WANG, JUN-LAN. Biochemical Studies of Galactokinase from Neurospora Crassa. (1970) Directed by: Dr. William K. Bates. pp. 62

Galactokinase (ATP: D-galactose 1-phosphotransferase, EC No. 2.7.1.6) was demonstrated in extracts prepared from the fungus Neurospora crassa. This enzyme was found to have maximal activity at pH 8.5 in Tris-HCl buffer, and optimal concentrations of magnesium chloride and adenosine triphosphate were determined.

An important part of the study was comparison of the galactokinase enzyme with the closely related enzyme glucokinase (ATP: D-glucose 6- phosphotransferase, EC No. 2.7.1.2) from Neurospora. Glucokinase was found to be much more thermostabile than galactokinase, and both enzyme activities sedimented at the rate of approximately 4 S on glycerol or sucrose gradients in the preparative ultracentrifuge.

Electrophoretic analysis of extracts containing both enzyme activities clearly demonstrated the migration pattern of glucokinase, but galactokinase was inactivated during this separation procedure. This electrophoretic study therefore provided no evidence concerning the separate identity of the proteins associated with the two activities. The process of extraction of protein from the mycelium, however, demonstrated that glucokinase is freely extractable, but a large portion of the total galactokinase activity is associated with some insoluble component of the cell debris. This suggests that separate proteins provide the two enzyme activities, but the evidence on this point is not definitive because of the possibility of activation, inhibition, or steric effects associated with attachment to a cellular organelle.

A purification of galactokinase of approximately 12-fold was obtained by salt fractionation and gel filtration, and this partially purified preparation was used for kinetic studies. In this way a Michaelis constant of 5.8×10^{-4} Molar galactose was determined for Neurospora galactokinase. Comparative studies of glucokinase demonstrated a Michaelis constant of 5.8×10^{-4} Molar glucose.

From these studies it is obvious that Neurospora crassa produces a galactokinase enzyme. This enzyme is present in crude extracts in an amount approximately 4% of the level of glucokinase. Although glucokinase and galactokinase could be separate activities of a single protein, the evidence suggests that these are separate, but very similar, proteins.

This thesis is

Dedicated to:

my Mother and Father

and

Dr. and Mrs. William K. Bates

BIOCHEMICAL STUDIES OF GALACTOKINASE FROM *NEUROSPORA CRASSA*

by

Jun-Lan Wang

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Arts

Greensboro
April, 1970

Approved by

_____
Thesis Adviser

APPROVAL SHEET

This thesis has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Thesis Adviser W. K. Bates

Oral Examination Committee Members Henry L Anderson II

Edward M. Cady IV

Bruce M Eberhart

April 9, 1970
Date of Examination

ACKNOWLEDGMENTS

Here I give my sincere gratefulness and appreciation to Dr. William K. Bates for his guidance, encouragement, criticism, and interest, and the hospitality generously given me by his family.

Without Dr. Bates' help it would have been impossible for me to come to the United States and study at The University of North Carolina at Greensboro. Without Dr. Bates' encouragement and guidance and the correction of the thesis, this thesis could never have been finished. All of the work was carried out in his laboratory and I appreciate very much his offering this good and comfortable studying and working circumstances.

I also wish to thank Dr. Bruce M. Eberhart for his many suggestions and helpful criticisms.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In 1856 lactose was first identified by Pasteur. In 1902 Tanet described the structure of lactose, a disaccharide which contains glucose as well as a previously unidentified sugar which he called galactose. D-galactose is an epimer of D-glucose at the hydroxyl group of the number four carbon atom. Glycolysis of glucose involves the phosphorylation of glucose to glucose-6-phosphate, and this reaction is catalyzed by the enzyme glucokinase (ATP: D-glucose 6-phosphotransferase, EC No. 2.7.1.2). The glycolysis of galactose requires phosphorylation by adenosine-5-triphosphate, catalyzed by the enzyme galactokinase (ATP: D-galactose 1-phosphotransferase, EC No. 2.7.1.6). In 1943 Kosterlitz reported the isolation of galactose-1-phosphate from livers of rabbits which had been fed galactose and on the basis of this he postulated the existence of galactokinase. The ultilization of galactose is now known in the Leloir, or uridine diphosphate-galactose pathway of galactose metabolism (Kalckar, et al., 1958; Robichon-Szulmajster, 1958; and Cardini and Leloir, 1961). Galactose metabolism includes the following steps:

1. Gal + ATP $\xleftrightarrow{\text{Galactokinase}}$ Gal-1-P + ADP

2. Gal-1-P + UDPG $\xleftrightarrow{\text{Gal-1-P, G-1-P uridyl transferase}}$ G-1-P + UDPGal

2a. Gal-1-P + UDP $\xleftrightarrow{\text{UDPGal pyrophosphorylase}}$ UDPGal + Pi

3. UDPGal $\xleftarrow{\text{UDPGal 4-epimerase}}$ UDPG

4. UDPG + Pi $\xleftarrow{\text{UDPG synthetase}}$ G-1-P + UTP

The abbreviations used are listed in the Methods Section.

Galactosemia is well known as a human hereditary disease. A lack of galactokinase can cause galactose to be excreted in the urine. The concentration of galactose in blood is elevated and also galactose-1-phosphate is accumulated in the red blood cells. The disease may also be caused by a lack of epimerase (Schwartz, et al., 1956). A person with galactosemia shows cataracts and may be mentally retarded, probably because of abnormal production of galactolipids in the brain. Folch-Pi (1955) and Kalckar, et al., (1956) found that galactosemia resulted primarily from a block in step 2.

A study of the UDPGal pathway in tumor cells showed that a number of tumor cells were defective in galactose metabolism (Robinson, et al., 1963).

Galactokinase has been identified in a large number of microorganisms. Kosterlitz (1943) postulated the existence of galactokinase in yeast. Subsequent studies were carried out by Trucco, et al., (1948) in Saccharomyces fragilis; by Cardini and Leloir (1953) in rat liver and brain; by Schwartz, et al., (1956) in human erythrocytes; by Weinberg and Segal (1960) in leukocytes; by Sherman and Adler (1963) in Escherichia coli; by Heinrich (1964) and Howard and Heinrich (1965) in yeast; by Ballard (1966) in pig liver; and by Gulbinsky and Cleland (1968) in Escherichia coli.

A manometric Method was used by Colowick and Kalckar (1943) and a titrametric Method was used by Kunitz and McDonald (1946) for assay of hexokinase. Robinson, et al., (1963) assayed galactokinase by paper chromatography using 1-^{14}C galactose with an automatic scanner. Colowick, et al., (1947) and Trucco, et al., (1948) determined hexokinase activity by using Nelson's procedure (1944) to measure the amount of the reducing sugars left in the solution after the protein and the phosphorylated sugars had been precipitated out by zinc sulphate and barium hydroxide.

Maxwell, et al., (1962) used Gal-1-P, G-1-P uridyl transferase, UDPG, phosphoglucomutase, TPN, and G-6-P dehydrogenase mixture to determine the Gal-1-P produced by galactokinase. The following reactions were involved:

(a) Gal-1-P + UDPG $\xleftarrow{\text{Gal-1-P, G-1-P, uridyl transferase}}$ G-1-P + UDPGal

(b) G-1-P $\xleftarrow{\text{Phosphoglucomutase}}$ G-6-P

(c) G-6-P + TPN $\xleftarrow{\text{G-6-P dehydrogenase}}$ 6-phosphogluconate + TPNH

The absolute molar amount of TPNH formed is a direct measurement of galactose-1-phosphate and thus of galactokinase activity.

Heinrich (1964), Howard and Heinrich (1965) and Ballard (1966) used a Spectrophotometric Method in which phosphoenol pyruvate, pyruvate kinase, DPNH, and lactic dehydrogenase were added to the solution under investigation. The ADP formed by galactokinase was then coupled to the following reaction: phosphoenol pyruvate $\longleftrightarrow$ pyruvate which is catalyzed by pyruvate kinase and which phosphorylates ADP to ATP. Then

in the presence of lactic dehydrogenase the pyruvate was reduced to lactic acid with the oxidation of DPNH. This oxidation was measured at 340 mμ.

Sherman (1963) has reported an assay for galactokinase using radioactive galactose as substrate. The product, ^{14}C-galactose-1-phosphate, was measured as the radioactivity remaining on DEAE-cellulose anion exchange paper after elution of the unreacted substrate, galactose-1-^{14}C, from the paper with water. This specific and sensitive method was chosen for the study described in this thesis.

Medina and Nicholas (1957) have reported that hexokinase in Neurospora crassa is zinc-dependent. This hexokinase catalyzes the phosphorylation of glucose and, at a slower rate, of mannose, fructose, and glucosamine. Galactose did not serve as substrate under their assay conditions. These workers also stated that chromatographic and enzymatic evidence indicated the phosphorylation product to be glucose-6-phosphate.

A search of the Chemical Abstracts, the Neurospora Bibliography and Index, and recent biochemical journals disclosed no previous studies of galactokinase from Neurospora. The objective of this study is to identify this enzyme and to compare the biochemical behavior of galactokinase with glucokinase from the same organism. The question of the existence of this and other enzymes of galactose metabolism in Neurospora is especially significant because of the importance of galactose derivatives in the structure of the cell wall (Crook and Johnston, 1962) and because of the importance of regulatory processes

which respond to galactose in this organism (Bates, et al., 1967).

METHODS

1. ABBREVIATIONS USED

ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; cm.: Centimeter; cpm: Counts per minute; DTT: Cleland's reagent, Dithiothreitol; g: Force of gravity; G-1-P: Glucose-1-phosphate; Gal.: Galactose; Gal-1-P: Galactose-1-phosphate; Gly.: Glycerol; gm.: Gram; IEU: International Enzyme Unit; M: Molar concentration, moles per liter; MCE: 2-Mercaptoethanol; mg.: Milligram; ml.: Milliliter; Pi: Inorganic phosphate ion; PPO: 2,5-diphenyl oxazole; rpm: Revolutions per minute; S: Sedimentation rate, Svedberg unit; TPN: Triphospho pyridine nucleotide; TPNH: Reduced triphospho pyridine nucleotide; Tris: Tris (hydroxymethyl) aminomethane; UDP: Uridine diphosphate; UTP: Uridine triphosphate; UDPG: Uridine diphosphate-glucose; UDPGal: Uridine diphosphate-galactose; V: Volume; W: Weight; Xyl.: Xylose.

2. CHEMICALS

All chemicals used were reagent grade and readily available unless otherwise noted. D-galactose-U-C-14, glucose-U-C-14, DTT, and MCE were from Calbiochem. Purified galactose was from SIGMA Chemical Company. Sucose used for gradients was Mann Crystalline, Density Gradient Grade, RNase free. PPO, galactose-1-C-14 and glucose-1-C-14 were from New England Nuclear Corporation. Sephadex is the trade name for a cross-linked dextran polymer made by Pharmacia, Inc.

3. GROWTH OF NEUROSPORA CRASSA

The strain 411-L5-A (Bates and Woodward, 1967) was chosen for study because it is a wild type with superior glycerol growth characteristics. Mycelia were cultured on Vogel's minimal medium (Vogel, 1956), with various carbon sources, in shaking or standing culture at 30° C. Diluted Vogel's medium and carbon sources were autoclaved separately and were mixed after cooling. Inoculum was 1.0×10^6 conidia per ml. Standing cultures were grown in one liter Roux bottles. Shaking cultures were grown in 500 ml. Erlenmeyer flasks on a rotary shaker at a speed of 172 cycles per minute. Mycelial pads were harvested using Buchner filtration on qualitative filter paper, and were washed with at least 500 ml. of deionized water for each flask. The resulting pads were squeezed dry in several thickness of paper towel, and the wet weight was determined. Mycelia were immediately frozen.

4. EXTRACTION OF MYCELIAL PROTEIN

Protein in Neurospora crassa can be extracted in many ways (Stine, et al., 1964; Bates, et al., 1967). The crude extract used in this paper was prepared by grinding mycelium in an OMNI MIXER (Ivan Sorvall, Inc.) with 10^{-2} M sodium phosphate buffer, pH 7.7, containing 10^{-2} M DTT or MCE and glass beads according to the following combinations:

Crude Extract	Mycelium Wet Weight (grams)	Buffer (ml.)	Glass Beads (grams)	Speed (rpm)	Extraction Time (minutes)
Micro diluted	0.2	3	1.00	4,600	10
Micro concentrated	1.2	3	1.05	4,600	20
Macro diluted	1.2	15	5.00	9,600	10
Macro concentrated	8.0	20	7.00	9,600	20

The reducing agents DTT and MCE were added to stabilize the thiol group on the enzymes in the reduced form (Cleland, 1964). After grinding the mycelium, the ground mixture was shaken for at least one hour. All the preparations were carried out in ice and the cell debris was spun down at 15,000 rpm (27,000 x g) for 20 minutes for diluted crude extracts, or 30 minutes for concentrated crude extracts, at 2^{o} C. The extract was used within 8 hours. For macro concentrated extracts, centrifugation was repeated three times.

Dialysis was for a total of 4 hours, or as otherwise indicated, against 10^{-2} M sodium phosphate buffer, pH 7.7, containing 10^{-2} M MCE or 3×10^{-4} M DTT, cooled in ice.

Debris from crude extracts was prepared by resuspending the centrifuged pellet in fresh buffer at the original extraction volume.

5. DETERMINATION OF PROTEIN

Protein was determined in crude extracts or trichloroacetic acid precipitates by the biuret method as described by Gornall, et al., (1948). Bovine serum albumin was used as standard. The samples containing DTT tended to turn the biuret reagent to black. This color

would disappear after about 24 hours depending on the amount of DTT in the solution. Therefore photometric readings were taken after this period of time. In the occasional samples in which a black precipitate occurred, this was removed by centrifugation prior to reading.

Protein was estimated in effluent fractions from columns by absorbance at 280 mμ (Layne, E., 1957). A Gilford 222 digital read out connected to a Beckman monochromomator was used for photometric readings. Photometric accuracy for this instrument was 0.5% A.

6. DETERMINATION OF ENZYME ACTIVITY

A modification of the procedure of Sherman (1963) was used through all the experiments. The procedures are the following: Stock solutions of Tris-HCl buffer, 0.182 gm./ml., pH 8.5; NaF, 6.06 mg./ml.; $MgCl_2.6H_2O$, 24.38 mg./ml.; MCE, 7.81 mg./ml.; and C-14 labelled substrate, galactose and glucose, were made and stored in the refrigerator. ATP, 16.5 mg./ml. and DTT, 7.7 mg./ml. were made fresh because of the unstable character of ATP and oxidation of DTT on exposure to the air. During the assay five stock solutions were involved: first was the buffer-metal ion mixture which is the mixture of equal volumes of Tris, Mg^{++} and F^- solution; second was the newly prepared ATP; third was the newly prepared DTT; fourth was the C-14 labelled substrate. After mixing those four stocks in equal volumes, an aliquot of 0.12 ml. of this mixture was pipetted to three inch test tubes. This solution was pre-incubated in a water bath at 30° C for about ten minutes before adding enzymes. The enzyme source was prepared by diluting the concentrated crude mycelial extract with distilled water just before use. A routine

dilution of 1 to 5 of concentrated crude extract or an original diluted crude extract was used for galactokinase and a dilution of 1 to 40 of concentrated crude extract or 1 to 8 of diluted crude extract was used for glucokinase assays. Effluent fractions from Sephadex columns were assayed directly for galactokinase, but a 1 to 10 or 1 to 20 dilution of these fractions was used for glucokinase assay. After preincubation, 0.03 ml. of the enzyme was added to start the reaction. One blank was formed in each set by adding 0.03 ml. of distilled water instead of enzyme. The final concentrations are: 0.1 millimole of Tris/ml.; 8.0 micromoles of $MgCl_2.6H_2O$/ml.; 9.6 micromoles of NaF/ml.; 5.3 micromoles of ATP/ml.; 10 micromoles of DTT/ml.; and 1 micromole of substrate/ml. After 10 minutes, the reaction was stopped by placing the tube in a boiling water bath for two minutes. The time for all steps was precisely controlled since the reaction was time dependent and excess evaporation during boiling should be avoided. After cooling, 0.03 ml. of the reacted mixture was applied to the 2 x 6.5 cm. DEAE cellulose paper strip at about 1 cm. from one end of the strip. Four strips or less were suspended in a 1 liter beaker containing double deionized water for two to five minutes. This process washes away the unreacted substrate, leaving the phosphorylated sugar on the paper. Three changes of deionized water were made during the wash (total of four washes). The strips were placed on aluminum foil and then dried in an oven at 50° C for at least 30 minutes. After the strips were dried, a 2.4 cm. section (1.2 cm. away from both sides of the center of the point of application) was cut off and placed in a counting vial (see counting

technique in Section 7). Net counting rates were obtained and the specific activities were calculated after determination of protein (see Data Processing in Section 8).

