81).		
Other NMPs			
Other NDPs			
P_i	Relieves ATP inhibition (81).		Relieves ATP inhibition (83).
Citrate	Inhibits (81).	Inhibits synergistically with ATP (82).	Inhibits synergistically with ATP; increases K_m for D-fructose-6-P (83).
NH_4^+		Protects enzyme from inactivation; relieves ATP inhibition (82).	
pH			
Sedimentation behavior and NW			

TABLE VII - Continued

Effector	Calf lens	Cockroach
ATP	Inhibits (84).	Noncompetitive inhibitor of D-fructose-6-P (85).
D-Fructose-6-P	Does not relieve ATP inhibition (84).	Does not relieve ATP inhibition (85).
FDP		
Mg^{++}		
Other NTPs	GTP inhibits (50% as effective as ATP) (84).	
Cyclic AMP	Relieves ATP inhibition; activates at non-inhibitory ATP levels (84).	Activates at low ATP levels; no effect at higher (2.6 mM) ATP levels (85).
AMP	Slight release of ATP inhibition (84).	Activates when added with P_i and NH_4^+ (85).
ADP	Slight release of ATP inhibition (84).	

Other NMPs	CMP, UMP, and GMP slightly relieves ATP inhibition (84).	
Other NDPs		
P_i	Slight relief of ATP inhibition (84).	Activates when added with AMP and NH_4^+ (85).
Citrate	Inhibits at concentrations above 2.5 mM (84).	
NH_4^+		Relieves ATP inhibition (85).
pH	Greater ATP inhibition below pH 9 (84).	
Sedimentation behavior and MW		

TABLE VII - Continued

Effector	Liver fluke	Brussels sprout	Carrot
ATP	Inhibits (86).	Inhibits (88).	Inhibits (89).
D-Fructose-6-P	Relieves ATP inhibition (86).	Relieves ATP inhibition in sprouts; non-sigmoidal kinetics in leaves (88).	Relieves ATP and citrate inhibition (89).
FDP			
Mg^{++}	Maximal activity at a Mg^{++} to ATP ratio of 10:1 (87).		At 20 mM, relieves ATP and citrate inhibition (89).
Other NTPs	GTP, UTP, and CTP have no effect (86).	GTP and ITP are substrates and less inhibitory than ATP (88).	
Cyclic AMP	Activates by lowering K_m for D-fructose-6-P; relieves ATP inhibition (86,87).		
AMP	Relieves ATP inhibition (87).	Inhibits synergistically with ATP (88).	Inhibits; enhances ATP and citrate inhibition (89).

ADP	No effect at 0.1 mM (86,87).	Inhibits synergistically with ATP (88).	Inhibits; enhances ATP and citrate inhibition (89).
Other NMPs			
Other NDPs	UDP and CDP do not activate (86).		
P_i		Activates in young and mature leaves; relieves ATP inhibition in sprouts (88).	Partially relieves ATP and citrate inhibition (89).
Citrate		Inhibits (88).	Inhibits synergistically with ATP (89).
NH_4^+			
pH			ATP more inhibitory below pH 7; Mg^{++} at 20 mM restores activity below pH 7 only (89).
Sedimentation behavior and MW			

TABLE VII - Continued

Effector	Avocado	Parsley	Slime mold
ATP	Inhibits (90).	Inhibits (90).	Does not inhibit; competitively overcomes ADP inhibition; affects K_m for D-fructose-6-P (91).
D-Fructose-6-P	Relieves ATP inhibition (90).	Relieves ATP inhibition (90).	Competitively overcomes FDP inhibition; affects K_m for ATP (91).
FDP			Competitive inhibitor of D-fructose-6-P; uncompetitive inhibitor of ATP (91).
Mg^{++}			
Other NTPs			GTP, CTP, ITP, and UTP can be used as phosphoryl donors (91).
Cyclic AMP	Inhibits (90).	No effect (90).	No effect (91).
AMP	Inhibits (90).	No effect (90).	No effect (91).

ADP	Inhibits (90).	Inhibits (90).	Competitive inhibitor of ATP; uncompetitive inhibitor of D-fructose-6-P (91).
Other NMPs			No effect with CMP, GMP, IMP, and UMP (91).
Other NDPs			GDP and UDP inhibit; almost no effect with CDP and IDP (91).
P_i	No effect (90).	Relieves ATP and ADP inhibition (90).	No effect (91).
Citrate			No effect (91).
NH_4^+			
pH			
Sedimentation behavior and MW			

TABLE VII - Continued

Effector	Yeast	*Clostridium perfringens*	*Staphylococcus aureus*
ATP	Inhibits (92-94).	Inhibits (90).	Inhibits (90).
D-Fructose-6-P	Relieves ATP inhibition (92-94).		Relieves ATP inhibition (90).
FDP	No effect (93).		
Mg^{++}			
Other NTPs	ITP, GTP, and CTP are substrates but not inhibitors (92-94).		
Cyclic AMP	No effect (92-94).		Activates (90).
AMP	Relieves ATP inhibition (92,94).		Activates (90).
ADP	No effect (92-94).	Relieves ATP and P_i inhibition (90).	Activates (90).

Other NMPs	No effect with CMP, GMP, and UMP (94).		
Other NDPs		GDP and IDP relieve ATP and P_i inhibition; UDP and CDP are slightly effective (90).	
P_i	No effect (92,93).	Inhibits (90).	Activates slightly (90).
Citrate			
NH_4^+			
pH	pH optimum with 1 mM ATP = pH 7.8; pH optimum with 0.4 mM ATP = pH 8.2 (94).		
Sedimentation behavior and MW	$S_{20,w}$ = 17.8 S MW = 5.8×10^5 (94).		

TABLE VII - Continued

Effector	Escherichia coli	A. aerogenes 6-PFK (this thesis)	A. aerogenes 1-PFK (this thesis)
ATP	Inhibits at Mg^{++} to ATP ratios below 2:1 (95,96).	Inhibits (more pronounced at low Mg^{++} levels).	Inhibits at Mg^{++} to ATP ratios below 2:1.
D-Fructose-6-P	Relieves ATP inhibition (95,96); lowers K_m for Mg^{++} (95).	Relieves ATP inhibition; does not relieve inhibition by other NTPs.	Competitive inhibitor of D-fructose-1-P; K_i = 1.5 mM.
FDP	No effect (97).		Competitive inhibitor of D-fructose-1-P; K_i = 7 mM.
Mg^{++}	Relieves ATP inhibition (95).	Relieves ATP inhibition.	Relieves ATP inhibition.
Other NTPs	ITP is a substrate but not an inhibitor (95).	CTP, GTP, ITP, UTP, and TTP are substrates and inhibit at Mg^{++}/NTP ratios below 2:1.	GTP and ITP are substrates and inhibit at Mg^{++}/NTP ratios below 2:1.
Cyclic AMP	No effect (95,97).	No effect.	No effect.
AMP	No effect (96).	Relieves ATP inhibition (adenylate kinase present in preparation).	No effect.

ADP	Relieves ATP inhibition (95, 96); competitive inhibitor of ATP at high levels (97).	Same as for AMP (see above).	No effect.
Other NMPs	No effect with IMP, UMP, CMP, and GMP (95).	No effect with CMP, GMP, IMP, and UMP.	No effect with CMP, GMP, IMP, and UMP.
Other NDPs	GDP, IDP, CDP, and UDP relieve ATP inhibition (96).	IDP, GDP, CDP, and UDP relieve ATP inhibition.	No effect with CDP, GDP, IDP, and UDP.
P_i	Inhibits (90).	No effect.	No effect.
Citrate	No effect (97).	No effect.	Competitive inhibitor of D-fructose-1-P; K_i = 0.85 mM.
NH_4^+			
pH		No effect on ATP inhibition.	
Sedimentation behavior and MW		MW ~ 100,000	MW ~ 75,000

of excess Mg^{++} (71-75, 80-83, 92-94), indicating that the MgATP complex can bind to a regulatory site and inhibit the reaction. Equilibrium binding studies by Kemp and Krebs (98) on skeletal muscle 6-PFK have indicated that three moles of ATP are bound per 90,000 g of the enzyme, which is probably the molecular weight of the protomer (77). E. coli 6-PFK is inhibited by ATP only when the Mg^{++} to ATP ratio falls below 2:1 (95, 96). Blangy et al (97) failed to detect ATP inhibition at a constant Mg^{++} to ATP ratio of 10:1.

Increased levels of D-fructose-6-P generally relieve ATP inhibition (70-74, 78, 80, 82, 83, 86, 88-90, 92-96). Exceptions are the 6-PFKs from cockroach (85) and calf lens (84). The rates of most 6-PFKs studied (71-74, 78, 80, 82, 83, 86, 88-90, 92-96) show a sigmoidal dependence on the D-fructose-6-P concentration. E. coli 6-PFK also shows a sigmoidal dependence of rate on the Mg^{++} concentration; increasing the D-fructose-6-P concentration from 1.2 mM to 4.8 mM serves to convert the sigmoidal curve to a hyperbolic one (95).

6-PFK is generally non-specific with regard to its phosphoryl donor (71, 72, 78, 88, 91-95). Further, several mammalian 6-PFKs are inhibited by other nucleoside triphosphates (71, 73, 78) in a manner similar to ATP inhibition. In yeast (92-94) and E. coli (95), however, other nucleoside triphosphates either inhibit

the enzyme very slightly or not at all even at low Mg^{++} concentrations.

Mammalian 6-PFKs in general have more positive effectors than those from plants or bacteria. AMP and cyclic AMP, which can relieve ATP inhibition of many mammalian 6-PFKs (71-75, 78, 80-82, 84) either have no effect (90-97) or even inhibit activity (88-90) of the enzyme from several plants and bacteria. ADP relieves ATP inhibition to various degrees in mammalian (71, 72, 74, 78, 80, 81, 84) and bacterial (90, 95, 96) 6-PFKs but was found to inhibit the enzyme from several plants (88-90).

Some mammalian 6-PFKs have been reported to be subject to control by pH. Calf lens (84) and rabbit muscle (73, 75) 6-PFKs are considerably inhibited at pH values near 7, but are inhibited very slightly or not at all at pH 9. In a similar manner, the enzyme from rat diaphragm (99) is more susceptible to ATP inhibition at pH 7.1 than at pH 7.6. Sheep heart 6-PFK exhibits regular Michaelis-Menten kinetics at pH 8.2 with respect to ATP, Mg^{++}, or D-fructose-6-P, but shows sigmoidal kinetics with respect to D-fructose-6-P at pH 6.9 (72). Increased concentrations of D-fructose-6-P shift the pH optimum of frog muscle 6-PFK towards the acid side, and this effect is further enhanced by the presence of AMP (100).

The only 6-PFK reported so far in the literature to exhibit non-sigmoidal kinetics is that from Dictyostelium discoideum (91). This enzyme is not inhibited by ATP, and is not activated by D-fructose-6-P, AMP, or P_i. ADP, PP_i, and FDP inhibit activity.

In addition to A. aerogenes, 1-PFK has now been reported to occur in Bacteroides symbiosus (58) and E. coli (59). However, no kinetic studies of this enzyme have yet been published.

MATERIALS AND METHODS

Bacteria

The organism used for most of the studies on 6-PFK is the wild-type Aerobacter aerogenes PRL-R3. Mutant DD31, lacking 1-PFK activity, was used for the inhibition study with D-fructose-1-P; this mutant was derived from the wild-type strain and was isolated by T. E. Hanson by the same procedure as for mutant 012 (56). Mutant A9-1, lacking 6-PFK activity, was used as the source of 1-PFK; this mutant was derived from the parental strain PRL-R3(U^-) and was isolated by Dr. R. L. Anderson (56).

Growth of Cells and Preparation of Extracts

The components of the mineral salts medium are as described in Part II, Section A. Strains PRL-R3 and DD31 were grown in 500 ml of glucose-mineral salts medium in Fernbach flasks on a rotary shaker at 32°C. The cells were harvested by centrifugation at 16,300 x g in a Servall refrigerated centrifuge.

Mutant A9-1 was grown in 100 liters of D-fructose-mineral salts medium supplemented with 0.005% uracil in a New Brunswick Model 130 Fermacell fermenter at 30°C

with an aeration rate of 6 cubic feet per minute and an agitation speed of 300 rpm. Dow Corning antifoam B (0.02%, sterilized separately) was added. The inoculum was 2 liters of an overnight culture in D-fructose-uracil-mineral salts medium. The cells were harvested with a Sharples AS-12 centrifuge 8 hr after inoculation. The yield was about 7.5 g (wet weight) of cells per liter.

Cells were washed once with 0.01 M Tris-HCl-0.03 M NaCl (pH 7.3) and centrifuged at 16,300 x g. Mutant A9-1 was taken up in water, while strain PRL-R3 and mutant DD31 were taken up in 0.05 M phosphate buffer (pH 7.5) containing 0.001 M EDTA (for the reason for the use of phosphate buffer and EDTA, see Properties, section on Stability). The resulting suspension was subjected to sonic oscillation for 10 min as described in Part I.

Chemicals

Crystalline α-glycerophosphate dehydrogenase and triose phosphate isomerase were from Calbiochem. Pig heart malic dehydrogenase and twice crystallized yeast alcohol dehydrogenase were from Worthington. D-Fructose-1-P, D-glucose-1-P, D-glucose-6-P, horse heart cytochrome c, and all nucleotides (except ATP) were from Sigma. L-Fructose-1-P was the preparation described by Mayo and Anderson (101). Calcium phosphate gel was prepared by D. P. Allison of this laboratory according to the method

described by O. Levin (102). All other chemicals were obtained as described in Part I or Part II, Section A.

Enzyme Assays

The routine assay for 6-PFK was as described in Part II, Section A, except that glycylglycine buffer (pH 8.0) was used. The aldolase-linked assay for 1-PFK was as described in Part II, Section A. The pyruvate kinase-lactate dehydrogenase-linked assay for 1-PFK was used for substrate specificity and FDP inhibition studies. The reaction mixture (0.15 ml) contained: 1.0 μmole of D-fructose-1-P, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.4 μmole of PEP, 0.05 μmole of NADH, 10.0 μmoles of glycylglycine buffer (pH 7.5), excess lactate dehydrogenase and pyruvate kinase, and limiting amounts of 1-PFK. The assays were done in microcuvettes with a 1-cm light path. The rate of oxidation of NADH was measured at 340 nm in a Gilford multiple sample absorbance-recording spectrophotometer thermostated at 25°C.

In all cases, the rates were directly proportional to the enzyme concentration. The rate of NADH oxidation with the aldolase-linked assay for 1-PFK was double that obtained with the pyruvate kinase-lactate dehydrogenase-linked assay. A unit of activity was defined as the number of μmoles of substrate phosphorylated per minute. Specific activity was defined as the number of units per milligram of protein.

The assay for mannitol-1-P dehydrogenase was the same as the routine assay for 6-PFK, except that ATP was omitted from the reaction mixture. The assay for FDPase was as described in Part II, Section A. The assay for adenylate kinase was the same as the aldolase-linked assay for 6-PFK or 1-PFK, except that ATP was replaced with 1.0 μmole of ADP.

The assays for malic dehydrogenase and alcohol dehydrogenase were done in microcuvettes with a 1-cm light path and monitored at 340 nm with a Gilford spectrophotometer. The reaction mixture for alcohol dehydrogenase contained, in a volume of 0.15 ml: 10 μmoles of ethanol, 0.1 μmole of NAD, 10.0 μmoles of glycylglycine buffer (pH 7.5), and limiting amounts of alcohol dehydrogenase. The reaction mixture for malic dehydrogenase contained, in a volume of 0.15 ml: 1.0 μmole of oxaloacetate (neutralized to pH 7.0), 0.05 μmole of NADH, 10.0 μmoles of glycylglycine buffer (pH 7.5), and limiting amounts of malic dehydrogenase.

Protein Determination

Total protein was estimated as described in Part I. Cytochrome c was determined by measuring absorbance at 550 nm.

RESULTS

Purification of 6-Phosphofructokinase

All operations were performed at 0-4°C. A summary of the purification procedure is given in Table VIII.

Protamine Sulfate Precipitation

The protein concentration of the crude extract was adjusted to 14 mg per ml. After the addition of solid ammonium sulfate to a final concentration of 0.2 M, the nucleic acids were precipitated by the slow addition of 20% by volume of a 2% aqueous solution of protamine sulfate (pH 7.0). The precipitate obtained by centrifugation was discarded.

Ammonium Sulfate Fractionation

Solid ammonium sulfate was added slowly with stirring to the above fraction. The precipitate that formed between 0 and 60% saturation was collected by centrifugation and dissolved in 0.05 M sodium phosphate buffer-0.001 M EDTA (pH 7.5).

Sephadex G-200 Chromatography

A 1-ml aliquot of the ammonium sulfate fraction

TABLE VIII

Purification of 6-phosphofructokinase from *Aerobacter aerogenes* PRL-R3

Fraction	Volume	Total protein	Total activity	Recovery	Specific activity[b]
	ml	mg	units[a]	%	
Crude extract	50	700	93	100	0.13
Protamine sulfate supernatant	56	672	104	100	0.16
0-60% $(NH_4)_2SO_4$ precipitate	8	336	94	100	0.28
Sephadex G-200 fractions 42-55	34[c]	68	51	55	0.75

[a]Units: μmoles of D-fructose-6-P phosphorylated per minute at 25°C and pH 8.0.

