ABSTRACT

METABOLISM OF D-FUCOSE AND
L-ARABINOSE IN A PSEUDOMONAD

by A. Stephen Dahms Jr.

The first known degradative pathway by which an organism utilizes D-fucose (6-deoxy-D-galactose) has been elucidated. Two unique, pyridine nucleotide-linked dehydrogenases have been found which are distinguishable, in part, by their ability to oxidize either the furanose or the pyranose hemiacetal of D-fucose to the γ- or the δ-lactone respectively. The NAD-linked D-aldohexose dehydrogenase oxidizes β-D-fucopyranose to D-fucono-δ-lactone which spontaneously hydrolyzes to D-fuconate. The NAD(P)-linked L-arabino-aldose dehydrogenase oxidizes β-D-fucofuranose to D-fucono-γ-lactone which is hydrolyzed to D-fuconate by a γ-lactonase. D-Fuconate is dehydrated by a specific dehydratase to form 3,6-dideoxy-D-threo-hexulosonic acid (2-keto-3-deoxy-D-fuconate) which, in turn, is cleaved by a specific aldolase, 2-keto-3-deoxy-D-fuconate aldolase, to yield pyruvate and D-lactaldehyde. No enzyme activity which modified D-fucose by means of isomerization, phosphorylation, epimerization, or reduction could be demonstrated. It was concluded that D-fucose was not degraded by a pathway analogous to those which occur in other organisms for the degradation of L-fucose

or L-rhamnose, which are the two other common 6-deoxy aldohexoses.

The five enzymes of the D-fucose pathway were purified and some of their properties determined and compared to similar enzymes in the literature. The specific activities of the enzymes in cell extracts and the K_m values are in physiologically significant ranges. The intermediates of the D-fucose pathway were also isolated and were identified by derivatization and chemical synthesis.

The NAD-dependent D-aldohexose dehydrogenase has been purified 327-fold, is NAD-specific, is not affected by thiols, thiol inhibitors, or metal ion activators, is induced by growth on D-fucose and D-glucose, and oxidizes D-fucose, D-galactose, 3,6-dideoxy-D-galactose, 2-deoxy-D-galactose, D-glucose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, D-allose, D-altrose, and D-mannose to the corresponding δ-lactones.

The NAD(P)-dependent dehydrogenase has been purified 276-fold, is operative equally well with either NAD or NADP, is not affected by thiols, thiol inhibitors, or metal ion activators, is induced by growth on D-fucose, D-galactose, L-arabinose, and 6-iodo-6-deoxy-D-galactose, and oxidizes D-fucose, D-galactose, 3,6-dideoxy-D-galactose, 2-deoxy-D-galactose, L-arabinose, L-mannose, and 6-iodo-6-deoxy-D-galactose to the corresponding γ-lactones.

The lactonase has been purified 16-fold, hydrolyzes γ-D-lactones, is not activated by thiols or metal ions, is not inactivated by thiol inhibitors or EDTA, and is induced by growth on D-fucose, L-arabinose, and 6-iodo-6-deoxy-D-galactose.

D-Fuconate dehydratase has been purified 30-fold, catalyzes the irreversible dehydration at C-2 and C-3 threo hydroxyl groups of its substrates, is specific for D-fuconate and L-arabonate out of 20 sugar acids tested, has an absolute requirement for a divalent cation, is activated by thiols and is inactivated by thiol inhibitors, and is induced by growth on D-fucose, L-arabinose, and D-galactose.

2-Keto-3-deoxy-D-fuconate aldolase has been purified 50-fold, catalyzes the C-3, C-4 cleavage of its substrate to form pyruvate and an aldehyde, is specific for 2-keto-3-deoxy-D-fuconate and 2-keto-3-deoxy-L-arabonate out of 9 deoxy sugar acids tested, has an absolute requirement for a divalent cation, is stabilized by thiols and is induced by growth on D-fucose and L-arabinose.

With the exception of the NAD-linked D-aldohexose dehydrogenase, all the enzymes of the D-fucose pathway act upon L-arabinose or its corresponding intermediates. Mutants which were lacking D-fuconate dehydratase and 2-keto-3-deoxy-D-fuconate aldolase showed defective growth on both D-fucose and L-arabinose and support the hypothesis that the enzymes of the D-fucose pathway also

function in the degradation of L-arabinose. 2-Keto-3-deoxy-L-arabonate, the product resulting from the D-fuconate dehydratase-catalyzed dehydration of L-arabonate, has been found to be degraded in other microorganisms by 2-keto-3-deoxy-L-arabonate dehydratase. The latter dehydratase could not be demonstrated in the pseudomonad under investigation, and it has been concluded that a new L-arabinose pathway has been found through which 2-keto-3-deoxy-L-arabonate is cleaved by 2-keto-3-deoxy-D-fuconate aldolase to yield pyruvate and glycolaldehyde.

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VITA

A. Stephen L. Dahms Jr. was born on September 12, 1943 in Mankato, Minnesota. He graduated from Central High School in Saint Paul, Minnesota in June, 1961, and then attended the College of Saint Thomas where his interests in chemistry were further enkindled by the beneficent goading of Drs. Morath, Ryan, and Allen, in that order. In the winter of his 3rd year, Mr. Dahms obtained employment as a chemist in the basic research laboratory of the Division of Internal Chemicals at Minnesota Mining and Manufacturing. During his 1½ years at 3M, Mr. Dahms became directly involved with research in the catalysis of photochemical reactions of organometallics, new syntheses of organometallics, development of unique analytical techniques for the quantitative analysis of organic peroxides and hydroperoxides, and formulation and analysis of new elastomers. Despite the spurious and malicious rumors that his research techniques and philosophy of chemistry were irreversibly coagulated while in the industrial environment, Mr. Dahms considers his duration at 3M as being one of his most didactic experiences. In June, 1965, he terminated his physical affiliations with 3M, received a B.S. in chemistry, and departed for East Lansing, Michigan to commence

graduate work in Biochemistry under the guidance of Dr. R. L. Anderson. On December 31, 1966, to gain a needed tax advantage, he married a fellow student, Judith Claire Daneault, and a year later, 3 days before his Preliminary Examinations, a daughter, Jacquelyne Kristin, was born.

Mr. Dahms was a National Institutes of Health predoctoral trainee for the first two years of his graduate experience and was awarded a National Institutes of Health predoctoral fellowship for the latter two years. Upon completion of his work at Michigan State University in May, 1969, the author will accept a postdoctoral position under Dr. Paul D. Boyer at UCLA. The research will concern the mechanism of action and site specificity of uncouplers of energy-linked phosphorylation. Mr. Dahms has been awarded National Science Foundation and Atomic Energy Commission postdoctoral fellowships for his duration at UCLA.

Mr. Dahms is a member of the American Chemical Society, the American Association for the Advancement of Science, Sigma Xi, Phi Kappa Phi, the American Philatelic Society, and the Faraday-Woodward Society.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ATP	adenosine triphosphate
Bicine	N,N-bis(2-hydroxyethyl)glycine
DEAE-cellulose	diethylaminoethyl-cellulose
EDTA	ethylenediamine tetraacetate
Hepes	N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid
KDA	2-keto-3-deoxy-L-arabonate
KDF	2-keto-3-deoxy-D-fuconate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine trinucleotide
NADPH	reduced nicotinamide adenine trinucleotide
TBA	2-thiobarbituric acid
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol

INTRODUCTION

D-Fucose (6-deoxy-D-galactose) is generally considered to be a rare carbohydrate whereas the L-enantiomorph is considered to be relatively abundant and widespread in nature. However, many published reports on the natural occurrence of β-D-fucosides (see Literature Survey in the appendix) indicate that the D-enantiomorph is more prevalent than is generally appreciated. Further, specific β-D-fucosidases have been found in several organisms, and a microbial membrane transport system active on D-fucose has been thoroughly documented. However, despite the plethora of information concerning D-fucose, its biodegradative pathway has not been described for any organism.

The purpose of this investigation was to elucidate the biodegradative pathway of D-fucose in a bacterium and to characterize the participating enzymes. The pathway was determined to be: D-fucose → D-fucono-γ-lactone + D-fucono-δ-lactone → D-fuconate → 2-keto-3-deoxy-D-fuconate → pyruvate + D-lactaldehyde. In addition, it was found that the enzymes instrumental in D-fucose degradation also function in a new pathway for L-arabinose degradation. An abstract on aspects of this work has been published (1).

EXPERIMENTAL PROCEDURES

Cultivation of Bacteria and Preparation of Cell Extracts- The organism used in this study was selected for its ability to utilize D-fucose as a sole carbon and energy source and was classified as a pseudomonad. Details of the isolation and identification are given in the Appendix B. The organism was grown aerobically in Fernbach flasks on a rotatory shaker at 32°C in a one liter medium consisting of 1.35% $Na_2HPO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeSO_4$, and 3.5% carbohydrate (autoclaved separately). Stepwise addition of the carbohydrate was necessary since 1% carbohydrate markedly reduced the growth rate. After inoculation, 20 ml 25% w/v of the carbohydrate was introduced per liter of medium; 6 additional 20 ml 25% w/v portions were added to the flasks at six hour intervals before harvesting. Very thick cell suspensions (absorbance at 600 nm, 18-22) were obtained using this technique. Turbidity measurements were made with a Coleman Junior Spectrophotometer on appropriate dilutions in 18 mm test tubes. The cells were harvested 4 hours after the final addition of the carbohydrate and approximately 40 hours after innoculation. The cells were resuspended in glass-distilled water and recentrifuged prior to

sonic disruption.

Extracts were prepared from washed cells in ice-cold 0.10 molar sodium phosphate buffer (pH 7.0) or 0.10 molar Bicine buffer (pH 7.4) by a 13 minute exposure to 10-kc sonic oscillation in a Raytheon sonic oscillator equipped with an ice-water cooling jacket. The supernatant fluid resulting from a 10 minute centrifugation at 40,000 x g was used as the cell extract.

Analytical Procedures- Reducing sugars were determined by the method of Sumner and Howell (1A). Aldonic acids were determined after conversion to the corresponding lactones by heating in 1 N HCl for 5 minutes. Lactones were determined as the hydroxamic acids by the method of Hestrin (2). Descending paper chromatography was performed using Whatman #1 filter paper in the following six solvent systems:

(1) water saturated 2-butanone
(2) n-propanol-formic acid-water (6:3:1)
(3) water saturated phenol
(4) pyridine-0.10 N HCl- n-butanol (3:2:5)
(5) n-butanol-water-95% ethanol (5:4:1)
(6) n-butanol-pyridine-water (6:4:3)

Carbohydrates were visualized by benzidine-HCl and periodate (3), N,N-dimethyl-p-phenylenediamine (4) and alkaline silver nitrate (5). Lactones were visualized by their formation of hydroxamic acids. 2-Keto-3-deoxy aldonic acids were visualized with the periodate-

thiobarbituric acid reagents of Warren (6). α-Keto acids were located by spraying with 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl followed by 10% KOH (18).

Protein was determined spectrophotometrically with the aid of a nomgraph (courtesy of Calbiochem) based on the data of Warburg and Christian (7). In crude extracts or preparations high in nucleic acid content, protein concentration was estimated by the biuret method (8), with bovine serum albumin as the standard. Light measurements were performed on a Gilford 2400 absorbance-recording spectrophotometer thermostated at 25.0°C using microcuvettes with a 1 cm light path or on a Gilford 300 digital spectrophotometer also with a 1 cm light path. Spectra of the quinoxaline derivatives, the β-formyl pyruvate-thiobarbituric acid chromogen, the malondialdehyde-thiobarbituric acid chromogen, the 3-methyl-2-benzothiazolinone hydrazone azine, and the tetraazopentamethine cyanine derivatives of 3-methyl-2-benzothiazolinone hydrazone were recorded with a Cary model 15 spectrophotometer.

L-Ribulose and D-fuculose were determined by the cysteine-carbazole method (9) with L-rhamulose as the standard. The cysteine-carbazole reagents were added to aliquots containing the ketoses and were incubated at 35°C for 10 minutes. Under the above conditions, using an 18 mm path length, 0.1 μmole of L-rhamulose in 7.4 ml of solution gave an absorbance of 0.28 at 540 nm (36).

Standard L-rhamulose was the gift of Dr. J. W. Mayo.

Pyruvate was determined with lactic acid dehydrogenase and NADH and by the method of Friedman and Haugen (10) as modified by Sayre and Greenberg (11). Acetaldehyde was determined by the method of Barker and Summerson (268) using periodate removal techniques of Itagaki and Susuki (12). Carbon dioxide from ceric sulfate decarboxylations of α-keto acids was measured manometrically according to the procedure of Meister (13). Sodium borohydride reductions were carried out according to the method of Ghalambor, Levine, and Heath (14). Carbonyl compounds were determined as the tetraazopentamethine cyanine derivatives with 3-methyl-2-benzothiazolinone hydrazone (15). Periodate consumption was determined by the method of Dixon and Lipkin as modified by Sugimoto and Okazaki (16, 269).

2-Keto-3-deoxy-D-fuconate and 2-keto-3-deoxy-D-galactonate were determined using modifications in the 2-thiobarbituric acid (TBA) assay of Weissbach and Hurwitz (17). The TBA assay for β-formyl pyruvate, which is liberated upon periodate oxidation of 2-keto-3-deoxy sugar acids, is influenced by unknown factors (18) and necessitated the utilization of different molar absorptivities for the respective 2-keto-3-deoxy sugar acids. The molar absorptivities for KDF and 2-keto-3-deoxy-D-galactonate were found to be 27,900 and 60,500

respectively by determining α-keto acid content in the semicarbazide assay (19) and by determining the amount of CO_2 released upon ceric sulfate decarboxylation. Microdetermination of α-keto acids was also carried out on the azines of the 3-methyl-2-benzothiazolinone hydrazones (20).

Thin-layer chromatography was performed in the following three solvent systems:

(i) pyridine-0.10 N HCl-n-butanol (3:2:5)

(ii) n-butanol-ethanol-0.5 N NH_4OH (70:10:20)

(iii) benzene-tetrahydrofuran-glacial acetic acid (57:35:8)

Reagents- D-Fucose was synthesized by modifications of the method of Freundenberg et al. (21) and Schmid et al. (22). Details for the preparation of this compound are given below. 6-Iodo-6-deoxy-D-galactose was prepared by modifications of the method of Raymond and Schroeder (23). Potassium salts of D-galactonic, L-arabonic, D-arabonic, D-xylonic, and D-lyxonic acids were prepared by the method of Moore and Link (24). Potassium salts of D- and L-fuconic and 6-iodo-6-deoxy-D-galactonic acids were prepared by hypoiodide oxidation of the corresponding aldoses. Potassium 3-deoxy-D-xylo-hexonate was prepared by KOH hydrolysis of D-galacto-α-metasaccharinic acid lactone synthesized by the method of Evans et al. (25). D-Glucarate and D-galactarate were obtained from Sigma and were crystallized as the dicyclohexammonium salts according to the procedure of Fish and

Blumenthal (26). Potassium salts of D-mannonic, L-rhamnonic and cellobionic acids were prepared by hypoiodide oxidation but were not crystallized. D-Ribonate and D-gluconate were prepared by alkaline hydrolysis (pH 8.5) of the corresponding γ- and δ-lactones, the gifts of Dr. W. A. Wood, L-Galactonate and L-gluconate were prepared by the alkaline hydrolysis of the corresponding γ- and δ-lactones, the gifts of Dr. J. W. Mayo. The free acids were generated by treatment with AG50W-X8-H^+, 50-100 mesh, and lyophilization. Pyridine nucleotides were obtained from P-L Biochemicals. Protamine sulfate and 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl buffer (Tris-HCl) were obtained from Sigma Chemical Co. D-Galactono-γ-lactone and standard D-fucose were obtained from General Biochemicals. $LiAlH_4$ was obtained from K and K Laboratories and was finely powdered under an anhydrous atmosphere before use. Thin-layer chromatography plates were Silica Gel F_{254} (Brinkman). N-2-Hydroxyethylpiperazine-N-2'-ethanesulfonic acid buffer (Hepes), N,N-bis(2-hydroxyethyl)glycine buffer (Bicine), N-tris(hydroxymethyl)methyl glycine buffer (Tricine), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (TES), DL-threonine and L-threonine were obtained from Calbiochem. Ethyl methanesulfonate and 2-thiobarbituric acid were obtained from Eastman; the latter compound was recrystallized by the procedure of Waravdekar and Sasalaw (27). 3-Methyl-2-benzothiazolinone

hydrazone-HCl was obtained from Aldrich Chemicals and was used without further purification.

Barium salts of 2-keto-3-deoxy-6-phospho-D-galactonate and 2-keto-3-deoxy-6-phospho-D-gluconate were the gifts of Dr. W. A. Wood. The barium salts were converted to the sodium form by treatment with AG50W-X8-Na^+, and portions were dephosphorylated with intestinal alkaline phosphatase (obtained from General Biochemicals). The phosphatase reaction mixture consisted of 2 μmoles of phosphate ester, 1 μmole $MgCl_2$, and 5 mg alkaline phosphatase in a volume of 5 ml. The reaction was followed titrimetrically with a Sargent recording pH stat at pH 8.3. The reaction was carried out at 28°C until proton release had ceased; the reaction mixture was then neutralized with a cation exchange resin and filtered.

Myo-inositol and dulcitol were the gifts of Dr. W. W. Wells. Glucose-free 2-deoxy-D-glucose, glucose-free D-mannose, galactose-free 2-deoxy-D-galactose, D-altrose, D-allose, adonitol, and xylitol were the gifts of Dr. W. A. Wood. L-Mannose, L-glucose, L-galactose, and L-fructose were the gifts of Dr. J. W. Mayo.

2-Keto-4-hydroxy glutarate was the gift of Dr. Eugene Dekker (University of Michigan). 2-Acetamido-2-deoxy-D-allose and 2-acetamido-2-deoxy-D-altrose were provided by Dr. M. B. Perry (National Research Council of Canada, Ottawa). 3,6-Dideoxy-D-galactose was the

gift of Dr. Otto Lüderitz (Max Planck Institute, Freiburg). N-Acetylneuraminic acid was provided by Dr. W. Jourdian (University of Michigan). 6-Deoxy-D-allose and 6-deoxy-D-glucose were the generous gifts of Dr. T. Reichstein (University of Basel, Basel, Switzerland).

Phosphate buffers were prepared from the sodium salts, and all melting points are uncorrected.

Assay for Aldose-Ketose Isomerization- Isomerase activity was measured colorimetrically by the cysteine-carbazole method. The reaction mixture consisted of 20 μmoles of the aldose, 2 μmoles $MgCl_2$, 5 μmoles phosphate buffer (pH 7.0), and 1-5 mg of the cell extract. The mixture was incubated at 30°C and aliquots were withdrawn at time intervals and assayed for ketose.

Assay for Kinase Activity- Kinase activity was determined spectrophotometrically at 340 nm and 25.0°C. The reaction mixture contained 10 μmoles of the aldose, 0.5 μmole ATP (pH 7.2), 1 μmole $MgCl_2$, 0.5 μmole phosphoenolpyruvate (pH 7.2), 0.07 μmole NADH, 5 μmoles glycylglycine buffer (pH 7.2), 13 μg lactate dehydrogenase-pyruvate kinase, and 0.05-0.1 mg of cell extract in a total volume of 0.15 ml. A control to correct for NADH oxidase and ATPase contained all the reaction components except the aldose.

Assay for Aldose Reductase Activity- Activity was measured spectrophotometrically at 340 nm by following the oxidation of NADH or NADPH. The assays consisted of

0.07 μmole NAD(P)H, 5 μmoles of glycylglycine buffer (pH 7.2), and 10 μmoles of the aldose in a volume of 0.15 ml. A control to correct for NADH oxidase contained all the reaction components except the aldose.

NAD(P)-Dependent Dehydrogenase Assay- Activity was measured spectrophotometrically at 340 nm and 25.0°C. The reaction mixture (0.15 ml) consisted of 2.5 μmoles D-galactose, 0.3 μmoles $NADP^+$, 15 μmoles Tris-HCl buffer (pH 8.1), and a limiting amount of the enzyme. The assay was linear with respect to time and enzyme concentration. All carbohydrate solutions used in the analysis of the NAD(P)-dependent and the NAD-dependent dehydrogenases were allowed to approach mutarotational equilibrium by incubation at room temperature for periods not less than two hours; this was done to minimize the possible effects resulting from the preferential enzymatic oxidation of one anomer at a rate greater than the rate of anomerization. A unit of NAD(P)-dependent dehydrogenase was defined as the amount of enzyme which catalyzed the reduction of 1 μmole of $NADP^+$ per hour.

NAD-Dependent Dehydrogenase Assay- Activity was measured spectrophotometrically at 340 nm and 25.0°C. The reaction mixture (0.15 ml) consisted of 2.5 μmoles D-glucose, 0.3 μmole NAD^+, 15 μmoles Tris-HCl buffer (pH 8.1), and a limiting amount of the NAD-dependent dehydrogenase. The assay was linear with respect to

enzyme concentration and time. A control to correct for NADH oxidase was necessary in the early steps of purification and contained all the components except D-glucose. NADH oxidase could be completely eliminated by preparing sonic extracts in a solution consisting of 0.10 molar Bicine and 0.147 mM 2-thioethanol (pH 7.4). A unit of NAD-dependent dehydrogenase activity was defined as the amount of enzyme which catalyzed the reduction of 1 μmole of NAD^+ per hour.

Lactonase Assay- Enzyme activity was measured spectrophotometrically at 540 nm using the hydroxamic acid procedure of Hestrin (2). The standard curve was prepared using D-galactono-γ-lactone. The assay consisted of 10 μmoles D-galactono-γ-lactone, 40 μmoles Tris-HCl buffer (pH 7.5), and a limiting amount of the lactonase in a total volume of 0.15 ml. The reaction mixture was incubated at 30°C before quenching with 1.0 ml of the alkaline hydroxylamine reagent. The assay was linear with respect to enzyme concentration and time. A control to correct for the non-enzymatic hydrolysis of lactones was necessary and contained all the components except the lactonase. All lactone solutions were freshly prepared before each assay. A unit of lactonase activity was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmole of D-galactono-γ-lactone per hour. All lactonase preparations were free

of contaminating NAD-dependent dehydrogenase, NAD(P)-dependent dehydrogenase, D-galactonate dehydratase, and D-fuconate dehydratase.

Dehydratase Assay- Two assays were used routinely for the detection of deoxy hexulosonic acids. The TBA assay was restricted to qualitative detection of KDF, 2-keto-3-deoxy-D-galactonate, and 2-keto-3-deoxy-L-arabonate. All enzyme-catalyzed dehydration reactions were assayed for α-keto acids by the semicarbazide method. The assay consisted of 20 μmoles Bicine buffer (pH 7.4), 0.147 μmole 2-thioethanol, 5 μmoles D-fuconic of L-arabonic acid (pH 7.4), 5 μmoles $MgCl_2$, and a limiting amount of the enzyme in a total volume of 0.15 ml. The reaction mixture was incubated at 30°C for 30 minutes and was quenched by the addition of 1.0 ml of the semicarbazide reagent. The mixture was incubated at 30°C for 15 minutes and diluted to 5.0 ml with water; the absorbance was determined at 250 nm in a 1 cm quartz cuvette against a diluted reagent blank. Controls for contributions to absorbance by protein, buffer, thiol, and carbohydrate were necessary. Under the above conditions, 0.5 μmole of the α-keto acid gave an absorbance of 1.02. The assay was linear with respect to enzyme concentration and time. A unit of dehydratase activity was defined as the amount of enzyme which catalyzed the formation of 1 μmole of α-keto acid per hour. D-Galactonate dehydratase was also assayed with the above procedure with the exception

that the 2-thioethanol was omitted.