7. COUNTING TECHNIQUE

Dry DEAE cellulose paper strips containing the labelled reaction product were placed in standard scintillation counting vials containing 10 ml. of nitrogen-flushed counting mixture (8 grams of PPO per liter of toluene), and counted in a full C-14 window at gain setting 3, in a Beckman LS-100 Liquid Scintillation Spectrometer. Efficiency for C-14 under these conditions was 91 per cent. Since this counting efficiency was high and was essentially the same for all samples, quench correction was not necessary. The paper strip was put in the vial with the 2.4 cm. side at the bottom. Samples were counted to 40,000 total counts, corresponding to a Standard Error of 0.5% with rejection of low activity samples after 200 minutes. After the counting was finished, the paper strip was taken away from the vial. The used counting mixture was filtered and pooled in an amber bottle, and flushed again with nitrogen for five minutes. This cocktail was then ready for re-use. The filter paper used was Rapid Filtering Paper, Arthur H. Thomas Company.

8. DATA PROCESSING

(a) UNIT DEFINITION: One International Enzyme Unit (IEU) is defined as that amount of enzyme that phosphorylates one micromole of D-galactose or D-glucose at 30° C in one minute under the conditions described in the experiment.

(b) TREATMENT OF THE ASSAYS: In the biuret method the absorbancies of a set of standards were determined at the same time as the samples. These standards formed a linear calibration curve which was used for calculation of protein concentration of the samples. Counting rates, as counts per minutes (cpm), were calculated from the Liquid Scintillation Counter. Routine quench determinations were carried out by the External Standard Channels Ratio Method using Cs-137. Net counting rates and standard deviations corrected for controls were calculated by appropriate computer programs.

(c) COMPUTER PROGRAMS: A program for linear least square fitting was used for calculation of protein content in the samples (Wm. K. Bates, unpublished). Additional programs were used for calculation of the net counting rate and its standard deviation and for specific activities.

(d) CONVERSION OF COUNTING RATE TO INTERNATIONAL ENZYME UNITS:

Most of the graphs in Results Section are presented as counting rates. This can be converted to International Enzyme Units (IEU) by the following formula: $IEU = R \times Mr \times t^{-1}$, where R is counting rate in cpm; Mr is the micromole of substrate per cpm; and t is reaction time, (10 minutes or as otherwise noted). Mr values for different substrates are listed in the following:

Substrate	Symbols	Mr (Micromoles/cpm, x 10^{-6})
Galactose-U-C-14	A-1	2.556
Glucose-U-C-14	B-1	2.209
Galactose-1-C-14	A-2	2.802
Glucose-1-C-14	B-2	2.867
Glucose-1-C-14	B-3	3.710

9. ISOLATION OF MUTANTS

Ultraviolet light was used as the mutagen. Conidia obtained from 8 day old cultures of the 411-L5-A strain at a concentration of 1×10^8 conidia /ml. (based on hemocytometer count) were placed in a Petri plate on a magnetic stirrer for exposure to ultraviolet light. Irradiation was done under a hood for twelve minutes. After irradiation, the conidial suspension was inoculated into a 500 ml. Erlenmeyer flask containing Fries minimal medium (Beadle and Tatum, 1945) with 1% (W/V) galactose. This was shaken for five days at 30° C. During these days the wild types were filtered off as they germinated. Then the mutant spores were preserved by plating onto supplemented media (Tatum, et al., 1950). All plating media included 0.5 or 1% sorbose, which caused colonial growth of the mycelium (Tatum, et al., 1949). All the colonies germinating on the sorbose plates (about 600) were transferred to three inch slants containing Vogel's minimal medium, plus 1% glycerol, 0.5% yeast extract and 0.5% Bacto Casitone. After growth and conidiation, these isolates were tested on liquid minimal medium plus 1.5% galactose. The ones which did not grow or which showed reduced growth were retained for further study. These isolates were then grown on slants of Vogel's medium plus sucrose. Ability to produce galactokinase and glucokinase was then tested under different conditions using wild types 411-L5-A as control.

10. SEDIMENTATION VELOCITY STUDIES

A Model L-2 Spinco preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Division Palo Alto, California) with a

swinging bucket rotor (SW-39) was used for this study. Gradients were made by using a linear gradient former as described by Britten and Roberts (1960) and modified by Martin and Ames (1961). Both sucrose, 5% (W/V; 0.146 M) and 20% (W/V; 0.548 M); and glycerol, 8.1% (W/V; 0.88 M) and 33% (W/V; 3.58 M), in 0.05 M Tris-HCl buffer, pH 7.5, containing 10^{-2} M DTT were used in the study. Linearity of the gradients was tested by adding methyl orange to the 20% sucrose solution used, and the absorbance of test fractions was determined photometrically at 505 mμ. Beef liver catalase, with a known sedimentation constant of 11.3 S, was used as a standard. Catalase (Worthington; diluted 1/100 with 0.05 M Tris-HCl buffer, pH 7.5) was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5. An aliquot of 0.02 ml. was applied to the top of the gradient, followed by 0.2 ml. of sample. After centrifugation, the tube was punctured at the bottom, and fractions of eight drops each were collected at room temperature, then transferred to an ice bath. The fractions were analyzed for catalase, galactokinase and glucokinase activities. Catalase activity was assayed by a photometric method, using H_2O_2 as substrate at 240 mμ (Beers and Sizer, 1952).

11. GEL FILTRATION

Sephadex G-75 or G-150 was packed in a column 28 cm. long and 1.6 cm. in diameter. To prepare the column, the gel was allowed to swell in the eluant without DTT for at least three days or heated in a boiling water bath for five hours followed by at least one overnight soaking. After the column was packed, a disc of Whatman No. 1 filter paper was placed on the surface of the gel bed. The column was used

within one day after it was prepared. The sample was applied to the top of the column directly or by layering under the buffer already present. The sample was the 40% (W/V) ammonium sulphate precipitate, redissolved in buffer at a total volume of 1/10 that of the original crude extract. The eluant used was 10^{-2} M sodium phosphate buffer, pH 7.7, containing 10 mg. of DTT per ml. The efluent was collected manually into three inch tubes. Each fraction was 2 or 5 ml. and the flow rate is described for each experiment.

12. DISC ELECTROPHORESIS

Disc electrophoresis was carried out as described by Davis (1964), except the lower gel had 1.0 to 1.5 mg. of DTT per ml. Running current was 3-2/3 milliamperes per tube. After electrophoresis, one gel was stained with amido black and de-stained with 7% acetic acid for protein analysis. The destained gel was then read in a recording densitometer with a light source collimated by a 0.1 millimeter slit and filtered through a #595 filter. Duplicate gels were sliced in 1.6 millimeter slices. Then the enzyme was extracted from each slice in 0.4 ml. of an equal volume mixture of buffer-metal ion mixture, DTT, and deionized water. The tubes were covered with corks and were shaken at room temperature for one to two hours. After centrifugation at 5,000 rpm (3,020 x g) at 2^{o} C, 0.09 ml. of supernate was transferred to the 0.06 ml. mixture of substrate and ATP used routinely for assay. The rest of the procedures were the same as that described in Section 6.

RESULTS

1. OPTIMUM CONDITIONS FOR ASSAY OF GALACTOKINASE AND GLUCOKINASE

(a) pH optimum: Both galactokinase (using substrate A-1) and glucokinase (using substrate B-1) have pH optima at pH 8.5 in Tris-HCl buffer as shown in Figure 1a and 1b. Therefore this was used routinely for assay of both enzymes. Citrate-phosphate buffer slightly interferes with the activity of both enzymes at pH 7.1, and glycine-NaOH buffer severely interfers with the activities at pH 8.5. It seems that galactokinase and glucokinase reach their pH optima at pH 10.0 or greater in glycine-NaOH buffer.

(b) ATP optimum: Figure 2a shows that 5 micromoles and 4 micromoles per ml. of reaction mixture are the optimal ATP concentrations for galactokinase (using substrate A-2) and glucokinase (using substrate B-2) respectively. The concentration used for both enzyme assays was 16.5 mg./ml. stock solution which gave a final concentration of 5.3 micromoles per ml. of reaction of mixture.

(c) Magnesium optimum: Mg^{++} ion is necessary for both galactokinase and glucokinase activities. Substrates A-2 and B-2 were used for the study. Figure 2b shows that both enzymes have a broad Mg^{++} optimum, 7.5-22.5 micromoles of $MgCl_2.6H_2O$ per ml. of reaction mixture, and a higher magnesium chloride concentration severely inhibits the phosphorylation reaction.

(d) Studies of incubation time and concentration of extracts:

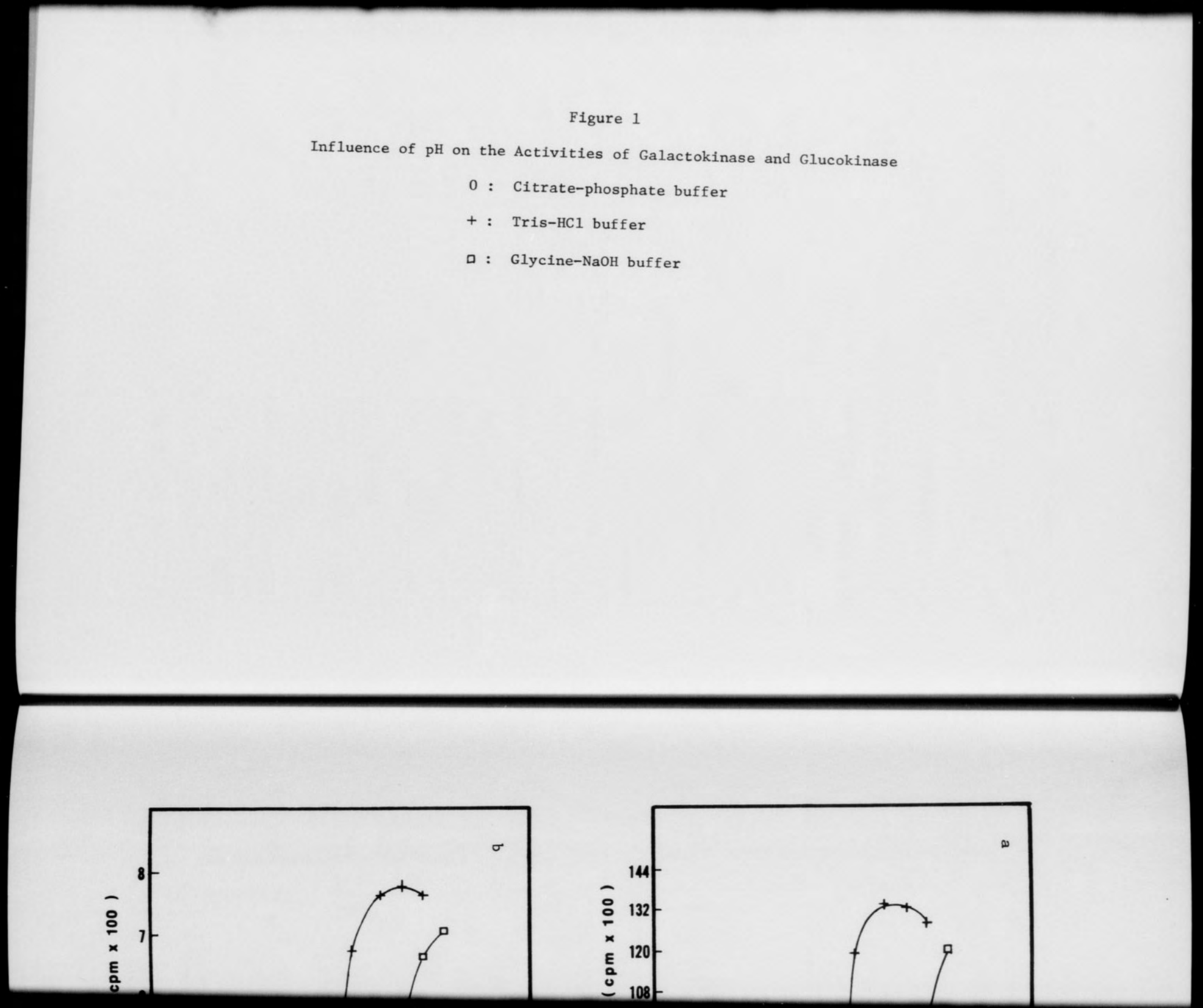
Figure 1

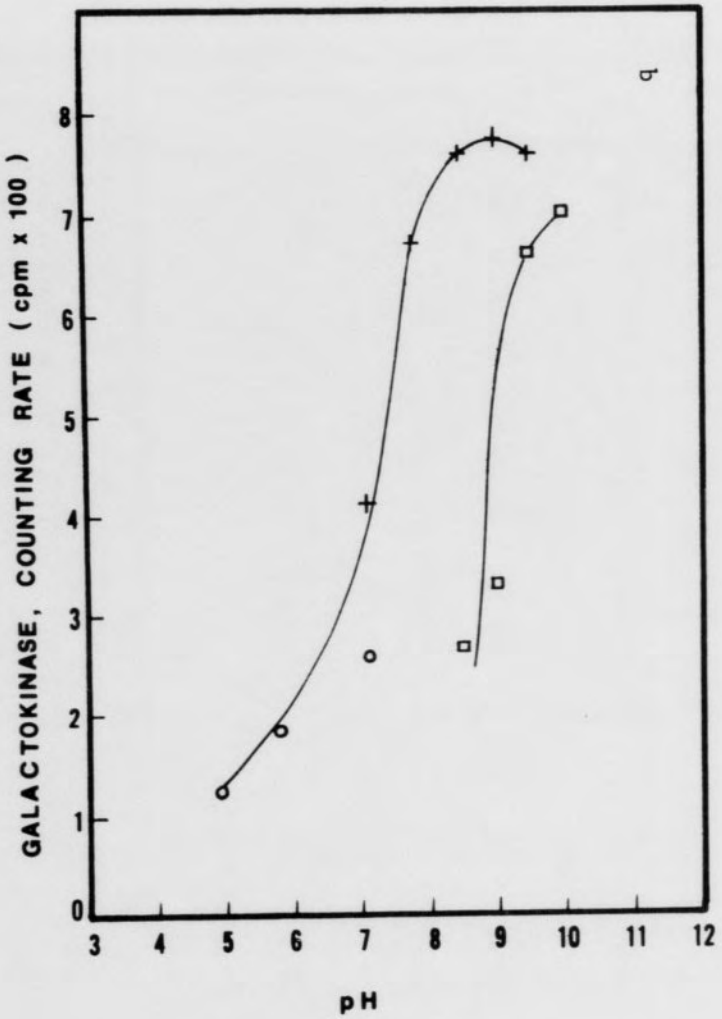
Influence of pH on the Activities of Galactokinase and Glucokinase