[b]Specific activity: units per milligram of protein.

[c]Extrapolated to a total of 8 ml put on Sephadex G-200 column.

was placed on a 23.5 x 1.2-cm column of Sephadex G-200 equilibrated with 0.05 M sodium phosphate buffer-0.001 M EDTA-0.1 M ammonium sulfate (pH 7.5). Fractions (15 drops) were eluted with the same solution and collected in a Gilson Medical Electronics linear fraction collector. The elution pattern is shown in Fig. 17. Fractions 42 to 55, which had the highest specific activity, were combined. The 6-PFK activity in the combined fraction was 6-fold purified over the crude extract.

Attempts to purify the enzyme further by DEAE-cellulose column chromatography, calcium phosphate gel adsorption, and pH fractionation led to a considerable loss of 6-PFK activity. The Sephadex fraction had contaminating activities of adenylate kinase (0.25 unit/mg), mannitol-1-P dehydrogenase (0.12 unit/mg), 1-PFK (0.06 unit/mg), and FDPase (0.27 unit/mg).

Purification of 1-Phosphofructokinase

The procedure described here for the purification of 1-PFK is a modification and extension of the procedure of Hanson and Anderson (54). All operations were performed at 0-4°C. A summary of the purification is given in Table IX.

Protamine Sulfate Precipitation

The protein concentration of the crude extract was adjusted to 8 mg per ml. Nucleic acids were precipi-

Figure 17: Elution pattern of 6-PFK on Sephadex G-200 column. Details are given in the text.

Figure 17

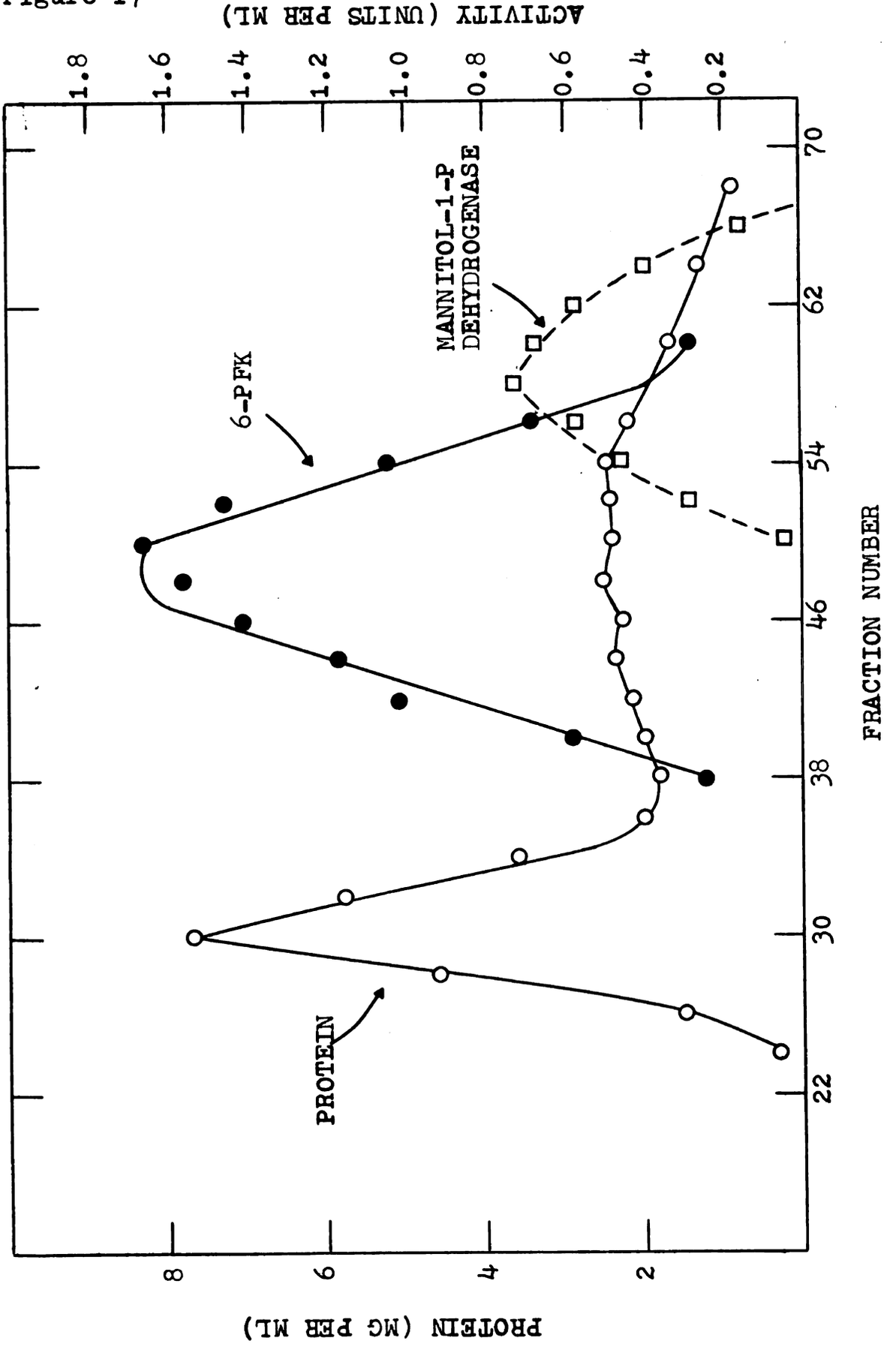

ACTIVITY (UNITS PER ML)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
MANNITOL-1-P DEHYDROGENASE
6-PFK
PROTEIN
70
62
54
46
38
30
22
FRACTION NUMBER
8
6
4
2
PROTEIN (MG PER ML)

TABLE IX

Purification of 1-phosphofructokinase from mutant A9-1

Fraction	Volume	Total protein	Total activity	Recovery	Specific activity[b]
	ml	mg	units[a]	%	
Crude extract	900	7200	105	100	0.15
Protamine sulfate supernatant	1050	8400	86	82	0.10
30-60% $(NH_4)_2SO_4$ precipitate	48	3464	103	99	0.30
G-200 fractions 43-52	595[c]	680	79	76	1.12
$Ca_3(PO_4)_2$ gel	336	54	66	63	12.2
0-70% $(NH_4)_2SO_4$ precipitate	2	20	48	46	17.0
pH 4.6 supernatant	4	9.2	43.5	41	47.0

[a]Units: μmoles of D-fructose-1-P phosphorylated per minute at 25°C and pH 7.5.

[b]Specific activity: units per milligram of protein.

[c]Extrapolated to a total of 48 ml put on Sephadex G-200 column.

tated by the slow addition, with constant stirring, of 20% by volume of 2% aqueous solution of protamine sulfate (pH 7). The precipitate obtained by centrifugation was discarded.

Ammonium Sulfate Fractionation

Solid $(NH_4)_2SO_4$ was added slowly with stirring to the protamine sulfate supernatant and the precipitate which formed between 0 and 30% saturation was removed by centrifugation and discarded. The fraction precipitating between 30 and 60% saturation was collected by centrifugation and dissolved in glass-distilled water.

Sephadex G-200 Chromatography

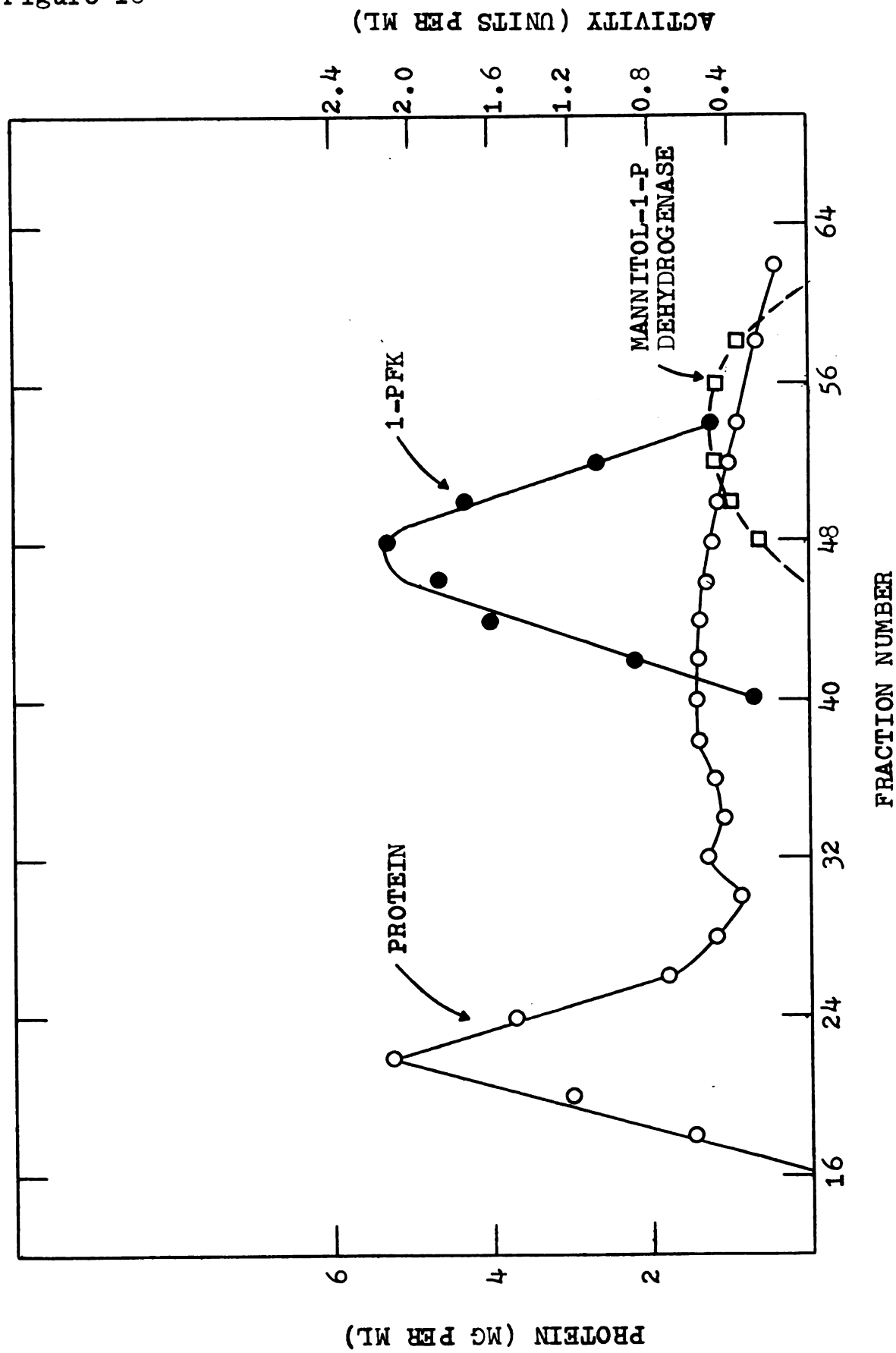
Sixteen ml of the above fraction was layered on a column (45 x 4.5 cm) of Sephadex G-200 equilibrated with 0.02 M sodium phosphate buffer (pH 7.5). The same buffer solution was used to elute 400-drop fractions, which were collected on a Gilson Medical Electronics fraction collector. The elution profile is shown in Fig. 18. Fractions 43 to 52, containing the highest 1-PFK specific activity, were combined.

Calcium Phosphate Gel Adsorption and Elution

The combined Sephadex fraction (1.2 mg protein per ml) was treated with 10% by volume of calcium phosphate

Figure 18: Elution pattern of 1-PFK on a Sephadex G-200 column. Details are given in the text.

Figure 18


ACTIVITY (UNITS PER ML)
2.4
2.0
1.6
1.2
0.8
0.4
MANNITOL-1-P DEHYDROGENASE
1-PFK
PROTEIN
64
56
48
40
32
24
16
FRACTION NUMBER
6
4
2
PROTEIN (MG PER ML)

gel containing 62 mg solids per ml. The suspension was stirred well for 5 minutes and centrifuged. The gel solids were washed with 0.02 M sodium phosphate buffer (pH 7.5), and treated twice (10 min per treatment) with 0.075 M sodium phosphate buffer (pH 7.5). The supernatant from each treatment was discarded. 1-PFK activity was eluted by mixing the gel solids well with 0.15 M sodium phosphate buffer (pH 7.5) for 15 min. The elution was repeated to recover about 80% of the adsorbed 1-PFK. The two eluates were combined.

Ammonium Sulfate Precipitation

The combined eluate was concentrated by precipitation with solid ammonium sulfate (70% saturation) and dissolving the precipitate in glass-distilled water.

pH Fractionation

The concentrated gel eluate (10 mg protein per ml) was diluted two-fold with glass-distilled water. The pH of the solution was carefully adjusted, with constant stirring, to pH 4.6 with 7.5% acetic acid. The supernatant obtained upon centrifugation was treated with 0.1 M NaOH to pH 7.5. 1-PFK activity in this preparation was approximately 315-fold purified over the crude extract and was essentially free from adenylate kinase (<0.0003 unit/mg), mannitol-1-P dehydrogenase (<0.0007 unit/mg),

and FDPase (< 0.0003 unit/mg).

Properties and Regulation of 6-Phosphofructokinase and 1-Phosphofructokinase

Stability

6-PFK readily lost activity in crude extracts prepared in water, but was stable at -20°C for approximately 1-2 weeks in 0.05 M sodium phosphate buffer-0.001 M EDTA (pH 7.5). The Sephadex G-200 fraction [in 0.05 M sodium phosphate buffer-0.001 M EDTA-0.1 M $(NH_4)_2SO_4$ (pH 7.5)] was stable at -20°C for about two months.

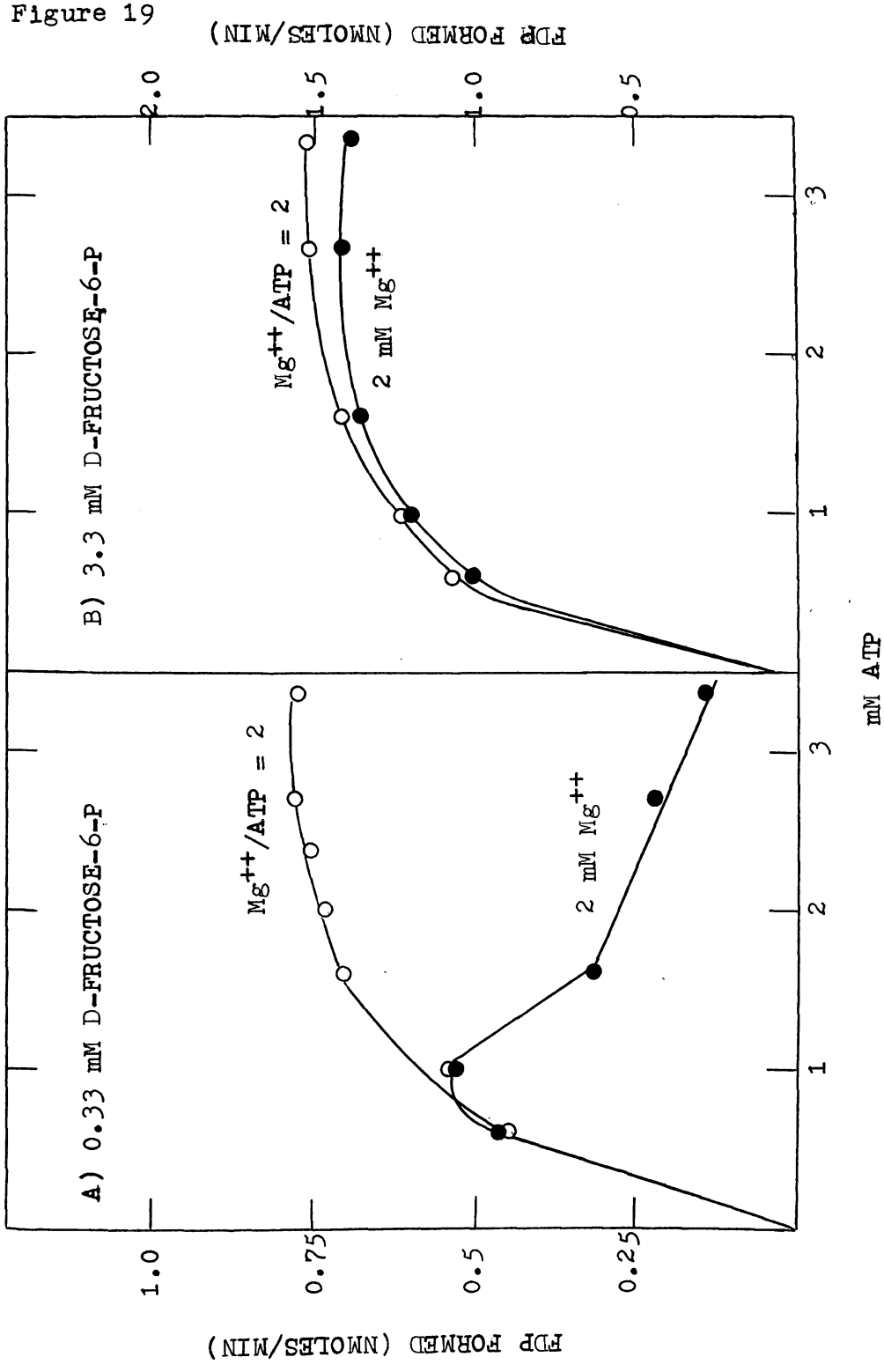
1-PFK was stable for weeks at -20°C in crude extracts prepared in water. The 315-fold purified enzyme preparation (pH 4.6 supernatant) in 0.1 M $(NH_4)_2SO_4$ (pH 7.5) was stable for at least 8 months at -20°C if not repeatedly thawed and frozen.