2-Keto-3-deoxy-D-fuconate Aldolase Endpoint Assay- Endpoint assays were used for the determination of the amount of pyruvate liberated upon enzymatic cleavage of 2-keto-3-deoxy-D-fuconate (KDF). Enzyme activity was measured spectrophotometrically at 340 nm and 25.0°C. The reaction mixture (0.15 ml) consisted of 13 µg lactic acid dehydrogenase, 0.07 µmole NADH, 1 µmole $MgCl_2$, 0.143 µmole 2-thioethanol, 15 µmoles Hepes buffer (pH 7.4), 0.02-0.04 µmole KDF, and a limiting amount of the aldolase. Levels of NADH oxidase and endogenous pyruvate were also determined and appropriate corrections made.

2-Keto-3-deoxy-D-fuconate Aldolase Standard Assay- Pyruvate liberated upon cleavage of KDF or KDA was measured in a coupled-assay using lactic acid dehydrogenase and NADH. Enzyme activity was measured spectrophotometrically at 340 nm and 25.0°C. The reaction mixture (0.15 ml) consisted of 13 µg lactic acid dehydrogenase, 0.07 µmole NADH, 1 µmole $MnCl_2$, 0.147 µmole 2-thioethanol, 15 µmoles Hepes buffer (pH 7.8), 5 µmoles KDF or KDA, and a limiting amount of the enzyme. The assay was linear with respect to enzyme concentration and time. Controls to correct for NADH oxidase and possible endogenous pyruvate were run.

Preparation of D-Fucose- Anhydrous zinc chloride (360 g) was added to a 6-liter Erlenmeyer flask equipped

with a polyethylene mixing blade and a mechanical stirrer. Acetone (3.75 liters) was rapidly added and the suspension stirred until the zinc chloride was dissolved. Concentrated sulfuric acid (12.0 ml) was added dropwise from a pipet, such that no acid would touch the inside of the neck. α-D-Galactose (200 g), doubly recrystallized according to the procedure of Wolfrom and Thompson (28) and chromatographically homogeneous on Whatman #1 paper in solvent systems 3, 4, and 5, was quickly added and the suspension stirred for 6 hours. A suspension of anhydrous sodium carbonate (600 g) in water (1.05 liters) was added slowly over a period of 30 minutes. The zinc carbonate suspension was collected by suction filtration and was washed several times by resuspending in acetone and refiltering. The filtrates were combined and reduced in volume to a syrup under reduced pressure. The acetal was extracted with three 400-ml portions of diethyl ether which were combined, dried over anhydrous sodium sulfate, and reduced in volume under vacuum to a syrup (bath temperature 30°C). The acetal was freed from condensation products of acetone by heating at 100°C and 0.05 mm Hg for 1 hour; yield 271.3 g (93.5%) of a crude, undistilled syrup, 1,2-3,4-di-O-isopropylidene-α-D-galactopyranose.

Di-O-isopropylidene-D-galactose (270 g) was stirred into anhydrous pyridine (520 ml) in a 1-liter, 3-necked flask fitted with a mechanical stirrer and a drying tube.

p-Toluenesulfonyl chloride (285 g), recrystallized from petroleum ether:ligroin (10:3), was added and the reaction stirred for 5.5 hours during which time the temperature was maintained below 60°C. Pyridine-HCl was immediately filtered out, following completion of the reaction, and was washed with pyridine and diethyl ether. Water (10 ml) was added to the filtrate to destroy excess tosyl chloride and the mixture was concentrated under reduced pressure (bath 25°C) to approximately 250 ml. The solution was immediately poured into cold water (2.5 liters, 15°C) while being stirred moderately fast by a mechanical stirrer. After 10 minutes a precipitate developed which solidified upon further standing. The solid residue was broken up manually into chunks, filtered, and washed with water. The product was suspended in distilled water by mixing in a large Waring blender at maximum speed for a period of 5 minutes after which time it was again filtered and washed; this procedure was repeated until both pyridine and colored impurities were removed. The product was dried over anhydrous calcium sulfate before dissolving in anhydrous methyl alcohol (1 liter) and treating with Norit (20 g). Norit treatment was repeated until the solution was free of all coloration. The filtrate (1400 ml) was left for approximately 12 hours at 4°C; crystallization began within 20 minutes. The product was collected by suction filtration, washed with cold water (1.5 liter)

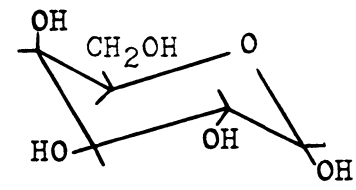
and dried in a vacuum desiccator over anhydrous calcium sulfate. The product, 1,2-3,4-di-O-isopropylidene-6-p-toluenesulfonyl-α-D-galactopyranose, was obtained in a yield of 88.2% (280 g); mp. 101-02°C; reported, 102-03°C (22).

The tosyl product (64.3 g) was dissolved in anhydrous benzene (100 ml) followed by distillation to a final volume of 70 ml. A suspension of anhydrous diethyl ether (140 ml) and 6.2 g lithium aluminum hydride were added through a dropping funnel, and the solution was stirred magnetically for 30 hours while refluxing under anhydrous conditions. After 12 hours of the 30 hour period had passed, additional lithium aluminum hydride (3.0 g) was added. Following completion of the reaction, the suspension was allowed to settle, and the supernatant was quickly decanted into a flask containing ethyl acetate (10 ml) to destroy excess hydride. Anhydrous diethyl ether was added to the reflux flask, the suspension was allowed to settle, and the solution was quickly decanted as above. This extraction procedure was repeated three times. The ether extracts were combined and extracted with 10% sodium chloride (2.5 liters) until all the aluminum hydroxide was removed. Following filtration through Hyflo Super-Cel, the sodium chloride-aluminum hydroxide solution was back-extracted with diethyl ether (two-350 ml portions). The ether extracts were combined and concentrated under vacuum to a syrup (50 ml) which

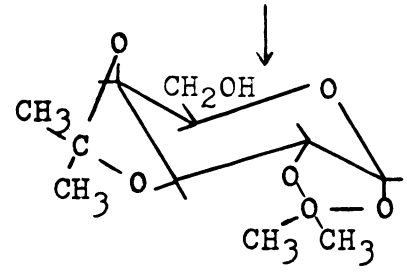
was distilled under vacuum (0.45 mm Hg); the 81-83°C fraction was collected. Extreme care had to be taken not to exceed 85°C at this pressure or else an unknown, highly exothermic reaction occurred. 1,2-3,4-Di-O-isopropylidene-α-D-fucopyranose distilled as a colorless oil which solidified after standing to form a solid melt; the yield was 23.0 g (58%); melting point 36-37°C, reported 36-37°C (22).

Di-O-isopropylidene-α-D-fucopyranose (23.0 g) was refluxed for 3 hours in 350 ml 1% sulfuric acid. The clear solution was neutralized while hot with a warm barium carbonate suspension. Following filtration of the mixed barium salts, the neutral solution was decolorized with Norit and was concentrated under vacuum. The resulting syrup was dehydrated by three treatments with absolute ethanol and was crystallized from 95% ethanol; yield 12.2 g (78%), overall yield 36.2%; m.p. 140-45°C, reported 140-45°C (21). No depression of the melting point was observed upon addition of authentic D-fucose. The product co-chromatographed with standard D-fucose in solvent systems 3, 4, and 5. The melting point of the phenylhydrazone was 171-72°C; reported 172°C (29, 30). Further evidence is supplied below where synthetic D-fucose is converted into four known derivatives of the free acid, D-fuconic acid. A summary of the reactions for the synthesis of D-fucose is presented in Figure 1.

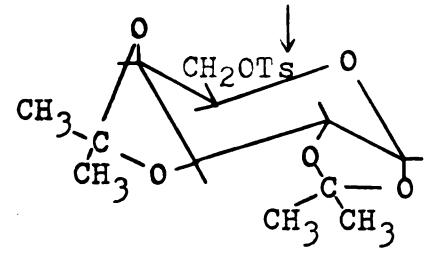
Figure 1. Reactions in the synthesis of D-fucose



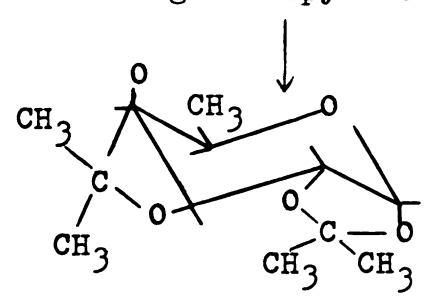
α-D-Galactopyranose



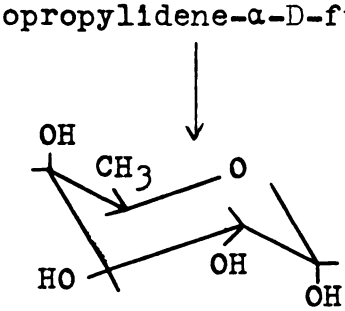
1,2-3,4-Di-O-isopropylidene-α-D-galactopyranose



1,2-3,4-Di-O-isopropylidene-6-p-toluenesulfonyl-α-D-galactopyranose



1,2-3,4-Di-O-isopropylidene-α-D-fucopyranose



D-Fucose

Preparation of Potassium D-Fuconate- Anhydrous D-fucose (2.0 g) was dissolved in 4 ml of distilled water and was added to 25 ml of absolute methanol. Resublimed iodine (6.3 g) was dissolved in absolute methanol (70 ml) in a 500 ml, 3-necked flask fitted with a mechanical stirrer, a drying tube, and a dropping funnel. The temperature was maintained at 40°C throughout the course of the reaction with an electronic thermoregulator. The methanolic D-fucose solution was quickly added to the methanolic iodine solution prior to the dropwise addition of methanolic KOH (115 ml, 4%). The resultant straw-yellow solution was set aside at room temperature and allowed to crystallize; it was complete within 24 hours. The product crystallizes as the monohydrate (31) in long, fine needles, m.p. 166-67°C. The product was recrystallized from methanol-water (97:3), m.p. 169-70°C, which remained unchanged upon further crystallization. The product was chromatographically homogeneous in solvent systems 1, 2, 3, and 5. D-Fucono-γ-lactone was prepared by treatment of the potassium salt with Dowex-50W-H^+, lyophilization, and crystallization from diethyl ether-ethanol (10:1); m.p. 104-05°C, reported 104-06°C (32). The amide was prepared through the lactone; m.p. 175-76°C, reported 180°C (33); further recrystallization did not affect the melting point of the amide. The benzimidazole derivative was prepared by the method of Moore and Link

(24); m.p. 252-53°C, reported 248-49°C (34).

Selection of Mutant Strains- An overnight culture of the pseudomonad in D-glucose-mineral medium was harvested by centrifugation in sterile tubes, and the resulting pellet was suspended in 7 ml mineral medium containing 1.4 mmole of ethyl methanesulfonate (35). After incubation for 3.5 hours at room temperature, 0.02 ml of the suspension was inoculated into 7 ml D-glucose-medium and incubated for 24 hours at 32°C. The cell mass increased in absorbancy at 600 nm from 0.050 to 0.60 (1.8 cm light path), as measured with a Coleman Junior Spectrophotometer. The cells were plated on D-glucose-mineral-agar after dilution by a factor of 110,000:1 (250 cells per plate). The resulting colonies were replicated on L-arabinose-mineral-agar and D-glucose-mineral-agar sequentially.

Mutant strain 5-1-10-1 was selected as a strain which grew well on D-glucose but failed to grow on L-arabinose. Further characterization indicated that the mutant possessed the following phenotype: D-fucose$^-$, L-arabinose$^-$, D-glucose$^+$, D-galactose$^+$, D-xylose$^+$, D-mannose$^-$, D-fructose$^-$, lactose$^-$ which was identical to the parental strain with the exception that the mutant was negative on L-arabinose and D-fucose. A revertant was obtained by incubation of mutant strain 5-1-10-1 with 0.5% D-fucose-mineral medium for 48 hours at 32°C. The revertant possessed the same phenotype as the parental strain.

Mutant strain 73-1-2 was selected as a strain which grew on L-arabinose and D-fucose at about 40% the wild-type rate but grew normally on D-glucose and D-galactose. This strain was identical to the parental strain in other respects.

RESULTS

A. INVESTIGATIONS OF VARIOUS POSSIBLE ENZYMATIC REACTIONS INVOLVING D-FUCOSE

The initial step in the metabolic pathway of an ω-deoxy aldose can potentially involve any of several enzymatic reactions. These include aldose-ketose isomerization, epimerization, phosphorylation at C-1, reduction at C-1, or oxidation. The results of a systematic investigation of these possible reactions are given below.

Isomerization or Epimerization- Using crude extracts of D-fucose-grown cells, attempts were made to chromatographically demonstrate either the epimerization of D-fucose to another deoxyaldose or the isomerization of D-fucose to D-fuculose. Using the procedure outlined in Table I, it was found that D-fucose was not modified to a detectable extent (< 0.03 μmole per hour per mg protein). D-Fucose was not isomerized to a ketose which would have yielded an orcinol positive spot nor was it epimerized to a deoxyaldose which exhibited partition coefficients differing from those of D-fucose in the two solvent systems employed. 6-Deoxy-D-gulose, 6-deoxy-D-talose, 6-deoxy-D-glucose, and 6-deoxy-L-idose, the possible epimerization products of D-fucose, were not available for use as standards, so it is not definitely known that their

Table I. Chromatographic analysis for epimerization or isomerization of D-fucose. Cell-free extracts of D-fucose-grown cells were prepared as described in Experimental Procedures. The reaction mixture consisted of 150 µmoles D-fucose, 10 µmoles $MgCl_2$, 10 µmoles sodium phosphate buffer (pH 7.0), and cell extract (15 mg of protein) in a volume of 1.0 ml. The mixture was incubated at 30°C, and 50 µl aliquots were withdrawn after 4 hours. The aliquots were deionized with Dowex-50W-H^+, and the eluate was spotted on Whatman #1 paper. The chromatograms were developed in solvent systems 3 and 6 and were visualized with alkaline silver nitrate for reducing compounds and with an orcinol spray for ketoses. A control consisted of a identical reaction mixture with the exceptions that L-arabinose-grown Aerobacter aerogenes (known to possess L-arabinose isomerase) was used as the enzyme source, and L-arabinose was used as the substrate.

		R_f Values	
Substrate	Enzyme Source	System #3	System #6
D-Fucose	*	.62	.44
L-Arabinose	*	.54	.40
D-Fucose	Pseudomonad	.62	.44
L-Arabinose	A. aerogenes	.54, .66**	.40, .50**

* No enzyme added

**Orcinol positive spot

chromatographic separation from D-fucose would have been achieved; however, the probability that D-fucose and its possible isomerization product possessed the same partition coefficients in the two solvent systems is very small, since other hexoses have R_f values ranging from .36 to .72 in solvent system 3 and from .37 to .55 in solvent system 6.

Aerobacter aerogenes is known to possess an inducible L-arabinose isomerase which converts L-arabinose to L-ribulose (37). In the chromatographic analysis a control experiment was run in which an identical reaction mixture was prepared with the exceptions that L-arabinose-grown Aerobacter aerogenes was used as the enzyme source and L-arabinose was used as the substrate. The cell extract effected the partial conversion of L-arabinose to a compound which exhibited the same chromatographic characteristics as ribulose and was orcinol positive (Table I). Thus, L-arabinose isomerase in Aerobacter aerogenes was detected using the same conditions employed for the investigation of possible D-fucose isomerization in extracts of the pseudomonad; this control provides an indication of the reliability of the techniques used in the detection of possible D-fucose isomerization.

A colorimetric assay was also used for the detection of possible isomerization of D-fucose to D-fuculose in extracts of the pseudomonad. The basic reaction mixture used in the colorimetric assay was identical to that

described in Table I. No ketose formation was detected (<0.004 μmole per hour per mg) either in the basic reaction mixture or in reaction mixtures supplemented with 2 mM $CoCl_2$, 10 mM ATP, 10 mM NAD^+, or 20 mM EDTA. The results of the chromatographic and colorimetric analyses indicated that cell-free extracts of D-fucose-grown cells did not isomerize D-fucose to D-fuculose.

Phosphorylation- The possible phosphorylation of D-fucose with ATP by extracts of D-fucose-grown cells was investigated using the assay described in Experimental Procedures. No ATP-dependent phosphorylation (<0.001 μmole per hour per mg) could be detected (Table II).

Reduction- The possible reduction of D-fucose by extracts of D-fucose-grown cells was investigated using the spectrophotometric assay described in Experimental Procedures. No D-fucose reductase activity (< 0.002 μmoles per hour per mg) could be detected (Table III).

Dehydrogenation- In attempts to detect an initial phosphorylation reaction for D-fucose, it was observed that if NAD^+ were added to the complete assay in the presence of D-fucose and cell extract, the endogenous rate due to NADH oxidase drastically decreased, thereby suggesting some reduction of NAD^+ was occurring. Further investigation showed that a dehydrogenase was present which could oxidize D-fucose, as well as a number of other carbohydrates, to aldonolactones (Table IV). It was also shown that $NADP^+$ could serve equally well in the

Table II. Phosphorylation of D-fucose. The standard assay was employed. The pH of all added components was 7.2.

Experiment	Rate of NADH Oxidation (μmoles per hour per mg protein)
Complete*	0.116
Complete minus D-fucose	0.116
Complete plus 1 μmole Mn^{2+}, Co^{2+}, or EDTA	0.115
NADH oxidase (cell extract plus NADH)	0.051
Endogenous ADP (PEP-coupling system plus cell extract)	0.000
Complete plus 0.1 μmole ADP	> 0.6
Complete minus cell extract	0.000
Complete plus 0.1 μmole NAD^+	0.012

*See Experimental Procedures

Table III. Spectrophotometric assay for D-fucose reductase activity. The standard assay was employed in which both NADH and NADPH were tested as electron donors. Cell-free extracts of D-fucose-grown cells were used as the source of the enzyme.

Experiment	Specific Activity (μmoles/hr/mg protein)
Complete*, using NADH	0.185
Complete, using NADPH	0.000
NADH oxidase (complete minus D-fucose)	0.183
Complete + 1 μmole $MgCl_2$; EDTA, or 2-thioethanol	0.185

*See Experimental Procedures.

Table IV. NAD-dependent dehydrogenase activity. Dehydrogenase activity was measured spectrophotometrically at 340 nm and 25.0°C. The reaction mixture consisted of 5 μmoles sodium phosphate buffer (pH 7.0), 0.1 μmole NAD^+, 10 μmoles aldose, and a limiting amount of the dehydrogenase in a total volume of 0.15 ml. Crude extract of D-fucose grown cells was used as the source of the enzyme. The control to correct for NADH oxidase contained all the above components with the exceptions that the aldose was eliminated and NADH was substituted for NAD^+. Those carbohydrates which were negative (<0.002 μmoles per hour per mg protein) were: L-galactose, D-xylose, L-fucose, D-lyxose, and D-arabinose.

Carbohydrate	Specific Activity (μmoles/hr/mg protein)
D-Galactose	2.1
D-Fucose	1.8
L-Arabinose	0.8
D-Glucose	0.5
D-Mannose	0.3
6-Iodo-6-deoxy-D-galactose	1.2
D-Galactose ($NADP^+$ substituted for NAD^+)	1.9

role as an electron acceptor in the oxidation of galactose and D-fucose. Crude purification involving ammonium sulfate fractionations and Sephadex G-200 chromatography resulted in NAD-dependent D-fucose dehydrogenase activity devoid of NAD-dependent D-glucose and D-mannose dehydrogenase activities. In addition, re-examination of crude extracts indicated that $NADP^+$ could not serve as an electron acceptor for D-mannose or D-glucose oxidation. These data suggested a minimum of two pyridine nucleotide-linked dehydrogenases. Further examination and purification described below revealed that there are only two dehydrogenases in D-glucose-, D-galactose-, L-arabinose-, and D-fucose-grown cells; they are, a NAD-dependent dehydrogenase and a NAD(P)-dependent dehydrogenase.

Using the NAD(P)-dependent dehydrogenase, a direct correlation was obtained between the amount of D-fucose oxidized, the amount of lactone formed, and the amount of NADH produced in the oxidation (Table V). Some hydrolysis of the lactone had occurred as evidenced by the higher lactone concentration after lactonization. Since all the D-fucose oxidized can be accounted for as lactone and since the previous experiments concerning alternative metabolic reactions for D-fucose were negative, it was concluded that D-fucose degradation by any other means in this microbe except by an initial oxidation was improbable.

Table V. Correlation between D-fucose oxidation, NADH production, and lactone formation. The reaction mixture consisted of 50.0 μmoles pyruvic acid (pH 7.0), 25.0 μmoles D-fucose, 50 μg lactic acid dehydrogenase-pyruvate kinase, 1 μmole NAD^+, 50 μmoles sodium cacodylate buffer (pH 7.0), and 12.5 mg cell extract of D-fucose-grown cells in a total volume of 1.00 ml. The reaction was incubated at 25°C and the pyruvate concentration monitored by the method of Friedman and Haugen. The pyruvate concentration became constant after one hour, and the lactone and fucose concentrations were then determined. An aliquot was lactonized to convert any free aldonic acids to the corresponding lactone. The cell extract contained no NAD-dependent dehydrogenase activity (<0.0002 μmoles per hour per mg of protein), as determined with glucose and NAD^+.

Component	Initial μmoles	Final μmoles	Change
D-Fucose	25.0*	0.00	25.0
Pyruvic Acid	50.0*	25.4	24.6
Lactone	00.0	20.8	20.8
Lactone (after lactonizing)	00.0	24.1	24.1

*Zero time aliquots were assayed.

B. CHARACTERIZATION OF THE NAD(P)-DEPENDENT DEHYDROGENASE

1. Purification

Cell extracts of D-galactose-grown cells were prepared as described in Experimental Procedures. Except where indicated otherwise, the fractionation procedures were carried out at 0-4°C. A summary of the purification is given in Table VI.

Protamine Sulfate Fractionation- The protein concentration of the cell extract was adjusted to 15 mg per ml by dilution with 0.10 molar sodium phosphate buffer (pH 7.0). Ammonium sulfate (13.7 g) was dissolved in 525 ml cell extract to a final concentration of 0.20 molar, and then 105 ml of a 2% protamine sulfate solution in 0.10 molar sodium phosphate buffer (pH 7.0) was added with stirring to a final concentration of 0.33%. After 30 minutes the suspension was centrifuged at 40,000 x g for 10 minutes, and the resulting precipitate was discarded.

Heat Step- The NAD-dependent dehydrogenase is heat labile with a half-life of 42 seconds at 55°C whereas the NAD(P)-dependent enzyme is somewhat more stable. Thus, the contaminating NAD-dependent dehydrogenase activity could be removed by a carefully controlled heating procedure. The 40,000 x g supernatant (630 ml) resulting from the protamine sulfate step was immersed in a 60°C bath

Table VI. Purification of the NAD(P)-dependent dehydrogenase.

Fraction	Units	Specific Activity	Fold	280/260 Ratio
Cell extract	101,000	11.8	1.0	0.620
Protamine sulfate	99,500	12.7	1.1	0.895
Heat step	82,500	23.4	1.89	0.960
Ammonium sulfate	55,500	69.5	5.90	1.22
Sephadex G-200	30,100	540.	45.7	1.31
Calcium phosphate gel	14,500*	3340.	276.	1.49

*Only a portion of the pooled Sephadex G-200 fractions was purified by calcium phosphate gel. This value has been corrected for the total volume of the pooled Sephadex fractions.

and stirred gently until the temperature reached 55°C (in about 20 minutes). The protein solution was heated for an additional 2 minutes at 55°C, cooled in an ice-water bath, and centrifuged at 40,000 x g. The consequent precipitate was discarded.

Ammonium Sulfate Fractionation- The 40,000 x g supernatant (620 ml) was brought to 40% of saturation by the addition of 122 g of ammonium sulfate and the resulting precipitate was centrifuged down and discarded. The supernatant was then brought to 60% of saturation with 81.3 g of ammonium sulfate and centrifuged, and the resulting precipitatewas collected by centrifugation and dissolved in 70 ml 0.10 molar sodium phosphate buffer (pH 7.0). The protein concentration at this stage was 28 mg per ml.