0 : Citrate-phosphate buffer

+ : Tris-HCl buffer

□ : Glycine-NaOH buffer




b
GALACTOKINASE, COUNTING RATE (cpm x 100)
0
1
2
3
4
5
6
7
8
3
4
5
6
7
8
9
10
11
12
pH

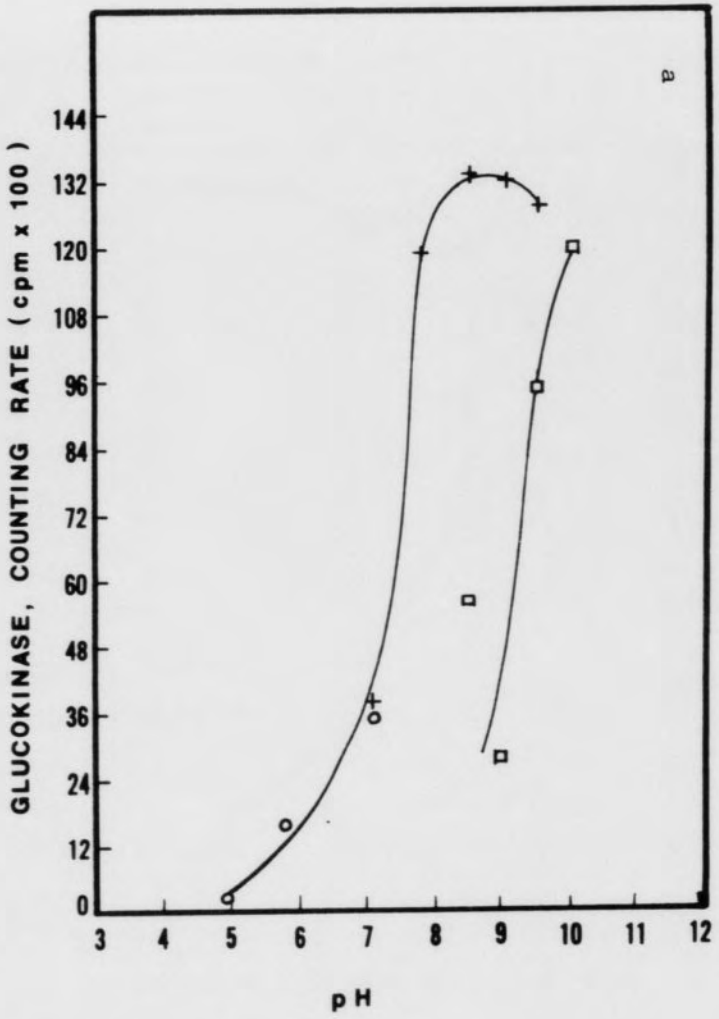
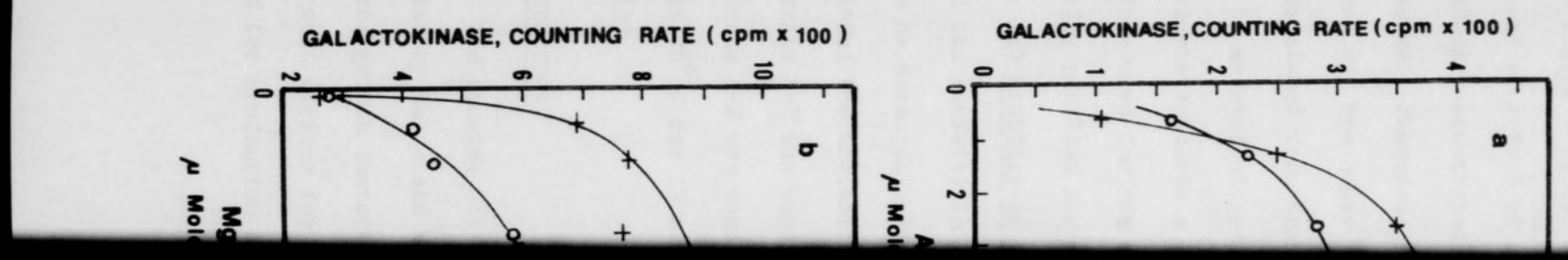

a
GLUCOKINASE, COUNTING RATE (cpm x 100)
0
12
24
36
48
60
72
84
96
108
120
132
144
3
4
5
6
7
8
9
10
11
12
pH

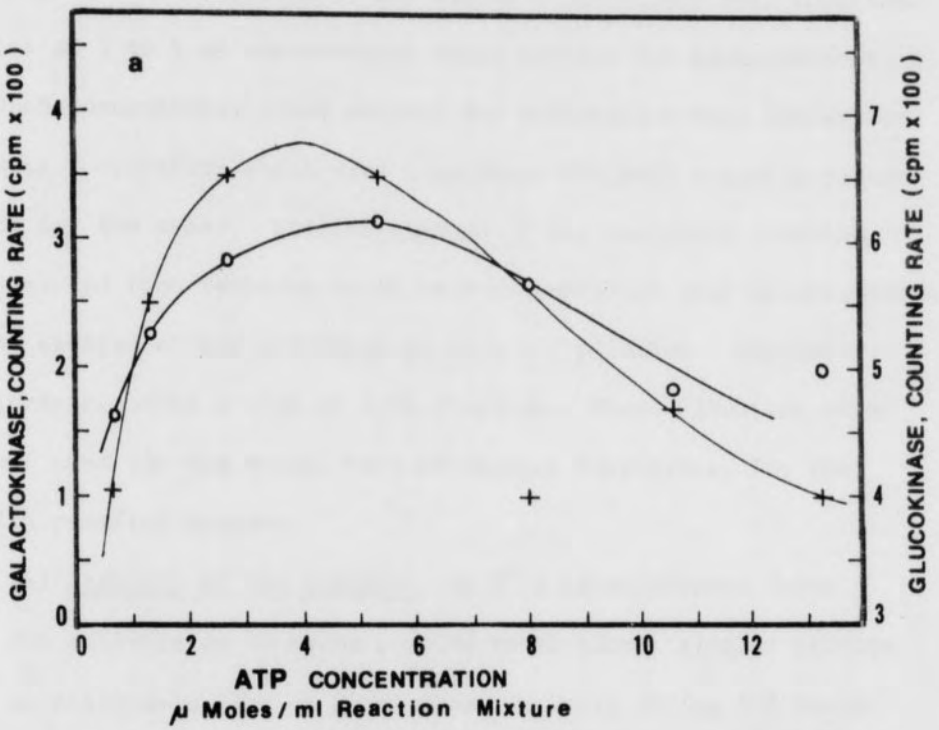
Figure 2

Influence of Mg^{++} ion and ATP Concentrations on the Activities of Galactokinase and Glucokinase

O———O : Galactokinase

+———+ : Glucokinase




a
GALACTOKINASE, COUNTING RATE (cpm x 100)
GLUCOKINASE, COUNTING RATE (cpm x 100)
ATP CONCENTRATION
μ Moles / ml Reaction Mixture

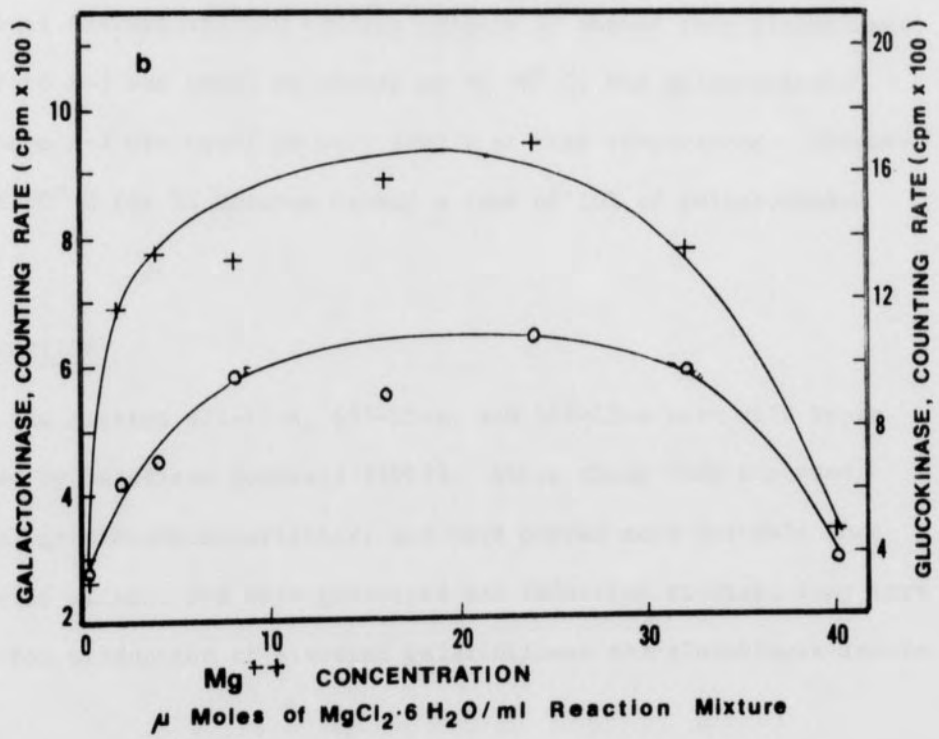

b
GALACTOKINASE, COUNTING RATE (cpm x 100)
GLUCOKINASE, COUNTING RATE (cpm x 100)
Mg++ CONCENTRATION
μ Moles of MgCl2·6 H2O / ml Reaction Mixture

Figure 3 (substrate A-1) and Figure 4 (substrate B-1) show that dilutions of 1 to 5 of concentrated crude extract for galactokinase and 1 to 40 of concentrated crude extract for glucokinase were linear for 10 minutes. Therefore these were considered the most suitable concentrations for the assay. Similar studies of the partially purified enzymes eluted from Sephadex G-150 columns indicated that galactokinase could be assayed either undiluted or at a 1/2 dilution. Similarly, glucokinase requires a 1/10 or 1/20 dilution. These dilutions were therefore used for the assay, with 10 minutes incubation, for the partially purified enzymes.

(e) Stablity of the enzymes: At 5^{o} C galactokinase loses 42% of its activity in 13 hours and 76% in 42 hours; similar studies showed no detectable loss of glucokinase activity during 103 hours. Additional thermostability studies (Figure 5) showed that glucokinase (substrate B-3 was used) is stable up to 40^{o} C, but galactokinase (substrate A-2 was used) is very labile at this temperature. Incubation at 30^{o} C for 10 minutes caused a loss of 10% of galactokinase activity.

2. INDUCTION

The strains 411-L5-A, 425-L5-A, and 417-L5-a were wild types isolated by Bates and Woodward (1967). Since these show improved glycerol growth characteristics, and have proven more suitable than other wild strains for beta-galactosidase induction studies, they were tested for production of elevated galactokinase and glucokinase levels

Figure 3

Phosphorylation of Galactose by Galactokinase and ATP in Crude Extracts as a Function of Reaction Time

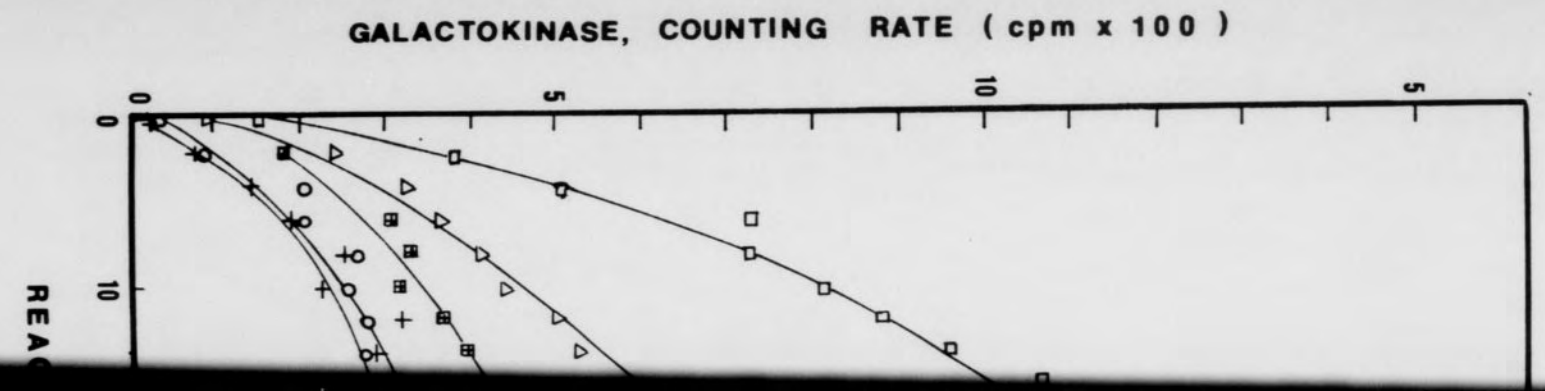
□———□ : Micro concentrated crude extract

△———△ : 1/5 dilution of micro concentrated crude extract

⊞———⊞ : 1/10 dilution of micro concentrated crude extract

O———O : 1/20 dilution of micro concentrated crude extract

+———+ : 1/40 dilution of micro concentrated crude extract



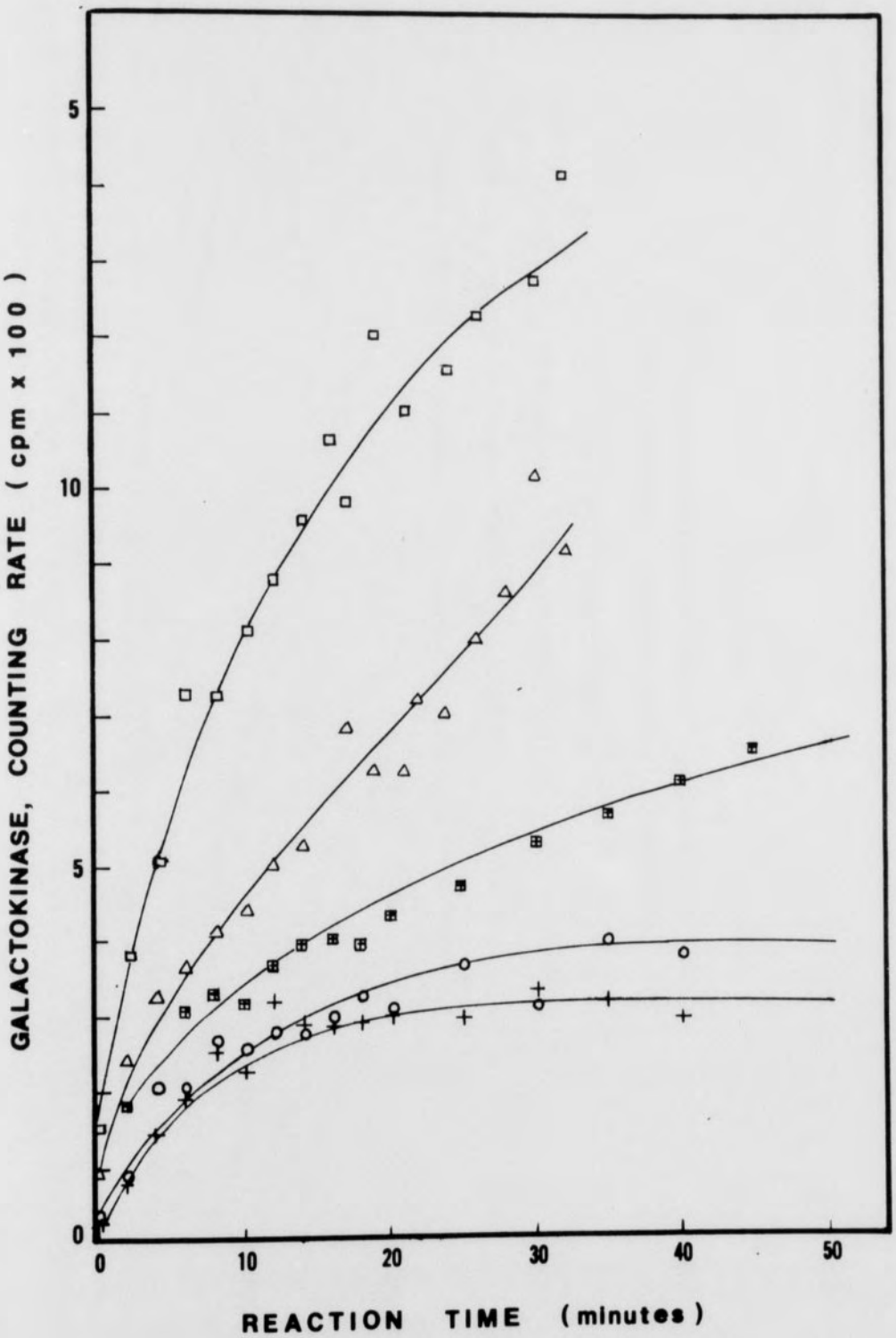

GALACTOKINASE, COUNTING RATE (cpm x 100)
5
10
5
0
0
10
20
30
40
50
REACTION TIME (minutes)