ATP Inhibition

At 0.33 mM D-fructose-6-P and 2.0 mM Mg^{++}, 6-PFK was inhibited by ATP at concentrations above 1.0 mM. The inhibition became undetectable when the Mg^{++} to ATP ratio was maintained at 2:1 from 0.6 mM to 3.3 mM ATP (Fig. 19 A & B). Raising the concentration of D-fructose-6-P to 3.3 mM while keeping the Mg^{++} constant at 2.0 mM also served to reverse the previously observed inhibition by ATP (Fig. 19B).

Figure 19: Inhibition of 6-PFK by ATP in the presence of various concentrations of Mg^{++} and D-fructose-6-P. Mg^{++} and ATP were varied as indicated in the plots. A, Effect of ATP in the presence of 0.33 mM D-fructose-6P. B, Effect of ATP in the presence of 3.3 mM D-fructose-6-P. The amount of enzyme in all assays was 0.0015 unit.

Figure 19



in the presence
of Mg^{++} and
ATP were varied
A, Effect of
3 mM D-fructose-
he presence of
e amount of
.0015 unit.

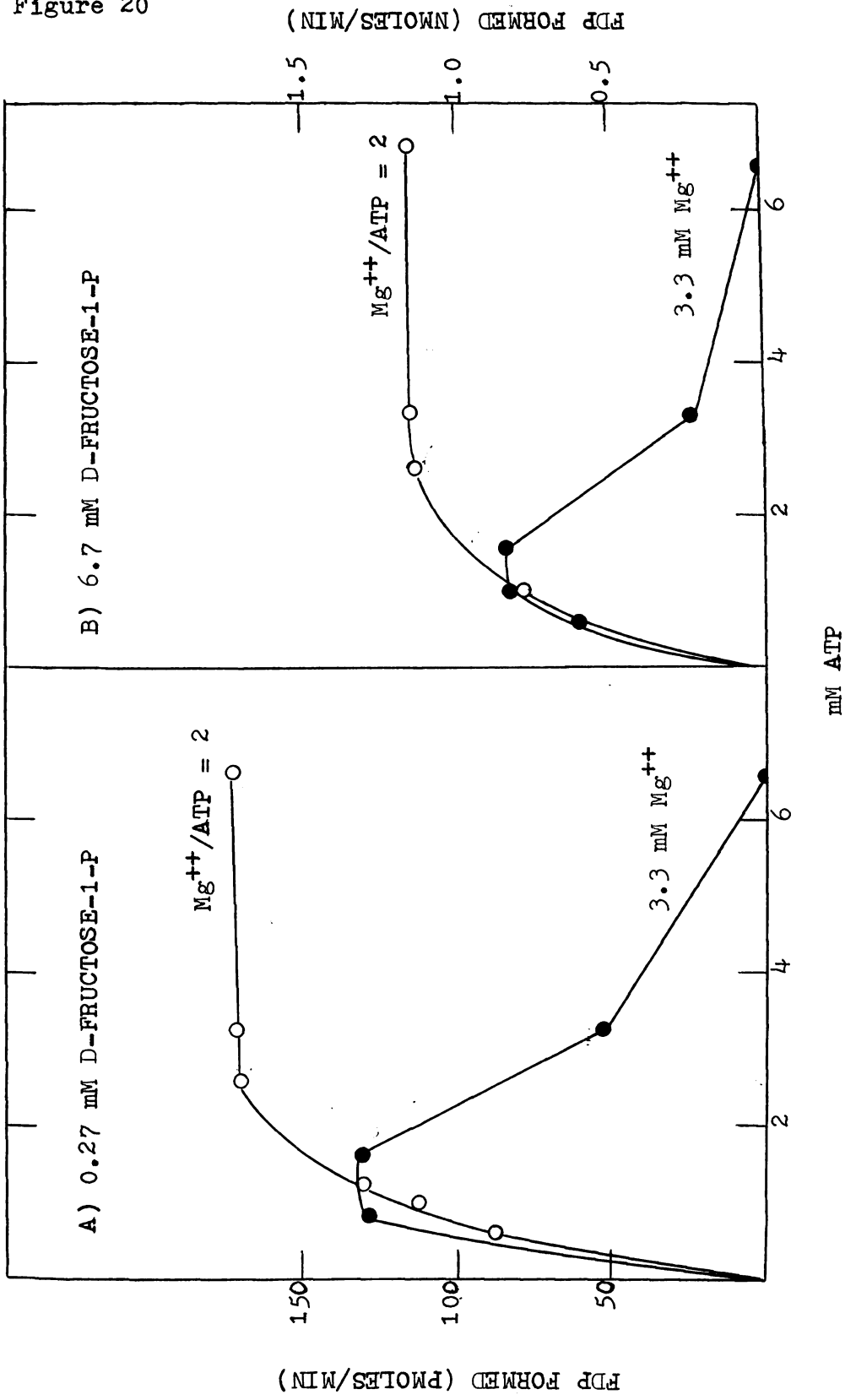
ATP at concentrations above 1.7 mM inhibited 1-PFK when the Mg^{++} and D-fructose-1-P concentrations were maintained at 3.3 mM and 0.27 mM, respectively (Fig. 20A). As observed with 6-PFK, the inhibition was prevented by the addition of Mg^{++} at twice the concentration of ATP in the assay throughout the range of ATP concentrations tested. However, raising the level of D-fructose-1-P from 0.27 mM to 6.7 mM and keeping the Mg^{++} constant at 3.3 mM had no effect on the inhibition of ATP at concentrations above 1.7 mM (Fig. 20B).

Interaction of Hexose Phosphate Substrate With ATP and Mg^{++}

A plot for 6-PFK of rate vs D-fructose-6-P concentration (Fig. 21A) gave a sigmoidal curve, which became more marked as the Mg^{++} concentration was decreased while ATP was maintained at 2 mM. The increase in the sigmoidal character of the curve at lowered Mg^{++} concentrations indicates a weakened affinity of the enzyme for D-fructose-6-P. As shown in the plot, the apparent K_m values for D-fructose-6-P depended on the Mg^{++} to ATP ratio and were approximately 0.3 mM at a ratio of 2:1, 0.6 mM at a ratio of 1.4:1, and 1.4 mM at a ratio of 0.7:1. Increased levels of D-fructose-6-P relieved ATP inhibition, with more D-fructose-6-P being required at low Mg^{++} concentrations. The shapes of the curves suggest a cooperative interaction between Mg^{++} and D-fructose-6-P (103).

Figure 20: Inhibition of 1-PFK by ATP in the presence of various concentrations of Mg^{++} and D-fructose-1-P. Mg^{++} and ATP were varied as indicated. The amount of enzyme in all assays was 0.0011 unit. A, Effect of ATP in the presence of 0.27 mM D-fructose-1-P. B, Effect of ATP in the presence of 6.7 mM D-fructose-1-P.

Figure 20



in the presence
f Mg^{++} and
TP were varied
f enzyme in all
Effect of ATP
D-fructose-1-P,
sence of 6.7 m

Figure 21: Dependence of initial velocity on the hexose phosphate concentration under conditions of varying Mg^{++} to ATP ratios. ATP was maintained at 2 mM throughout the determination. Mg^{++} was varied as indicated. A, Plot of rate vs D-fructose-6-P concentration for the 6-PFK reaction. The amount of enzyme in all assays was 0.0018 unit. B, Plot of rate vs D-fructose-1-P concentration for the 1-PFK reaction. The amount of enzyme in all assays was 0.0016 unit.

Figure 21

city on the
tion under
to ATP
d at 2 mM
on. Mg^{++}
A, Plot of
centration
he amount of
.0018 unit.
ose-i-P con-
eaction.
l assays was

On the other hand, a plot of rate vs D-fructose-1-P concentration with 1-PFK (Fig. 21B) shows a hyperbolic dependence of initial velocity on D-fructose-1-P concentration under conditions of varying Mg^{++} and a constant ATP level of 2 mM. The apparent K_m of 1-PFK remained constant at about 0.7 mM with varying amounts of Mg^{++} in the assays. The absence of sigmoidicity in the Michaelis-Menten plot for 1-PFK suggested the inability of D-fructose-1-P at the concentrations tested to relieve ATP inhibition.

The K_m of 1-PFK for D-fructose-1-P (0.75 mM) did not vary with ATP concentration (Fig. 22). D-Fructose-1-P concentration had no effect on the K_m for ATP, which remained constant at approximately 0.7 mM (Fig. 23).

Effect of Mg^{++}

Fig. 24A shows that with 6-PFK, a sigmoidal curve of rate vs Mg^{++} concentration was obtained at a low D-fructose-6-P concentration (0.33 mM). An increase in the D-fructose-6-P level to 1.33 mM led to a shift from a sigmoidal to a regular hyperbolic curve, indicating that increased D-fructose-6-P concentrations could decrease the requirement of the reaction for Mg^{++}.

A similar plot for 1-PFK also shows a sigmoidal dependence of rate on the Mg^{++} concentration (Fig. 24B). However, D-fructose-1-P could not substitute for Mg^{++}, as

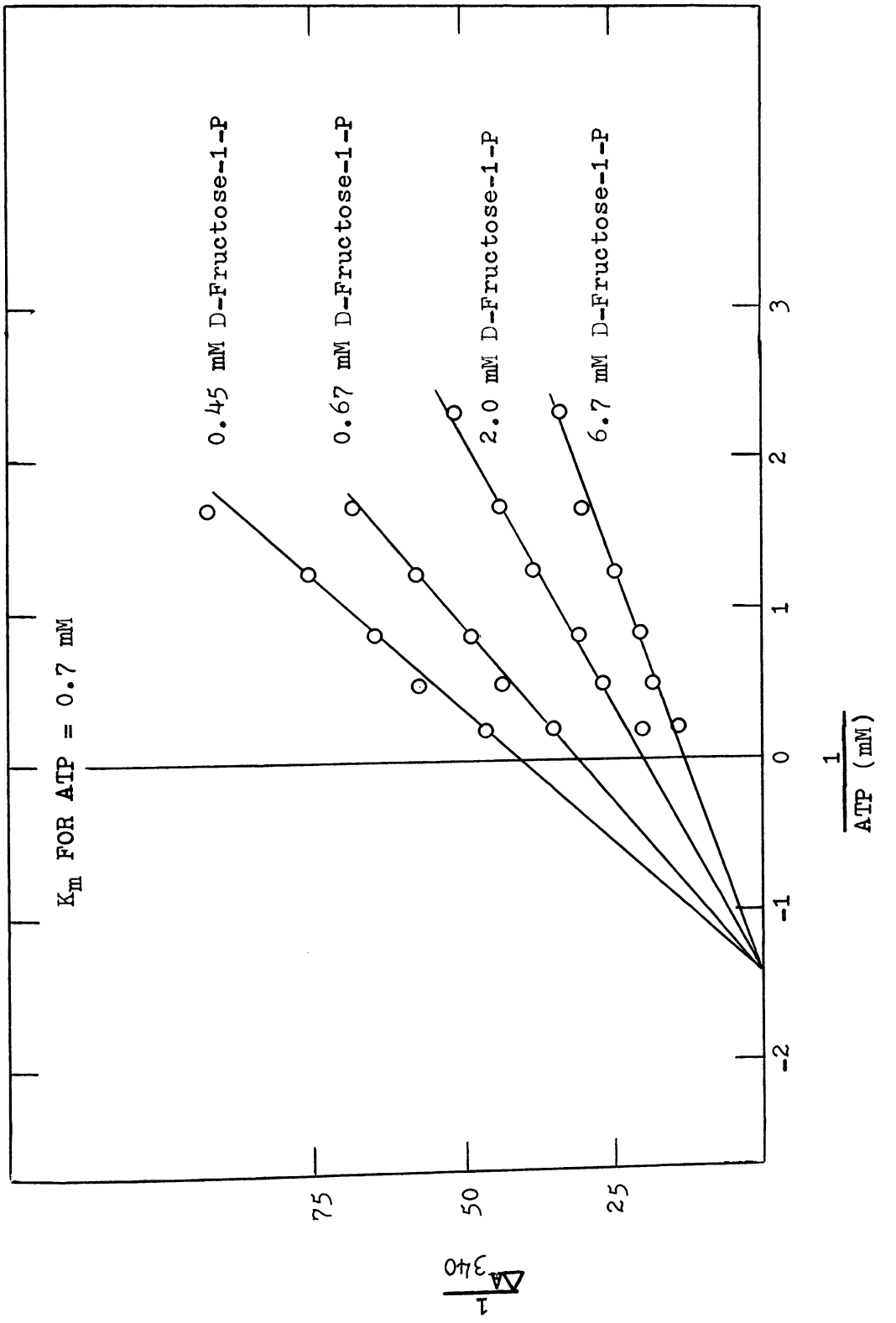
Figure 22: Lineweaver-Burk plot for the determination of the K_m of 1-PFK for D-fructose-1-P in the presence of various ATP concentrations. ATP and D-fructose-1-P were varied as indicated, and Mg^{++} was maintained at twice the ATP concentration throughout the determination. The amount of enzyme in all assays was 0.001 unit.

Figure 22

che determina-
or D-fructose-
rious ATP con-
ructose-1-P
and Mg^{++} was
TP concentra-
ination. The
ssays was 0.00

Figure 23: Lineweaver-Burk plot for determining the K_m of 1-PFK for ATP in the presence of various concentrations of D-fructose-1-P. ATP and D-fructose-1-P were varied as indicated, and Mg^{++} was maintained at twice the ATP concentration. The amount of enzyme in all assays was 0.001 unit.

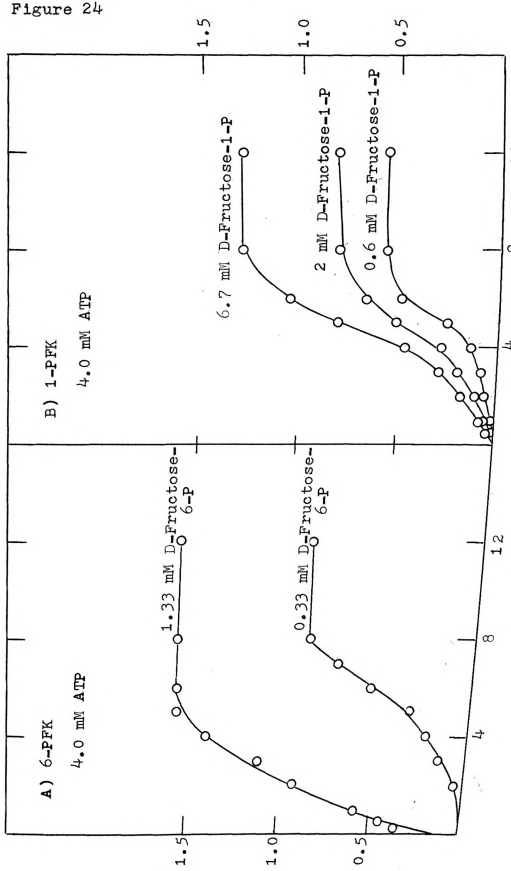
Figure 23



etermining the
presence of
D-fructose-1-P.
e varied as
intained at
n. The amount
s 0.001 unit.

Figure 24: Dependence of initial velocity on Mg^{++} concentration in the presence of various concentrations of the hexose phosphate substrate. ATP was maintained at 4 mM, and Mg^{++} and hexose phosphate concentrations were varied as indicated. A, Plot of rate of the 6-PFK reaction vs Mg^{++} concentration. The amount of enzyme in all assays was 0.0015 unit. B, Plot of rate of the 1-PFK reaction vs Mg^{++} concentration. The amount of enzyme in all assays was 0.0013 unit.

Figure 24



city on Mg^{++}
nce of vari-
hexose phos-
maintained
se phosphate
l as indicated.
PFK reaction
he amount of
0.0015 unit.
PFK reaction
he amount of
0.0013 unit.

shown by the absence of a shift from a sigmoidal to a hyperbolic curve when the D-fructose-1-P level was raised from 0.6 mM to 6.7 mM.