Sephadex G-200 Chromatography- The above 40-60% fraction was placed on a column (6 x 60 cm) of Sephadex G-200 equilibrated with 0.01 molar sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer. Fractions (15 ml) were collected, and those which contained the most activity were pooled (90 ml total).

Calcium Phosphate Gel- The pooled Sephadex G-200 fractions containing the NAD(P)-dependent dehydrogenase activity were adjusted to pH 6.5 with 0.05 molar HCl and dialyzed for 24 hours against 0.01 molar sodium cacodylate buffer (pH 6.5). A portion of the dialyzed protein (5.0 ml) was then treated with 20% v/v of calcium phosphate

gel prepared according to the method of Wood (49). The gel suspension was centrifuged for one minute in a clinical centrifuge and then successively eluted with 1 ml each of 0.01, 0.02, 0.03, 0.04, and 0.05 molar sodium phosphate buffer (pH 7.0). Approximately 50% of the activity eluted in the 0.01-0.02 molar range whereas 100% of the activity was recoverable in the 0.01-0.05 molar range.

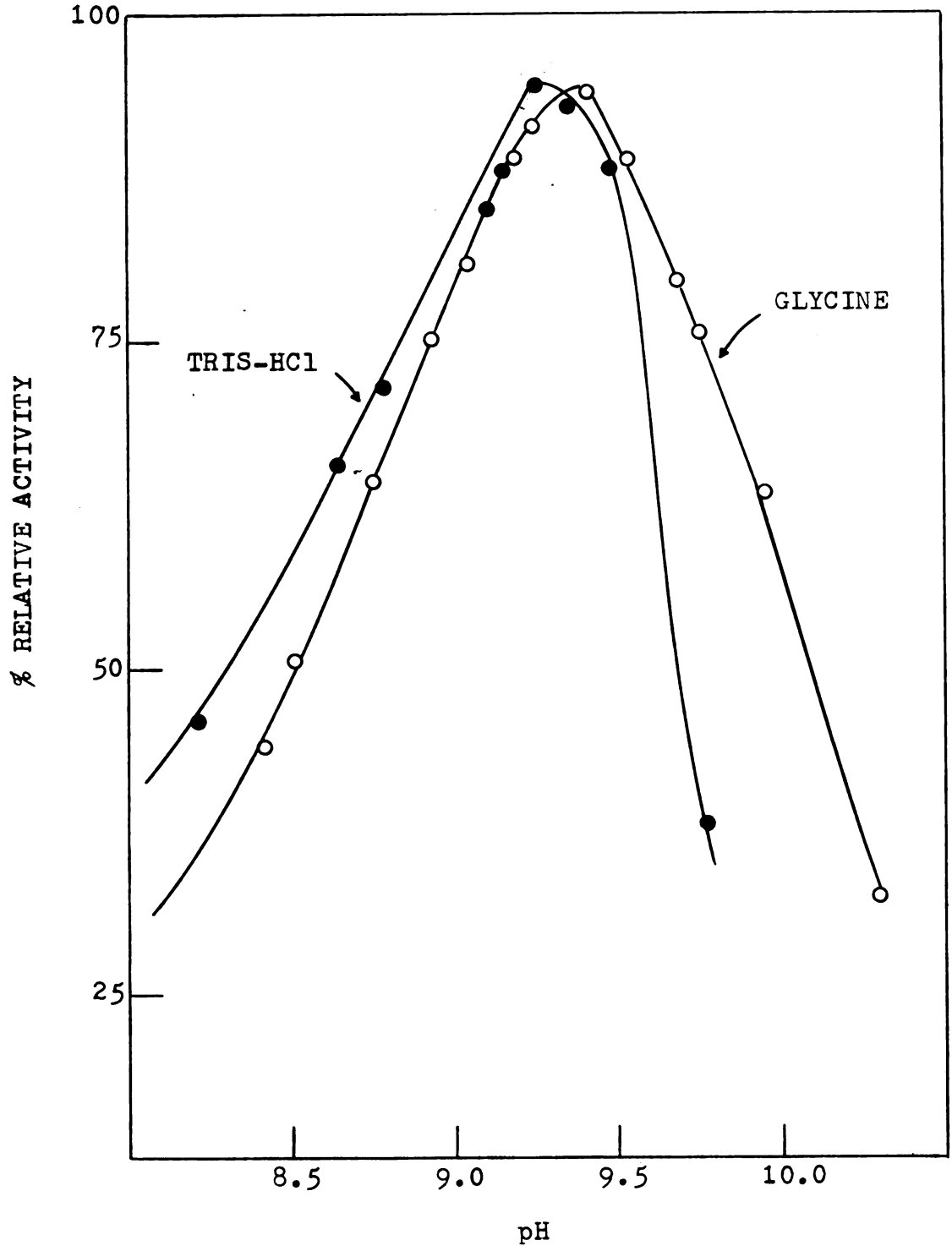
2. Properties

pH Optimum- NAD(P)-dependent dehydrogenase activity as a function of pH was maximal at pH 9.4 in Tris-HCl and glycine buffers (Figure 2). However, maintenance of the dehydrogenase at pH 9.4 in an ice-water bath in the presence of 0.10 molar Tris-HCl buffer resulted in an irreversible loss of 10% of the starting activity within 12 minutes. In addition, at pH values >9, reaction rates were not linear beyond 10 minutes and were observed to decrease exponentially. Further, the K_m values for L-arabinose, D-fucose, and D-galactose at pH 9.4 were found to be 5-14 fold higher than at pH 8.1 (see below). For these reasons, the standard assay employed a buffer at pH 8.1 rather than at the pH optimum.

Substrate Specificity- A large number of carbohydrates were examined as possible substrates for the enzyme. The carbohydrates which were found to be oxidized

Figure 2. pH Optimum of the NAD(P)-dependent dehydrogenase. The standard assay was employed except that the pH and the buffer composition were varied with the dehydrogenase concentration constant. The calcium phosphate fraction with the highest specific activity was used. Each buffer was 0.10 molar. The pH measurements were determined on duplicate reaction mixtures. The pH of the reaction mixture did not vary during the 10 minute reaction period.

Figure 2


100
75
50
25
% RELATIVE ACTIVITY
TRIS-HCl
GLYCINE
8.5
9.0
9.5
10.0
pH

by the enzyme were D-fucose, D-abequose (3,6-dideoxy-D-galactose), D-galactose, 2-deoxy-D-galactose, L-arabinose, 6-iodo-6-deoxy-D-galactose, and L-mannose (Table VII). The carbohydrates which did not serve as substrates also did not reduce the rate of oxidation of 33.3 mM D-galactose when added at equimolar concentrations.

Lineweaver-Burk plots for D-galactose, D-fucose, and L-arabinose are presented in Figures 3, 4, and 5. The reactions were performed at two pH values: (i) the standard assay (pH 8.1); (ii) the pH optimum (pH 9.4). A tabulation of the K_m and V_{max} values derived from the plots in Figures 3-5 is presented in Table VIII. All rates with mixed substrates were non-additive (Table IX), suggesting that the activity in the Sephadex G-200 fraction represented one enzyme.

Nucleotide Specificity- NAD and NADP served equally well as cofactors for the dehydrogenase reaction at pH 8.1. From the Lineweaver-Burk plots shown in Figures 6 and 7, the K_m values for NAD^+ and $NADP^+$ were found to be 14.8 μM and 67.6 μM respectively.

Reversal of the Dehydrogenation- The product of D-fucose oxidation by this enzyme has been identified as D-fucono-γ-lactone (see Product Identification, this section). The dehydrogenation reaction for similar enzymes has been found by other investigators to be reversible when using a high concentration of the respective lactone and a low pH (38-45). The reversibility of

Table VII. Substrate specificity of the NAD(P)-dependent dehydrogenase. The standard assay was employed with the exception that the carbohydrate concentration was 33.3 mM.

Carbohydrate	Relative Rate %
2-Deoxy-D-galactose	142
D-Galactose	110
D-Fucose	100
L-Arabinose	47
3,6-Dideoxy-D-galactose (abequose)	42
6-Iodo-6-deoxy-D-galactose	37
L-Mannose*	34

Carbohydrates which were not active as substrates (i.e., less than 1% the activity on D-fucose) are: galactose-6-P, glucose-6-P, fructose-6-P, D-xylose, lactose, melezitose, maltose, cellobiose, DL-glyceraldehyde, D-ribose, D-lyxose, 2-deoxy-D-ribose, L-rhamnose, sucrose, D-arabinose, L-xylose, N-acetyl-D-glucosamine, D-glucosamine, D-mannitol, D-sorbitol, myo-inositol, L-sorbose, raffinose, turanose, melibiose, trehalose, L-fucose, L-arabitol, α-methyl-glucoside, inulin (8.3 mM), L-galactose, L-glucose, L-mannose, L-fructose, D-fructose, xylitol, D-arabitol, adonitol, glucuronic acid, galacturonic acid, D-xylulose, galactaric acid, glucaric acid, 6-deoxy-D-glucose, D-glucose, 2-deoxy-D-glucose, D-allose, D-altrose, 2-acetamido-D-allose, 2-acetamido-D-altrose, 6-deoxy-D-allose, and D-mannose.

*L-Mannose was not saturating at 33.3 mM. The K_m value was later determined to be about 100 mM. L-Mannose at 250 mM was oxidized at the same rate as D-galactose at 33.3 mM.

Figure 3. Lineweaver-Burk plots relating NAD(P)-dependent dehydrogenase activity to D-galactose concentration at two pH values. With the exception of the differences in buffers, the routine assay was employed. The calcium phosphate fraction with the highest specific activity was used.

Figure 3A

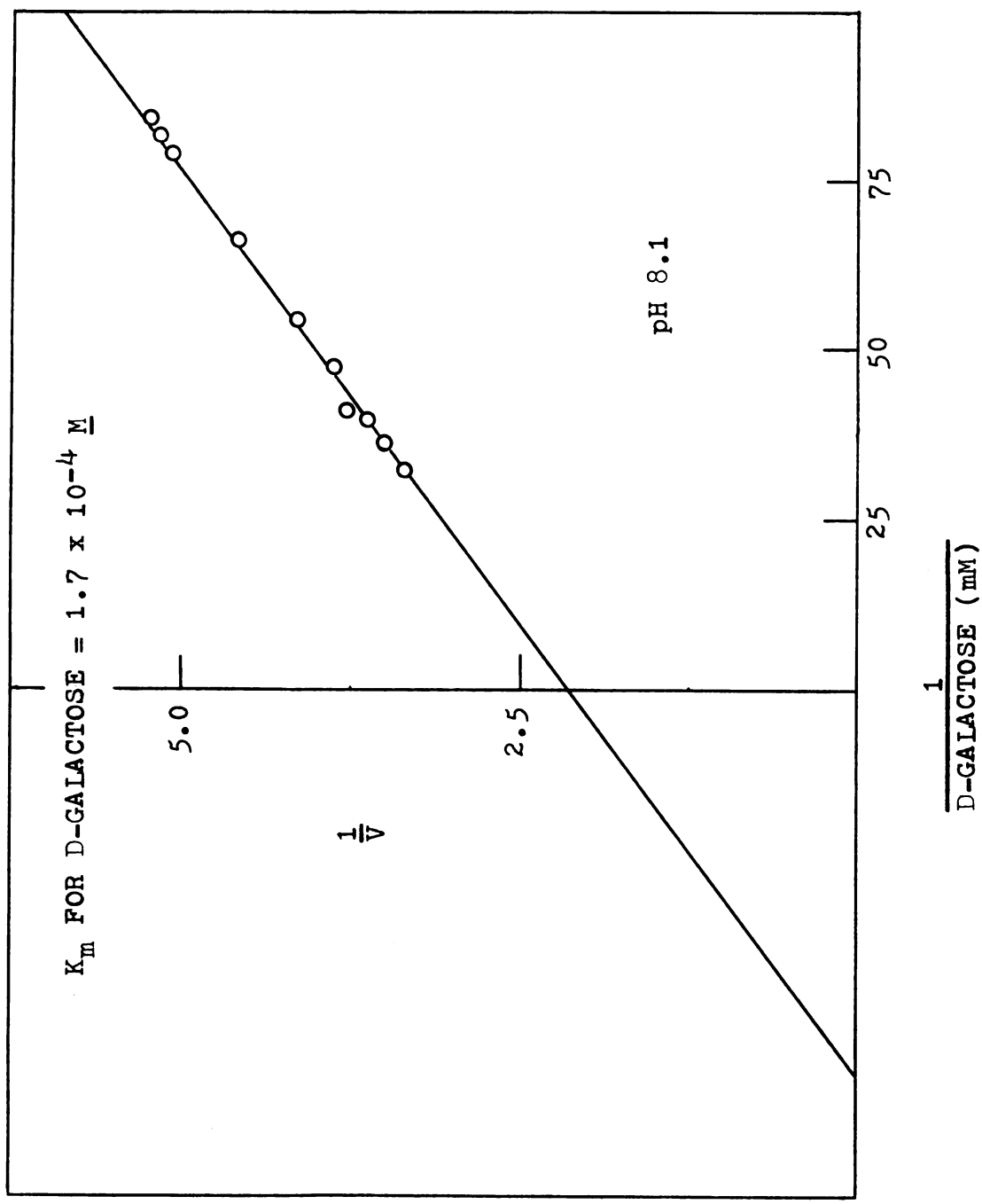

K_m FOR D-GALACTOSE = 1.7 x 10^{-4} M
pH 8.1
$\frac{1}{V}$
5.0
2.5
25
50
75
$\frac{1}{\text{D-GALACTOSE (mM)}}$

Figure 3B

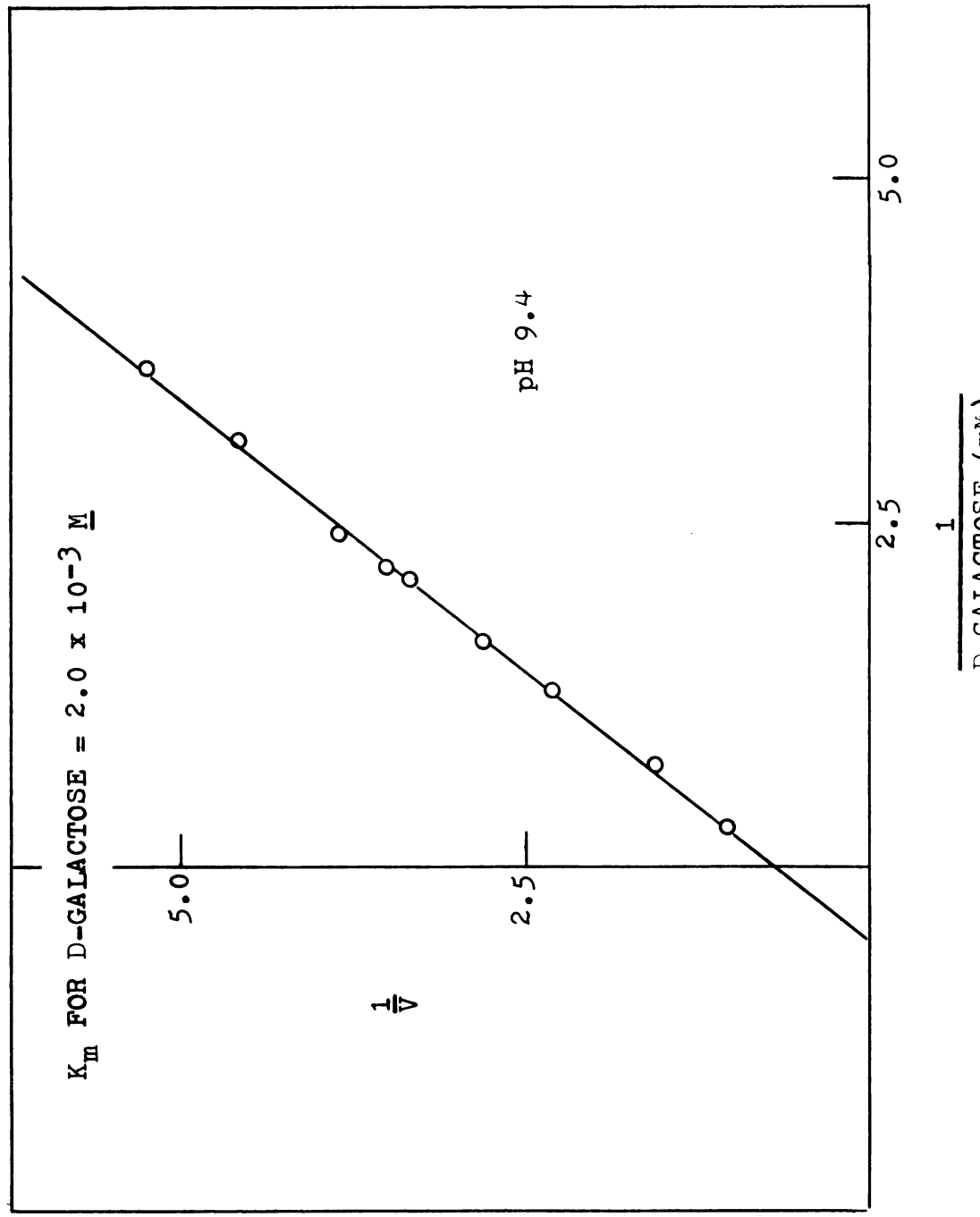

K_m FOR D-GALACTOSE = 2.0 x 10^{-3} M
pH 9.4
$\frac{1}{V}$
5.0
2.5
5.0
2.5
$\frac{1}{\text{D-GALACTOSE (mM)}}$

Figure 4. Lineweaver-Burk plots relating NAD(P)-dependent dehydrogenase activity to D-fucose concentration at two pH values. Other conditions were the same as described in Figure 3.

Figure 4A

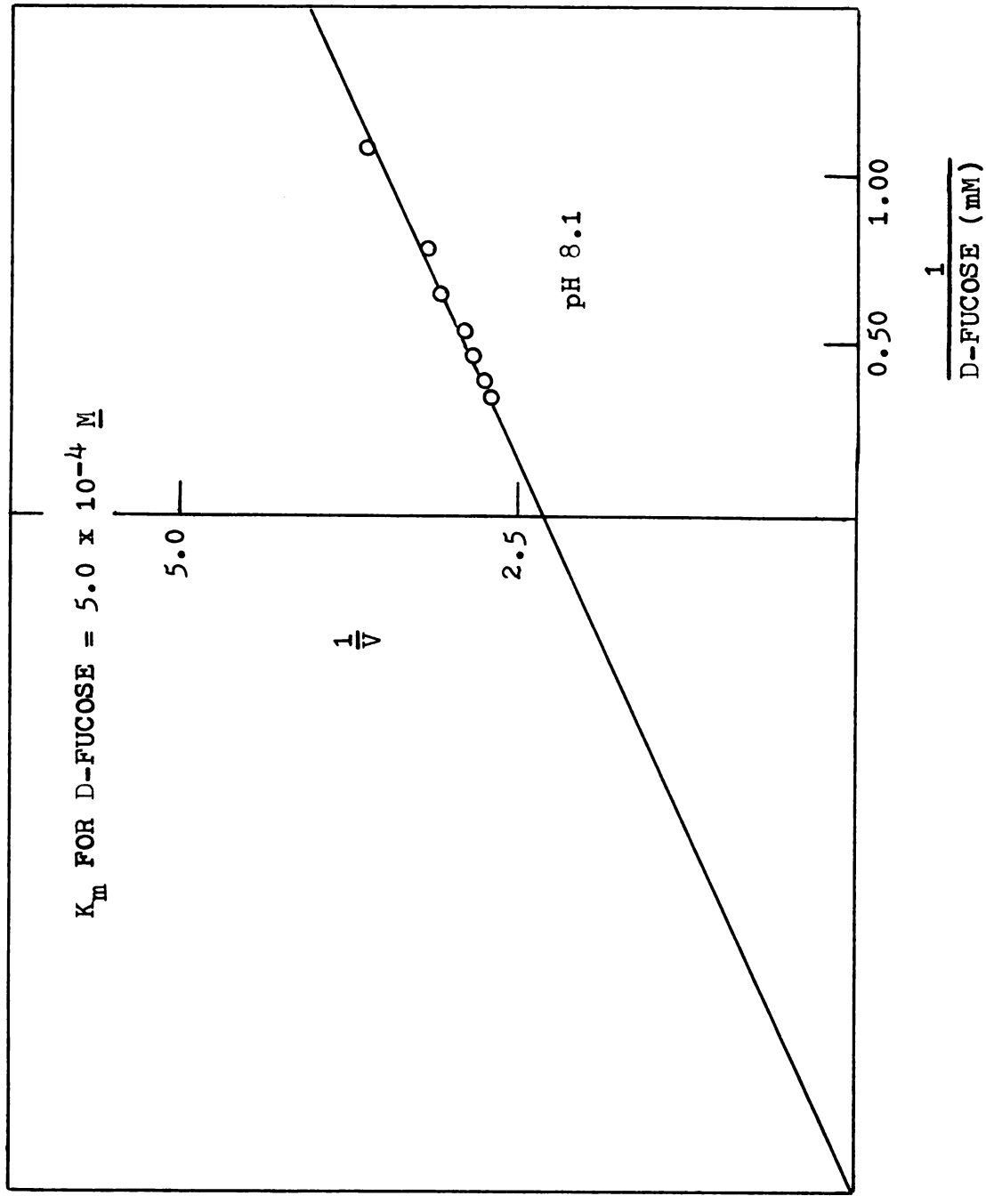

K_m FOR D-FUCOSE = 5.0 x 10^{-4} M
5.0
2.5
$\frac{1}{V}$
pH 8.1
0.50
1.00
$\frac{1}{\text{D-FUCOSE (mM)}}$

Figure 4B

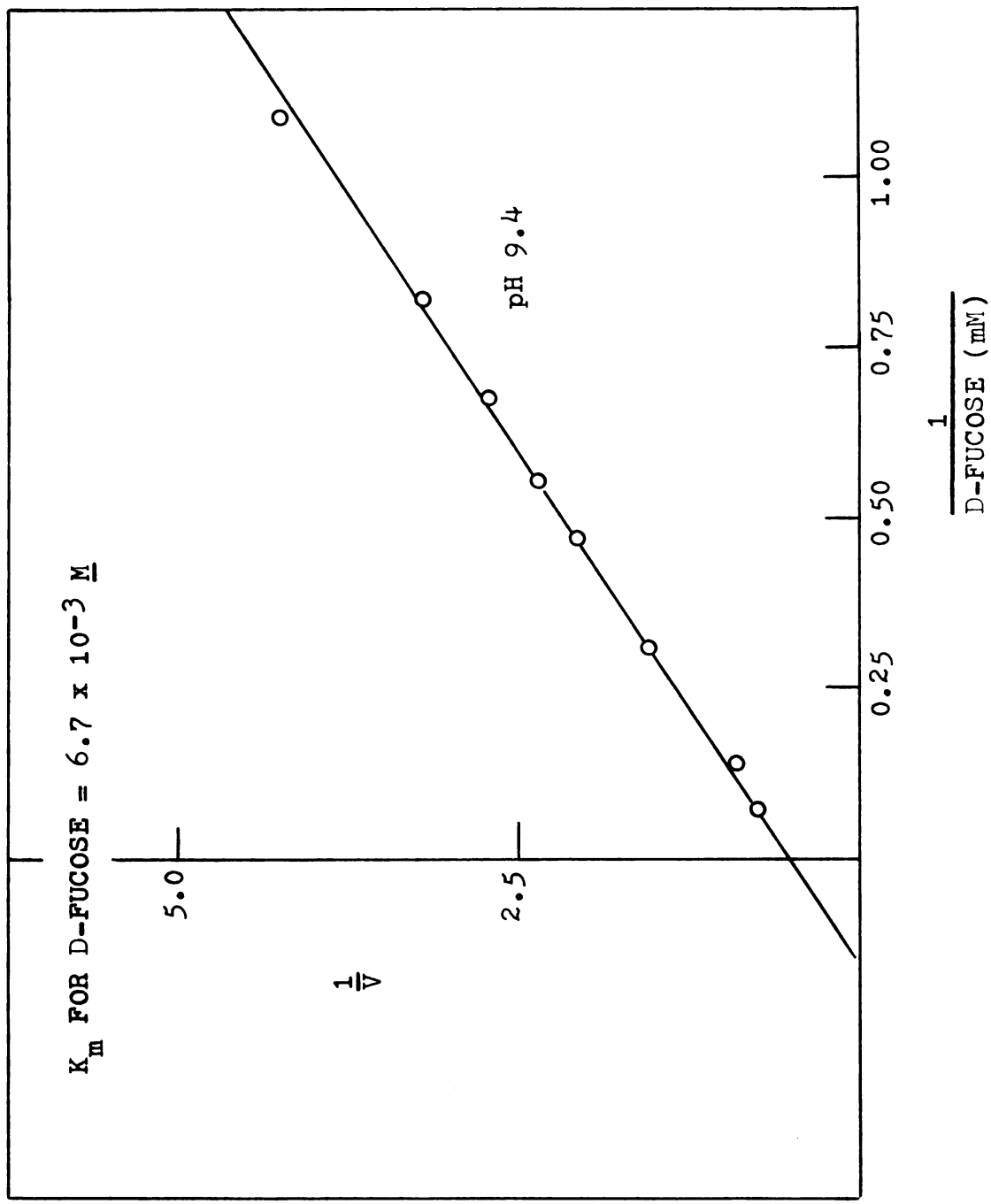

K_m FOR D-FUCOSE = 6.7 x 10^{-3} M
pH 9.4
$\frac{1}{V}$
5.0
2.5
0.25
0.50
0.75
1.00
$\frac{1}{\text{D-FUCOSE (mM)}}$

Figure 5. Lineweaver-Burk plots relating NAD(P)-dependent dehydrogenase activity to L-arabinose concentration at two pH values. Other conditions were the same as described in Figure 3.