Figure 4

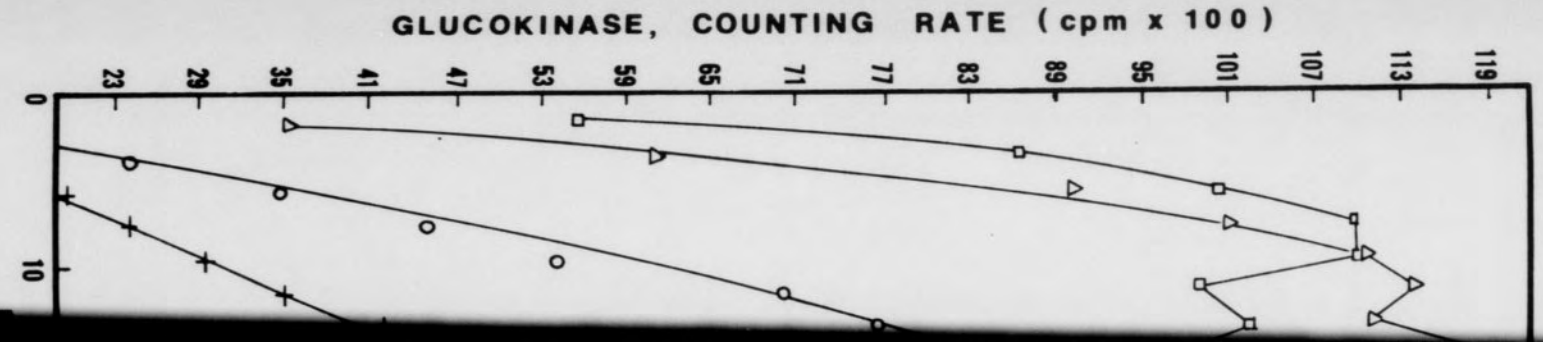
Phosphorylation of Glucose by Glucokinase and ATP in Crude Extracts as a Function of Reaction Time

□——□ : Micro concentrated crude extract

△——△ : 1/5 dilution of micro concentrated crude extract

o——o : 1/20 dilution of micro concentrated crude extract

+——+ : 1/40 dilution of micro concentrated crude extract



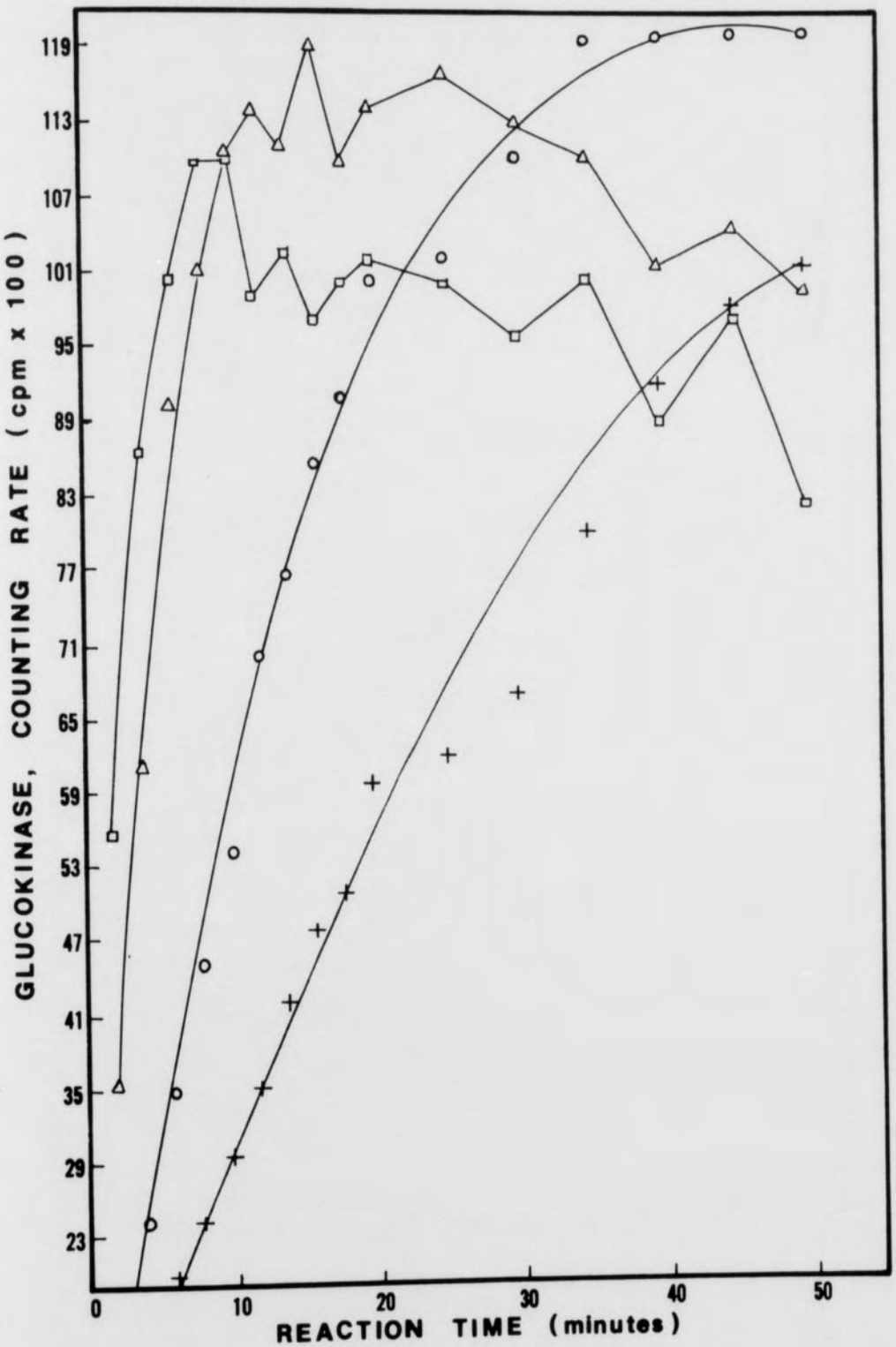

GLUCOKINASE, COUNTING RATE (cpm x 100)
119
113
107
101
95
89
83
77
71
65
59
53
47
41
35
29
23
0
10
20
30
40
50
REACTION TIME (minutes)

Figure 5

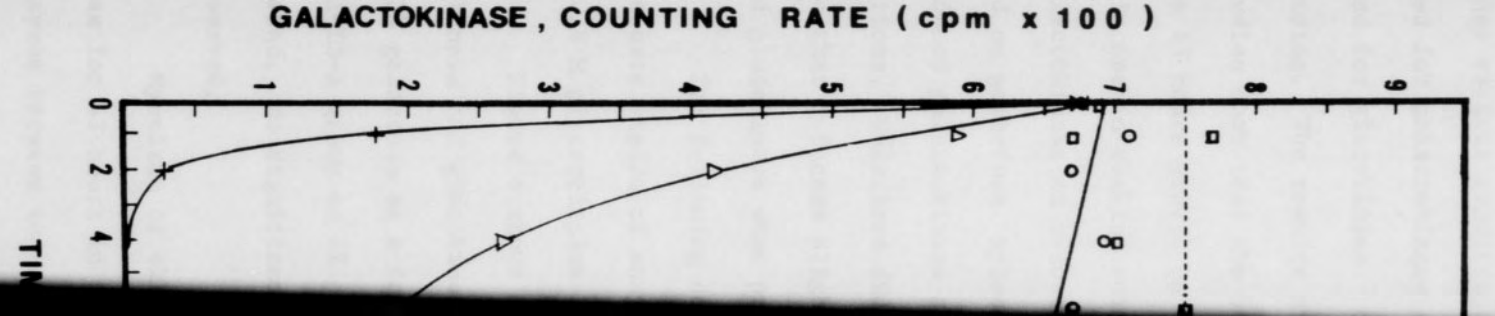
Thermostability of Galactokinase and Glucokinase in Concentrated Crude Extracts

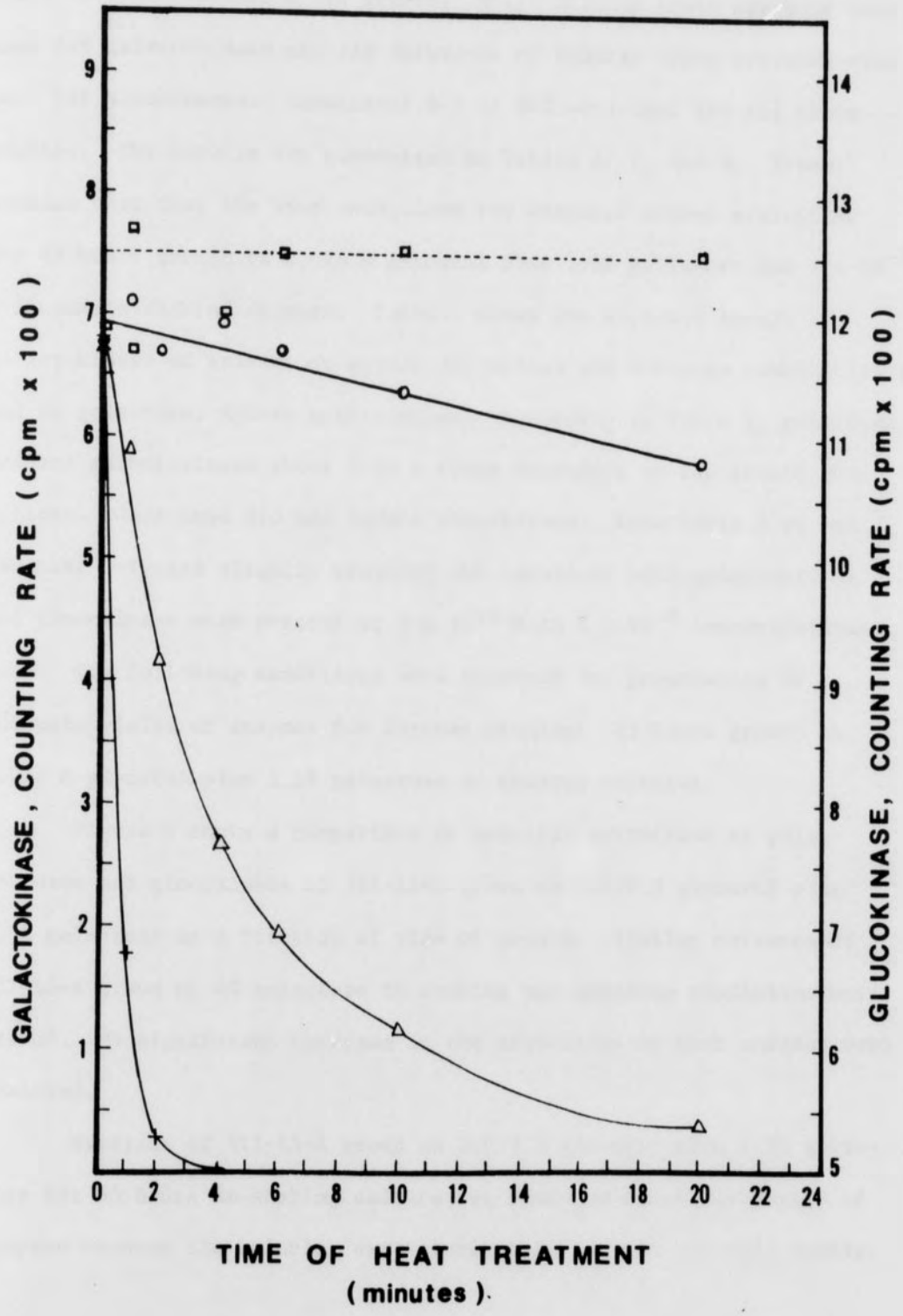
□——□ : 40° C Glucokinase

o——o : 30° C Galactokinase

Δ——Δ : 40° C Galactokinase

+——+ : 50° C Galactokinase




GALACTOKINASE, COUNTING RATE (cpm x 100)
GLUCOKINASE, COUNTING RATE (cpm x 100)
TIME OF HEAT TREATMENT
(minutes)
0
2
4
6
8
10
12
14
16
18
20
22
24
1
2
3
4
5
6
7
8
9
5
6
7
8
9
10
11
12
13
14

under various conditions for growth. Micro diluted crude extracts were used for galactokinase and 1/8 dilutions of diluted crude extracts were used for glucokinase. Substrates A-2 or B-2 were used for all these studies. The results are summarized in Tables 1, 2, and 3. These studies show that the best conditions for elevated enzyme activities are 43 hours growth on 0.018 M glycerol plus 1.5% galactose and 1×10^{-3} M D-fucose in shaking culture. Table 1 shows the elevated levels of galactokinase on xylose; on galactose, xylose and D-fucose combinations; and on galactose, xylose combinations. According to Table 2, galactose induced galactokinase about 3 to 6 times depending on the growth conditions. Galactose did not induce glucokinase. From Table 3 we can see that D-fucose slightly elevated the levels of both galactokinase and glucokinase when present at 5×10^{-4} M to 1×10^{-3} concentrations.

The following conditions were selected for preparation of adequate yields of enzymes for further studies: 43 hours growth on 0.018 M glycerol plus 1.5% galactose in shaking cultures.

Figure 6 shows a comparison of specific activities of galactokinase and glucokinase of 411-L5-A grown on 0.018 M glycerol plus 1.5% galactose as a function of time of growth. Similar cultures of 411-L5-A grown on 4% galactose in shaking and standing conditions were tested. No significant increase in the activities of both enzymes were observed.

Mycelium of 411-L5-A grown on 0.018 M glycerol plus 1.5% galactose for 43 hours in shaking culture was examined for distribution of enzymes between the soluble, extractable fraction and the cell debris.