Effect of Other Nucleoside Triphosphates

6-PFK is non-specific with regard to its phosphoryl donor (Table X). The purine nucleotides, GTP and ITP, seem to be better donors than the pyrimidine nucleotides, CTP, UTP, and TTP. The enzyme showed a hyperbolic dependence of rate on D-fructose-6-P concentration with all the nucleoside triphosphates tested (Fig. 25A). At a Mg^{++} to nucleotide ratio of 0.7:1, the apparent K_m for D-fructose-6-P with all the nucleoside triphosphates tested was approximately 0.3 mM, which is the same as that with ATP as a phosphoryl donor at a Mg^{++} to ATP ratio of 2:1.

CTP inhibited the activity when the Mg^{++} to CTP ratio was less than 2:1, and failed to do so when the Mg^{++} was present at twice the concentration of CTP (Fig. 26 A & B). D-Fructose-6-P, when increased from 0.33 mM to 3.3 mM, failed to substitute for Mg^{++} (Fig. 26B). This is in contrast with the finding with ATP as a phosphoryl donor (Fig. 19 A & B), in which a high concentration of D-fructose-6-P relieved ATP inhibition at a Mg^{++} to ATP ratio below 2:1. All the other nucleoside triphosphates gave results similar to those obtained with CTP.

TABLE X

Phosphoryl donor specificity of 6-phosphofructokinase and 1-phosphofructokinase

Nucleoside triphosphate	Relative 6-PFK activity	Relative 1-PFK activity
	%	%
ATP	100	100
ITP	93	43
GTP	85.5	35
UTP	57	3.9
CTP	50	9.8
TTP	50	8.8

All nucleoside triphosphates were tested at a concentration of 3.3 mM.

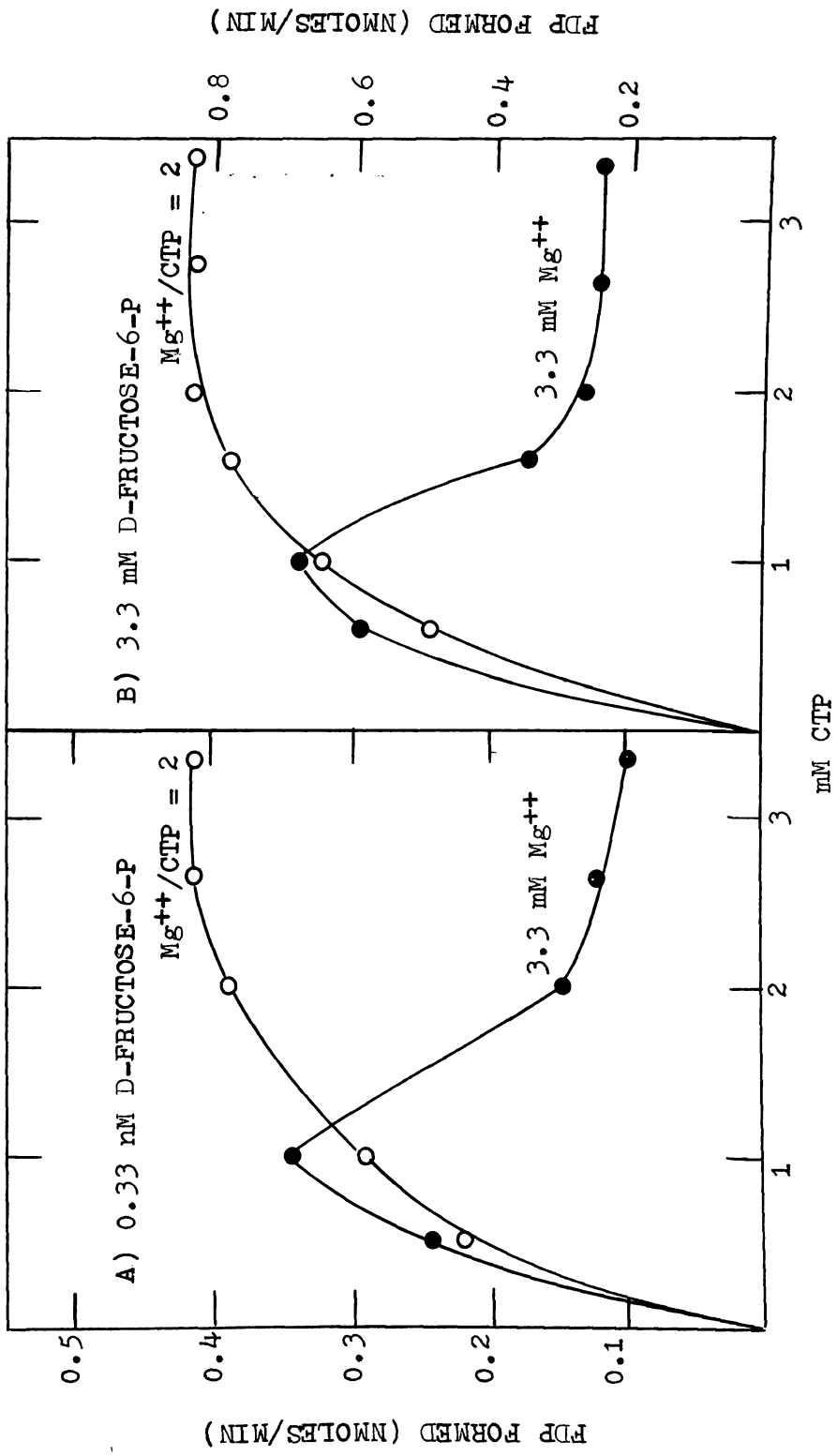
Figure 25: Dependence of initial velocity on the concentration of the hexose phosphate substrate with other nucleoside triphosphates as phosphoryl donors. Mg^{++} and nucleoside triphosphate were maintained at 1.33 mM and 2 mM, respectively, and the hexose phosphates were varied as indicated. A, Plot of the rate of the 6-PFK reaction vs D-fructose-6-P concentration with ITP, GTP, UTP, TTP, and CTP as phosphoryl donors. The amount of enzyme in all assays was 0.0015 unit. B, Plot of rate of the 1-PFK reaction vs D-fructose-1-P concentration with GTP and ITP as phosphoryl donors. The amount of enzyme in all assays was 0.022 unit.

Figure 25

A) 6-PFK
1.33 mM Mg++
2.0 mM NUCLEOSIDE TRIPHOSPHATE
ITP
GTP
UTP
TTP
CTP
mM D-FRUCTOSE-6-P
B) 1-PFK
1.33 mM Mg++
2.0 mM NUCLEOSIDE TRIPHOSPHATE
ITP
GTP
mM D-FRUCTOSE-1-P
FDP FORMED (NMOLES/MIN)

Figure 26: Inhibition of 6-PFK by CTP under conditions of varying D-fructose-6-P and Mg^{++} concentrations. CTP and Mg^{++} were varied as indicated. The amount of enzyme in all assays was 0.0015 unit. A, CTP inhibition in the presence of 0.33 mM D-fructose-6-P. B, CTP inhibition in the presence of 3.3 mM D-fructose-6-P.

Figure 26



P under condi-
se-6-P and Mg^{++}
Mg^{++} were
amount of
0.0015 unit.
presence of
B, CTP inhibi-
3.3 mM D-fructos-

GTP and ITP served as substrates for the 1-PFK reaction at 35% and 43%, respectively, of the rate observed with ATP as the phosphoryl donor (Table X). Only a slight activity (<10% of the rate with ATP) was observed when ATP was replaced with CTP, TTP, or UTP. As with ATP, the rate showed a hyperbolic dependence on D-fructose-1-P concentration with GTP or ITP as phosphoryl donor at a Mg^{++} to nucleotide ratio of 0.7:1 (Fig. 25B).

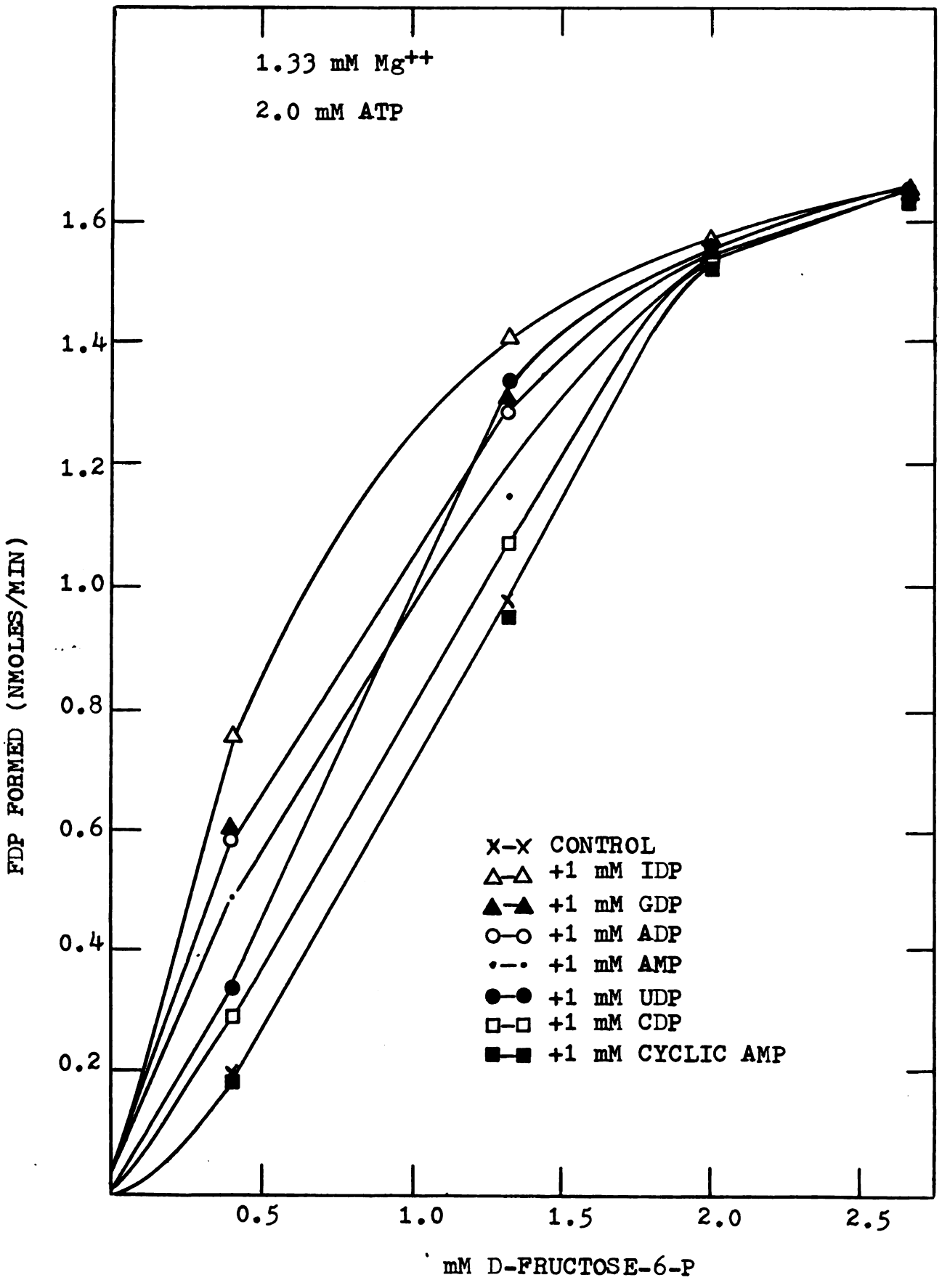
Effect of Nucleoside Diphosphates

At a Mg^{++} to ATP ratio of 0.7:1, all of the nucleoside diphosphates tested at 1.0 mM shifted to the left the sigmoidal curve for 6-PFK of rate vs D-fructose-6-P concentration (Fig. 27). The curves with ADP, IDP, and GDP were more hyperbolic than those with CDP and UDP; therefore the purine nucleotides seem to be more effective in relieving ATP inhibition at low D-fructose-6-P concentrations. None of the nucleotides inhibited or activated the enzyme at concentrations of D-fructose-6-P sufficient to give maximal rate.

None of the nucleoside diphosphates (3.3 mM) exhibited any effect on 1-PFK activity when the Mg^{++} concentration was maintained at twice the total concentration of the nucleotides in the assay. No relief of ATP inhibition was observed at 2.0 mM ATP, 1.34 mM Mg^{++}, and 0.2 mM D-fructose-1-P.

Figure 27: Dependence of the rate of the 6-PFK reaction on D-fructose-6-P concentration in the presence of various nucleotides. All the nucleotides were tested at a concentration of 1.0 mM. Mg^{++} and ATP were maintained at 1.3 mM and 2 mM, respectively, and D-fructose-6-P was varied as indicated. The amount of enzyme in all assays was 0.0017 unit.

Figure 27


1.33 mM Mg++
2.0 mM ATP
FDP FORMED (NMOLES/MIN)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.5
1.0
1.5
2.0
2.5
mM D-FRUCTOSE-6-P
X–X CONTROL
△–△ +1 mM IDP
▲–▲ +1 mM GDP
○–○ +1 mM ADP
•–• +1 mM AMP
●–● +1 mM UDP
□–□ +1 mM CDP
■–■ +1 mM CYCLIC AMP

Effect of Nucleoside Monophosphates

AMP was found to relieve ATP inhibition of 6-PFK (Fig. 27). However, due to the presence of a considerable adenylate kinase activity in the partially purified enzyme preparation, it is not certain whether AMP itself or ADP was the compound responsible for reversing the inhibition. Atkinson and Walton (95) reported a similar reversal of ATP inhibition in E. coli 6-PFK by both ADP and AMP; however, it was later established (96) that, with an adenylate kinase-free enzyme preparation, only ADP was effective. Therefore, the previously observed effect of AMP was actually an artifact due to the conversion of AMP to ADP catalyzed by the adenylate kinase contaminant in the partially purified E. coli preparation.

The effect of other nucleoside monophosphates (cyclic AMP, CMP, GMP, IMP, and UMP) on 6-PFK was tested under different conditions. At inhibiting ATP levels (0.4 mM D-fructose-6-P, 1.3 mM Mg^{++}, and 2.0 mM ATP), none of the above nucleotides, when tested at 3.3 mM, released ATP inhibition. With a non-inhibiting ATP level of 0.67 mM, neither inhibition nor activation was detected.

None of the above nucleoside monophosphates, when tested at 3.3 mM, had any effect on 1-PFK activity when the Mg^{++} concentration was maintained at twice the total concentration of the nucleotides in the assay. A

slight apparent enhancement of ATP inhibition was detected under conditions of inhibiting ATP level (1.3 mM Mg^{++}, 2.0 mM ATP, and 0.2 mM D-fructose-1-P). This effect was likely due to the unavailability of some of the Mg^{++} for the formation of MgATP complex since Mg^{++} can also form a complex with other nucleotides.

Substrate Specificity

The non-specific pyruvate kinase-lactate dehydrogenase-linked assay for 1-PFK was used to test for possible phosphorylation of D-fructose and several sugar phosphates other than D-fructose-1-P. With 0.0056 unit of 1-PFK, no phosphorylation (<0.03% of the rate with D-fructose-1-P) was observed with the following compounds: D-fructose, L-fructose-1-P, D-mannose-6-P, D-fructose-6-P, D-glucose-6-P, and D-glucose-1-P. L-Fructose-1-P was tested at a concentration of 10 mM, while the rest were tested at 33.4 mM.

The specific aldolase-linked assay was used to test for inhibition of phosphorylation of 0.53 mM D-fructose-1-P. The following compounds, when added at 33.4 mM, did not inhibit: D-mannose-6-P, D-glucose-6-P, D-glucose-1-P, and D-fructose. No inhibition was observed with 10 mM L-fructose-1-P. A concentration of 33.4 mM D-fructose-6-P, however, totally inhibited the reaction.

The 6-PFK preparation had not been purified enough to permit meaningful substrate specificity studies to be conducted.

Test for Inhibition of 6-PFK by D-Fructose-1-P

Mutant DD31, lacking 1-PFK activity, was employed to test for possible inhibition of D-fructose-6-P phosphorylation by D-fructose-1-P. The mutant was grown on D-glucose, and 6-PFK was purified 6-fold as described for the wild type. D-Fructose-1-P at 6.7 mM did not inhibit the phosphorylation of 0.33 mM D-fructose-6-P in an aldolase-linked assay.

Inhibition of 1-PFK by D-Fructose-6-P

The specific aldolase-linked assay was used to test for inhibition of the phosphorylation of D-fructose-1-P by D-fructose-6-P. The inhibition was competitive with D-fructose-1-P (Fig. 28) and the K_i for D-fructose-6-P, as determined from a kinetic plot (Fig. 29), is approximately 1.5 mM.

FDP Inhibition

The pyruvate kinase-lactate dehydrogenase-linked assay for 1-PFK was used for the inhibition study with FDP. As shown in Fig. 30, FDP competitively inhibited D-fructose-1-P phosphorylation. The K_i for FDP was approximately 7 mM (Fig. 31).

The presence of FDPase contaminant in the 6-PFK preparation precluded the determination of the effect of FDP on 6-PFK activity.

Figure 28: Lineweaver-Burk plot showing the relationship of D-fructose-1-P concentration to 1-PFK reaction velocity in the presence of various concentrations of D-fructose-6-P. The routine aldolase-linked assay was used, except that D-fructose-1-P and D-fructose-6-P were varied as indicated. The amount of enzyme in all assays was 0.001 unit.