Figure 5A

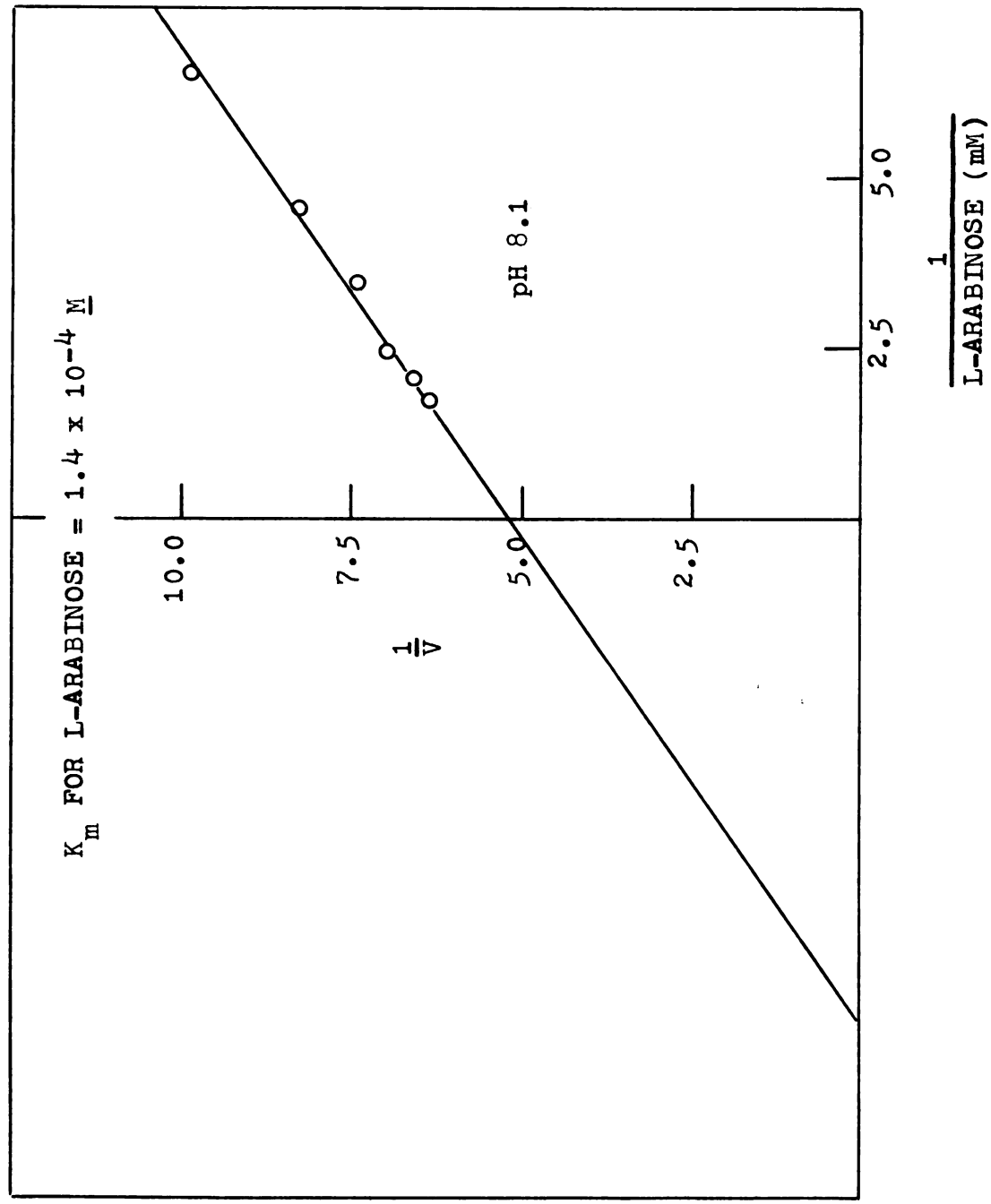

K_m FOR L-ARABINOSE = 1.4×10^{-4} M
pH 8.1
$\frac{1}{V}$
10.0
7.5
5.0
2.5
2.5
5.0
$\frac{1}{\text{L-ARABINOSE (mM)}}$

Figure 5B

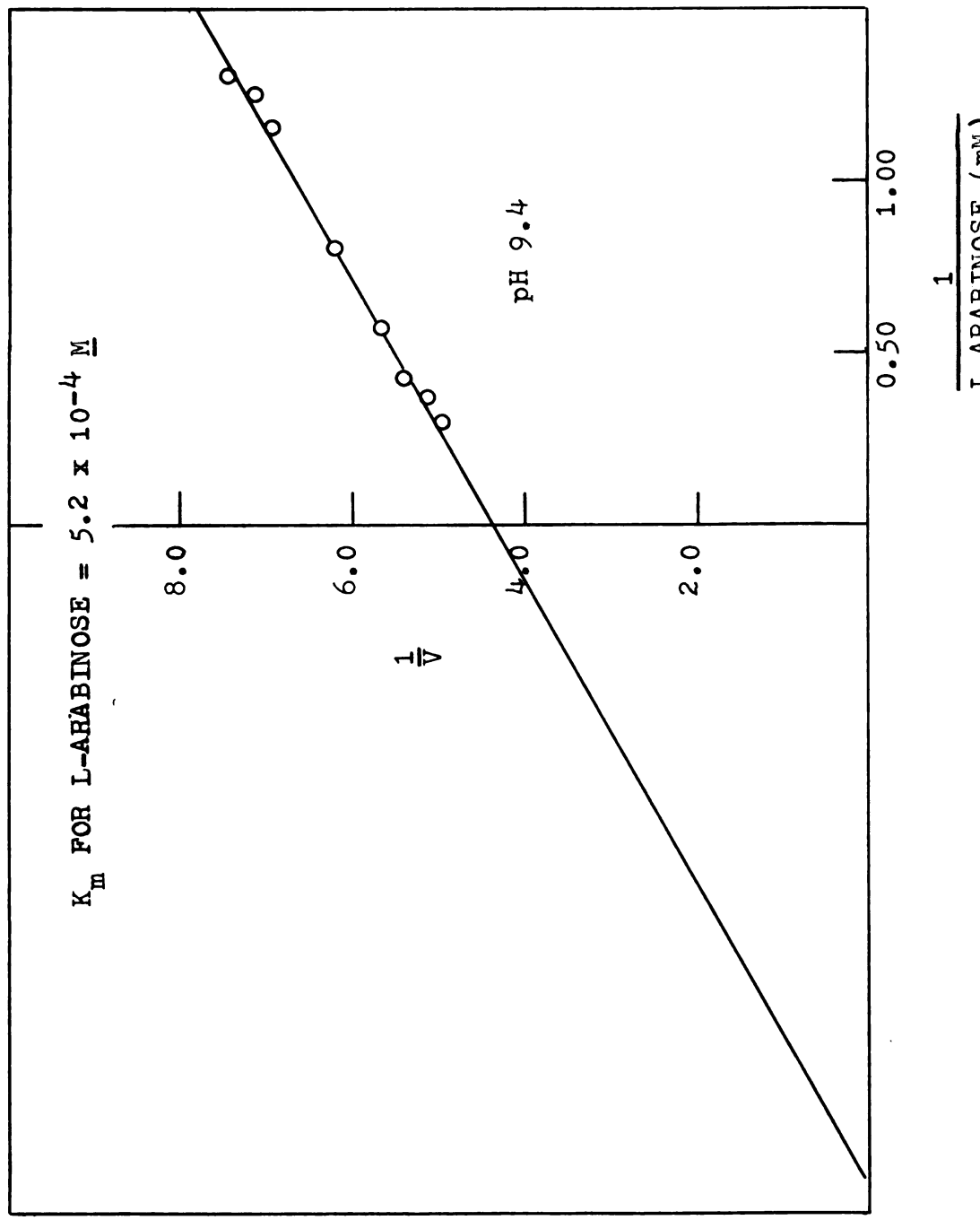

K_m FOR L-ARABINOSE = 5.2 x 10^{-4} M
pH 9.4
$\frac{1}{V}$
8.0
6.0
4.0
2.0
0.50
1.00
$\frac{1}{\text{L-ARABINOSE (mM)}}$

Table VIII. A tabulation of the K_m and V_{max} values for the NAD(P)-dependent dehydrogenase. The data were derived from the data depicted in Figures 3-5.

	pH 8.1		pH 9.4	
Carbohydrate	K_m (mM)	Relative V_{max}	K_m (mM)	Relative V_{max}
D-Galactose	0.17	110	2.0	72
D-Fucose	0.50	100	6.6	100
L-Arabinose	0.14	47	0.52	12

Table IX. Effect of mixed substrates on NAD(P)-dependent dehydrogenase activity. The standard assay was employed with the exception that the carbohydrate concentration was 6.7 mM. When both substrates were mixed, the concentration of each substrate was the same as that used above. A Sephadex G-200 fraction was used.

Substrate	Specific Activity (μmoles/hour/mg)
D-Galactose	310
D-Fucose	285
L-Arabinose	132
D-Galactose + D-Fucose	291
D-Galactose + L-Arabinose	215
D-Fucose + L-Arabinose	198

Figure 6. Lineweaver-Burk plot relating NAD(P)-dependent dehydrogenase activity to NAD^+ concentration. The routine assay was employed with the exception that the NAD^+ concentration was varied, as indicated, with the dehydrogenase concentration constant. D-Galactose concentration was 33.3 mM. The calcium phosphate fraction with the highest specific activity was utilized. The reaction rate was linear for the 10 minute reaction period.

Figure 6

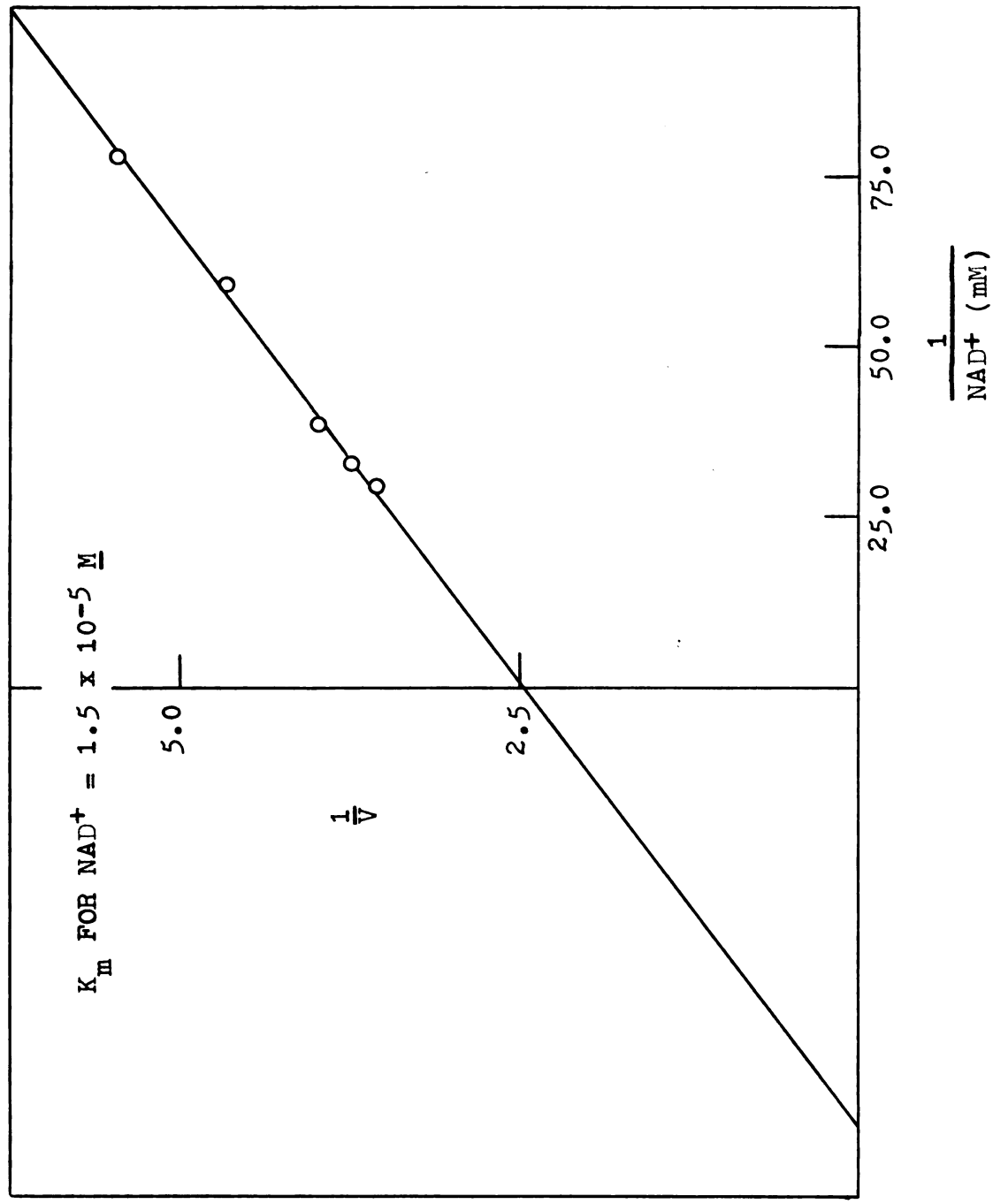
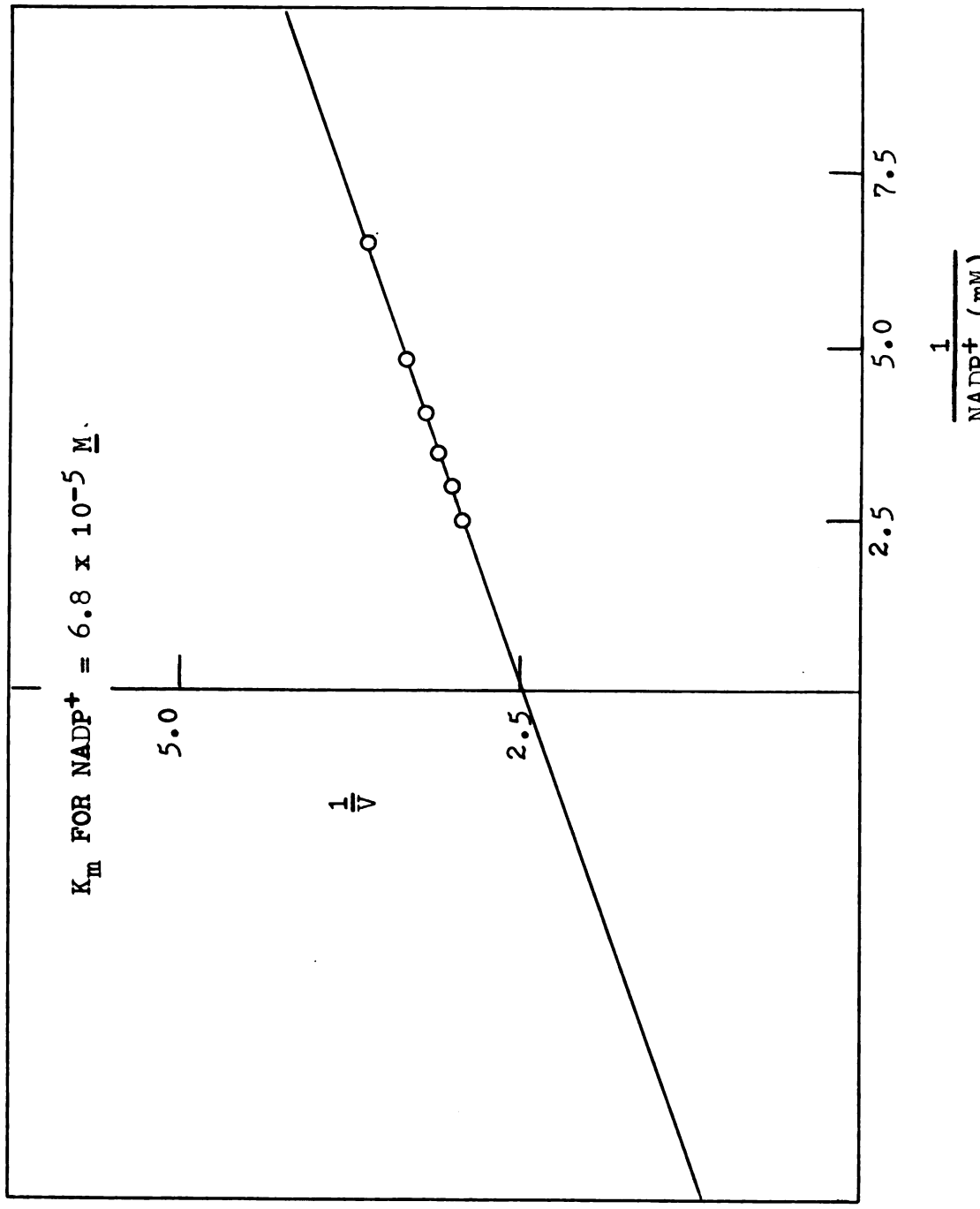


Figure 7. Lineweaver-Burk plot relating NAD(P)-dependent dehydrogenase activity to $NADP^+$ concentration. Other conditions were the same as described in Figure 6.

Figure 7



the NAD(P)-dependent dehydrogenase was tested with both δ- and γ-lactones. The data, shown in Table X, indicate that the reaction is reversible with γ-lactones of D-fuconic and D-galactonic acids but not with D-glucono-δ-lactone; further, the reaction was not inhibited by D-glucono-δ-lactone. The δ-lactones of D-fuconic and D-galactonic acids could not be tested because of their instability (46-48). Since the product was identified as D-fucono-γ-lactone and since the reversibility of the reaction was demonstrated with the γ-lactones of the respective substrates, it may be inferred that the dehydrogenase is operative on the furanose form of the carbohydrate.

Stability- The half-life of the dehydrogenase has been found to be 13 minutes at 55°C. The enzyme, in Sephadex G-200 fractions, is stable to freezing at -20°C in 0.01 molar sodium phosphate buffer (pH 7.0) for periods up to two months. Lyophilization of pooled Sephadex G-200 fractions containing the enzyme in 0.01 molar sodium phosphate buffer (pH 7.0) resulted in a 60-80% loss of activity.

The effects of various metal ions, thiols, and thiol group inhibitors are shown in Table XI. The enzyme is not affected by 1 mM 2-thioethanol or 1 mM dithiothreitol. Similarly, 0.5 mM iodoacetate or 0.5 mM p-chloromercuribenzoate is not inhibitory to enzyme activ-

Table X. Reversibility of the NAD(P)-dependent dehydrogenase. The assay consisted of 0.26 μmole NADPH, 10 μmoles of the respective lactone, 30 μmoles Tris-maleate buffer (pH 6.5) and a limiting amount of the dehydrogenase in a total volume of 0.15 ml. A Sephadex G-200 fraction was used. The pH of the reaction mixture did not vary during the 15 minute reaction period. The lactone solutions were prepared before each assay in 0.20 molar Tris-maleate (pH 6.5).

Lactone	Specific Activity (μmoles/hour/mg)
D-Galactono-γ-lactone	13.5
D-Fucono-γ-lactone	8.4
D-Glucono-δ-lactone	< 0.02
D-Galactono-γ-lactone + D-glucono-δ-lactone	13.4
D-Fucono-γ-lactone + D-glucono-δ-lactone	8.2

Table XI. Effect of various reagents on NAD(P)-dependent dehydrogenase activity. The standard assay was utilized in which the enzyme was added to the cuvette containing the reagent. The Sephadex G-200 fraction with the highest specific activity was used. The pH of the reagents was 7.0.

Reagent	Concentration	% of Control Activity
p-Chloromercuribenzoate	0.5 mM	100
Iodoacetic acid	0.5 mM	100
2-Thioethanol	1.0 mM	100
Dithiothreitol	1.0 mM	100
EDTA	6.6 mM	100
$MgCl_2$	6.6 mM	100
$CoCl_2$	6.6 mM	95
$(NH_4)_2SO_4$	6.6 mM	95
$MnCl_2$	6.6 mM	90
$NiCl_2$	6.6 mM	70
$FeSO_4$	6.6 mM	10
$ZnCl_2$	6.6 mM	10
$CaCl_2$	6.6 mM	5

ity. There was no observed inhibition by 6.6 mM EDTA or activation by metal ions, also at 6.6 mM.

Induction- The inducibility of the NAD(P)-dependent dehydrogenase was tested by growth on various substrates. The results (Table XII) indicate that D-fucose, D-galactose, L-arabinose, and 6-iodo-6-deoxy-D-galactose induce the NAD(P)-dependent dehydrogenase to a level 35-120 fold over the non-induced level present in nutrient broth-grown cells. The data suggest that the enzyme is instrumental in the metabolism of D-fucose, D-galactose, L-arabinose, and 6-iodo-6-deoxy-D-galactose.

3. Product Identification

The enzymatic reaction product resulting from the NAD(P)-dependent oxidation of D-fucose, D-fucono-γ-lactone, was prepared on a small scale and was identified by chromatography, derivatization, and by comparison with authentic, chemically prepared D-fucono-γ-lactone, as described below. Also, a large scale reaction was carried out at a high pH to hydrolyze the D-fucono-γ-lactone, formed from the oxidation of D-fucose, to the free acid, which was subsequently crystallized as the potassium salt.

Enzymatic Preparation of D-Fucono-γ-lactone- The reaction mixture consisted of 200 μmoles D-fucose, 250 μmoles pyruvic acid (pH 6.4), 125 μg lactic acid dehydro-

Table XII. Induction of the NAD(P)-dependent dehydrogenase by various growth substrates. The standard assay was employed. Cell extracts were prepared by sonic disruption in 0.01 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). Protein was estimated by the biuret assay.