Table 1

Galactokinase and Glucokinase levels in 411-L5-A grown in Standing Culture

on Various Carbon Sources

Experiment Number	Sugars	Growth Time (hours)	Wet Weight (grams)	Protein (mg./ml.)	Specific Activities (units/mg. protein)	
					Galactokinase x 10^{-4}	Glucokinase x 10^{-2}
48	1.5% Glucose	48	1.84	3.35	1.74	3.09
48	1.5% Arabinose	96	1.24	3.92	1.26	2.19
48	1.5% Xylose	96	2.50	3.02	6.99	2.21
48	1.5% Glycerol	96	1.56	4.93	2.84	1.50
48	1.5% Galactose	98	0.16	4.70	5.38	2.79
48	1.5% Gly. + 1.5% Gal.	96	1.51	3.80	5.33	2.94
50	1.5% Gal. + 0.15% Xyl. + 0.15% Fucose	72	0.82	6.95	15.50	2.43
50	1.5% Gal. + 0.15% Xyl. + 0.15% Fucose	97	0.71	7.10	7.14	2.09
50	1.5% Gal. + 0.15% Xyl.	97	0.85	7.21	8.00	1.96
51	1.5% Gal. + 0.15% Fucose	72	0.17	6.23	5.18	2.97
51	0.18 M Gly. + 0.15% Xyl. + 0.15% Fucose	72	0.75	7.34	1.80	3.19
51	0.18 M Gly. + 0.15% Gal. + 0.15% Fucose	72	0.72	7.74	1.62	2.99

Table 2

Induction of Galactokinase and Glucokinase by Galactose in Shaking and Standing Culture

Experiment Number	Strains	Sugars		Growth Condition	Growth Time (hours)	Wet Weight (grams)	Protein (mg./ml.)	Specific Activities (units/mg. protein)	
		Glycerol (M)	Galactose (%)					Galactokinase x 10^{-4}	Glucokinase x 10^{-2}
54	411-L5-A	0.018	---	Shaking	44	1.07	2.69	3.43	1.71
54	411-L5-A	0.018	1.5	Shaking	44	4.17	4.17	13.10	3.08
54	425-L5-A	0.018	---	Shaking	44	0.99	2.89	3.09	3.20
54	425-L5-A	0.018	1.5	Shaking	44	2.74	4.94	9.29	2.86
51	417-L5-a	0.18	---	Standing	72	0.65	6.29	0.88	2.58
51	417-L5-a	----	1.5	Standing	72	0.11	4.15	6.01	2.70
51	425-L5-A	0.18	---	Standing	72	0.58	6.73	1.12	3.21
51	525-L5-A	----	1.5	Standing	72	0.18	7.21	6.79	2.40

Table 3

Induction of Galactokinase and Glucokinase by D-fucose in the 411-L5-A Strain in Shaking Culture, 43 Hours

Sugars			Specific Activities (units/mg. protein)	
Glycerol (M)	Galactose (%)	D-fucose (M)	Galactokinase x 10^{-4}	Glucokinase x 10^{-2}
0.018	---	---	9.0	3.47
0.018	1.5	---	19.0	3.86
0.018	1.5	3.0×10^{-4}	23.1	3.93
0.018	1.5	5.0×10^{-4}	21.9	4.69
0.018	1.5	1.0×10^{-3}	25.6	4.57
0.018	1.5	2.0×10^{-3}	22.9	3.94
0.018	1.5	5.0×10^{-3}	25.3	4.11

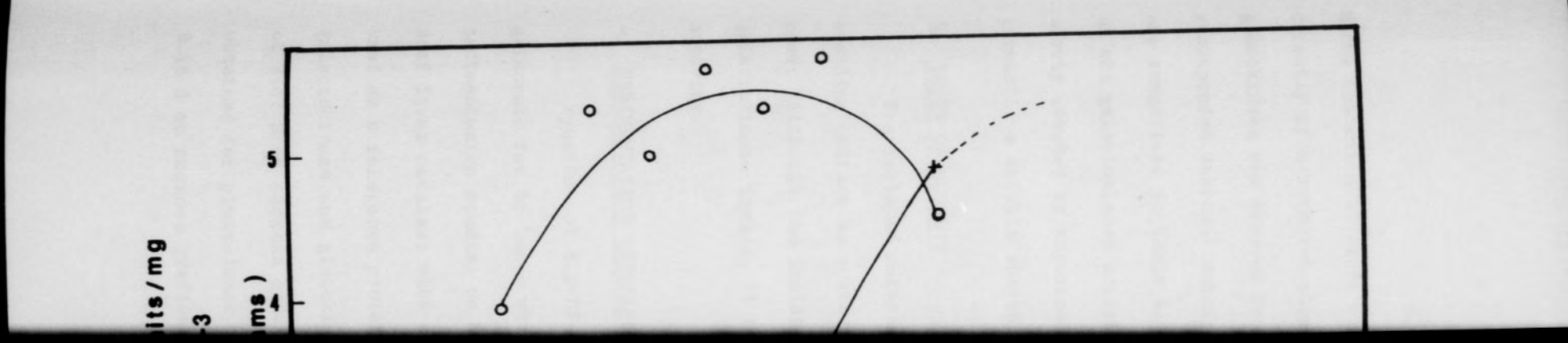
Figure 6

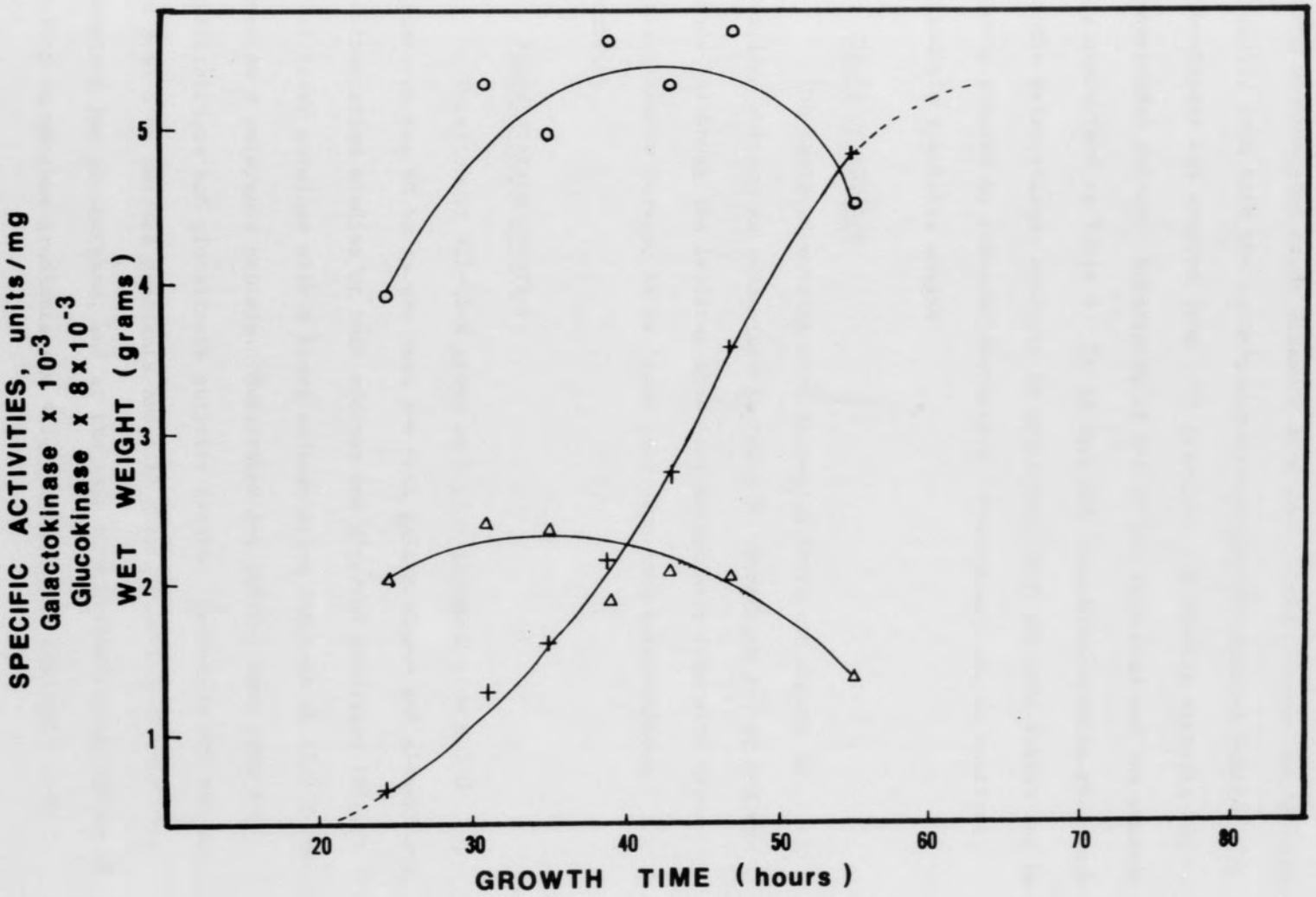
Specific Activities of Galactokinase and Glucokinase of 411-L5-A Grown on 0.018 M Glycerol Plus 1.5% Galactose as a Function of Time of Growth

O———O : Specific activity of glucokinase

△———△ : Specific activity of galactokinase

+———+ : Wet weight of mycelium




SPECIFIC ACTIVITIES, units/mg
Galactokinase x 10^{-3}
Glucokinase x 8×10^{-3}
WET WEIGHT (grams)
1
2
3
4
5
20
30
40
50
60
70
80
GROWTH TIME (hours)

Micro concentrated crude extracts were used. Galactokinase was assayed directly from both the soluble extracts and the resuspended debris, and glucokinase was assayed from 1/40 dilutions for soluble extracts and resuspended debris. Substrates of A-2 or B-3 were used and the studies are summarized in Table 4. It is apparent from these studies that much of the galactokinase activity is associated with the cell debris and is slowly removed by repeated extraction. Glucokinase is, in contrast, primarily a soluble enzyme.

3. TESTS OF MUTANTS

The isolated mutants were tested on different sugars in standing culture as summarized in Table 5. Substrate A-2 or B-2 was used. Although the isolates show some differences from wild type galactokinase levels, it is clear that none lack galactokinase activity.

4. SEDIMENTATION CONSTANTS

Mycelium of 411-L5-A grown on 0.18 M glycerol plus 0.5 M galactose for 48 hours was used for both galactokinase and glucokinase sedimentation studies on both sucrose and glycerol gradients (Figure 7). Beef liver catalase with a known sedimentation constant of 11.3 S was used as a reference protein. Substrates A-1 and B-1 were used for galactokinase and glucokinase activity assays. Sedimentation values of 3.67 S on sucrose gradients and 4.10 S on glycerol gradients were obtained for glucokinase, and at the same time sedimentation values of 4.26 S on sucrose gradients and 4.34 S on glycerol gradients were

Table 4

Distribution of Enzymes Between the Soluble, Extractable Fraction and the Cell Debris

Samples		Galactokinase Net Counting Rate, cpm	Glucokinase Net Counting Rate, cpm
First Extraction	Soluble Extract*	704.5 ± 10.0	1158.8 ± 14.4
	Resuspended Debris**	1459.6 ± 17.3	240.4 ± 5.8
Second Extraction	Soluble Extract	957.4 ± 12.4	211.6 ± 5.6
	Resuspended Debris	1235.7 ± 15.1	50.0 ± 4.3
Third Extraction	Soluble Extract	644.2 ± 9.4	34.1 ± 4.2
	Resuspended Debris	457.0 ± 7.7	6.4 ± 4.0
Fourth Extraction	Soluble Extract	166.1 ± 5.2	-1.5 ± 4.0
	Resuspended Debris	131.6 ± 4.9	-20.7 ± 3.9

* Activity remaining in the supernant fraction after centrifugation at 15,000 rpm (27,000 x g) for 30 minutes

** Debris was resuspended in the same sodium phosphate buffer containing 10^{-2} M DTT used for extraction. The total volume of the resuspended debris was equal to that of the soluble extract.

Table 5

Comparison of the Growth and of the Spcific Activities of Galactokinase and Glucokinase on Different Sugars in the 411-L5-A Strain and the Mutants (GKL-numbers) in Standing Culture

Strain	On 0.018 M Glycerol for 72 Hours			On 1.5% Xylose for 48 Hours			On 1.5% Xylose for 72 Hours		
	% Growth of 411-L5-A (a)-1	Specific Activities (units/mg. protein)		% Growth of 411-L5-A (a)-2	Specific Activities (units/mg. protein)		% Growth of 411-L5-A (a)-3	Specific Activities (units/mg. protein)	
		Galacto-kinase, x 10^{-4}	Gluco-kinase, x 10^{-2}		Galacto-kinase, x 10^{-4}	Gluco-kinase, x 10^{-2}		Galacto-kinase, x 10^{-4}	Gluco-kinase, x 10^{-2}
411-L5-A	100	0.64	2.25	100	0.81	2.10	100	3.64	2.44
GKL-1	15	1.25*	2.65*	100	2.93	2.31	71	5.10	2.60
GKL-3	61	0.81*	2.63*	114	3.58	2.48	85	7.24	1.92
GKL-6	86	0.39	2.60	100	2.31	2.21	94	2.78	2.52
GKL-7	65	0.35	2.00	104	1.17	1.89	83	2.04	2.40
GKL-13	29	0.36	2.95	---	----	----	74	1.86	2.21
GKL-14	82	0.69	1.78	102	1.02	2.35	88	1.85	1.73
GKL-15	31	0.80	2.45	44	0.64	1.68	61	4.84	1.88
GKL-16	56	0.59*	3.38*	0	----	----	0	----	----

Table 5 Continued

Strain	On 1.5% Galactose for 96 Hours			On 1.5% Xylose + 1.5% Galactose for 72 Hours		
	% Growth of 411-L5-A (a)-4	Specific Activities (units/mg. protein)		% Growth of 411-L5-A (a)-5	Specific Activities (units/mg. protein)	
		Galactokinase x 10^{-4}	Glucokinase x 10^{-2}		Galactokinase x 10^{-4}	Glucokinase x 10^{-2}
411-L5-A	100	0.66	2.23	100	1.20	1.68
GKL-1	$\ll$ 17	----	----	$<$ 70	1.39(b)-3	1.85(b)-3
GKL-3	$<$ 17	10.10(b)-1	1.78	$<$ 70	1.42(b)-3	1.95(b)-3
GKL-6	39	5.08	2.15	101	0.61	2.10
GKL-7	61	5.16	2.76	70	1.87	2.36
GKL-13	96	5.04	2.38	78	1.36	1.96
GKL-14	17	----	----	103	2.36	2.18
GKL-15	$\ll$ 17	----	----	34(b)-2	----	----
GKL-16	$<$ 17	----	----	24(b)-2	----	----

(a) Wet weight of 411-L5-A: -1, 1.01 gram; -2, 0.50 gram; -3, 1.18 gram; -4, 0.23 gram; -5, 0.67 gram.

(b) Growth time, hours: -1, 113 hours; -2, 160 hours; -3, 97 hours.

* Protein was determined in trichloroacetic acid precipitates by the biuret method.