Figure 28

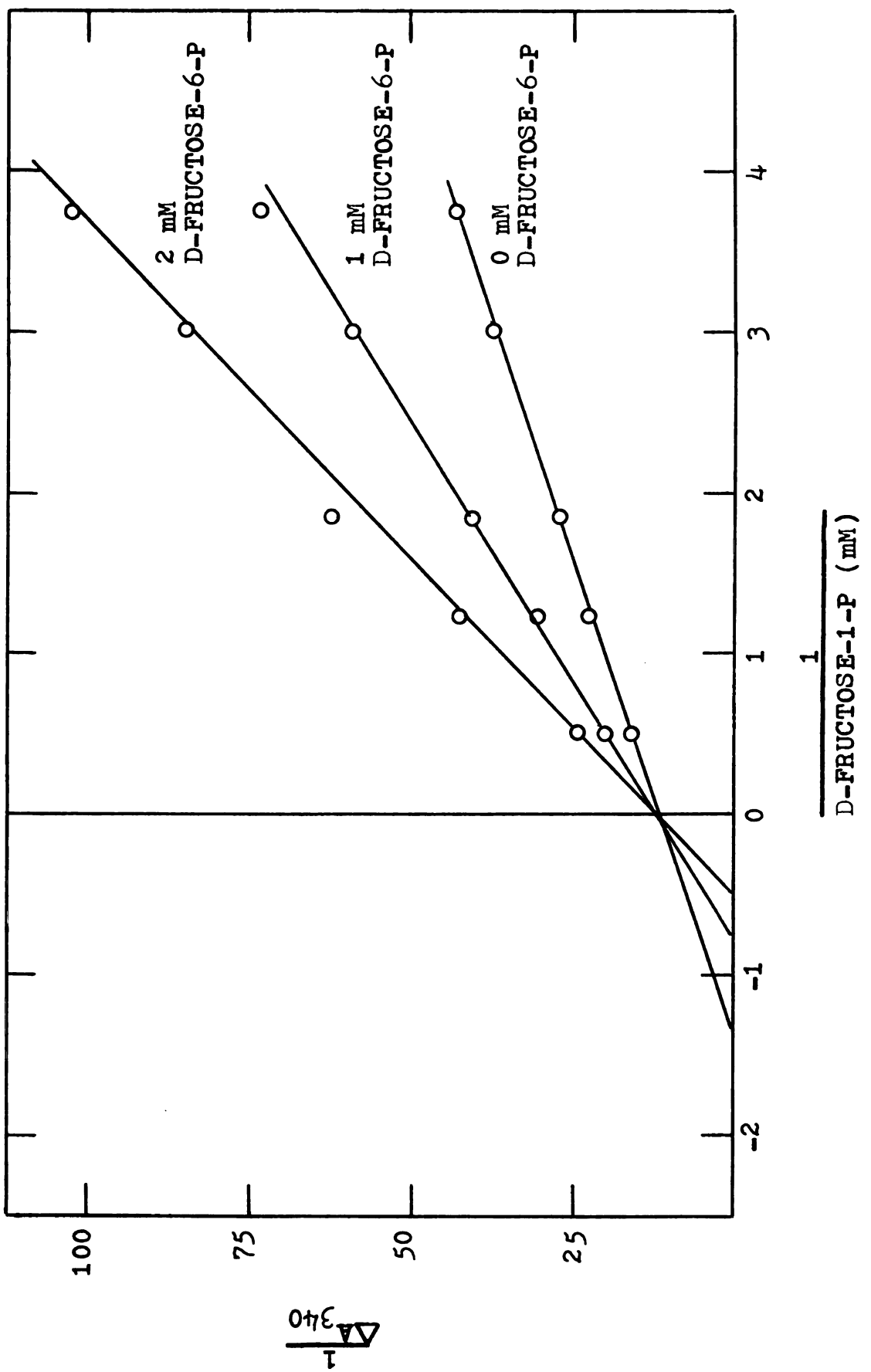

2 mM D-FRUCTOSE-6-P
1 mM D-FRUCTOSE-6-P
0 mM D-FRUCTOSE-6-P
100
75
50
25
-2
-1
0
1
2
3
4
1/ΔA340
1/D-FRUCTOSE-1-P (mM)

Figure 29: Kinetic plot for the determination of the K_i of 1-PFK for D-fructose-6-P. The data are taken from the experiment described in Figure 28.

Figure 29

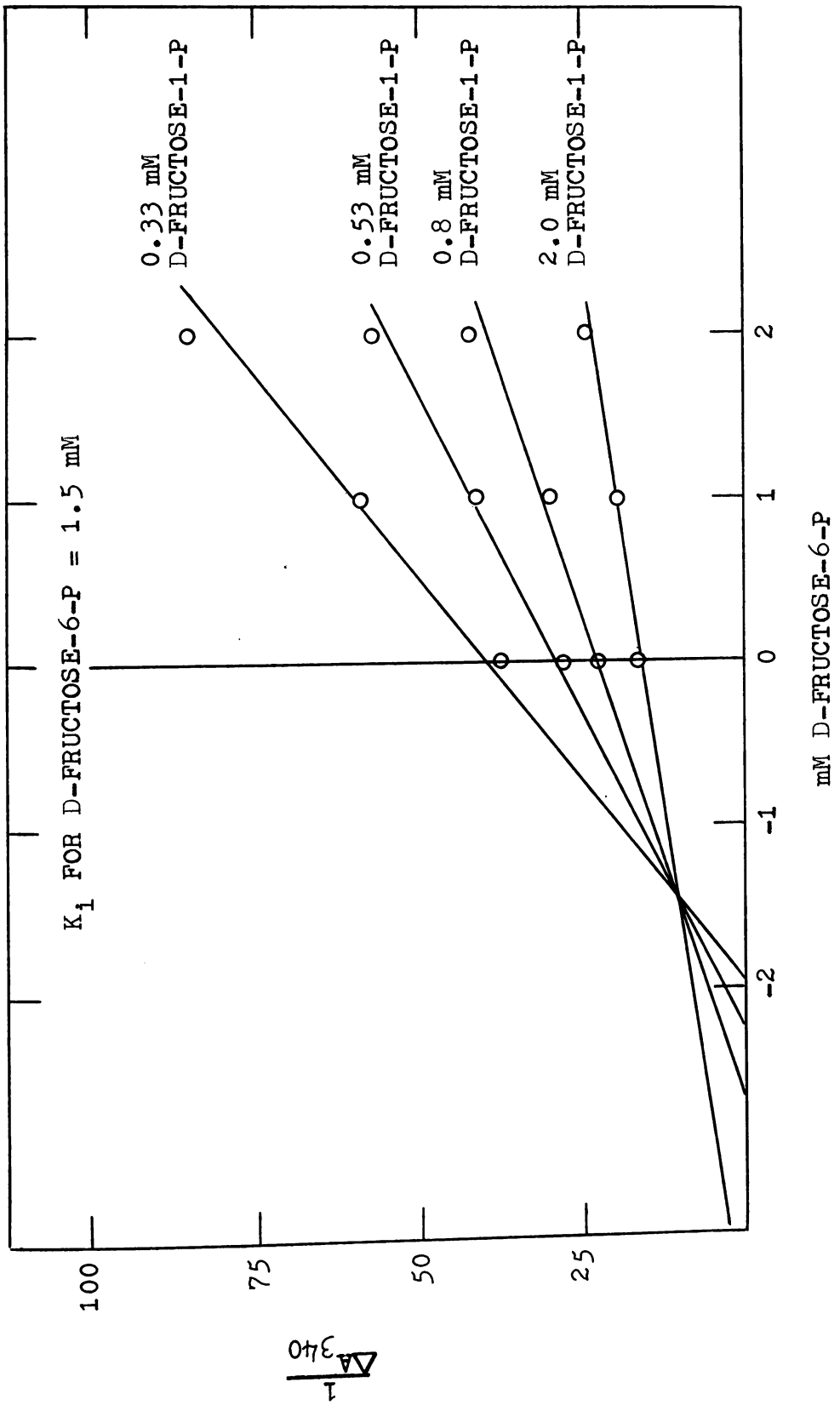

0.33 mM D-FRUCTOSE-1-P
0.53 mM D-FRUCTOSE-1-P
0.8 mM D-FRUCTOSE-1-P
2.0 mM D-FRUCTOSE-1-P
K_i FOR D-FRUCTOSE-6-P = 1.5 mM
100
75
50
25
$\frac{1}{\Delta A_{340}}$
2
1
0
-1
-2
mM D-FRUCTOSE-6-P

Figure 30: Lineweaver-Burk plot showing the relationship of D-fructose-1-P concentration to 1-PFK reaction velocity in the presence of various concentrations of FDP. The routine pyruvate kinase-lactate dehydrogenase-linked assay was used, except that D-fructose-1-P and FDP were varied as indicated in the plot. The amount of enzyme in all assays was 0.001 unit.

Figure 30

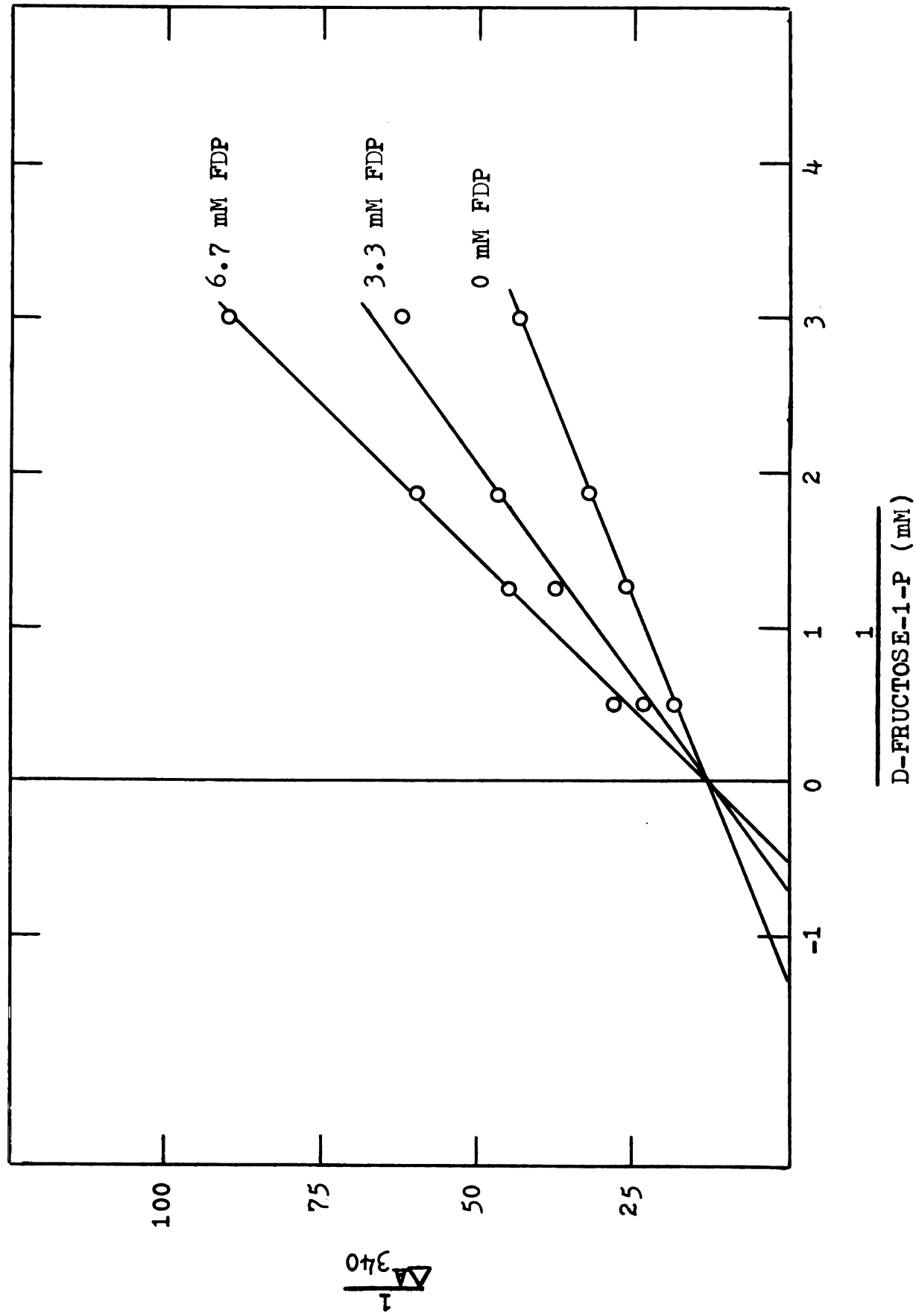
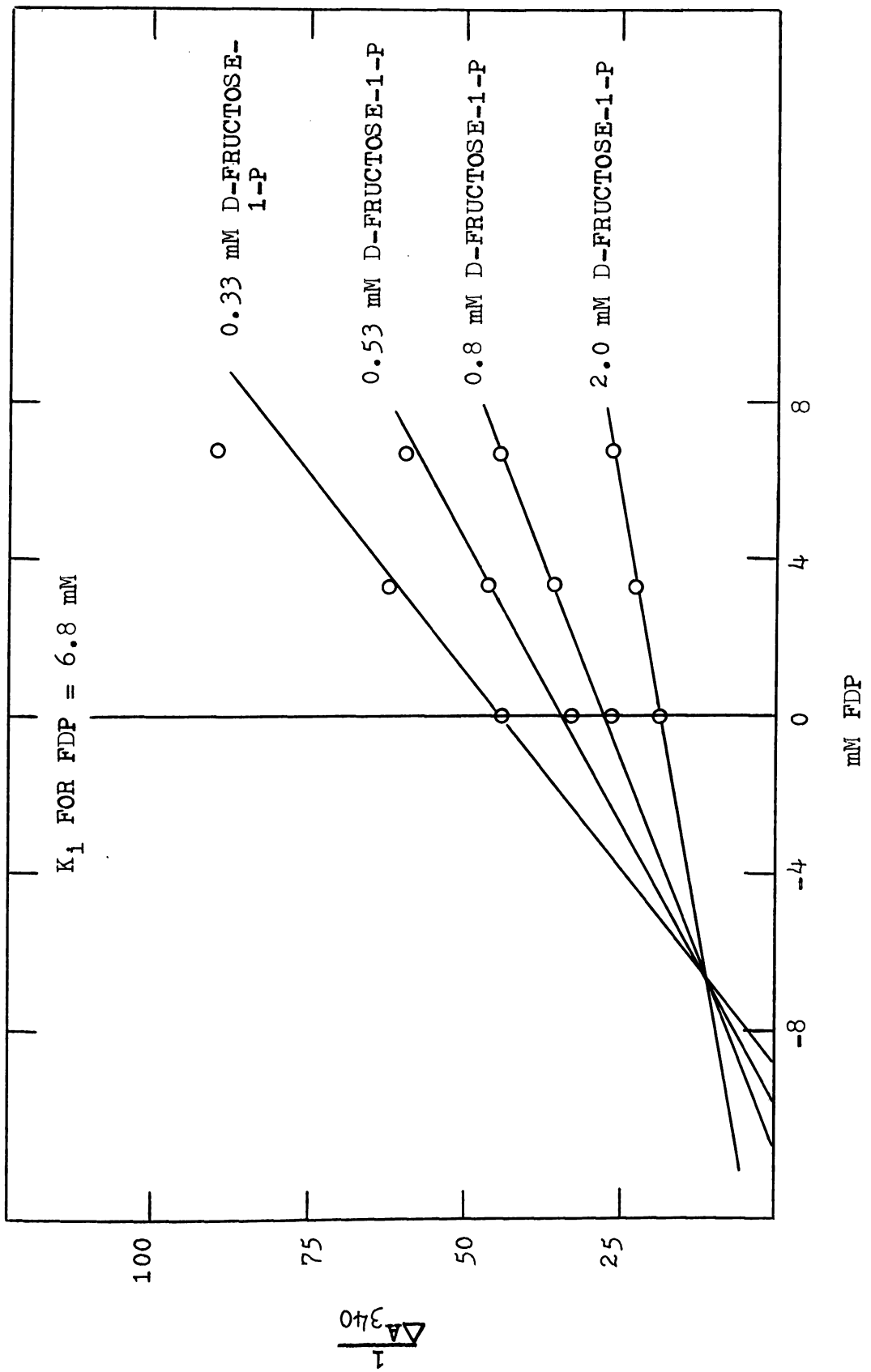

6.7 mM FDP
3.3 mM FDP
0 mM FDP
100
75
50
25
-1
0
1
2
3
4
1/ΔA340
1/D-FRUCTOSE-1-P (mM)

Figure 31: Kinetic plot for the determination of the K_i of 1-PFK for FDP. The data are taken from the experiment described in Figure 30.