Growth Substrate	Specific Activity (μmoles/hour/mg)
D-Fucose	11.8
L-Arabinose	15.7
D-Glucose	0.250
D-Galactose	11.8
Nutrient Broth	0.130
6-Iodo-6-deoxy-D-galactose	4.69

genase, 1 μmole NAD^+, and 2 ml of a Sephadex G-200 fraction (3.5 mg protein). The NAD(P)-dependent dehydrogenase contained no NAD-dependent dehydrogenase, γ-lactonase, D-fuconate dehydratase, on 2-keto-3-deoxy-D-fuconate aldolase, and was prepared in the same manner described in the purification procedure with the exception that the enzyme was isolated from L-arabinose-grown cells. The pH was maintained at 6.4 by automatic titration with a Sargent recording pH stat using 0.10 molar NaOH as the titrant or by incorporating 400 μmoles Tris-maleate buffer (pH 6.4) into the reaction mixture. The reaction mixture was maintained at 25°C and was judged to be complete after 2 hours as measured by the complete loss of reducing sugar. The reaction mixture was then deionized by passage through a mixed bed resin (50:50, Dowex-50W-H^+, Dowex-1-OH^-) and chromatographed on Whatman #1 paper in solvent systems 1, 2, 3, and 5; the lactone co-chromatographed with authentic D-fucono-γ-lactone prepared by lactonization of D-fuconic acid. The yield of the enzymatically prepared lactone was 150 μmoles (75%). A comparison of the properties of the enzymatically and chemically prepared lactones is presented in Table XIII.

Enzymatic Preparation of Potassium D-Fuconate- The reaction mixture (80 ml) consisted of 20 mmoles of D-fucose, 25 mmoles of pyruvic acid (pH 8.9), 500 μg lactic acid dehydrogenase, 1 μmole NAD^+, and 20 ml of a

Table XIII. A comparison of the physical properties potassium D-fuconate and D-fucono-γ-lactone prepared by chemical and enzymatic routes.

	Preparation			
Derivatives*	Enzymatic	Chemical	Lit.	Ref.
m.p. K^+-D-Fuconate	169-70°C	169-70°C	169-70 °C	(31)
m.p. Lactone	105-06°C	104-05°C	105-06 °C	(32)
m.p. Amide	176-77°C	175-76°C	180 °C	(33)
m.p. Benzimidazole	251-53°C	252-53°C	248-49 °C	(34)

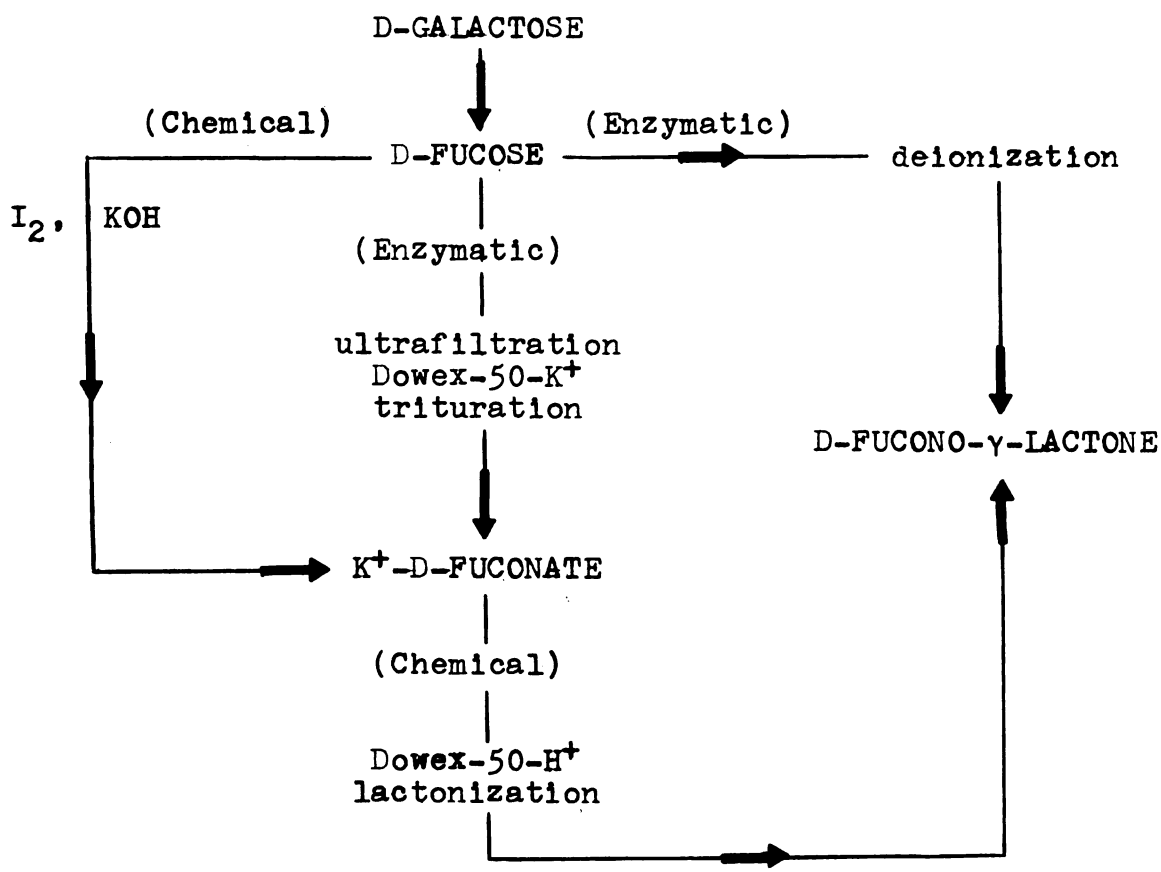
Chromatography (Whatman #1)	R_f Values Enzymatic	Chemical
Solvent System #1:		
D-Fuconic acid	.10	.098
D-Fucono-γ-lactone	.61	.62
Solvent System #2:		
D-Fuconic acid	.47	.47
D-Fucono-γ-lactone	.61	.61
Solvent System #3:		
D-Fuconic acid	.70	.68
D-Fucono-γ-lactone	-	-
Solvent System #5:		
D-Fuconic acid	.26	.25
D-Fucono-γ-lactone	.56	.55

*No depressions of the melting points were observed upon mixing.

Sephadex G-200 fraction (35 mg protein) containing the NAD(P)-dependent dehydrogenase prepared in the same manner as described in the purification procedure with the exception that the enzyme was isolated from L-arabinose-grown cells. The enzyme preparation was devoid of D-fuconate dehydratase, 2-keto-3-deoxy-D-fuconate aldolase, γ-lactonase, and NAD-dependent dehydrogenase activity. The pH was maintained at 8.9 by automatic titration with a Sargent recording pH stat, using 0.50 molar KOH as the titrant. The temperature was maintained at 25°C for 6 hours after which time the reaction was judged to be complete as measured by the complete loss of reducing sugar and the constancy of the pyruvate concentration. The reaction mixture (100 ml) was then passed through a Diaflow ultrafiltration cell equipped with a 10,000 molecular weight cut-off ultrafiltration membrane to remove protein. The eluate from the ultrafiltration cell was reduced in volume under vacuum to 15 ml, applied to a AG50W-X8-K^+ column (1 x 12 cm) and eluted with deionized water (350 ml). The eluate from the column was reduced in volume under vacuum to 4.0 ml. The salt-syrup slurry was then triturated with two portions of absolute methanol (110 ml, 45°C) and filtered. The filtrate was seeded with authentic potassium D-fuconate (see Experimental Procedures); crystallization commenced immediately. The long, fine needles characteristic of

this salt were collected by suction filtration and washed with diethyl ether (25 ml); yield 4.21 g. The supernatant yielded an additional 0.56 g after repeating the above procedure. Total yield was 4.67 g (97%) after recrystallizing from methanol-water (100:2). A comparison of the properties of the enzymatically and chemically prepared sugar acids is presented in Table XIII. Enzymatically prepared potassium D-fuconate was found to be identical in all respects to the chemically prepared material. A general outline for the chemical and enzymatic preparations for D-fucose, potassium D-fuconate, and D-fucono-γ-lactone is presented in Figure 8.

Figure 8. A general outline for the preparation of D-fucose, D-fuconic acid, and D-fuconolactone.



C. CHARACTERIZATION OF THE NAD-DEPENDENT DEHYDROGENASE

1. Purification

Cell extracts of D-glucose-grown cells were prepared as described in Experimental Procedures. Except where indicated otherwise, the fractionation procedures were carried out at 0-4°C. A summary of the purification is given in Table XIV.

Protamine Sulfate Fractionation- The protein concentration was adjusted to 6.5 mg per ml by dilution with 0.10 molar sodium phosphate buffer (pH 7.0). Ammonium sulfate (17.2 g) was dissolved in 650 ml cell extract to a final concentration of 0.20 molar, and then 130 ml of a 2% protamine sulfate solution in 0.10 molar sodium phosphate buffer (pH 7.0) was added with stirring to a final concentration of 0.33%. After 30 minutes the suspension was centrifuged at 40,000 x g for 10 minutes, and the resulting precipitate was discarded.

Ammonium Sulfate Fractionation- The 40,000 x g supernatant (800 ml) from the protamine sulfate step was brought to 30% of saturation by the addition of 110 g of ammonium sulfate and centrifuged. The supernatant was then brought to 40% of saturation with 48.1 g of ammonium sulfate. The resulting precipitate was collected by centrifugation and was dissolved in 53 ml 0.01 molar sodium phosphate (pH 7.0). The protein concentration was 20 mg per ml. It might be noted that the 0-30% fraction,

Table XIV. Purification of NAD-dependent dehydrogenase.

Fraction	Units	Specific Activity	Fold	280/260 Ratio
Cell extract	50,140	12.2	1.0	.633
Protamine sulfate	45,130	11.1	1.0	.859
Ammonium sulfate	27,500	27.2	2.24	1.17
Sephadex G-200	14,600	134	11.0	1.25
DEAE-cellulose	11,700*	830	68	1.48
Calcium phosphate gel	6,750	3980	327	1.58

*Only a portion of the pooled Sephadex G-200 fractions was purified by DEAE-cellulose. This value and the units recovered from the calcium phosphate gel step have been corrected for the total volume of the pooled Sephadex fractions.

which contains 30-40% of the total soluble protein present in crude extracts of the pseudomonad, contains the rust-colored protein which bestows the characteristic pigmentation to the crude extract and the microorganism.

Sephadex G-200 Chromatography- The 30-40% ammonium sulfate fraction (53 ml) was placed on a column (6 x 60 cm) of Sephadex G-200 equilibrated with 0.01 molar sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer. Fractions (15 ml) were collected, and those which contained the most activity were pooled (135 ml total).

DEAE-Cellulose Chromatography- DEAE-cellulose (Sigma, exchange capacity = 0.9 meq per g) was pretreated as recommended by Sober et al. (50) and was equilibrated with 0.02 molar sodium phosphate buffer (pH 7.0). The pooled Sephadex G-200 fractions were reduced in volume to 15 ml using a Diaflow ultrafiltration cell equipped with a 10,000 molecular weight cut-off ultrafiltration membrane (Amicon Corporation). A portion of the Sephadex G-200 concentrate (2 ml) was applied to a DEAE-cellulose column (3 x 5 cm) which was then washed with 60 ml of the above buffer, and then successively eluted with a step-wise gradient composed of 60 ml each of 0.10, 0.20, 0.30, 0.40, and 0.80 molar sodium chloride in 0.02 molar sodium phosphate buffer (pH 7.0). Of the two dehydrogenases, only the NAD-dependent dehydrogenase remains active fol-

lowing the above DEAE-cellulose fractionation, and thus, contaminating NAD(P)-dependent dehydrogenase activity was removed. The NAD-dependent enzyme elutes in the 0.20-0.30 molar sodium chloride range. The fractions containing the most activity were pooled (44 ml total).

Calcium Phosphate Gel- The procedures used were the same as described for the calcium phosphate purification of the NAD(P)-dependent dehydrogenase. The NAD-dependent dehydrogenase exhibited the same elution characteristics as the NAD(P)-dependent dehydrogenase.

2. Properties

pH Optima- NAD-Dependent dehydrogenase activity as a function of pH was maximal at pH 8-8.5 in Tris-HCl buffer and at pH 9-10 in glycine buffer (Figure 9).

Substrate Specificity- A large number of carbohydrates were examined as possible substrates for the dehydrogenase. The following carbohydrates were oxidized by the enzyme: D-glucose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, D-galactose, 2-deoxy-D-galactose, D-fucose, 3,6-dideoxy-D-galactose, D-altrose, D-allose, and D-mannose (Table XV). The carbohydrates which did not serve as substrates also did not reduce the rate of oxidation of 33.3 mM D-glucose when added at equimolar concentrations.

The Lineweaver-Burk plots for eight of the ten

Figure 9. pH Optima of the NAD-dependent dehydrogenase. The standard assay was employed except that the pH and the buffer composition were varied with the dehydrogenase concentration constant. The calcium phosphate fraction with the highest specific activity was used. Each buffer was 0.10 molar, and the pH measurements were determined on duplicate reaction mixtures. The pH did not change during the 10 minute reaction period.

Figure 9

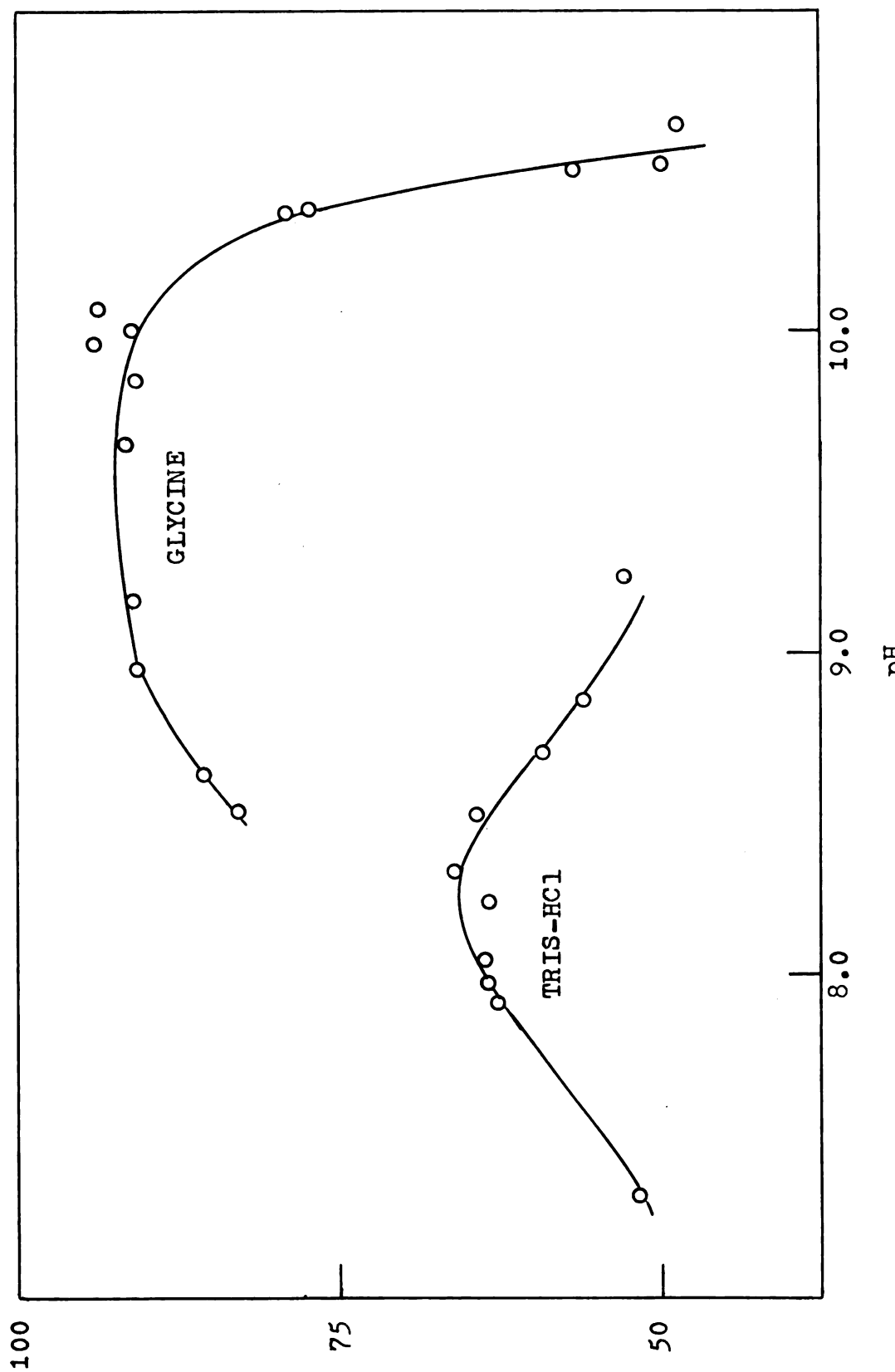


Table XV. A tabulation of the K_m and V_{max} values for the NAD-dependent dehydrogenase. The data were derived from the data depicted in Figures 10-17.

Carbohydrate	Relative V_{max} %	K_m (mM)
D-Glucose	188	0.86
D-Galactose	142	1.6
D-Mannose	124	4.5
2-Deoxy-D-glucose	107	1.6
D-Fucose	100	5.8
2-Deoxy-D-galactose	94	6.3
D-Altrose	79	2.4
D-Allose	47	13.0
6-Deoxy-D-glucose	61	*
3,6-Dideoxy-D-galactose	23	*

The carbohydrates which were not active as substrates (i.e. <1% the activity on D-fucose) are: galactose-6-P, glucose-6-P, fructose-6-P, D-xylose, lactose, melezitose, maltose, cellobiose, DL-glyceraldehyde, D-ribose, D-lyxose, 2-deoxy-D-ribose, L-rhamnose, sucrose, D-arabinose, L-xylose, N-acetyl-D-glucosamine, D-glucosamine, D-mannitol, D-sorbitol, myo-inositol, L-sorbose, 6-iodo-6-deoxy-D-galactose, raffinose, turanose, melibiose, trehalose, L-fucose, L-arabitol, α-methyl-glucoside, inulin (1.25 μmoles), L-galactose, L-glucose, L-mannose, L-fructose, D-fructose, xylitol, D-arabitol, adonitol, glucuronic acid, galacturonic acid, D-xylulose, galactaric acid, glucaric acid, 6-deoxy-D-allose, 2-acetamido-D-allose, and 2-acetamido-D-altrose.

*Not determined.

carbohydrates active as substrates are presented in Figures 10 through 17. A tabulation of the K_m and V_{max} values derived from these kinetic plots is also presented in Table XV. All rates with mixed substrates were non-additive thereby suggesting that the activity in the DEAE-cellulose fraction represented one enzyme (Table XVI).

Nucleotide Specificity- From the Lineweaver-Burk plot presented in Figure 18, the K_m value for NAD^+ was found to be 7.7×10^{-5} M. $NADP^+$ was absolutely ineffective as a cofactor for the dehydrogenase reaction using concentrations up to 20 mM. In addition, $NADP^+$ (4 mM) was not a competitive inhibitor of the standard assay.

Reversal of the Dehydrogenation- The fact that the isolation of a lactone resulting from D-fucose oxidation by the NAD-dependent dehydrogenase was consistently unsuccessful (see Product Identification, this section) suggested that the product was the unstable δ-lactone rather than the stable γ-lactone. The ring size of the lactone resulting from the oxidation of D-galactose, D-glucose, and D-fucose by the NAD-dependent dehydrogenase was determined by observing the reversal of the dehydrogenation reaction using δ- and γ-lactones (38-45). The reduction of D-fucono-γ-lactone, D-galactono-γ-lactone, or D-glucono-δ-lactone in the presence of the purified enzyme and NADH was tested, and the results are

Figure 10. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-fucose concentration. The standard assay was employed using the calcium phosphate fraction with the highest specific activity. The carbohydrate concentration was varied, as indicated, with the dehydrogenase concentration constant.

Figure 10

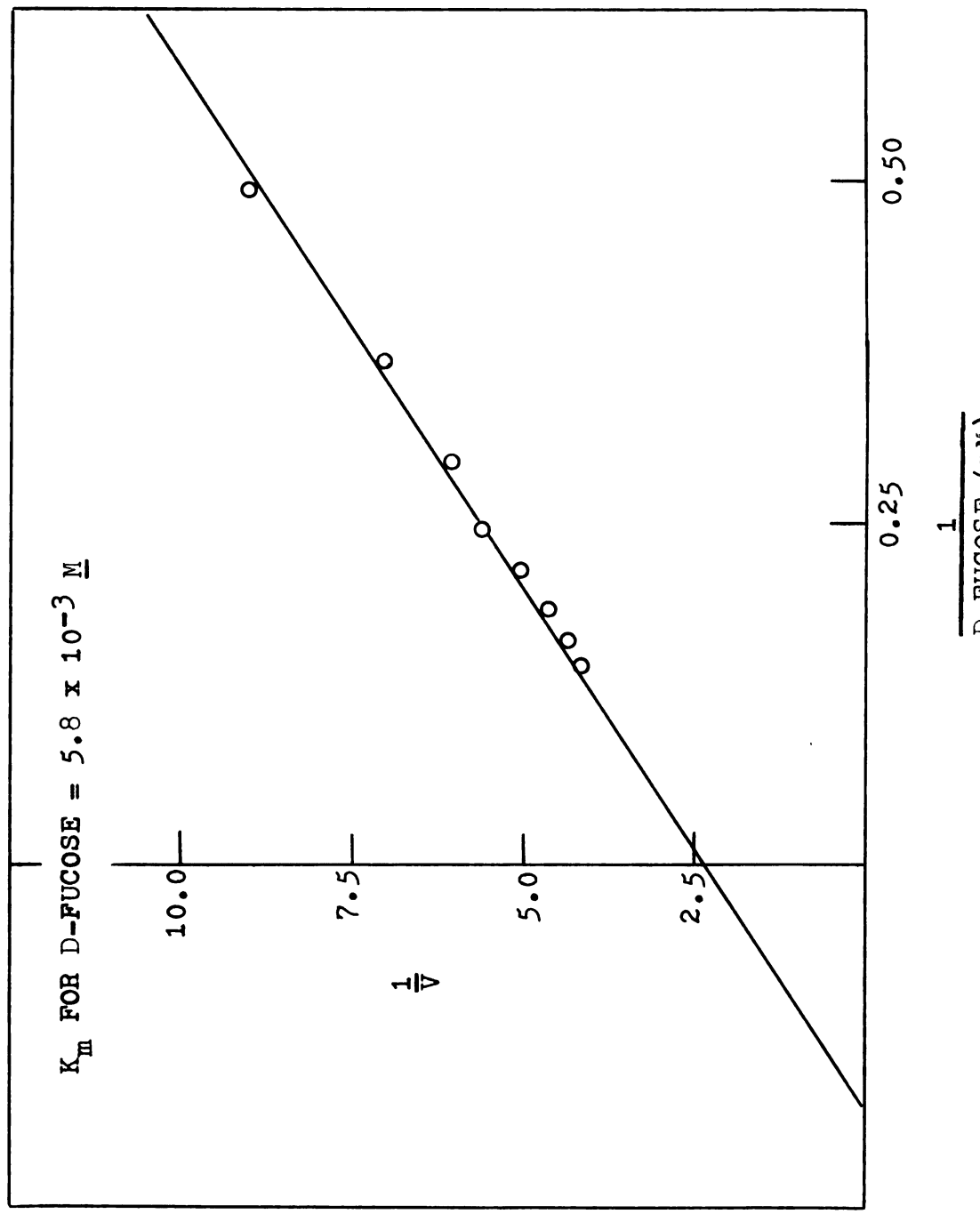

K_m FOR D-FUCOSE = 5.8 x 10^{-3} M
10.0
7.5
5.0
2.5
$\frac{1}{V}$
0.25
0.50
$\frac{1}{\text{D-FUCOSE (mM)}}$

Figure 11. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-glucose concentration. Other conditions were the same as described in Figure 10.