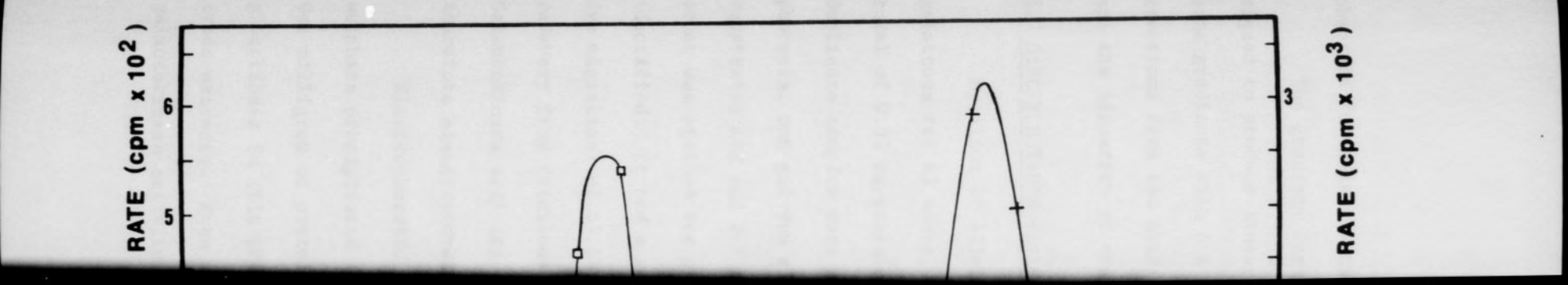
Figure 7

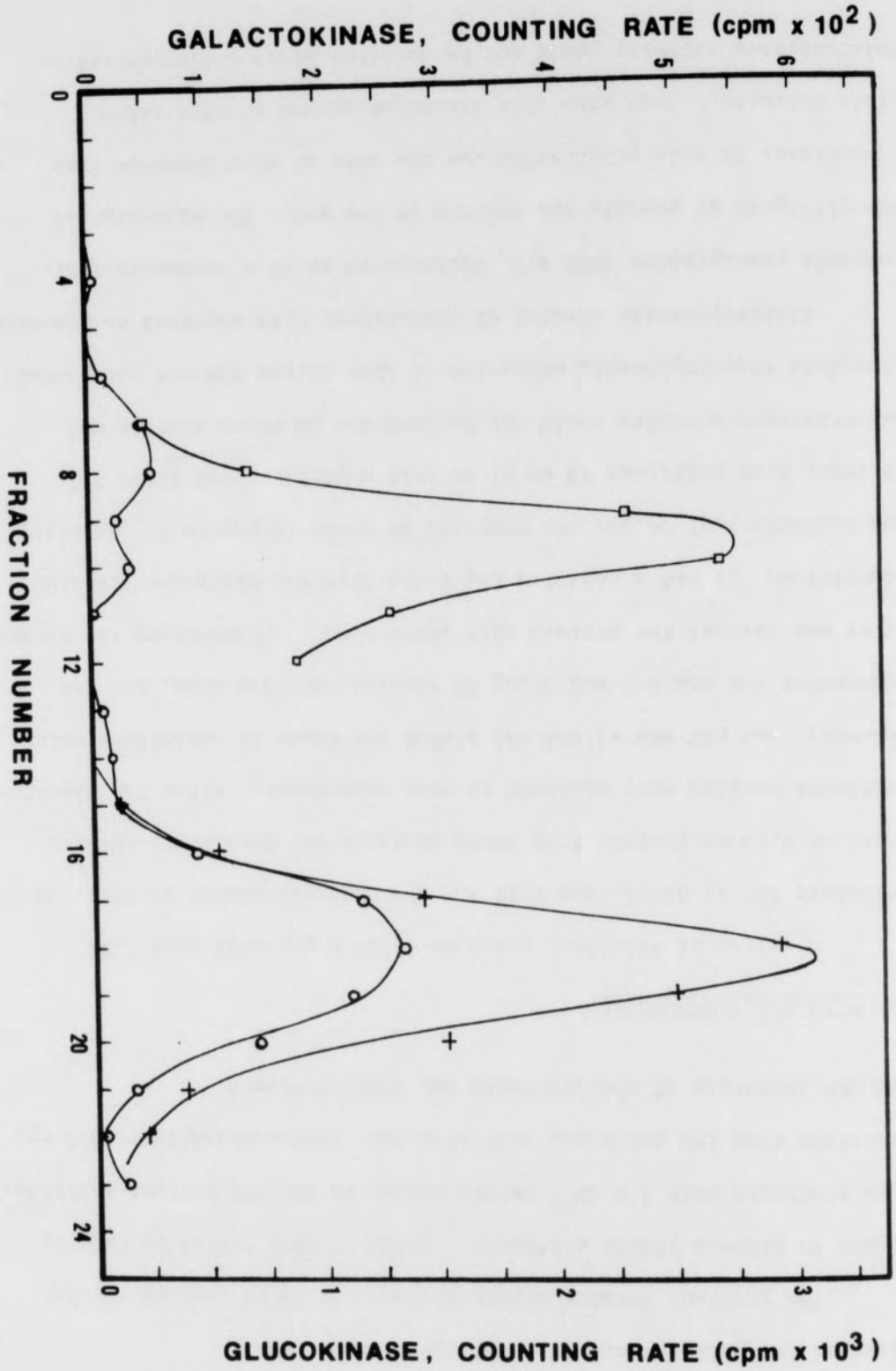
Sedimentation Velocities of Galactokinase and Glucokinase on Glycerol Density Gradients

□——□ : Catalase

O——O : Galactokinase

+——+ : Glucokinase




GALACTOKINASE, COUNTING RATE (cpm x 10²)
0
1
2
3
4
5
6
FRACTION NUMBER
0
4
8
12
16
20
24
GLUCOKINASE, COUNTING RATE (cpm x 10³)
0
1
2
3

observed for galactokinase (33,000 rpm; 14.82 hours).

The gradient forming apparatus used for these studies was designed to produce linear gradients. Linearity was tested by forming some gradients with 5×10^{-3} methyl orange in the 20% sucrose solution. Fractions from the gradients were then read photometrically at 505 mμ, and the linearity of the gradients was thus confirmed.

5. DISC ELECTROPHORESIS

Mycelium of 411-L5-A, grown on 0.018 M glycerol plus 1.5% galactose for 43 hours, was used for the electrophoretic studies. A total of 0.33 mg. protein from crude extracts was applied to the gel. Duplicate samples were analyzed in each experiment. After the electrophoresis, one gel was sliced and eluted for assay of activities using substrates A-2 and B-2 and using 20 minutes reaction time, and the other was stained for protein with amido black. Glucokinase was clearly identified: it had a relative migration (Protein migration/Tracking dye migration) of 64.5%; the position is shown in Figure 8. Activity recovery from fractions 18 to 21 in this experiment was about 57%. Galactokinase activity could not be detected in these samples and therefore electrophoretic migration of this enzyme was not determined.

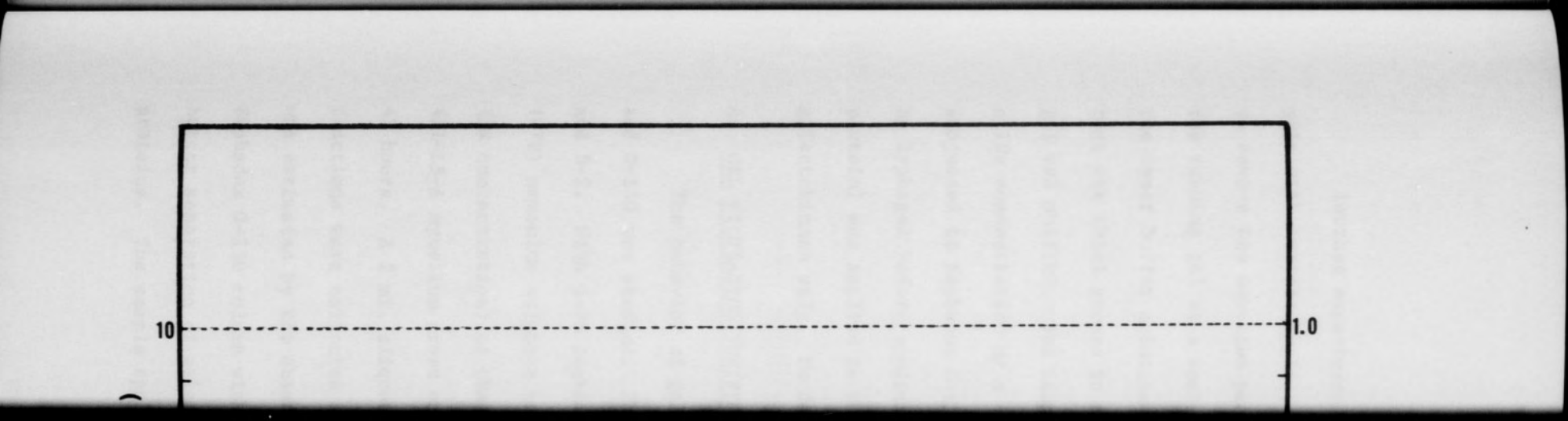
Electrophoretic studies of glucokinase were repeated on ammonium sulphate precipitates (40% W/V, redissolved at 10 x concentration). One milligram of protein was applied to the gel. The migration of glucokinase in this preparation was the same as that observed from crude extracts. Even with this increased enzyme concentration, galactokinase activity could not be detected after electrophoresis.

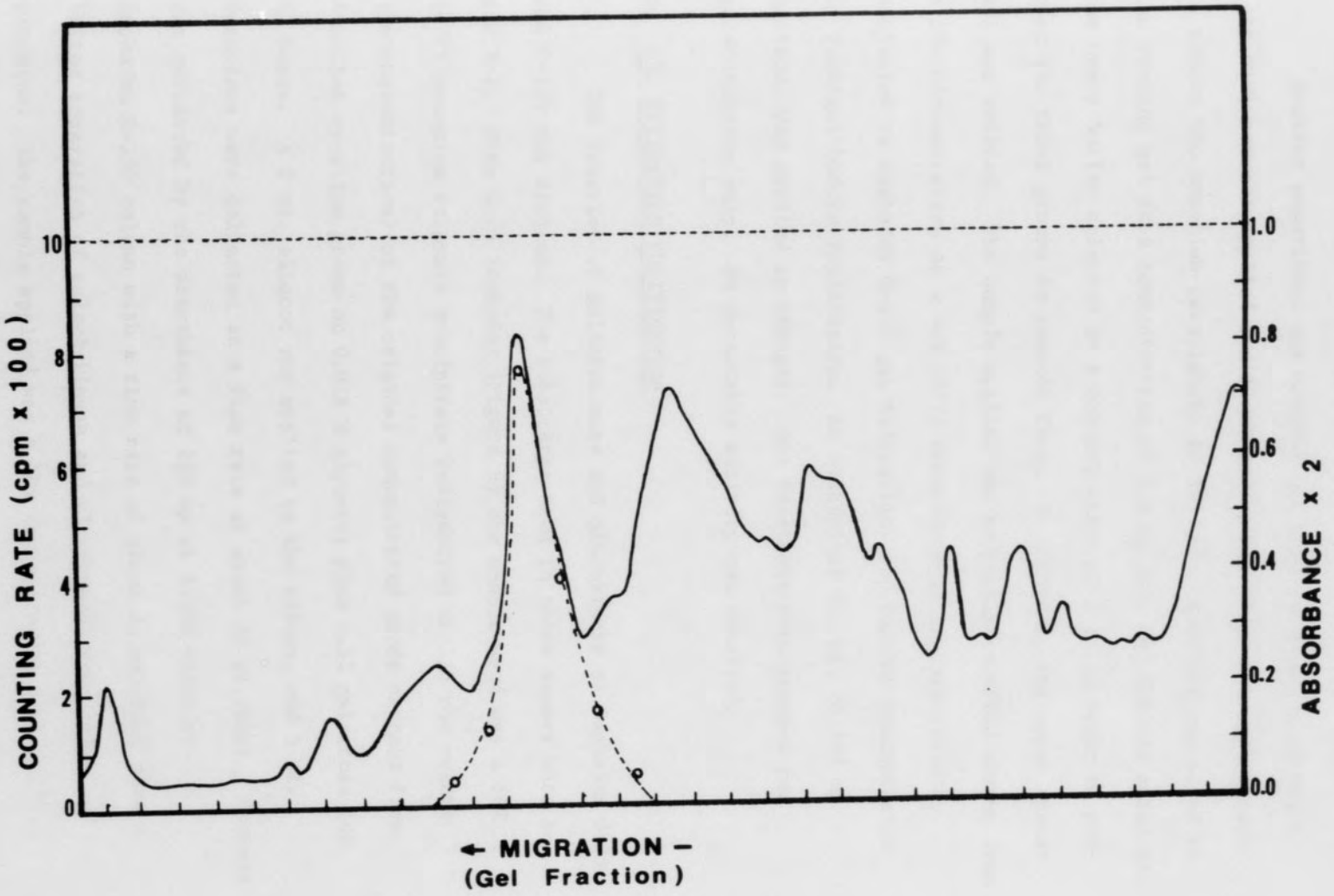
Figure 8

Disc Electrophoresis of Glucokinase in Crude Extract

——— : Protein (Amido Black Stain)

O———O : Glucokinase activity




COUNTING RATE (cpm x 100)
10
8
6
4
2
0
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE x 2
← MIGRATION —
(Gel Fraction)

Another experiment was carried out with carefully pre-cooled gels with current applied for 30 minutes prior to sample application to remove the ammonium persulphate in the gel. Also DTT was added to the running gel at a concentration of 1.5 mg./ml. and MCE was added to the upper buffer solution to a concentration of 5 mM in order to protect the thiol groups in reduced form. In addition, the upper spacer gel was omitted. The sample applied was partially purified enzyme from a 10x concentration of a 40% (W/V) ammonium sulphate precipitate, subjected to Sephadex G-150 gel filtration, and further concentrated by lyphogel before application. An aliquot of 0.1 ml. (0.788 mg. protein) was applied to the gel. Gel fractions were assayed for galactokinase only. No detectable activity was observed.

6. GEL FILTRATION PURIFICATION

The behavior of galactokinase and glucokinase on Sephadex G-75 and G-150 was studied. The substrates used in these assays were A-2 and B-2. With G-75 Sephadex (Figure 9) the sample used was a 60% (W/V) ammonium sulphate precipitate redissolved in 1/5 the volume (5x concentration) of the original concentrated crude extract from 411-L5-A mycelium grown on 0.018 M glycerol plus 1.5% galactose for 43 hours. A 2 ml. aliquot was applied to the column, and 5 ml. fractions were collected at a flow rate of about 17 ml./hour. Protein was estimated by the absorbance at 280 mμ at 1/100 dilution. A Sephadex G-150 column with a flow rate of about 12 ml./hour gave a better separation of galactokinase and glucokinase from other proteins. The sample applied was a 10x concentration from a 40%

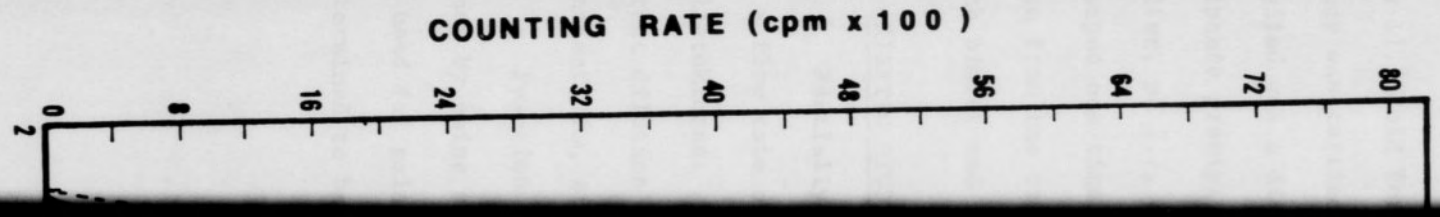
Figure 9

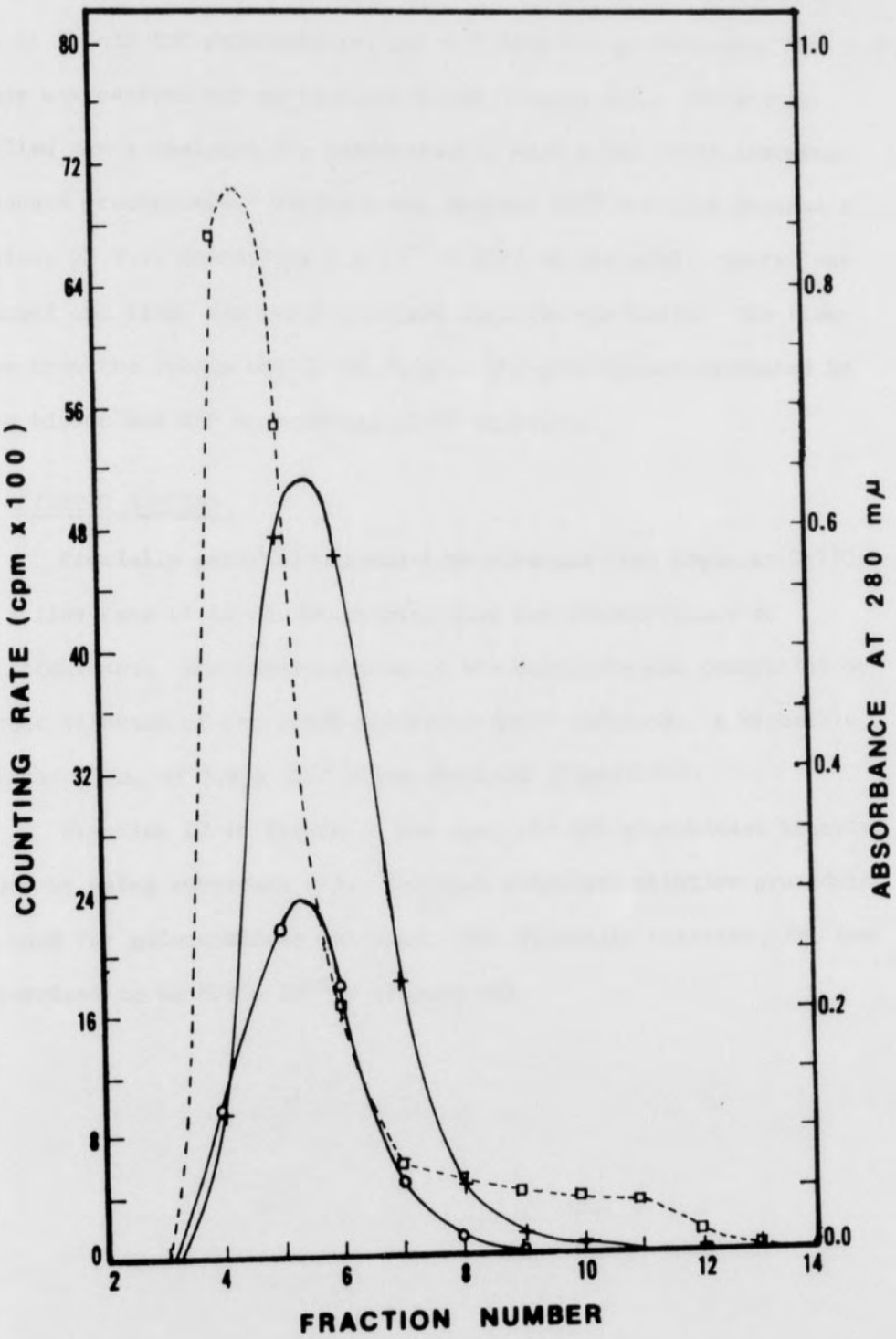
Galactokinase and Glucokinase Activities from Sephadex G-75 Gel Filtration

O———O : Galactokinase activity

+———+ : Glucokinase activity

□———□ : Absorbance at 280 mμ




COUNTING RATE (cpm x 100)
ABSORBANCE AT 280 mμ
FRACTION NUMBER
80
72
64
56
48
40
32
24
16
8
0
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
10
12
14

(W/V) ammonium sulphate precipitate. The maxium purification obtained was 11.7 fold for galactokinase and 4.2 fold for glucokinase. Another study was carried out on Sephadex G-150 (Figure 10). The sample applied was a dialyzed 10x concentration from a 40% (W/V) ammonium sulphate precipitate. Dialysis was against 10^{-2} M sodium phosphate buffer, pH 7.7, containing 3 x 10^{-4} M DTT, in the cold. Buffer was changed one time, and total dialysis time was two hours. The flow rate from the column was 20 ml./hour. The protein was estimated by both biuret and 280 mμ readings (1/40 dilution).