Figure 31


0.33 mM D-FRUCTOSE-1-P
0.53 mM D-FRUCTOSE-1-P
0.8 mM D-FRUCTOSE-1-P
2.0 mM D-FRUCTOSE-1-P
K_1 FOR FDP = 6.8 mM
$\frac{1}{\Delta A_{340}}$
100
75
50
25
8
4
0
-4
-8
mM FDP

Effect of P_i

P_i at 2.6 mM did not relieve ATP inhibition of 6-PFK when the Mg^{++} to ATP ratio was 0.7:1; neither inhibition nor activation could be detected when the Mg^{++} concentration was twice the ATP concentration.

P_i had no effect also on 1-PFK activity when tested at 2.7 mM under conditions of inhibiting or non-inhibiting ATP levels.

Effect of Citrate

Citrate at 6.7 mM had no effect on 6-PFK activity under conditions of limiting ATP and D-fructose-6-P (0.67 mM ATP, 0.67 mM D-fructose-6-P, and 1.3 mM Mg^{++}).

In contrast, citrate was found to be a competitive inhibitor of D-fructose-1-P in the 1-PFK reaction (Fig. 32). The K_i for citrate was approximately 0.85 mM (Fig. 33).

Effect of pH

A pH-activity profile of 6-PFK (Fig. 34A) gave a broad curve, with optimum activity at about pH 8. No deviation from sigmoidal kinetics was observed over a pH range from pH 7 to pH 9 (Fig. 35). There was no apparent increase in ATP inhibition at low pH values, and the effectivity of D-fructose-6-P in relieving ATP inhibition did not seem to be affected by the pH of the reaction.

Figure 32: Lineweaver-Burk plot showing the relationship of D-fructose-1-P concentration to 1-PFK reaction velocity in the presence of various concentrations of citrate. The routine aldolase-linked assay was used, except that D-fructose-1-P and citrate were varied as indicated. The amount of enzyme in all assays was 0.0018 unit.

Figure 32

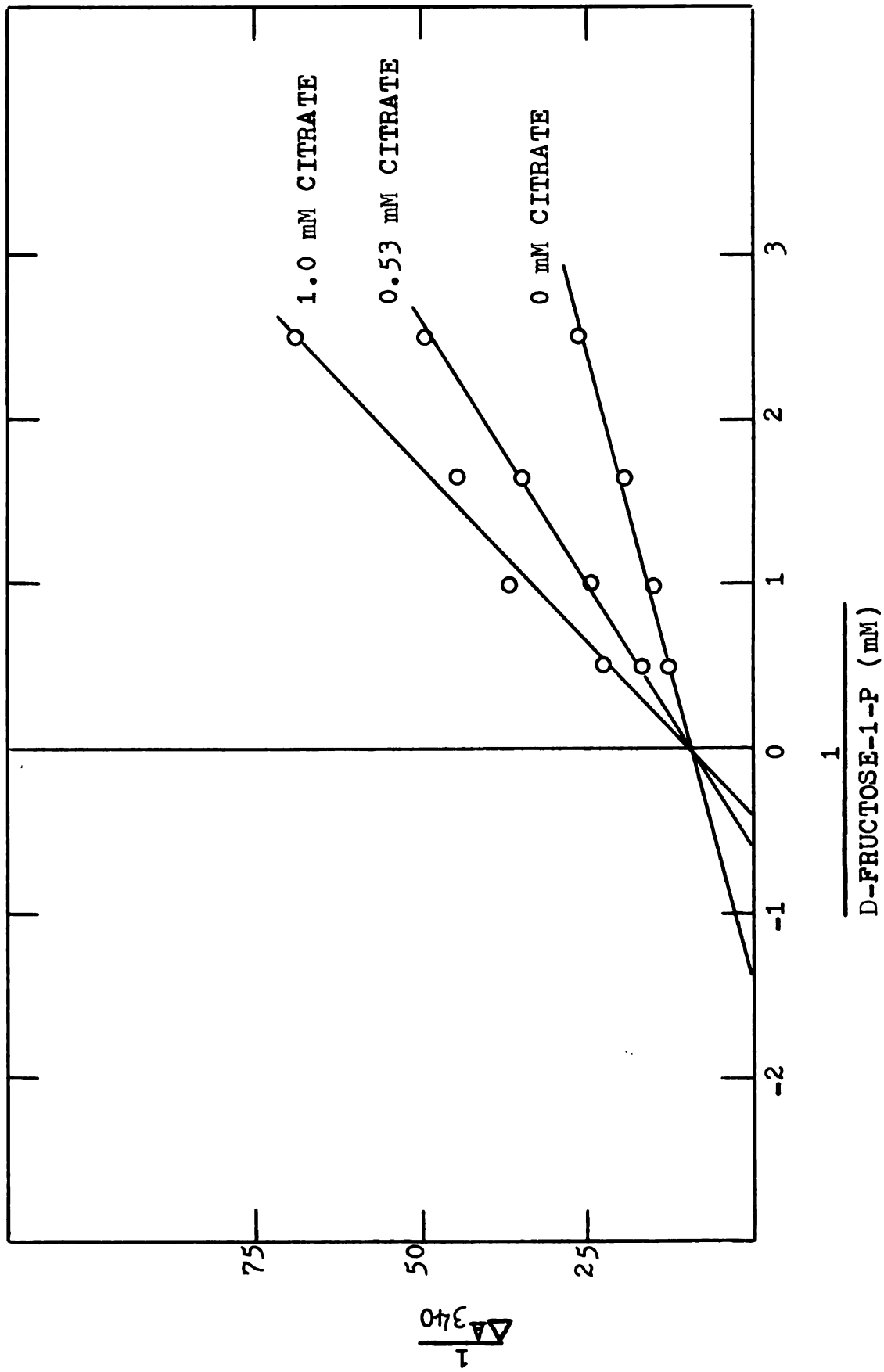

1.0 mM CITRATE
0.53 mM CITRATE
0 mM CITRATE
75
50
25
-2
-1
0
1
2
3
$\frac{1}{\Delta A_{340}}$
$\frac{1}{\text{D-FRUCTOSE-1-P (mM)}}$

Figure 33: Kinetic plot for the determination of the K_i of 1-PFK for citrate. The data are taken from the experiment described in Figure 32.

Figure 33

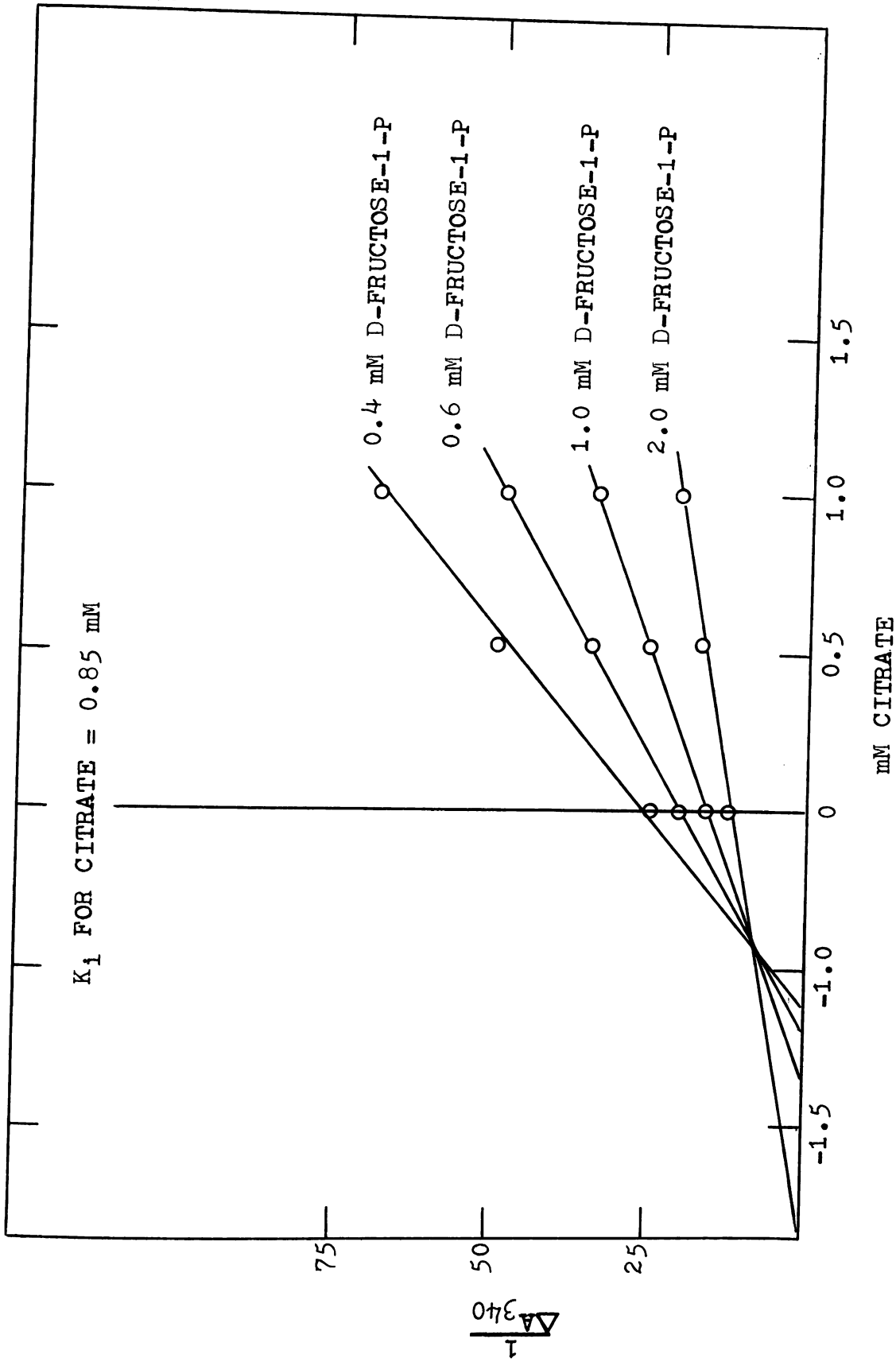

K_i FOR CITRATE = 0.85 mM
0.4 mM D-FRUCTOSE-1-P
0.6 mM D-FRUCTOSE-1-P
1.0 mM D-FRUCTOSE-1-P
2.0 mM D-FRUCTOSE-1-P
mM CITRATE
$\frac{1}{\Delta A_{340}}$
75
50
25
-1.5
-1.0
0
0.5
1.0
1.5

Figure 34: pH-activity profiles of 6-PFK and 1-PFK. The buffers (0.067 M) used were cacodylate, pH 6.0 to 7.0, glycylglycine, pH 7.0 to 8.0, and glycine, pH 8.5 and 9.0. pH measurements were made in duplicate reaction mixtures. A, pH-activity profile of 6-PFK. B, pH-activity profile of 1-PFK.

Figure 34

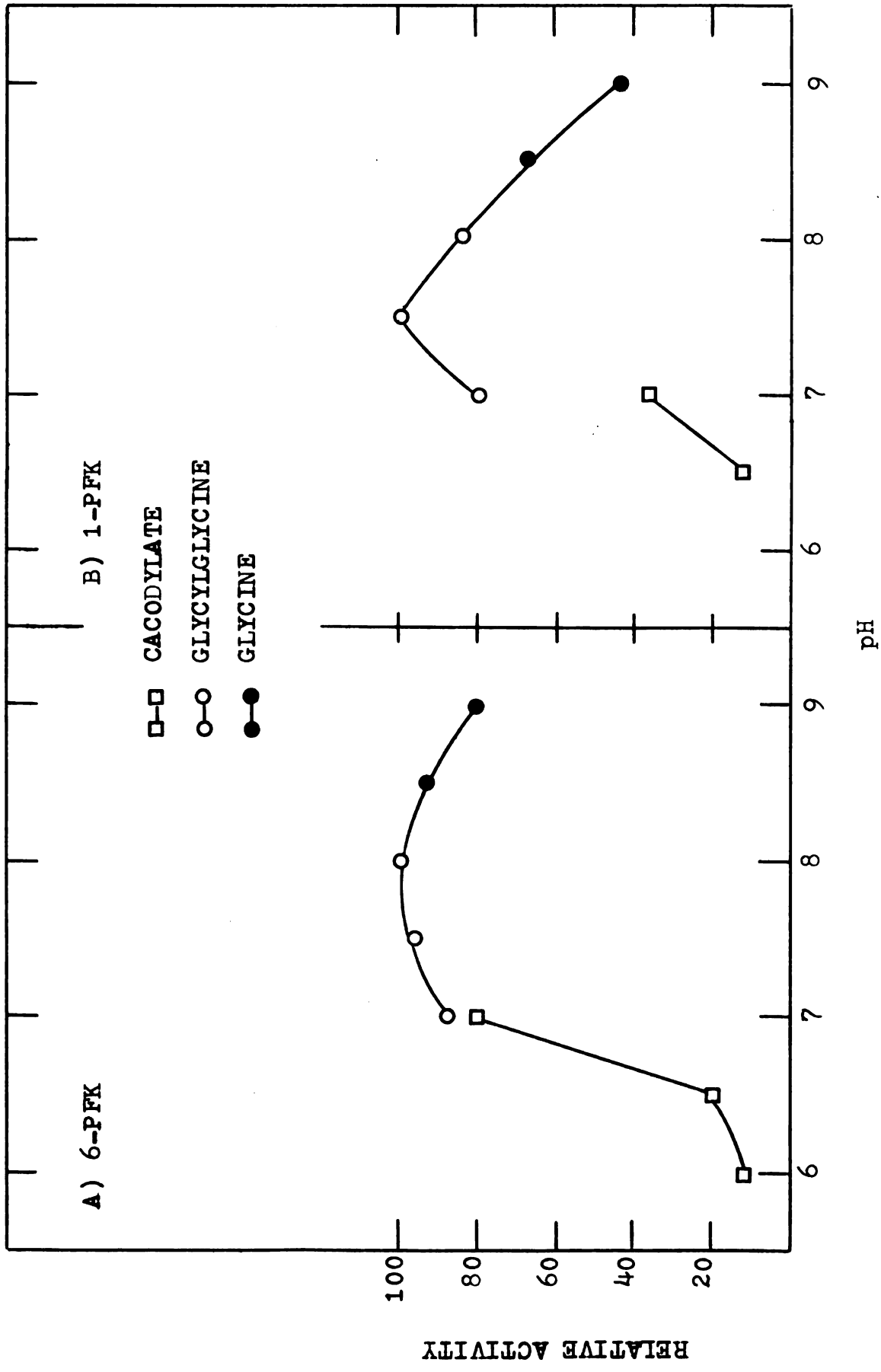
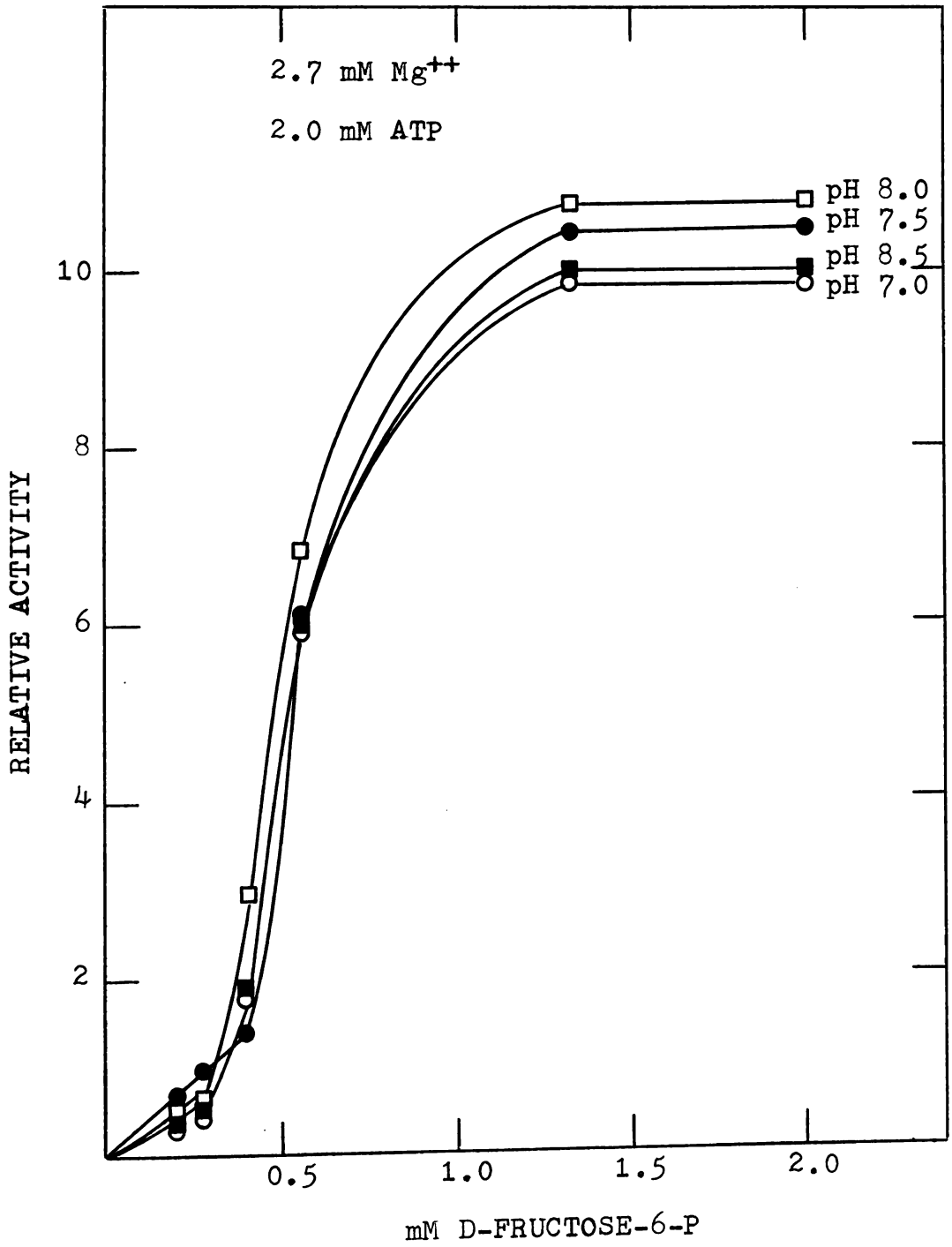

A) 6-PFK
B) 1-PFK
CACODYLATE
GLYCYLGLYCINE
GLYCINE
RELATIVE ACTIVITY
100
80
60
40
20
6
7
8
9
pH

Figure 35: Dependence of rate of the 6-PFK reaction on D-fructose-6-P concentration at different pH values. The buffers (0.067 M) used were glycylglycine, pH 7.0 to 8.0, and glycine, pH 8.5. ATP and Mg^{++} were maintained at 2 mM and 2.7 mM, respectively.