Figure 11

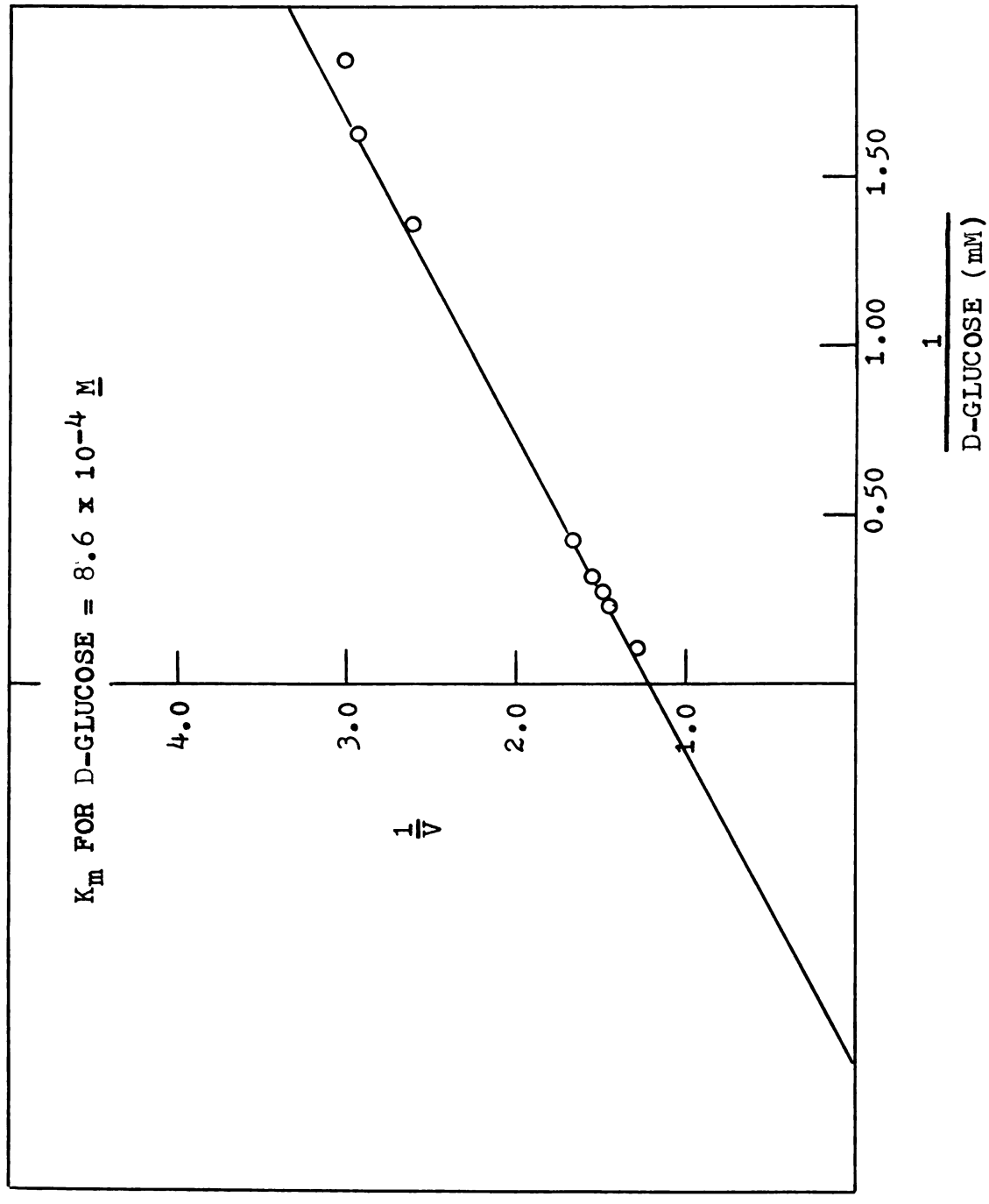

K_m FOR D-GLUCOSE = 8.6 x 10^{-4} M
$\frac{1}{V}$
4.0
3.0
2.0
1.0
0.50
1.00
1.50
$\frac{1}{\text{D-GLUCOSE (mM)}}$

Figure 12. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-galactose concentration. Other conditions were the same as described in Figure 10.

Figure 12

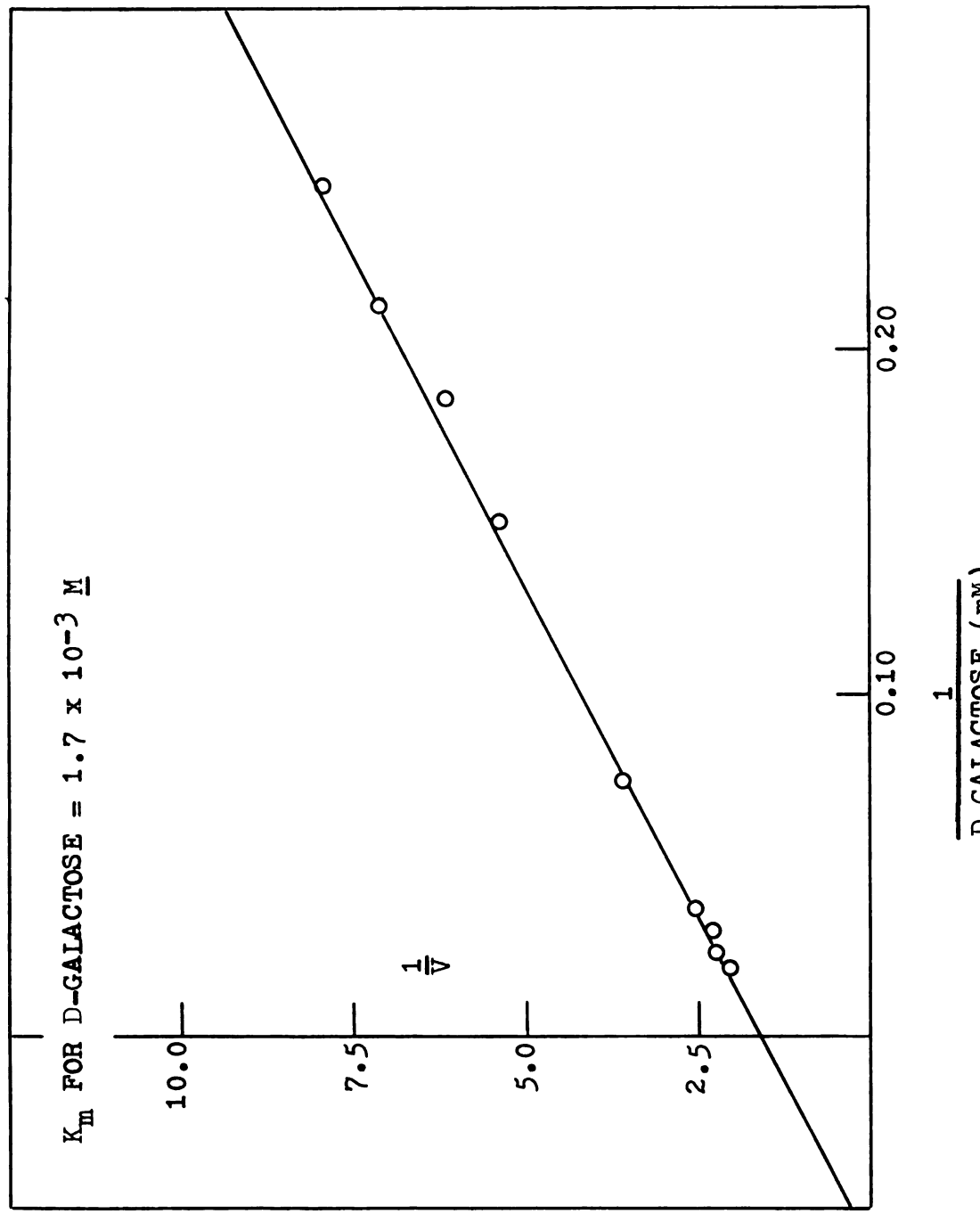

K_m FOR D-GALACTOSE = 1.7 x 10^{-3} M
10.0
7.5
5.0
2.5
$\frac{1}{V}$
0.10
0.20
$\frac{1}{\text{D-GALACTOSE (mM)}}$

Figure 13. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-mannose concentration. Other conditions were the same as described in Figure 10.

Figure 13

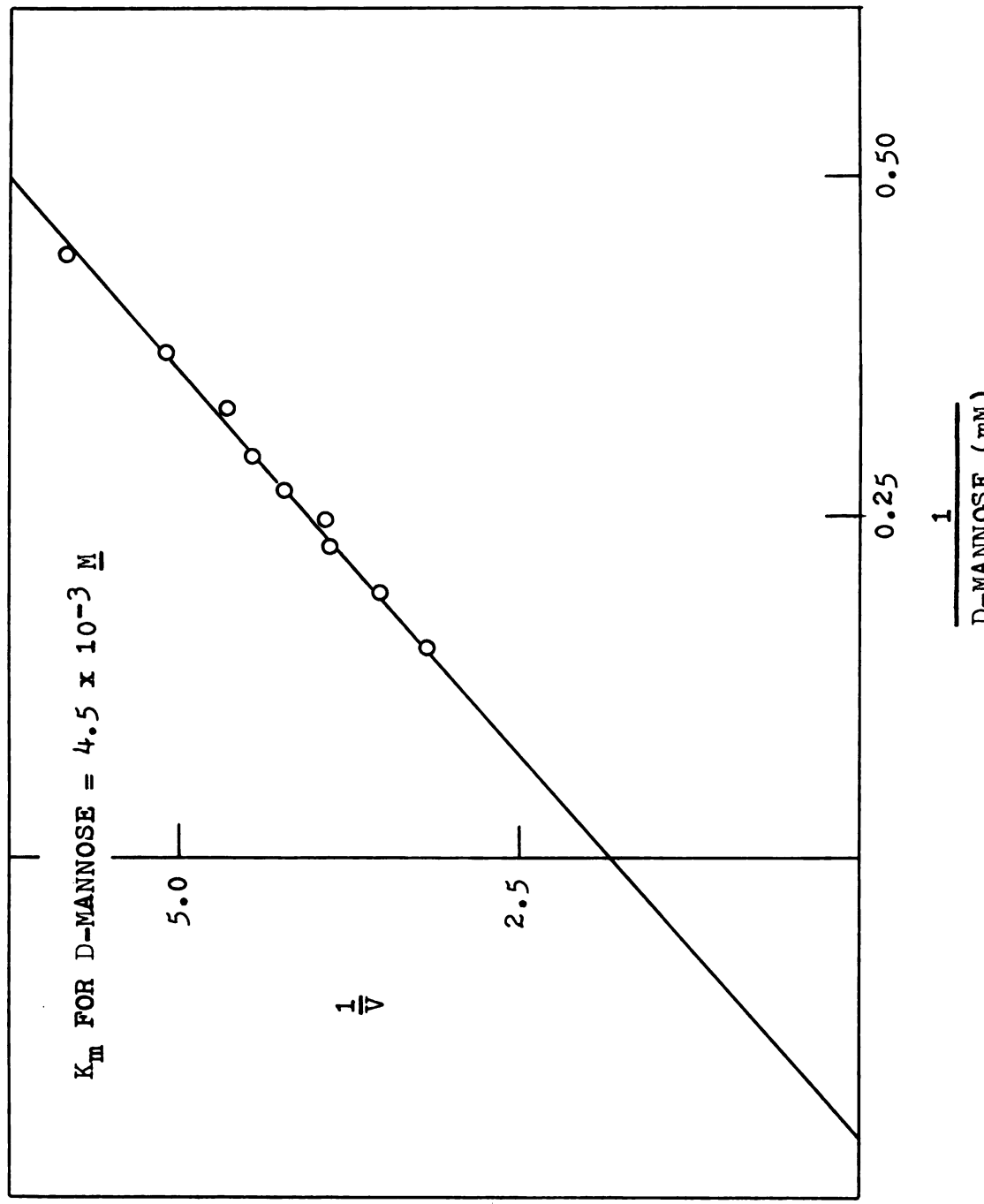

K_m FOR D-MANNOSE = 4.5 x 10^{-3} M
5.0
2.5
$\frac{1}{V}$
0.25
0.50
$\frac{1}{\text{D-MANNOSE (mM)}}$

Figure 14. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to 2-deoxy-D-glucose concentration. Other conditions were the same as described in Figure 10.

Figure 14

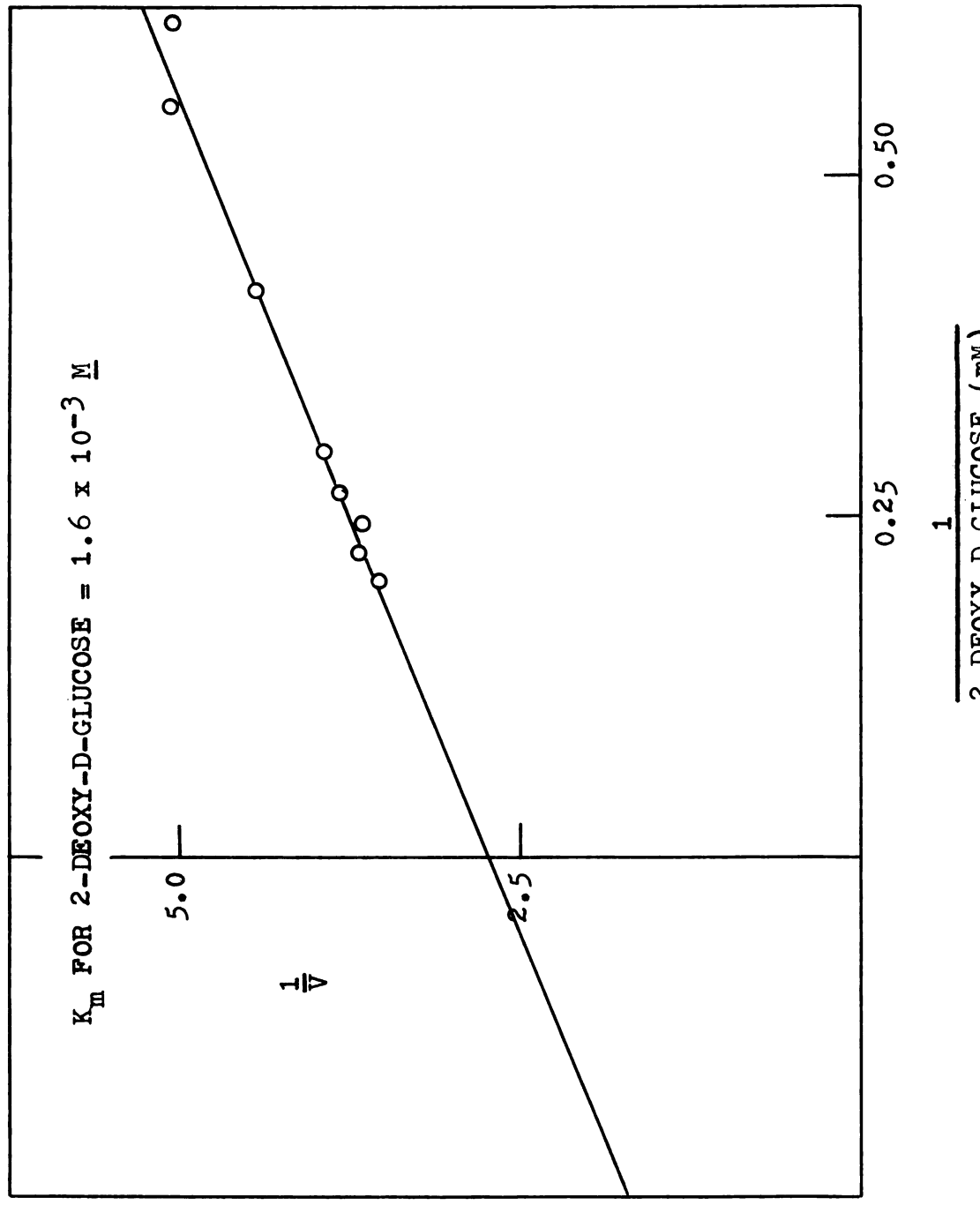

K_m FOR 2-DEOXY-D-GLUCOSE = 1.6 x 10^{-3} M
5.0
2.5
$\frac{1}{V}$
0.25
0.50
$\frac{1}{\text{2-DEOXY-D-GLUCOSE (mM)}}$

Figure 15. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to 2-deoxy-D-galactose concentration. Other conditions were the same as described in Figure 10.

Figure 15

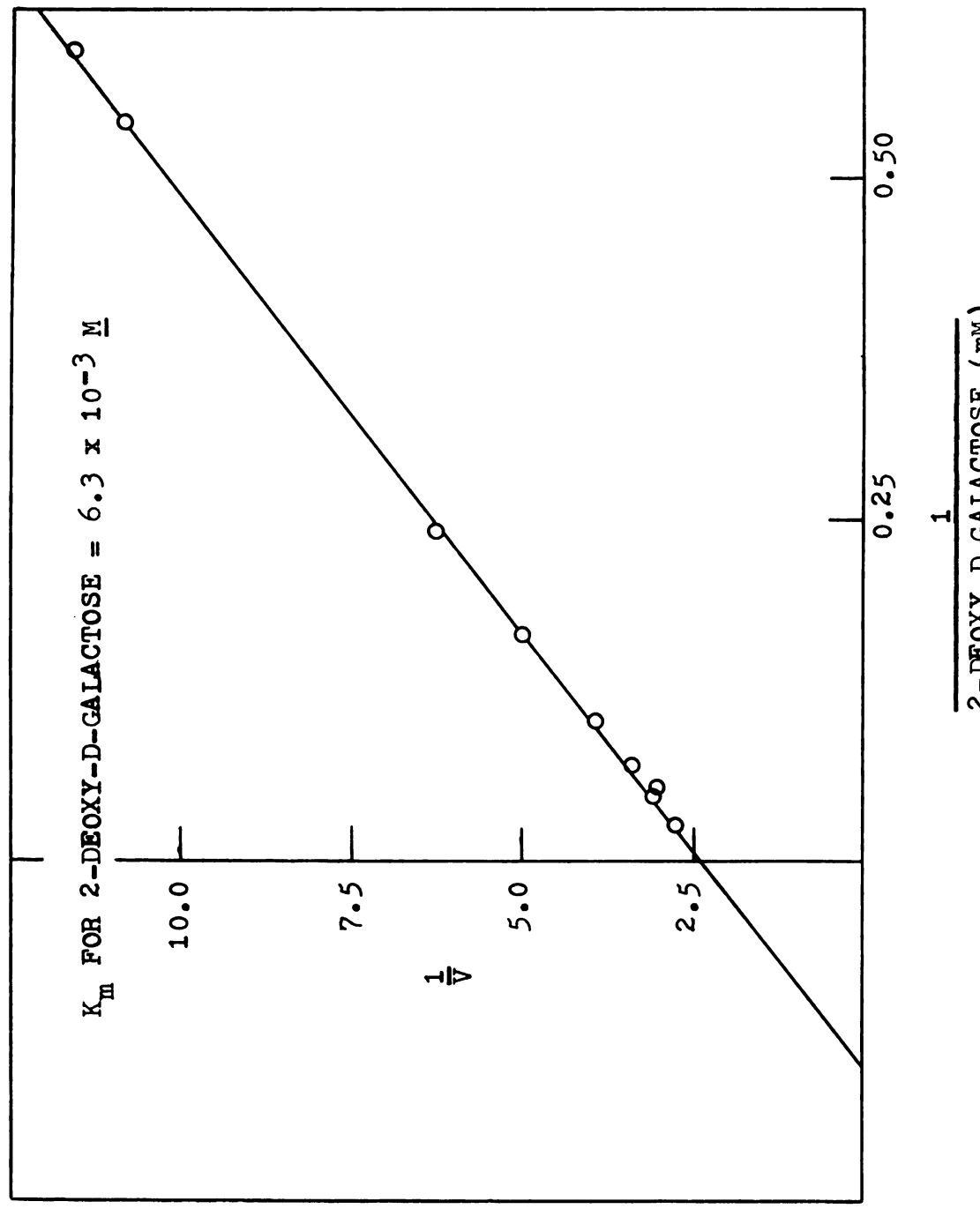

K_m FOR 2-DEOXY-D-GALACTOSE = 6.3 x 10^{-3} M
10.0
7.5
5.0
2.5
$\frac{1}{V}$
0.25
0.50
$\frac{1}{\text{2-DEOXY-D-GALACTOSE (mM)}}$

Figure 16. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-altrose concentration. Other conditions were the same as described in Figure 10.

Figure 16

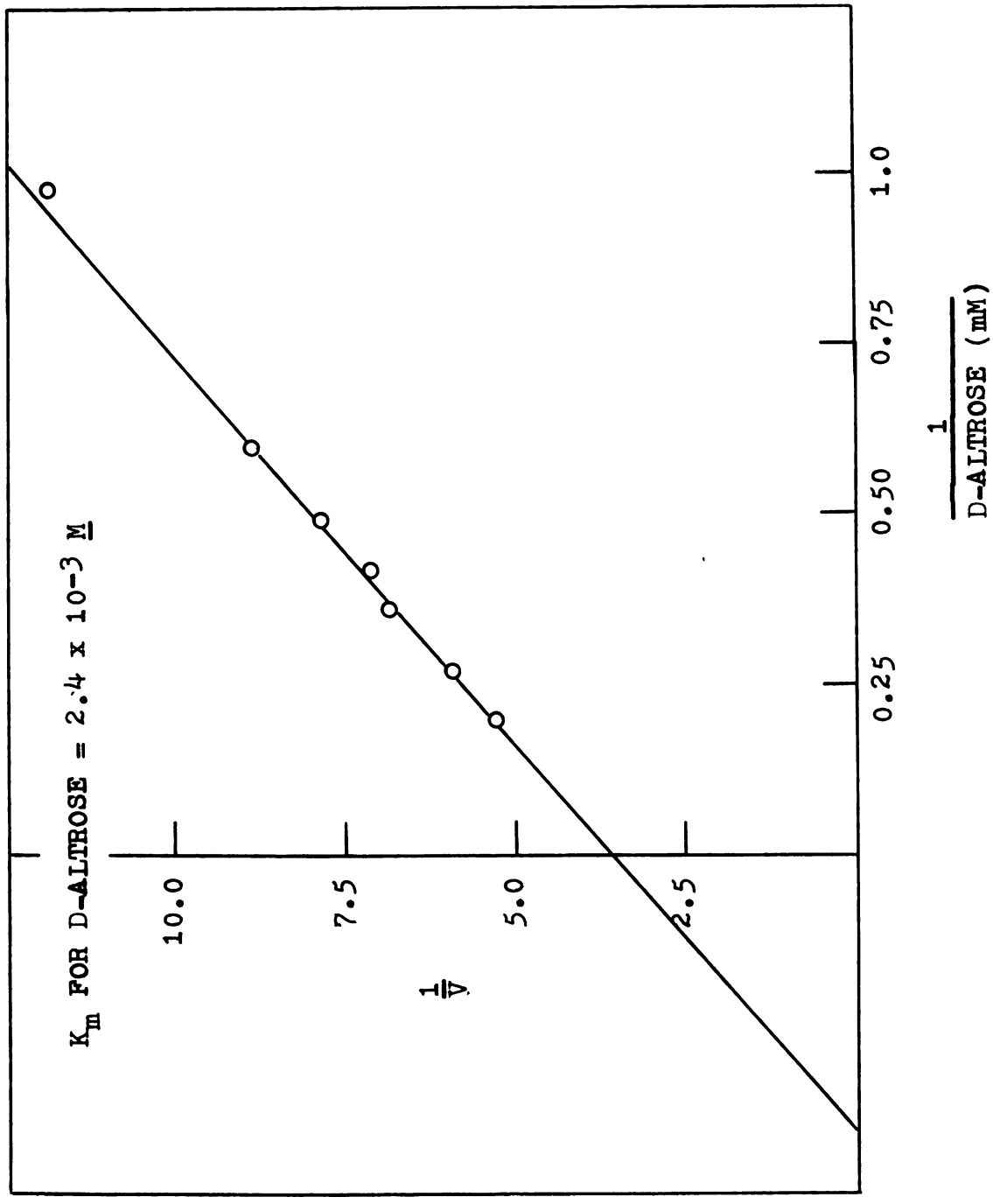

K_m FOR D-ALTROSE = 2.4 x 10-3 M
10.0
7.5
5.0
2.5
1/V
0.25
0.50
0.75
1.0
1
D-ALTROSE (mM)

Figure 17. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-allose concentration. Other conditions were the same as described in Figure 10.