7. KINETIC STUDIES

Partially purified preparations obtained from Sephadex G-150, at a flow rate of 12 ml./hour, were used for kinetic study of galactokinase. The concentration of the substrate was controlled by direct dilution of the stock substrate (A-2) solution. A Michaelis constant, Km, of 5.8 x 10^{-4} M was obtained (Figure 11).

Fraction 12 in Figure 10 was used for the glucokinase kinetic study by using substrate B-3. The same substrate dilution procedure as used for galactokinase was used. The Michaelis constant, Km, was determined to be 5.8 x 10^{-4} M (Figure 11).

Figure 10

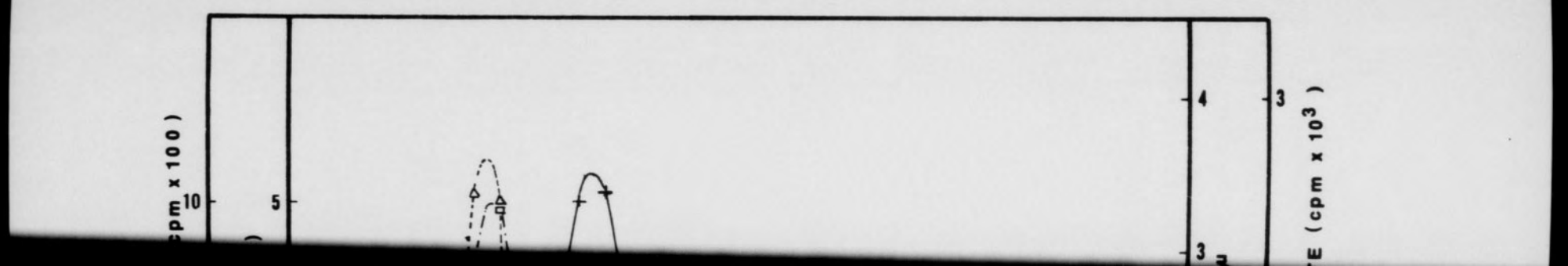
Purification of Galactokinase and Glucokinase by Gel Filtration on Sephadex G-150

O——O : Galactokinase activity

+——+ : Glucokinase activity

△——△ : Absorbance at 280 mμ

□——□ : Protein (mg./ml., Biuret)



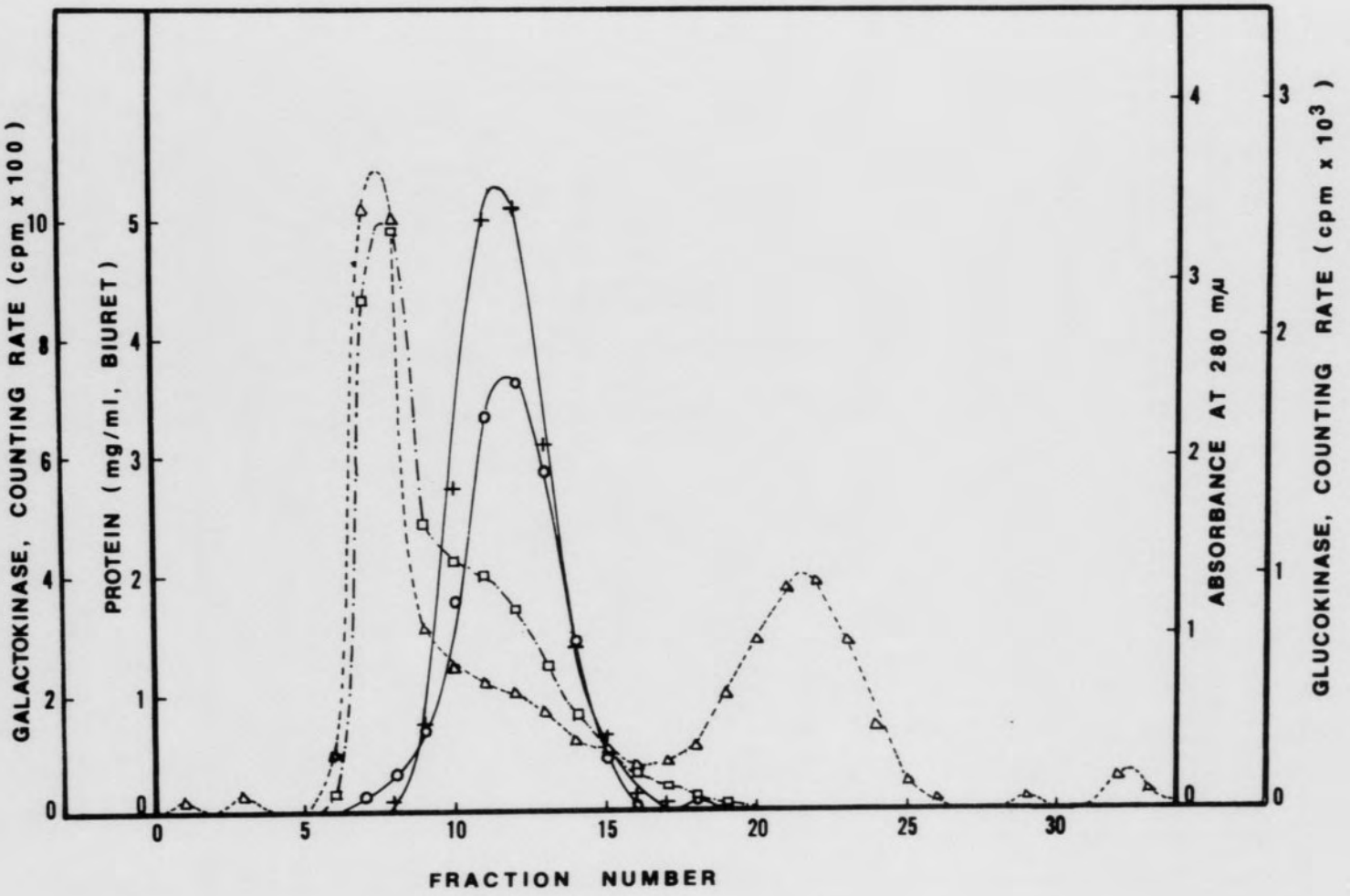
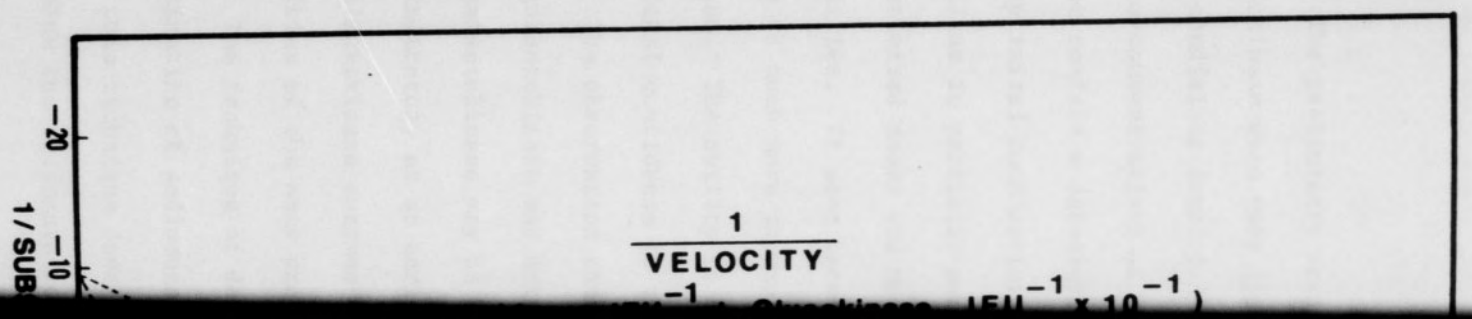

GALACTOKINASE, COUNTING RATE (cpm x 100)
PROTEIN (mg/ml, BIURET)
ABSORBANCE AT 280 mμ
GLUCOKINASE, COUNTING RATE (cpm x 10³)
FRACTION NUMBER
0
5
10
15
20
25
30

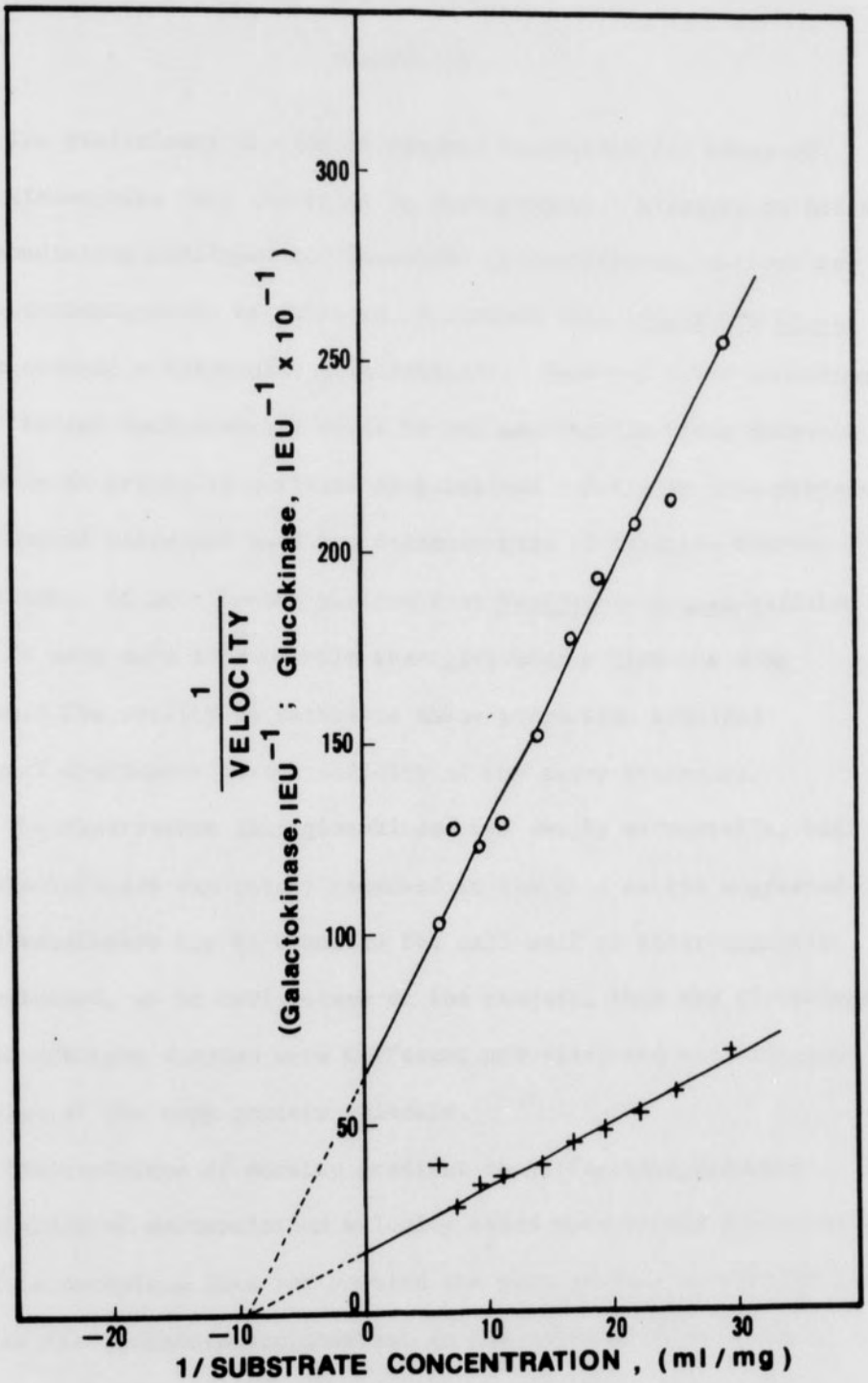
Figure 11

Lineweaver-Burk Plot for Galactokinase and Glucokinase

O———O : Galactokinase

+———+ : Glucokinase




300
250
200
150
100
50
0
−20
−10
0
10
20
30
1/VELOCITY
(Galactokinase, IEU^{-1} ; Glucokinase, IEU^{-1} x 10^{-1})
1/SUBSTRATE CONCENTRATION , (ml/mg)

DISCUSSION

The preliminary studies of optimal conditions for assay of galactokinase were very important in this project. Attempts to use the assay conditions developed for bacterial galactokinases, without regard to concentrations of extracts, indicated that Neurospora crassa did not contain a detectable galactokinase. However, after conditions were optimized such activity could be demonstrated in crude extracts as well as in partially purified preparations. Early in this project, the optimized assay was used for determination of relative thermostabilities. It soon became obvious that Neurospora crassa galactokinase is much more thermolabile than glucokinase from the same organism. The ability to determine these properties provided additional confidence in the validity of the assay procedure.

The observation that glucokinase was easily extractable, but that galactokinase was mainly retained in the cell debris suggested that galactokinase may be bound to the cell wall or other organelle. This indicated, at an early stage of the project, that the glucokinase and galactokinase enzymes were different proteins, and not different activities of the same protein molecule.

The technique of density gradient centrifugation allows a determination of sedimentation velocity based upon enzyme activity. Since this technique does not require the pure enzyme, we were able to determine the sedimentation constant in the crude extract. These

determinations indicated that the galactokinase and glucokinase are approximately 4 S molecules. This suggests a molecular weight of approximately 50,000.

By using ammonium sulfate fractionation and gel filtration, a partially purified preparation of both enzymes was obtained. The exclusion from Sephadex G-75 and the partial inclusion into G-150 was consistent with the molecular weight estimate obtained from the sedimentation velocity studies.

The ability to obtain a partially purified preparation allowed the assay of galactokinase in a preparation free of low molecular weight contaminants. This greatly reduced the chance that the observed activity was an artifact of phosphorylation of sugars present in the extracts. These partially purified enzymes were also used for studies of kinetic properties.