Figure 35


2.7 mM Mg++
2.0 mM ATP
pH 8.0
pH 7.5
pH 8.5
pH 7.0
RELATIVE ACTIVITY
10
8
6
4
2
0.5
1.0
1.5
2.0
mM D-FRUCTOSE-6-P

A pH-activity profile of 1-PFK (Fig. 34B) showed that the pH optimum of the reaction is pH 7.5. No shift in the pH curve was observed when the D-fructose-1-P concentration was varied in the range of 0.2 mM to 6.7 mM.

Molecular Weight Determination

The molecular weights of 6-PFK and 1-PFK were estimated by Sephadex G-100 chromatography as described by P. Andrews (104). Pig heart malic dehydrogenase (MW 70,000) (105), yeast alcohol dehydrogenase (MW 150,000) (106, 107), and horse heart cytochrome c (MW 12,400) (108) were used as molecular weight standards. Sodium phosphate buffer (0.02 M, pH 7.5) was used to equilibrate the column (25 x 1.2 cm) of Sephadex G-100 and to elute 20-drop fractions. The elution pattern of the proteins is shown in Fig. 36. From a plot of elution volume, V_e, versus log MW of the standards (Fig. 37), the molecular weight of 6-PFK was estimated to be approximately 100,000 and that of 1-PFK to be approximately 75,000.

Figure 36: Elution profile of 1-PFK, 6-PFK, malic dehydrogenase, alcohol dehydrogenase, and cytochrome c on a Sephadex G-100 column. Details are as described in the text.

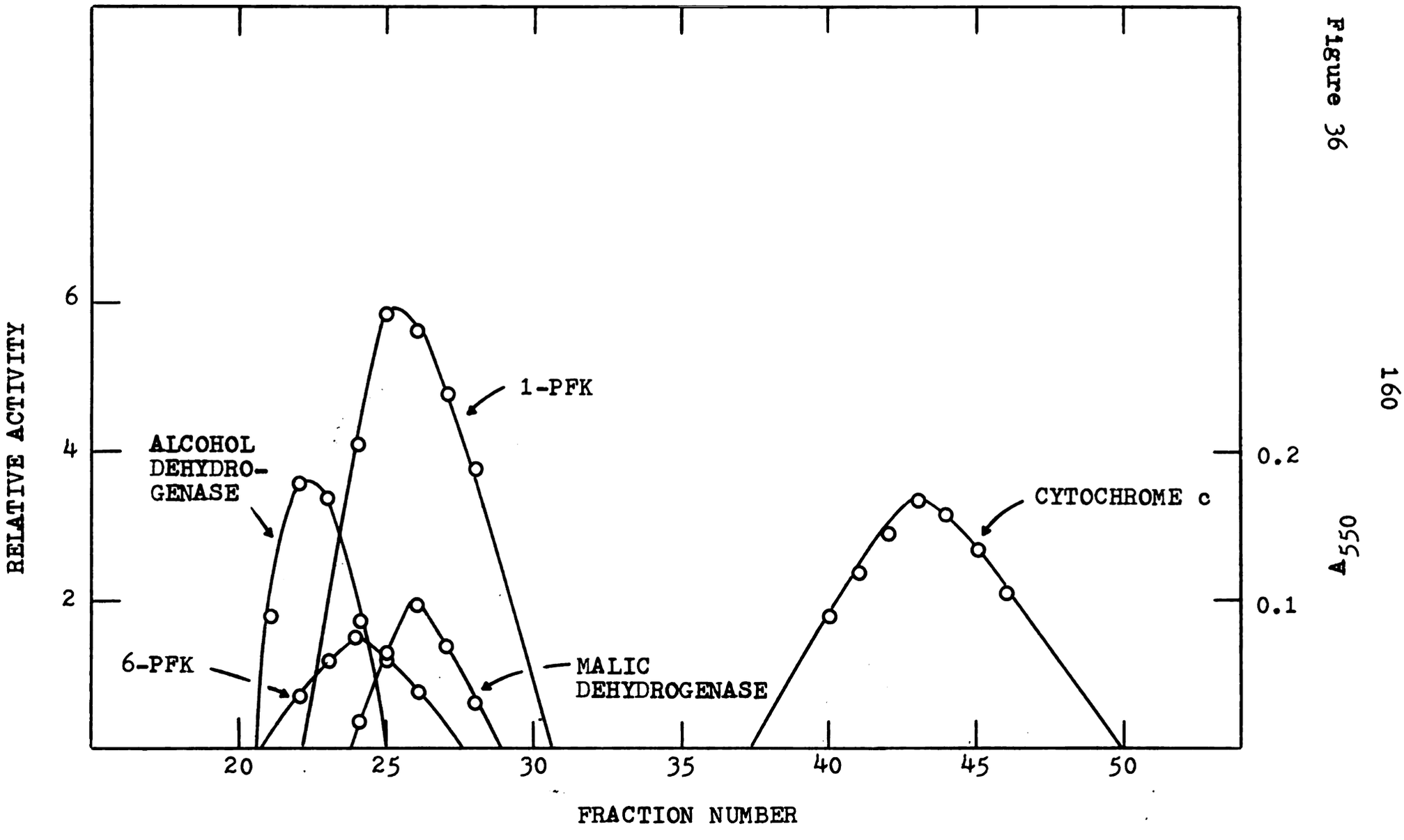
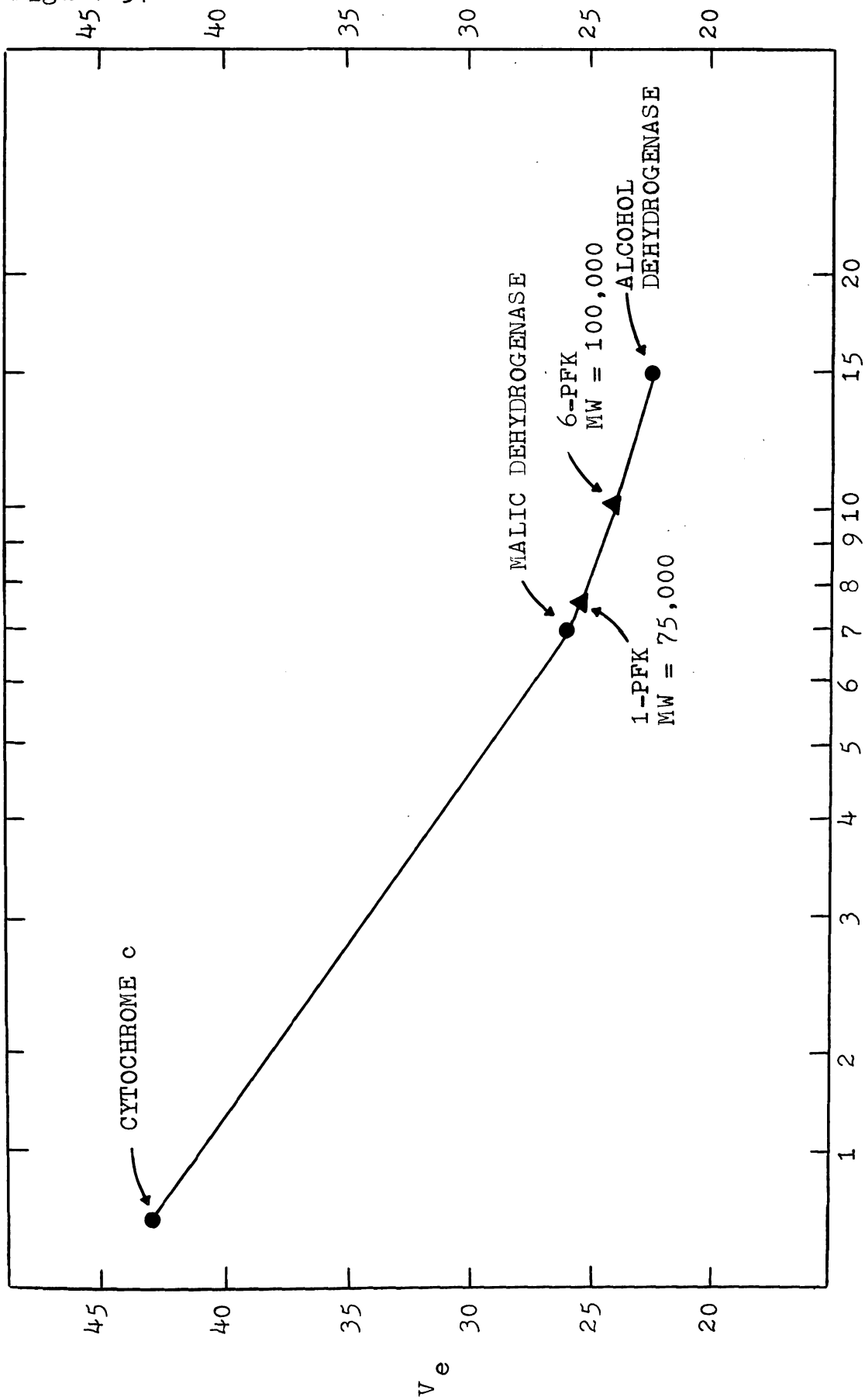

RELATIVE ACTIVITY
6
4
2
1-PFK
ALCOHOL
DEHYDRO-
GENASE
6-PFK
MALIC
DEHYDROGENASE
CYTOCHROME c
0.2
0.1
A_{550}
20
25
30
35
40
45
50
FRACTION NUMBER

Figure 36

Figure 37: Plot of elution volume V_e vs log MW of the standards for the estimation of the molecular weights of 1-PFK and 6-PFK.

Figure 37


45
40
35
30
25
20
CYTOCHROME c
MALIC DEHYDROGENASE
6-PFK
MW = 100,000
ALCOHOL
DEHYDROGENASE
1-PFK
MW = 75,000
1 2 3 4 5 6 7 8 9 10 15 20
MOLECULAR WEIGHT x 10^{-4}
V_e

DISCUSSION

6-PFK functions in D-glucose metabolism of A. aerogenes presumably in a manner similar to that of analogous 6-PFKs from a variety of sources. A constitutive FDPase hydrolyzes the product back to D-fructose-6-P. For this reason, the D-fructose-6-P-FDP cycle may function as a net ATPase if not controlled (57). If properly controlled, the cycle can regulate both glycolytic and gluconeogenic rates and maintain the delicate balance of nucleotides within the cell. It is therefore necessary for the 6-PFK reaction to be regulated. On the other hand, the inducible 1-PFK, which functions in D-fructose metabolism of A. aerogenes, is not known to participate in such a "futile" cycle. It is therefore highly probable that 1-PFK is not regulated by the mechanisms governing 6-PFK activity. The present studies were conducted to characterize 1-PFK and to compare its properties and control mechanisms with those of 6-PFK from the same organism. Since 6-PFKs from a number of organisms have already been purified and characterized, an extensive purification of the enzyme from A. aerogenes was not attempted.

The findings described in this investigation are consistent with the central role played by 6-PFK in

glycolytic control and in maintaining the delicate balance of nucleotides within the cell. More important, this thesis establishes that 1-PFK has kinetic properties very different from those of 6-PFK.

ATP inhibits both enzymes when the Mg^{++} to ATP ratio in the assay falls below 2:1. The reaction rate with 6-PFK, like that of the enzyme from E. coli (95), exhibits a sigmoidal dependence on D-fructose-6-P concentration which becomes more pronounced as the Mg^{++} to ATP ratio is decreased. The apparent K_m for D-fructose-6-P is dependent on the relative amounts of Mg^{++} and ATP in the assay. On the other hand, 1-PFK exhibits hyperbolic kinetics with respect to the D-fructose-1-P concentration even under conditions of inhibiting ATP levels, and the apparent K_m for D-fructose-1-P remains constant with varying ATP and Mg^{++} concentrations.

A sigmoidal curve of rate vs Mg^{++} concentration is obtained with both enzymes. Increased concentrations of D-fructose-6-P shift the curve of the 6-PFK reaction from a sigmoidal to a hyperbolic one, whereas, in the 1-PFK reaction, increased D-fructose-1-P levels have no effect on the sigmoidal character of the curve or on the apparent K_m for Mg^{++}. Since Mg^{++} and ATP form a MgATP complex, which is the real substrate of the reaction (113), it is not certain whether the relief of ATP inhibition by Mg^{++} in the 1-PFK reaction is due to the formation of the complex or to the binding of Mg^{++} to a separate site.

Neither enzyme is strictly specific for ATP as phosphoryl donor. 6-PFK utilizes ITP, GTP, CTP, UTP, and TTP, while 1-PFK utilizes GTP and ITP. The mechanism of inhibition of the nucleoside triphosphates seem to be similar for both enzymes. In this respect A. aerogenes 6-PFK behaves differently from those from mammalian sources, where inhibition by other nucleoside triphosphates exhibits a pattern similar to ATP inhibition (71, 73, 78). In yeast (92-94) and E. coli (95), other nucleoside triphosphates either inhibit the activity very slightly or not at all even at low Mg^{++} concentrations.

Both AMP and ADP were found to relieve ATP inhibition of 6-PFK, but since a considerable amount of adenylate kinase activity was present as a contaminant in the partially purified preparation, it is not clear whether both compounds are in fact effective. A similar relief of ATP inhibition by other nucleoside diphosphates and the absence of any effect with other nucleoside monophosphates suggest that ADP, rather than AMP, is the active compound. The absence of any effect on 6-PFK with cyclic AMP and other nucleoside monophosphates is in agreement with the results obtained with the enzyme from yeast (92-94) and E. coli (95, 97). Mammalian 6-PFKs are generally not affected by most nucleoside monophosphates (71, 73, 78); cyclic AMP, however, is known to be very effective in relieving ATP inhibition (71-75, 78, 81, 84).

ADP, AMP, cyclic AMP, and various other nucleoside mono- and diphosphates have no effect on the 1-PFK reaction when the Mg^{++} to total nucleotide ratio in the assay is maintained at 2:1. In some cases, a slight inhibition is observed at lower ratios; this effect may be due to ATP alone, rather than to enhancement of ATP inhibition by the other nucleotides.

pH has no effect on the extent of ATP inhibition of 6-PFK or on the sigmoidicity of the curve of rate vs D-fructose-6-P concentration. This finding is in contrast with those for several mammalian 6-PFKs (72, 73, 75, 84, 99, 100). To my knowledge, no studies have as yet been reported on the effect of pH on 6-PFKs from yeast and other bacteria.

According to Monod, Wyman, and Changeaux (103), allosteric proteins may be classified into either the K system or the V system. In the K system, both substrate and effector have differential affinities for the two states of the protein, and the presence of one will modify the apparent affinity of the protein for the other. In the V system, the substrate has the same affinity for the two states, while the effector has differential affinities. The two states of the protein differ in their catalytic activity. The effector will therefore act as an inhibitor if it has maximum affinity for the inactive state, or as an activator if it has maximum affinity for the active state. The effect of

Mg^{++} and various nucleoside diphosphates on the K_m of 6-PFK for D-fructose-6-P and the constancy of the maximal velocity suggest that A. aerogenes 6-PFK belongs to the K system type of allosteric proteins.

Aside from A. aerogenes 1-PFK, Dictyostelium discoideum 6-PFK is the only PFK known to deviate from the general regulatory pattern of a typical 6-PFK (91). Previous investigations on this organism have revealed that proteins and amino acids are the primary energy sources for growth (109-111), and that the main role of D-glucose seems to be to supply hexose units for cell wall synthesis (112). It was suggested, therefore, that the unusual regulatory pattern of the 6-PFK from this organism may reflect an altered physiological function, that is, as an enzyme in a supplementary energy-yielding metabolism under conditions of high glycogen and excess D-glucose (91).