Figure 17

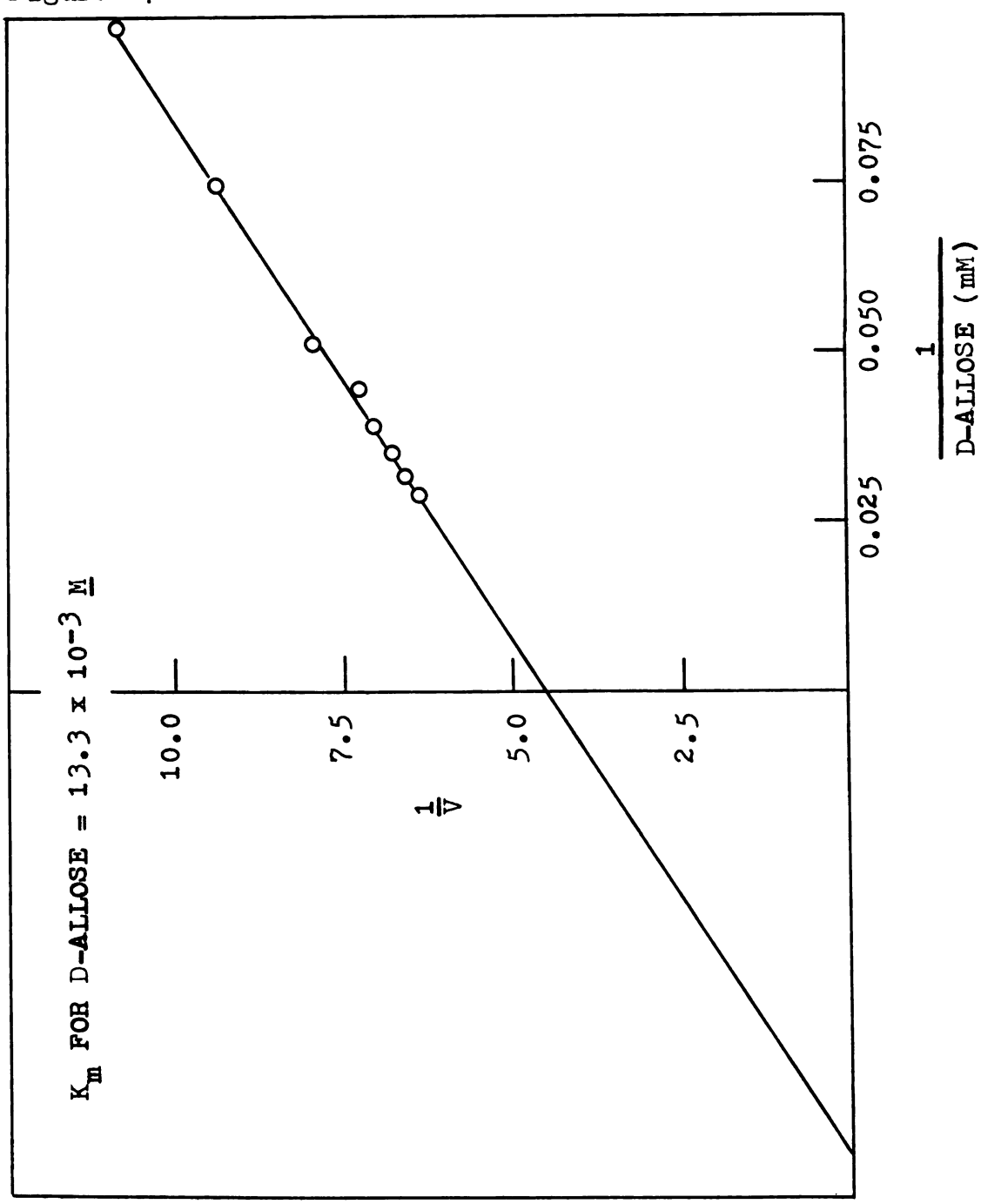
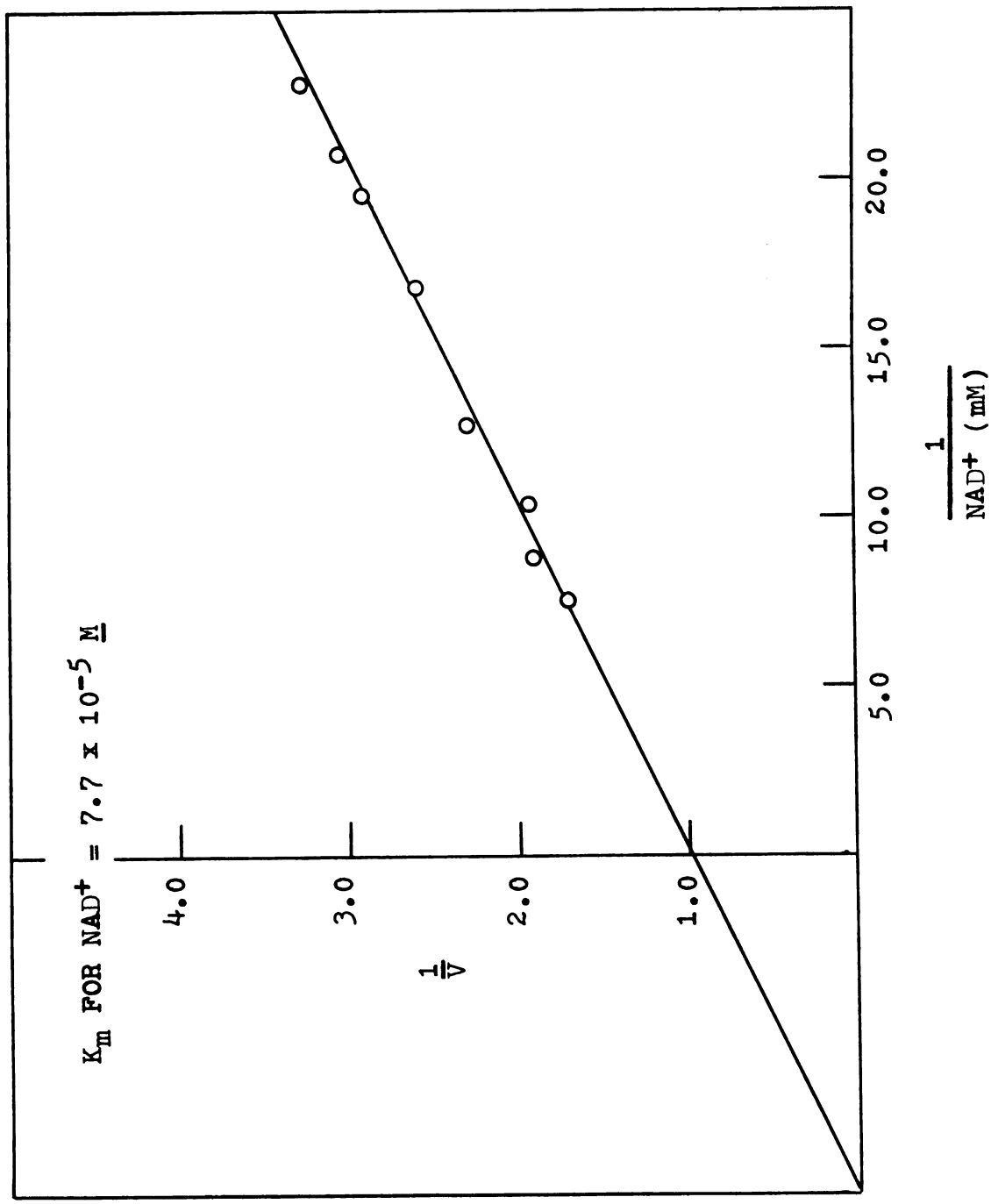

K_m FOR D-ALLOSE = 13.3 x 10^{-3} M
10.0
7.5
5.0
2.5
$\frac{1}{V}$
0.025
0.050
0.075
$\frac{1}{\text{D-ALLOSE (mM)}}$

Table XVI. Effect of mixed substrates on NAD-dependent dehydrogenase activity. The standard assay was employed with the exception that the carbohydrate concentration was 33.3 mM. When both substrates were mixed, the concentration of each substrate was the same as that used above. A DEAE-cellulose fraction which contained no NAD(P)-dependent dehydrogenase was used.

Substrate	Specific Activity (μmoles/hour/mg protein)
D-Glucose	410
D-Galactose	306
D-Mannose	271
D-Fucose	216
D-Mannose + D-Fucose	243
D-Mannose + D-Glucose	342
D-Mannose + D-Galactose	287
D-Glucose + D-Galactose	350
D-Glucose + D-Fucose	303
D-Fucose + D-Galactose	259

Figure 18. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to NAD^+ concentration. The routine assay was employed except that the NAD^+ concentration was varied, as indicated, with the dehydrogenase concentration constant. D-Glucose concentration was 33.3 mM. The calcium phosphate fraction with the highest specific activity was utilized.

Figure 18


K_m FOR NAD^+ = 7.7 x 10^{-5} M
$\frac{1}{V}$
4.0
3.0
2.0
1.0
5.0
10.0
15.0
20.0
$\frac{1}{NAD^+}$ (mM)

presented in Table XVII. It may be seen that the reaction is reversible only with the δ-lactone which suggests, in turn, that the enzyme is operative on the pyranose form of the carbohydrate. The data also indicate that the γ-lactones are not competitive inhibitors of the reverse reaction under the conditions described in Table XVII. The δ-lactones of D-fuconic and D-galactonic acids could not be tested because of their instability (46-48).

From the Lineweaver-Burk plot presented in Figure 19, the K_m value for D-glucono-δ-lactone was found to be about 10 mM. The half-life for D-glucono-δ-lactone under the conditions of the assay (pH 6.5) is approximately 10 minutes (51, 52), and for this reason the K_m value must be considered to be an approximation. Using conditions under which the lactone was saturating (66.6 mM) and varying the NADH concentration, a K_m value for NADH was obtained. From the Lineweaver-Burk plot presented in Figure 20, the K_m value was found to be 39 μM.

Glucose has been identified as the product of the reduction of D-glucono-δ-lactone by trapping with hexokinase and measuring the resulting glucose-6-phosphate with glucose-6-phosphate dehydrogenase. The results are presented in Figure 21.

Anomer Preference- Experiments were carried out to determine whether both pyranose forms were equally effective as substrates. The rate of oxidation of an

Table XVII. Reversibility of the NAD-dependent dehydrogenase. The assay consisted of 10 μmoles of the respective lactone, 0.26 μmole NADH, 30 μmoles sodium phosphate buffer (pH 6.5) and a limiting amount of the dehydrogenase in a volume of 0.15 ml. A DEAE-cellulose fraction was used. The pH of the reaction mixture did not change during the 15 minute reaction period. The lactones solutions were prepared before each assay in 0.10 molar phosphate buffer (pH 6.5).

Lactone	Specific Activity (μmoles/hr/mg)
D-Glucono-δ-lactone	18.6
D-Galactono-γ-lactone	< 0.02
D-Fucono-γ-lactone	< 0.02
D-Glucono-δ-lactone + D-galactono-γ-lactone	18.4
D-Glucono-δ-lactone + D-fucono-γ-lactone	18.4

Figure 19. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to D-glucono-δ-lactone concentration. The assay was identical with that utilized in Table XVII with the exception that the lactone concentration was varied. Fresh lactone solutions were prepared before each assay in 0.10 molar sodium phosphate (pH 6.5). The pH of the reaction mixture did not vary in the course of the reaction.

Figure 19

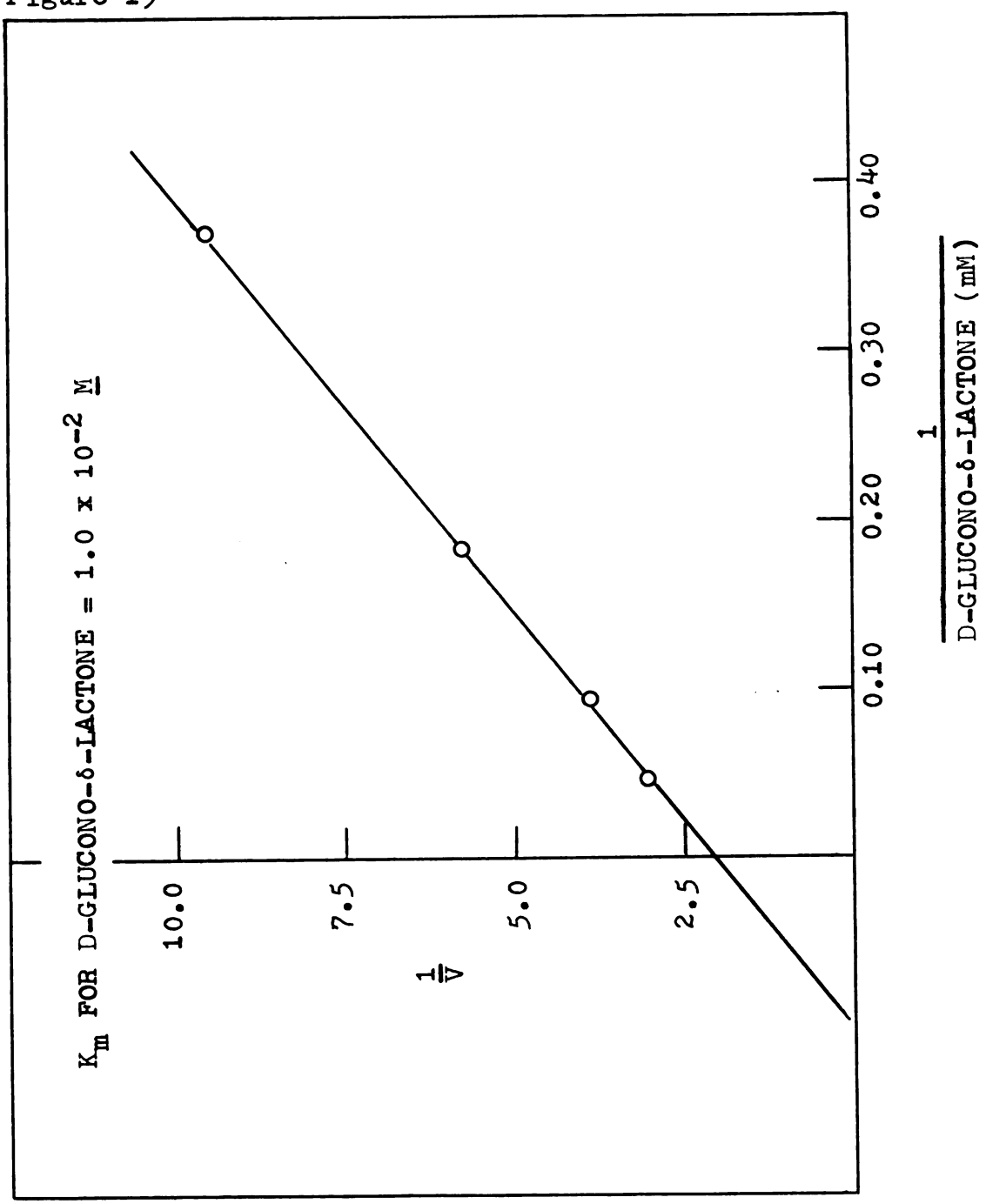

K_m FOR D-GLUCONO-δ-LACTONE = 1.0 x 10^{-2} M
$\frac{1}{V}$
10.0
7.5
5.0
2.5
0.10
0.20
0.30
0.40
$\frac{1}{\text{D-GLUCONO-δ-LACTONE (mM)}}$

Figure 20. Lineweaver-Burk plot relating NAD-dependent dehydrogenase activity to NADH concentration. The assay was identical to that presented in Table XVII with the exception that the NADH concentration was varied. The pH of the reaction mixture did not vary in the course of the reaction.

Figure 20

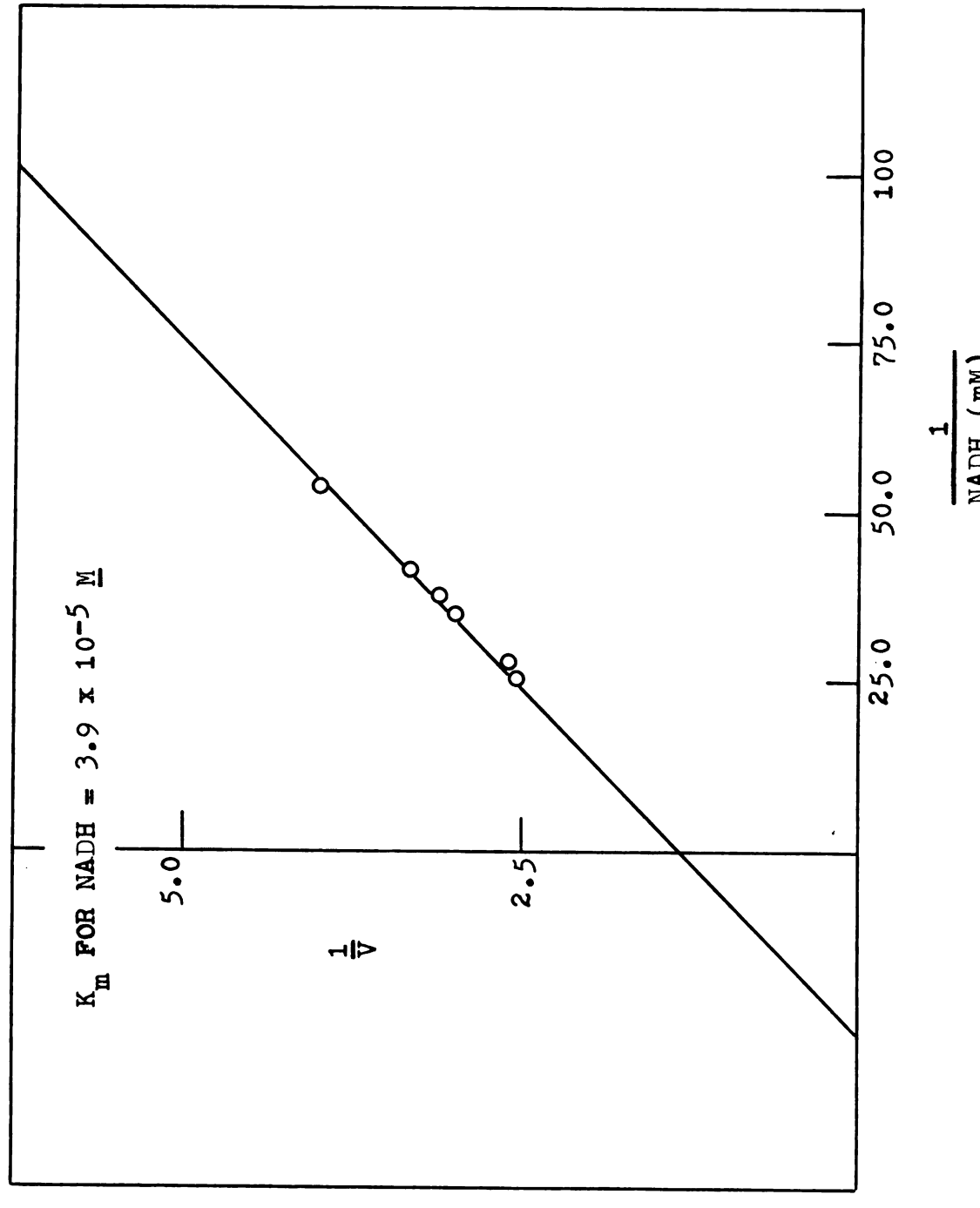

K_m FOR NADH = 3.9 x 10^{-5} M
5.0
2.5
$\frac{1}{V}$
25.0
50.0
75.0
100
$\frac{1}{\text{NADH (mM)}}$

Figure 21. Identification of D-glucose as the product of D-glucono-δ-lactone reduction by the NAD-dependent dehydrogenase. The complete assay consisted of 1.25 μmoles D-allose, 0.75 μmole ATP, 1.5 μmole $MgCl_2$, 20 μmoles sodium cacodylate buffer (pH 6.5), 0.001 μmole NAD^+, 0.3 μmole $NADP^+$, 10 μmoles D-glucono-δ-lactone (freshly prepared in 0.10 molar sodium cacodylate buffer, pH 6.5), excess hexokinase and glucose-6-phosphate dehydrogenase, and a limiting amount of the NAD-dependent dehydrogenase. A calcium phosphate fraction was used. D-Allose and NAD^+ were used as a NADH regenerating system.

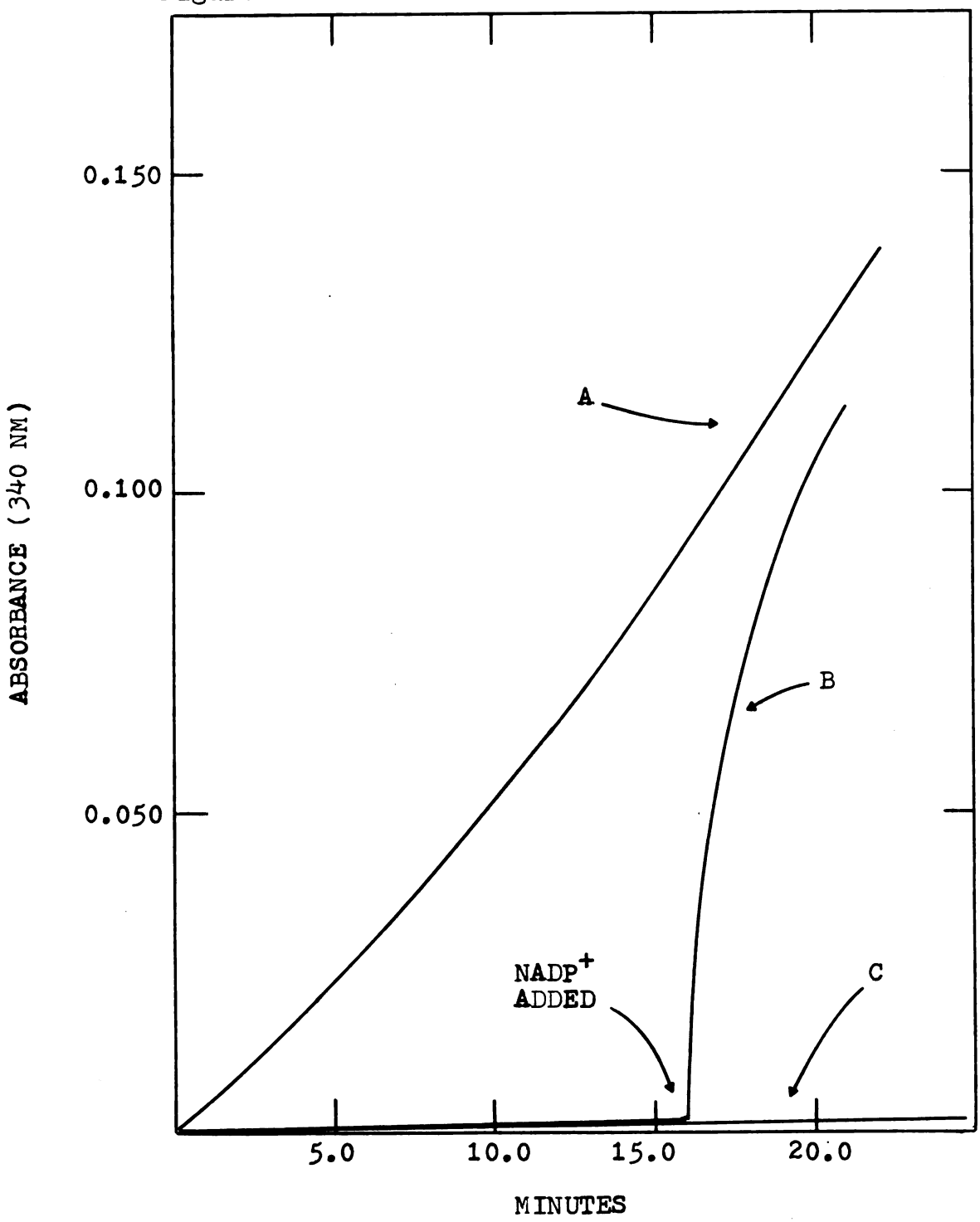
Curve A: Complete

Curve B: Complete minus $NADP^+$

Curve C: Complete minus any one of the following components:

NAD-dependent dehydrogenase
hexokinase
glucose-6-phosphate dehydrogenase
NAD^+
ATP, $MgCl_2$
D-allose
D-glucono-δ-lactone

Figure 21


0.150
0.100
0.050
ABSORBANCE (340 NM)
A
B
C
NADP+
ADDED
5.0
10.0
15.0
20.0
MINUTES

equilibrium solution of α,β-D-glucose was compared to the rate of oxidation of freshly prepared α-D-glucose at 15.6°C and pH 7.5. Under the above conditions, in which the mutarotational step is the rate limiting step in the oxidation (38, 53), α,β-D-glucose was preferentially oxidized at a rate 5-fold that of α-D-glucose (Figure 22). It may be inferred that β-D-glucose is probably the true substrate for the enzyme.