Kinetic studies for both enzymes indicated that they have the same Km value of 5.8×10^{-4} M. This may be compared to the values of 7×10^{-4} M galactose for galactokinase from Escherichia coli obtained by Sherman and Adler (1963); 1×10^{-4} to 3×10^{-4} M galactose for galactokinase from pig liver obtained by Ballard (1966); 1×10^{-4} M glucose for glucokinase from Baker's yeast by Darrow and Colowick (1962); 2×10^{-2} M glucose for glucokinase from rat liver by Parry and Walder (1966); 8×10^{-5} M glucose for glucokinase from Aerobacter aerogenes PRL-R3 by Anderson and Kamel (1966); 1.5×10^{-4} M glucose for glucokinase from yeast by McDonald (1955). Although the Km values are identical for galactokinase and glucokinase from Neurospora crassa,

the amounts of the two activities are quite different. Crude extracts contain glucokinase activity approximately 23 times that of the total extractable galactokinase activity.

In electrophoresis studies the migration of glucokinase was clearly demonstrated in the gels. Galactokinase, however, could not be demonstrated. In view of the thermolability of galactokinase, it is likely that most of the activity was lost during the separation.

The attempt to obtain mutants deficient in galactokinase activity were successful to the extent that some mutants showed a sharply reduced rate of growth on galactose. These isolates, however, still retained significant levels of galactokinase activity and were therefore not suitable for a direct gene-enzyme study. From the growth studies of these mutants, it appears that glucokinase, like the D-glucokinase in Aerobacter aerogenes PRL-R3 (Anderson and Kamel, 1966), is a constitutive enzyme, as its level remains about the same whether the cells are grown on glucose, galactose, xylose, or glycerol. This suggests that galactokinase and glucokinase are controlled by two different genes.

The general question of separate identity of the two enzymes cannot be answered on the basis of the data presented in this thesis. The similar sedimentation and gel filtration characteristics demonstrate that if the two activities are associated with different proteins, the two are almost identical in size. The different extraction properties support the conclusion that these are two different proteins, but it could be argued that galactokinase is activated, or glucokinase is inhibited when attached to the cell debris.

The indications that galactokinase and glucokinase in Neurospora crassa are two different proteins are consistent with observations in other organisms, in which each activity is associated with a single protein. It is clear that the two proteins must have very similar properties.

This study suggests that further work with preparative disc electrophoresis, on DEAE ion exchange separation, and on further purification would be valuable. Such studies could provide conclusive evidence of whether different proteins are involved. Similar studies of related enzymes of galactose metabolism would also be valuable, in view of the importance of galactose derivatives in the Neurospora crassa cell wall structure.

SUMMARY

The studies described in this thesis have demonstrated the existence of the enzyme galactokinase (ATP: D-galactose 1-phosphotransferase, EC No. 2.7.1.6) in extracts prepared from mycelium of the fungus Neurospora crassa. This enzyme was found to have maximal activity at pH 8.5 in Tris-HCl buffer, and optimal concentrations of magnesium chloride and adenosine triphosphate were determined.

An important part of the study was comparison of the galactokinase enzyme with the closely related enzyme glucokinase (ATP: D-glucose 6-phosphotransferase, EC No. 2.7.1.2) from Neurospora. Glucokinase was found to be much more thermostabile than galactokinase, and both enzyme activities sedimented at the rate of approximately 4 S on glycerol or sucrose gradients in the preparative ultracentrifuge.

Electrophoretic analysis of extracts containing both enzyme activities clearly demonstrated the migration pattern of glucokinase, but galactokinase was inactivated during this separation procedure. This electrophoretic study therefore provided no evidence concerning the separate identity of the proteins associated with the two activities. The process of extraction of protein from the mycelium, however, demonstrated that glucokinase is freely extractable, but a large portion of the total galactokinase activity is associated with some insoluble component of the cell debris. This suggests that separate proteins provide the two enzyme activities, but the evidence

on this point is not definitive because of the possibility of activation, inhibition, or steric effects associated with attachment to a cellular organelle.

A purification of galactokinase of approximately 12-fold was obtained by salt fractionation and gel filtration, and this partially purified preparation was used for kinetic studies. In this way a Michaelis constant of 5.8×10^{-4} Molar galactose was determined for Neurospora galactokinase. Comparative studies of glucokinase demonstrated a Michaelis constant of 5.8×10^{-4} Molar glucose.

From these studies it is obvious that Neurospora crassa produces a galactokinase enzyme. This enzyme is present in crude extracts in an amount approximately 4% of the level of glucokinase. Although glucokinase and galactokinase could be separate activities of a single protein, the evidence suggests that these are separate, but very similar, proteins.

BIBLIOGRAPHY

Anderson, R. L., and M. Y. Kamel. 1966. Glucokinase, p. 388-392. In: W. A. Wood (ed.), Methods in Enzymology, Vol. IX, Carbohydrate Metabolism. Acad. Press, Inc., New York.

Atkinson, M. R., R. M. Burton, and R. K. Morton. 1961. Equilibrium constant of phosphoryl transfer from adenosine triphosphate to galactose in the presence of galactokinase. Biochem. J. 78: 813-820.

Ballard, F. J. 1966. Purification and properties of galactokinase from pig liver. Biochem. J. 98: 347-352.

Bates, Wm. K., R. S. Beck, and B. M. Eberhart. 1967. Extraction of mycelial protein: Some specific comparisons. Neurospora Newsletter, No. 12, p. 16.

Bates, Wm. K., and D. O. Woodward. 1967. Origin of the "Accelerated growth on lactose" ("AGL") trait. Neurospora Newsletter, No. 12: 11.

Bates, Wm. K., S. C. Hedman, and D. O. Woodward. 1967. Comparative inductive responses of two β-galactosidases of Neurospora. J. Bacteriol. 93: 1631-1637.

Beadle, G. W., and E. L. Tatum. 1945, Neurospora II: Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Botany, 32: 678-686.

Beers, R. F., and I. W. Sizer. 1952. A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase. J. Biol. Chem., 195: 133-140.

Britten, R. J., and R. B. Roberts. 1960. High-resolution density gradient sedimentation analysis. Science 131: 32-33.

Buttin, G. 1963. Mécanismes régulateurs dans la biosynthesése des enzymes du métabolisme du galactose chez Escherchia coli K-12, I. La biosynthese induite de la galactokinase et l'induction simultance de la séquence enzymatique (in French, English summary). J. Mol. Biol. 7: 164-183.

Cardini, C. E., and L. F. Leloir. 1953. Enzymic phosphorylation of galactosamine and galactose. Arch. Biochem. Biophys. 45: 55-64.

Cardini, C. E., and L. F. Leloir. 1961. In: E. J. King and W. M. Sperru (ed.) Biochemist's Handbook. New York, N. Y., p. 404, 439.

Cleland, W. W., and E. P. Kennedy. 1960. Enzymic synthesis of psychosine. J. Biol. Chem. 235: 45-51.

Cleland, W. W. 1964. Dithiothreitol, a new protective reagent for SH group. Biochemistry 3: 480.

Cohn, S. S. 1955. Glucokinase, p. 350-355. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, Vol. I. Acad. Press, Inc., New York.

Colowick, S. P., G. T. Cori, and M. W. Slein. 1947. The effect of adrenal cortex and anterior pituitary extracts and insulin on the hexokinase reaction. J. Biol. Chem., 168: 583-596.

Colowick, S. P., and H. M. Kalckar. 1943. The role of myokinase in transphosphorylations I. The enzymatic phosphorylation of hexose by adenyl pyrophosphate. J. Biol. Chem., 148: 117-126.

Crane, P. K. 1961. In: C. Long (ed.), Biochemist's Handbook. Van Nostrand, Prinston, New Jersey, p. 401.

Crane, R. K., and A. Sols. 1955. Animal tissue hexokinase, p. 277-286. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, Vol. I. Acad. Press, Inc., New York.

Crook, E. M., and I. R. John. 1962. The qualitative analysis of the cell walls of selected species of fungi. Biochem. J. 83: 325-331.

Darrow, R. A., and S. P. Colowick. 1962. Hexokinase from baker's yeast, p. 226-235. In: S. P. Colowick and N. O. Kaplan (ed.) Methods in Enzymology, Vol. V. Acad. Press, Inc., New York.

Davis, B. J. 1964. Disc electrophoresis-II: Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404-427.

Flodin, P. 1962. Dextran gels and their applications in gel filtration, Halmstad: Meijels Bokindustri. (Pharmacia; Uppsals Swedon).

Flodin, P., and J. Porath. 1961. Molecular sieve processes, Chromatography, Heftman, ed., New York: Reinhold.

Folch-Pi, J. 1955. Composition of the brain in relation to maturation, p. 121. In: H. Waelsch (ed.), Biochemistry of Developing Nervous System. Acad. Press, Inc., New York.

Galzer, A. N., and D. Wellner. 1962. Adsorption of proteins on Sephadex, Nature 194: 862.

Geiger, J. W., and L. B. Wright. 1960. Liquid scintillation counting of radioautograms. Biochem. Biophys. Res. Comm. 2: 282.

Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.

Gulbinsky, J. S., and W. W. Cleland. 1968. Kinetic studies of Escherichia coli galactokinase. Biochemistry, Vol. 7, No. 2: p. 566-575.

Heinrich, M. R. 1964. The purification and properties of yeast galactokinase. J. Biol. Chem. 239: 50-53.

Heinrich, M. R., and S. M. Howard. 1966. Galactokinase, p. 407-412. In: W. A. Wood (ed.), Methods in Enzymology, Vol. IX, Carbonhydrate Metabolism. Acad. Press, Inc., New York.

Howard, S. M., and M. R. Heinrich. 1965. The anomeric specifity of yeast galactokinase. Arch. Biochem. Biophys. 110: 395-400.

Kalckar, H. M. 1965. Galactose metabolism and cell "Sociology". Science, Vol. 150, No. 3694: 305-313.

Kalckar, H. M., and E. S. Maxwell. 1956. Nature of the enzymic galactose-glucose conversion. Biochem. Biophys. Acta. 22: 588-589.

Kalckar, H. M. 1963. Interrelation of enzyme syntheses in the galactose sequence. Proc. Intern. Congr. Biochem. 5th, Moscow, 1961 3: 245-247.

Kalckar, H. M., E. P. Anderson, and K. J. Isselbacher. 1956. Galactosemia, a congenital defect in a nucleotide transferase. Proc. Nat. Acad. Sci. U. S., 42: 49-51, and Biochem. Biophys. Acta. 20: 262-268.

Kalckar, H. M., H. de Robichon-Szulmajster, and K. Kurahashi. 1958. Galactose metabolism in mutants of man and micro-organisms. Proc. Intern. Symposium Enzyme Chem. Tokyo and Kyoto 2: 1957, p. 52-56.

Kurahashi, K. 1957. Enzyme formation in galactose-negative mutants of Escherichia coli. Science 125: 114-116.

Kosterlitz, H. W. 1943. The fermentation of galactose and galactose-1-phosphate. Biochem. J. 37: 322-326.

Kunitz, M., and M. R. McDonald. 1946. Isolation of crystalline hexokinase and other proteins from yeast. J. Gen. Physiol. 29: 143-147.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 447-454. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, Vol. III. Acad. Press, Inc., New York.

Leloir, L. F. 1962. In: P. D. Boyer, H. Lardy, and K. Myrback (ed.), the Enzymes. Acad. Press, Inc., New York, p. 317.

Lederberg, E. M. 1960. In microbial genetics, W. Hsyes and R. S. Glowes, eds. (Cambridge University Press, New York), p. 115.

Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzyme: Application to protein mixtures. J. Biol. Chem. 236, No. 5: 1372-1379.

Maxwell, E. S., K. Kurahashi, and H. M. Kalckar. 1962. Enzymes of the Leloir pathway, p. 174. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology. Acad. Press, Inc., New York.

McDonald, M. R. 1955. Yeast hexokinase, p. 269-276. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, Vol. I. Acad. Press, Inc., New York.

Medina, A., and D. J. D. Nicholas. 1957. A zinc-dependent hexokinase from Neurospora crassa. Nature 179: 87-88.

Medina, A., and D. J. D. Nicholas. 1957. Some properties of a zinc-dependent hexokinase from Neurospora crassa. Biochemistry 66:573-8.

More, N. L., E. M. Lederberg, and J. Lederg. 1966. Transductional heterogenotes in Escherichia. Genetics 41: 758-779.

More, N. L., E. M. Lederberg, and J. Lederg. 1956. Transduction in Escherichia coli K-12. Genetics 41: 143-156.

Munch-Peterson, A., H. M. Kalckar, E. Cutolo, and E. B. Smith. 1953. Uridyl transferases and the formation of uridine triphosphate, enzymic production of uridine triphosphate: Uridine diphosphoglucose pyrophosphoroylsis. Nature 172: 1036-1037.

Nelson, N. J. 1944. A photometric adaptation of the Somogyi Method for determination of glucose. J. Biol. Chem. 153: 375-380.

Ornstein, L. 1964. Disc electrophoresis, Part I: Theory. Annals. of New York Academy of Sciences 121 (2), 321.

Parry, M. J., and D. G. Walker. 1966. Purification and properties of adenodine 5'-triphosphate-D-glucose 6-phosphotransferase from rat liver. Biochem. J. 99: 266-274.

Perkins, D. D. 1962. Preservation of Neurospora stock cultures with anhydrous silica gel. Can. J. Microbiol. Vol. 8: 591-594.

de Robichon-Szulmajster, H. 1958. Induction of enzymes of the galactose pathway in mutants of Saccharomyces Cerevisiae. Science 127: 28-29.

Robinson, H. W., and C. G. Hogden. 1940. The Biuret reaction in the determination of serum proteins. J. Biol. Chem. 135: 707-725.

Robinson, E. A., H. M. Kalckar, and H. Troedsson. 1963. On the loss of uridine diphosphogalactose-4-epimerase activity in L cell cultures and in tumor cells. Biochem. Biophys. Res. Commun. 13: 313-319.

Schwartz, V., L. Golberg, G. M. Komrower, and A. Holzel. 1956. Some disturbances of erythrocyte metabolism in galactosemia. Biochem. J. 62: 34-40.

Sherman, J. R. 1963. Rapid enzyme assay technique utilizing radioactive substrate, ion-exchange paper, and liquid scintillation counting. Ana. Biochem. 5: 548-554.

Sherman, J. R., and J. Adler. 1963. Galactokinase from Escherichia coli. J. Biochem. 238. 873-878.

Smith, I. 1968. Chromatographic and electrophoretic techniques, Vol. II Zon electrophoresis, 2nd ed., Interscience Publishers, New York.

Stine, G. L., W. N. Strickland, and R. W. Barratt. 1964. Method of protein extraction from Neurospora crassa. Can. J. Microbiol. 10: 29-35.

Tatum, E. L., R. W. Barratt, and V. M. Cutter, Jr. 1949. Chemical induction of colonial paramorphs in Neurospora and Syncephalastrum. Science, 109: 509-511.

Tatum, E. L., R. W. Barratt, N. Fries, and D. Bonner. 1950. Biochemical strains of Neurospora produced by physical and chemical treatment. Am. J. Botany 37: 38-46.

Trucco, R. E., R. Caputto, L. F. Leloir, and N. Mittelman. 1948. Galactokinase. Ana. Biochem. 18: 137-146.

Vogel, H. J. 1956. A convenient growth medium for Neurospora. Microbiol. Genetic Bulletin 13: 42-43.

Wang, C. H., and D. E. Jones. 1959. Liquid scintillation counting of paper chromatograms. Biochem. Biophys. Res. Communs. 1: 203.

Weichselbaum, T. E. 1946. Accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Path., Tech. Sect. 10: 40-49.

Weinberg, A. N., and S. Segal. 1960. Effect of galactose-1-phosphate on glucose oxidation by normal and galactosemic leukocytes. Science 132: 1015-1016.

Wilson, D. B. 1966. Ph. D. Dissertation, Stanford University, Palo Alto, Calif.