Although A. aerogenes 1-PFK is not controlled by mechanisms similar to those operative for 6-PFK, the observed in vitro inhibition of the former enzyme by D-fructose-6-P, citrate, and, to some extent, FDP, may suggest possible in vivo control. Citrate, which is an intermediate in the tricarboxylic acid cycle, is a feedback inhibitor of the enzyme. Although the inhibition seems to be competitive with D-fructose-1-P, it is difficult to visualize citrate as binding to the D-fructose-1-P site, since its structural formula is very different from

that of D-fructose-1-P. Further studies are needed to elucidate the real mechanism of citrate inhibition. FDP inhibits competitively with D-fructose-1-P, but the rather high K_i of approximately 7 mM suggests that the inhibition may not be of major physiological significance. D-Fructose-6-P, the product of the FDPase reaction, is a more potent (K_i = 1.5 mM) inhibitor of 1-PFK activity.

A. aerogenes 6-PFK, like that from E. coli, was not inhibited by citrate. 6-PFKs from various organisms are known to be inhibited by citrate (70, 80-84, 88, 89). The inhibition of sheep brain (80) and rat heart 6-PFKs (83) is competitive with D-fructose-6-P.

It is not known at this time whether the 1-PFK reaction is a rate-controlling step in D-fructose metabolism of A. aerogenes. But if it is, its control mechanisms are very different from those of 6-PFK from the same organism.

The K_m of A. aerogenes 1-PFK for either substrate is not affected by the concentration of the other substrate. Such kinetics are consistent with a sequential mechanism of substrate binding (41). Similar findings have been reported for sheep brain 6-PFK at pH 8 (71). In contrast, Dictyostelium discoideum 6-PFK (91) exhibits parallel kinetics characteristic of the so-called ping-pong mechanism of substrate binding (41).

SUMMARY OF PART II

The relative significance of the D-fructose-1-P and D-fructose-6-P pathways in D-fructose metabolism of A. aerogenes PRL-R3 was assessed by mutant analysis. Mutant A9-1, which grew well on both D-fructose and glycerol but not on D-glucose, lacked 6-PFK activity but showed normal levels of 1-PFK and FDPase activities. On the other hand, mutant 012, which grew well on D-glucose but not on D-fructose or glycerol, lacked FDPase but had a normal level of 6-PFK activity. The data thus indicate that the pathway of D-fructose metabolism is primarily through D-fructose-1-P, and that the D-fructose-6-P pathway is operational in D-glucose metabolism.

Comparative studies on the properties and regulation of partially purified 6-PFK and 1-PFK from A. aerogenes were conducted. A plot of rate vs substrate concentration revealed that 6-PFK exhibits a sigmoidal dependence of rate on D-fructose-6-P concentration, whereas 1-PFK shows a hyperbolic dependence of rate on D-fructose-1-P concentration. ATP inhibited both enzymes under conditions of Mg^{++} to ATP ratios below 2:1. The inhibition of 6-PFK was relieved by Mg^{++}, D-fructose-6-P, ADP, and various other nucleoside diphosphates. In contrast, the inhibition of 1-PFK could be relieved by Mg^{++}

only. Both enzymes showed a sigmoidal dependence of rate on Mg^{++} concentration. Increased levels of D-fructose-6-P shifted the 6-PFK curve from sigmoidal to hyperbolic, whereas D-fructose-1-P had no effect on a similar plot for 1-PFK. Other nucleoside triphosphates were used as phosphoryl donors by both enzymes, and inhibited activity when the Mg^{++} to ATP ratio was below 2:1. In contrast with the finding with ATP, the inhibition of 6-PFK by other nucleoside triphosphates was not relieved by D-fructose-6-P, and the rate of the reaction exhibited hyperbolic dependence on D-fructose-6-P concentration. Citrate, FDP, and D-fructose-6-P inhibited the 1-PFK reaction competitively with D-fructose-1-P, suggesting possible in vivo control of activity. The data indicated that whereas 6-PFK exhibits allosteric properties and a regulatory pattern typical of 6-PFKs from a variety of organisms, 1-PFK behaves more like a non-allosteric kinase. The two enzymes were further characterized with respect to substrate specificity, pH optimum, and molecular weight. The K_m of 1-PFK for either substrate did not vary with the concentration of the other. This finding is consistent with a sequential mechanism of substrate binding to the enzyme.

REFERENCES

1. DeMoss, R. D., Bard, R. C., and Gunsalus, I. C., J. Bacteriol., 62, 499 (1951).

2. Gunsalus, I. C., and Gibbs, M., J. Biol. Chem., 194, 871 (1952).

3. DeMoss, R. D., J. Cell. Comp. Physiol., 41, Suppl. 1, 207 (1953).

4. Busse, M., Kindel, P. K., and Gibbs, M., J. Biol. Chem., 236, 2850 (1961).

5. Eltz, R. W., and Vandemark, P. J., J. Bacteriol., 79, 763 (1960).

6. Cifferi, O., Blakley, E. R., and Simpson, F. J., Can. J. Microbiol., 5, 277 (1959).

7. Palleroni, N. J., and Doudoroff, M., J. Biol. Chem., 218, 535 (1956).

8. Anderson, R. L., and Allison, D. P., J. Biol. Chem., 240, 2367 (1965).

9. Bueding, E., and MacKinnon, J. A., J. Biol. Chem., 215, 495 (1955).

10. Agosin, M., and Aravena, L., Biochim. Biophys. Acta, 34, 90 (1959).

11. Moore, D., and O'Kane, D. J., J. Bacteriol., 86, 766 (1963).

12. Medina, A., and Sols, A., Biochim. Biophys. Acta, 19, 378 (1956).

13. Sapico, V., and Anderson, R. L., J. Biol. Chem., 242, 5086 (1967).

14. DeMoss, R. D., J. Bacteriol., 95, 1692 (1968).

15. Rogosa, M., Mitchell, J. A., and Wiseman, R. F., J. Bacteriol., 62, 132 (1951).

16. Costilow, R. N., Etchells, J. L., and Anderson, T. E., Appl. Microbiol., 12, 539 (1964).

17. Kamel, M. Y., Allison, D. P., and Anderson, R. L., J. Biol. Chem., 241, 690 (1966).

18. Mayo, J. W., and Anderson, R. L., Carbohyd. Res., 8, 344 (1968).

19. Isbell, H. S., and Frush, H. L., in R. L. Whistler and M. L. Wolfrom (Editors), Methods in Carbohydrate Chemistry, Vol. I, Academic Press, N. Y., 1962, p. 146.

20. Kamel, M. Y., Hart, R. R., and Anderson, R. L., Anal. Biochem., 18, 270 (1967).

21. Anderson, R. L., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. 9, Academic Press, N. Y., 1966, p. 48.

22. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).

23. Cecil, R., and Ogston, A. G., Biochem. J., 49, 105 (1951).

24. Martin, R. G., and Ames, B. N., J. Biol. Chem., 236, 1372 (1961).

25. Tanford, C., Physical Chemistry of Macromolecules, John Wiley and Sons, Inc., N. Y., 1961, p. 381.

26. Sols, A., and Crane, R. K., J. Biol. Chem., 210, 581 (1954).

27. Sols, A., de la Fuente, G., Villar-Palasi, C., and Asencio, C., Biochim. Biophys. Acta, 30, 92 (1958).

28. Davidson, E. A., J. Biol. Chem., 235, 23 (1960).

29. Seed, J. R., and Baquero, M. A., J. Protozool., 12, 427 (1965).

30. Salas, J., Salas, M., Viñuela, E., and Sols, A., J. Biol. Chem., 240, 1014 (1965).

31. Parry, M. J., and Walker, D. G., Biochem. J., 99, 266 (1966).

32. Grossbard, L., and Schimke, R. T., J. Biol. Chem., 241, 3546 (1966).

33. Reeves, R. E., Montalvo, F., and Sillero, A., Biochemistry, 6, 1752 (1967).

34. Sebastian, J., and Asencio, C., Biochem. Biophys. Res. Commun., 28, 197 (1967).

35. Crane, R. K., in P. D. Boyer, H. Lardy, and K. Myrbäck (Editors), The Enzymes, Vol. 6, Academic Press, N. Y., 1962, p. 47.

36. Andersen, B., and Degn, H., Acta Chem. Scand., 16, 215 (1962).

37. Eliel, E. L., Allinger, N. L., Angyal, S. J., and Morrison, G. A., Conformational Analysis, Interscience Publishers, N. Y., 1965, p. 405.

38. Isbell, H. S., and Pigman, W. W., J. Res. Natl. Bur. Stand., 18, 141 (1937).

39. Pontremoli, S., Luppis, B., Wood, W. A., Traniello, S., and Horecker, B. L., J. Biol. Chem., 240, 3464 (1965).

40. Bitensky, M. W., Yielding, K. L., and Tompkins, G. M., J. Biol. Chem., 240, 663 (1965).

41. Cleland, W. W., Biochim. Biophys. Acta, 67, 104 (1963).

42. Fromm, H. J., and Zewe, V., J. Biol. Chem., 237, 3027 (1962).

43. Zewe, V., Fromm, H. J., and Fabiano, R., J. Biol. Chem., 239, 1625 (1964).

44. Fromm, H. J., Silverstein, E., and Boyer, P. D., J. Biol. Chem., 239, 3645 (1964).

45. Gulbinsky, J. S., and Cleland, W. W., Biochemistry, 7, 566 (1968).

46. Hanson, T. L., and Fromm, H. J., J. Biol. Chem., 242, 501 (1967).

47. Mourad, N., and Parks, R. E., Jr., Biochem. Biophys. Res. Commun., 19, 312 (1965).

48. Garces, E., and Cleland, W. W., Biochemistry, 8, 633 (1969).

49. Hanson, T. L., and Fromm, H. J., J. Biol. Chem., 240, 4133 (1965).

50. Fromm, H. J., and Zewe, V., J. Biol. Chem., 237, 1661 (1962).

51. Copley, M., and Fromm, H. J., Biochemistry, 6, 3503 (1967).

52. Fromm, H. J., Biochem. Biophys. Res. Commun., 32, 672 (1968).

53. Fromm, H. J., Fed. Proc., 28, 852 (1969).

54. Hanson, T. E., and Anderson, R. L., J. Biol. Chem., 241, 1644 (1966).

55. Hanson, T. E., and Anderson, R. L., Proc. Natl. Acad. Sci. U. S., 61, 269 (1968).

56. Sapico, V., Hanson, T. E., Walter, R. W., and Anderson, R. L., J. Bacteriol., 96, 51 (1968).

57. Scrutton, M. C., and Utter, M. F., in P. D. Boyer (Editor), Ann. Rev. Biochem., Vol. 37, Annual Review, Inc., Palo Alto, Calif., 1968, p. 249.

58. Reeves, R. E., Warren, L. G., and Hsu, D. S., J. Biol. Chem., 241, 1257 (1966).

59. Fraenkel, D. G., J. Biol. Chem., 243, 6458 (1968).

60. Magasanik, B., Cold Spring Harbor Symp. Quant. Biol., 26, 249 (1961).

61. Neidhart, F. C., J. Bacteriol., 80, 536 (1960).

62. Fraenkel, D. G., and Horecker, B. L., J. Bacteriol., 90, 837 (1965).

63. Morrissey, A. T. E., and Fraenkel, D. G., Biochem. Biophys. Res. Commun., 32, 467 (1968).

64. Wood, W. A., in P. D. Boyer (Editor), Ann. Rev. Biochem., Vol. 35, Annual Review, Inc., Palo Alto, Calif., 1966, p. 521.

65. Atkinson, D. E., in P. D. Boyer (Editor), Ann. Rev. Biochem., Vol. 35, Annual Review, Inc., Palo Alto, Calif., 1966, p. 85.

66. Stadtman, E. R., in F. F. Nord (Editor), Advances in Enzymology, Vol. 28, Interscience Publishers, N. Y., 1966, p. 41.

67. Caputto, R., Barra, H. S., and Cumar, F. A., in P. D. Boyer (Editor), Ann. Rev. Biochem., Vol. 36, Annual Review, Inc., Palo Alto, Calif., 1967, p. 211.

68. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W., J. Biol. Chem., 239, 18 (1964).

69. Williamson, J. R., in B. Chance, R. W. Estabrook, and J. R. Williamson (Editors), Control of Energy Metabolism, Academic Press, N. Y., 1965, p. 333.

70. Passonneau, J. V., and Lowry, O. H., Biochem. Biophys. Res. Commun., 13, 372 (1963).

71. Lowry, O. H., and Passonneau, J. V., J. Biol. Chem., 241, 2268 (1966).

72. Mansour, T. E., and Ahlfors, C. E., J. Biol. Chem., 243, 2523 (1968).

73. Uyeda, K., and Racker, E., J. Biol. Chem., 240, 4682 (1965).

74. Passonneau, J. V., and Lowry, O. H., Biochem. Biophys. Res. Commun., 7, 10 (1962).

75. Parmeggiani, A., Luft, J. H., Love, D. S., and Krebs, E. G., J. Biol. Chem., 241, 4625 (1966).

76. Paetkau, V. E., and Lardy, H. A., J. Biol. Chem., 242, 2035 (1967).

77. Paetkau, V. E., Younathan, E. S., and Lardy, H. A., J. Mol. Biol., 33, 721 (1968).

78. Mansour, T. E., J. Biol. Chem., 238, 2285 (1963).

79. Mansour, T. E., J. Biol. Chem., 240, 2165 (1965).

80. Underwood, A. H., and Newsholme, E. A., Biochem. J., 104, 296 (1967).

81. Denton, R. M., and Randle, P. J., Biochem. J., 100, 420 (1966).

82. Underwood, A. H., and Newsholme, E. A., Biochem. J., 95, 868 (1965).

83. Pogson, C. I., and Randle, P. J., Biochem. J., 100, 683 (1966).

84. Lou, M. F., and Kinoshita, J. H., Biochim. Biophys. Acta, 141, 547 (1967).

85. Grasso, A., and Natalizi, G. M., Comp. Biochem. Physiol., 26, 979 (1968).

86. Mansour, T. E., and Mansour, J. M., J. Biol. Chem., 237, 629 (1962).

87. Stone, D. B., and Mansour, T. E., Mol. Pharmacol., 3, 177 (1967).

88. Dennis, D. T., and Coultate, T. P., Biochim. Biophys. Acta, 146, 129 (1967).

89. Dennis, D. T., and Coultate, T. P., Biochem. Biophys. Res. Commun., 25, 187 (1966).

90. Lowry, O. H., and Passonneau, J. V., Arch. Expt. Path. Pharmak., 248, 185 (1964).

91. Baumann, P., and Wright, B. E., Biochemistry, 7, 3653 (1968).

92. Ramaiah, A., Hathaway, J. A., and Atkinson, D. E., J. Biol. Chem., 239, 3619 (1964).

93. Viñuela, E., Salas, M. L., and Sols, A., Biochem. Biophys. Res. Commun., 12, 140 (1963).

94. Lindell, T. J., and Stellwagen, E., J. Biol. Chem., 243, 907 (1968).

95. Atkinson, D. E., and Walton, G. M., J. Biol. Chem., 240, 757 (1965).

96. Griffin, C. E., Houck, B. N., and Brand, L., Biochem. Biophys. Res. Commun., 27, 287 (1967).

97. Blangy, D., Buc, H., and Monod, J., J. Mol. Biol., 31, 13 (1968).

98. Kemp, R. G., and Krebs, E. G., Biochemistry, 6, 423 (1967).

99. Ui, M., Biochim. Biophys. Acta, 124, 310 (1966).

100. Trivedi, B., and Danforth, W. H., J. Biol. Chem., 241, 4110 (1966).

101. Mayo, J. W., and Anderson, R. L., J. Biol. Chem., 243, 6330 (1968).

102. Levin, O., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. V, Academic Press, N. Y., 1962, p. 27.

103. Monod, J., Wyman, J., and Changeux, J. P., J. Mol. Biol., 12, 88 (1965).

104. Andrews, P., Biochem. J., 96, 595 (1965).

105. Thorne, C. J. R., and Kaplan, N. O., J. Biol. Chem., 238, 1861 (1963).

106. Kägi, J. H. R., and Vallee, B., J. Biol. Chem., 235, 3188 (1960).

107. Hayes, J. E., and Velick, S. F., J. Biol. Chem., 207, 225 (1954).

108. Margoliash, E., J. Biol. Chem., 237, 2161 (1962).

109. Sussman, M., and Bradley, S. G., Arch. Biochem. Biophys., 51, 428 (1954).

110. Gezelius, K., Physiol. Plantarum, 15, 587 (1962).

111. Wright, B. E., Bacteriol. Rev., 27, 273 (1963).

112. Pannbacker, R. G., and Wright, B. E., in M. Florkin and B. T. Scheer (Editors), Chemical Zoology, Vol. 1, Academic Press, N. Y., 1967, p. 610.

113. Lardy, H. A., and Parks, R. E., Jr., in O. H. Gaebler (Editor), Enzymes: Units of Biological Structure and Function, Academic Press, N. Y., 1956, p. 584.