Stability- The half-life of the NAD-dependent dehydrogenase at 55°C was found to be about 40 seconds (Figure 23). The NAD-dependent enzyme is not heat stable relative to the NAD(P)-dependent enzyme as shown also in Figure 23. Inactivation profiles of the enzymes with their respective substrates were superimposable and were linear, suggesting that the activities were due to single enzymes.

The enzyme, in Sephadex G-200 fractions, is completely stable to freezing at -20°C in 0.01 molar sodium phosphate buffer (pH 7.0) for six months. Lyophilization of pooled Sephadex G-200 fractions containing the enzyme in 0.02 molar sodium phosphate buffer (pH 7.0) resulted in a 50% loss of activity.

The effects of various metal ions, thiols, and thiol group inhibitors are shown in Table XVIII. The enzyme is not affected by 1 mM 2-thioethanol or 1 mM reduced glutathione. Similarly, 0.5 mM iodoacetate of

Figure 22. Comparison of the rate of oxidation of α- and α,β-D-glucose by the NAD-dependent dehydrogenase. The standard assay was employed with the exceptions that 0.026 μmoles D-glucose and 15 μmoles Tris-HCl buffer (pH 7.5) were used. The cuvettes containing the reaction mixture minus glucose were equilibrated at 15.6°C before adding the equilibrated α,β-D-glucose or the freshly prepared α-D-glucose solution. The reaction was maintained at 15.6°C. Experiments were identical in all respects with the exception of the state of mutarotational equilibrium of the D-glucose solutions.

Figure 22

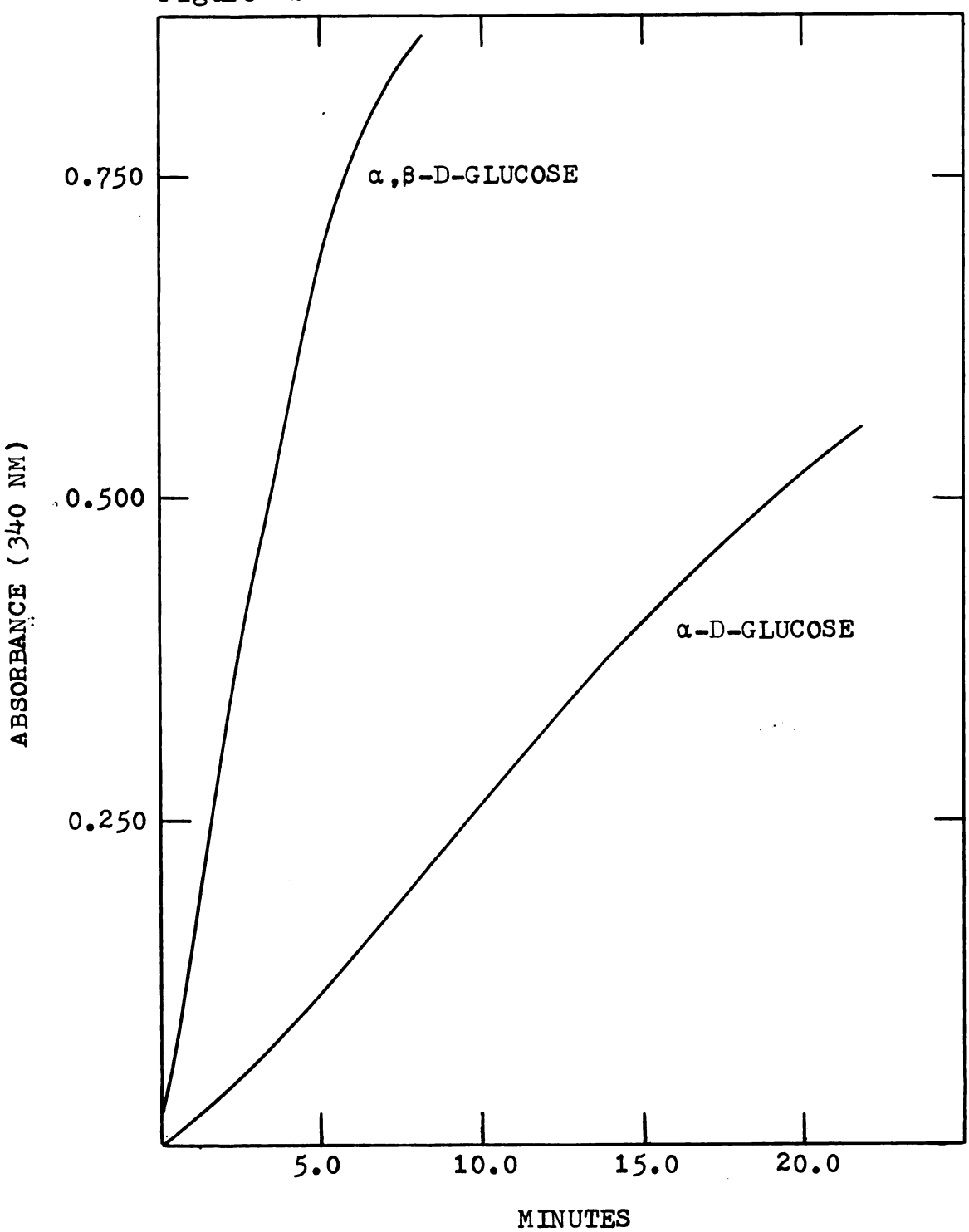

0.750
0.500
0.250
ABSORBANCE (340 NM)
α,β-D-GLUCOSE
α-D-GLUCOSE
5.0
10.0
15.0
20.0
MINUTES

Figure 23. Line A: Thermal denaturation of the NAD(P)-dependent dehydrogenase at 55°C. The protein solution (1.08 mg per ml) was heated in 0.01 molar sodium phosphate buffer (pH 7.0). Aliquots were withdrawn at the indicated times and assayed with the standard assay. The thermal denaturation profiles were superimposable using D-fucose, L-arabinose, and D-galactose with either NAD or NADP as the electron acceptor. A calcium phosphate fraction was used. Line B: Thermal denaturation of the NAD-dependent dehydrogenase at 55°C. A DEAE-cellulose fraction was used. The protein solution (1.8 mg per ml) was heated in 0.01 molar sodium phosphate (pH 7.0). Aliquots were withdrawn at the indicated times and assayed with the standard assay. The heat inactivation profiles using D-mannose, D-galactose, and D-fucose, in addition to D-glucose, were superimposable.

Figure 23

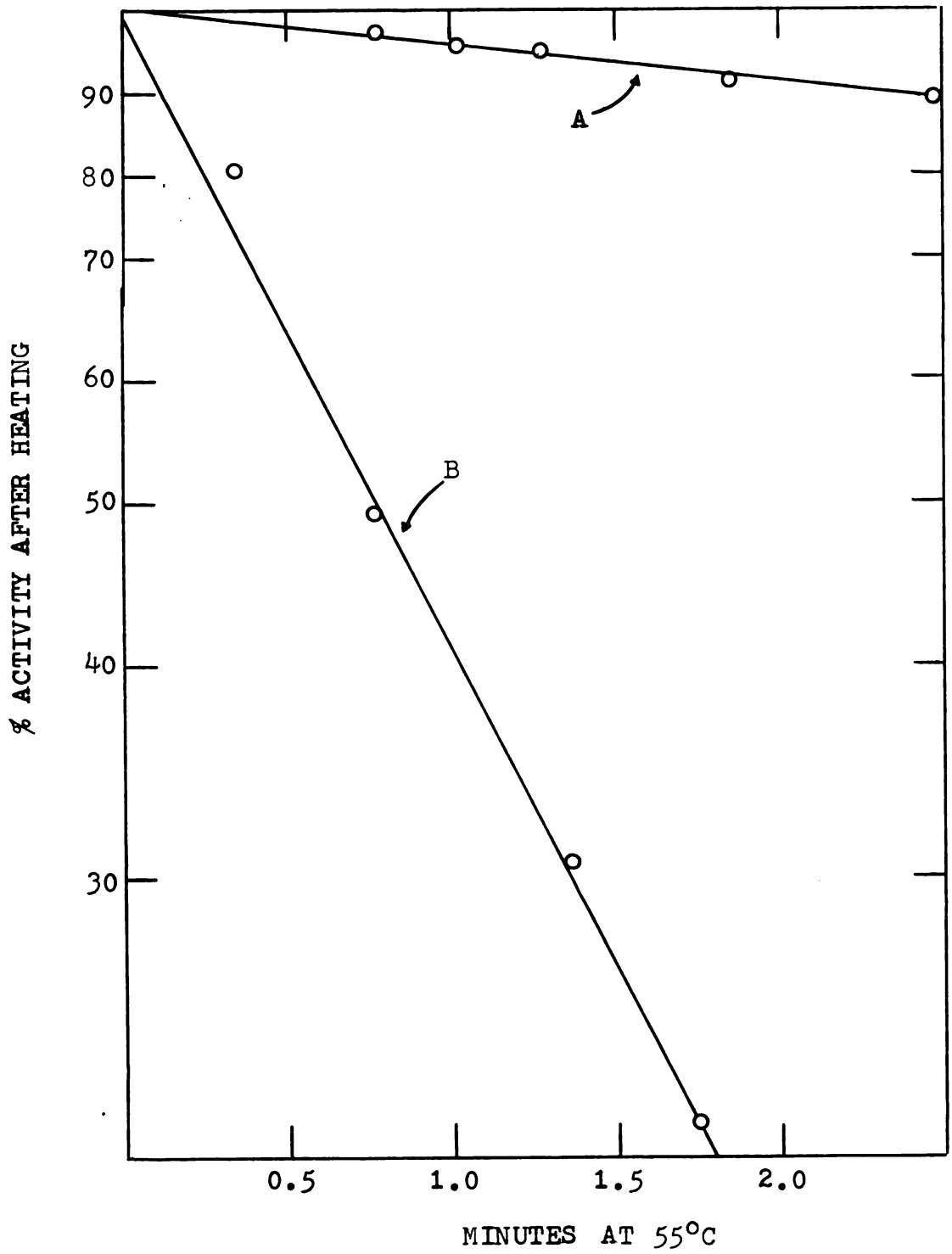

90
80
70
60
50
40
30
A
B
0.5
1.0
1.5
2.0
% ACTIVITY AFTER HEATING
MINUTES AT 55°C

Table XVIII. Effect of various reagents on NAD-dependent dehydrogenase activity. The standard assay was utilized in which the enzyme was added to the cuvette containing the reagent. The calcium phosphate gel fraction with the highest specific activity was used. The pH of all reagents was adjusted to 7.0.

Reagent	Concentration	% of Control Activity
p-Chloromercuribenzoate	0.5 mM	100
Iodoacetic acid	0.5 mM	100
2-Thioethanol	1.0 mM	100
Reduced glutathione	1.0 mM	100
EDTA	6.6 mM	100
$MgCl_2$	6.6 mM	100
$MnCl_2$	6.6 mM	97
NH_4Cl, $(NH_4)_2SO_4$	6.6 mM	86
$CoCl_2$	6.6 mM	51
$NiCl_2$	6.6 mM	35
$CuSO_4$	6.6 mM	16
$FeSO_4$	6.6 mM	16
$CaCl_2$	6.6 mM	12
$ZnCl_2$	6.6 mM	10

0.5 mM p-chloromercuribenzoate were not inhibitory to enzyme activity. There was no observed inhibition by 6.6 mM EDTA or activation by metal ions at 6.6 mM.

Induction- The inducibility of the NAD-dependent dehydrogenase was tested by growth on various substrates. The data (Table XIX) indicate that D-glucose and D-fucose induced the dehydrogenase to a level 48-146 fold over the non-induced level as present in L-arabinose-grown cells and suggest that the enzyme is instrumental in the metabolism of D-fucose and D-glucose..

3. Product Identification

The isolation of a lactone resulting from D-fucose oxidation by the NAD-dependent dehydrogenase, using the same techniques employed for the isolation of D-fucono-γ-lactone resulting from D-fucose oxidation by the NAD(P)-dependent dehydrogenase, was consistently unsuccessful. This was concluded as being due to the instability of the δ-lactone as compared to the γ-lactone (46-48). In support of this conclusion the reversibility studies indicated that the product of the NAD-dependent dehydrogenase was a δ-lactone rather than a γ-lactone. Thus, due to the instability of D-fucono-δ-lactone, the apparent product of D-fucose oxidation by the NAD-dependent dehydrogenase is D-fuconate which was identified by co-chromatography and derivatization.

Table XIX. Induction of the NAD-dependent dehydrogenase by growth on various substrates. The cell extracts were prepared by sonic disruption in 0.01 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4) to eliminate NADH oxidase activity. The standard assay was employed. Protein was estimated by the biuret assay.

Growth Substrates	Specific Activity (μmoles/hr/mg)
D-Fucose	5.55
L-Arabinose	0.115
D-Glucose	16.3
D-Galactose	0.690
Nutrient Broth	0.204
6-Iodo-6-deoxy-D-galactose	0.330

Product Isolation- The reaction mixture consisted of 100 μmoles D-fucose, 125 μmoles pyruvic acid (pH 6.5), 400 μmoles Tris-maleate buffer (pH 6.5), 50 μg lactic acid dehydrogenase, 1 μmole NAD^+, and 2 ml of a DEAE-cellulose fraction containing the NAD-dependent dehydrogenase (1.5 mg protein) in a total volume of 2.5 ml. The NAD-dependent dehydrogenase fraction contained no NAD(P)-dependent dehydrogenase, D-fuconate dehydratase, γ-lactonase, or 2-keto-3-deoxy-D-fuconate aldolase. No lactone was detectable after one hour although the D-fucose concentration, as measured by reducing sugar, was zero and the pyruvate concentration had become constant. The reaction mixture was then deionized by passage through a Dowex-50W-H^+ column (0.5 x 2 cm) and chromatographed on paper in solvent systems 1 and 5. The product co-chromatographed with authentic D-fuconate; no D-fucono-γ-lactone was detected. A portion of the Dowex-50W-H^+ eluate was lactonized and chromatographed in solvent systems 1 and 5. The lactonization product was found to co-chromatograph with authentic D-fucono-γ-lactone in both systems. It was concluded that D-fuconate was the apparent product of the NAD-dependent dehydrogenation of D-fucose and that the δ-lactone was probably the immediate product which spontaneously hydrolyzed to form D-fuconate.

D. CHARACTERIZATION OF A D-FUCONO-γ-LACTONASE

1. Preliminary Experiments

D-Fucono-γ-lactone is a stable lactone which hydrolyzes slowly, even at pH 9 (48). Since this γ-lactone is stable, since D-fucono-γ-lactone was identified as the product of D-fucose oxidation by one of the dehydrogenases (Section B), and since hydrolysis of D-fucono-γ-lactone was noted in the initial experiments (Table V), the presence of a lactonase was postulated. The presence of a lactonase was later confirmed by assaying the enzymatic enhancement of the rate of proton release in the hydrolysis of various aldonolactones. Evidence is presented in Figure 24 which shows the participation of a lactonase in the hydrolysis of D-fucono-γ-lactone.

2. Purification

Cell extracts of D-galactose-grown cells were prepared as described in Experimental Procedures. Except where indicated otherwise, the fractionation procedures were carried out at 0-4°C. A summary of the purification is given in Table XX.

Protamine Sulfate Fractionation- The protein concentration was adjusted to 17.5 mg per ml by dilution with 0.10 molar sodium phosphate buffer (pH 7.0).

Figure 24. The participation of a lactonase in the hydrolysis of D-fucono-γ-lactone determined by automatic titration using a Sargent recording pH stat. The assay consisted of cell extract (5 mg of protein) and 40 μmoles D-fucono-γ-lactone in a volume of 1.0 ml. The rate of proton release was monitored in an unbuffered solution by automatic titration at pH 7.0 with 0.005 M NaOH. The controls consisted of determining the rate of proton release in the separate cell extract and lactone solutions. The cell extracts were from D-fucose-grown cells.

Curve A: Complete

Curve B: Complete minus fucono-γ-lactone

Curve C: Complete minus enzyme

Figure 24

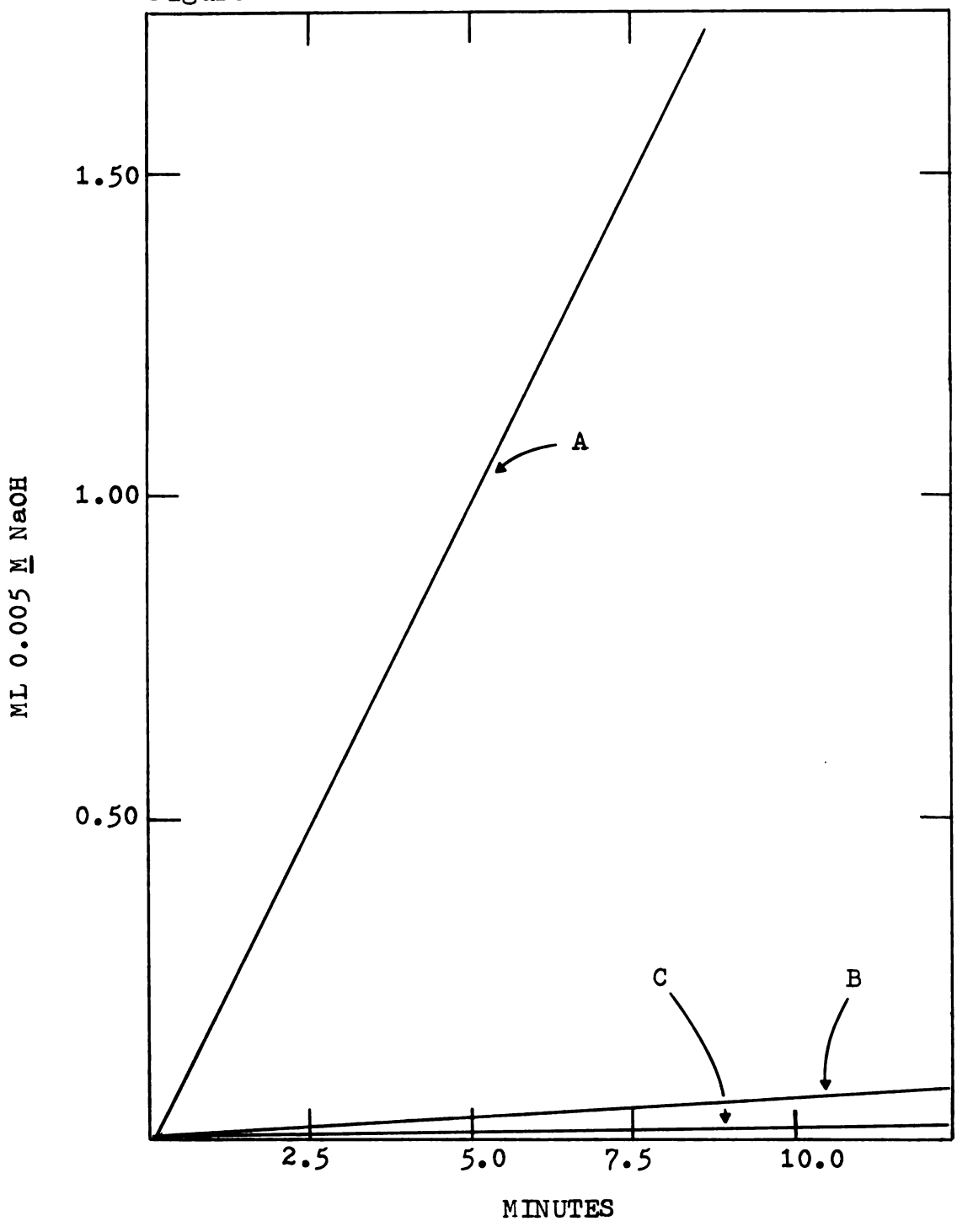


Table XX. Purification of the γ-lactonase.

Fraction	Units	Specific Activity	Fold
Cell extract	178,000	20.9	1
Protamine sulfate	142,000	13.9	0.69
Ammonium sulfate	85,500	80.1	4
Sephadex G-200	17,000	333	16

Ammonium sulfate (13.7 g) was dissolved in 525 ml cell extract to a final concentration of 0.20 molar, and then 105 ml of a 2% protamine sulfate solution in 0.10 molar sodium phosphate buffer (pH 7.0) was added with stirring to a final concentration of 0.33%. After 30 minutes the suspension was centrifuged at 40,000 x g for 10 minutes, and the resulting precipitate was discarded.

Ammonium Sulfate Fractionation- The 40,000 x g supernatant (620 ml) was brought to 40% of saturation by the addition of 122 g of ammonium sulfate and the resulting precipitate was centrifuged down and discarded. The supernatant was then brought to 60% of saturation with 81.3 g of ammonium sulfate and centrifuged, and the resulting precipitate was collected by centrifugation and dissolved in 35 ml 0.05 molar Bicine buffer (pH 7.5). The protein concentration at this stage was approximately 42 mg per ml.

Sephadex G-200 Chromatography- The 40-60% ammonium sulfate fraction was placed on a column (6 x 60 cm) of Sephadex G-200 equilibrated with 0.05 molar Bicine buffer (pH 7.4). The enzyme was eluted with the same buffer. Fractions (15 ml) were collected, and those which contained the most activity were pooled (60 ml total).

3. Properties

pH Optimum- Lactonase activity as a function of pH was maximal at pH 7.6 in Tris-HCl and sodium phosphate

buffers (Figure 25).

Substrate Specificity- The lactonase catalyzed the hydrolysis of γ-D-lactones but failed to catalyze the hydrolysis of L-galactono-γ-lactone or D-glucono-δ-lactone as indicated in Table XXI.

The data in Table XXI also indicate that the K_m values for D-fucono-γ-lactone, D-galactono-γ-lactone, and D-ribono-γ-lactone are approximately equal to or less than 20 mM since the lactones were saturating at 40 mM. From the Lineweaver-Burk plot presented in Figure 26, the K_m value for D-galactono-γ-lactone was determined to be 2.1×10^{-2} M.

Stability- The half-life at 60°C was determined to be 3 minutes (Figure 27). The thermal denaturation profile of the pooled Sephadex G-200 fractions was linear, suggesting that the observed activity was due to a single enzyme.

The effects of various metal ions, thiols, and thiol group inhibitors are shown in Table XXII. The enzyme is not affected by 1 mM 2-thioethanol, 1 mM reduced glutathione, or 1 mM sodium sulfide. Similarly, 0.50 mM iododacetate or 0.50 mM p-chloromercuribenzoate was not inhibitory to enzyme activity. There was no observed activation or inhibition with 5 mM EDTA or 5 mM metal ion.

Figure 25. pH Optimum of the lactonase. The standard assay was utilized with the exception that the pH and the buffer concentration were varied, as indicated. The pH measurements were determined on duplicate reaction mixtures, and the pH of the reaction mixtures did not vary during the reaction period. The Sephadex G-200 fraction with the highest specific activity was used. All rates were corrected for non-enzymatic hydrolysis of the lactone.

Figure 25

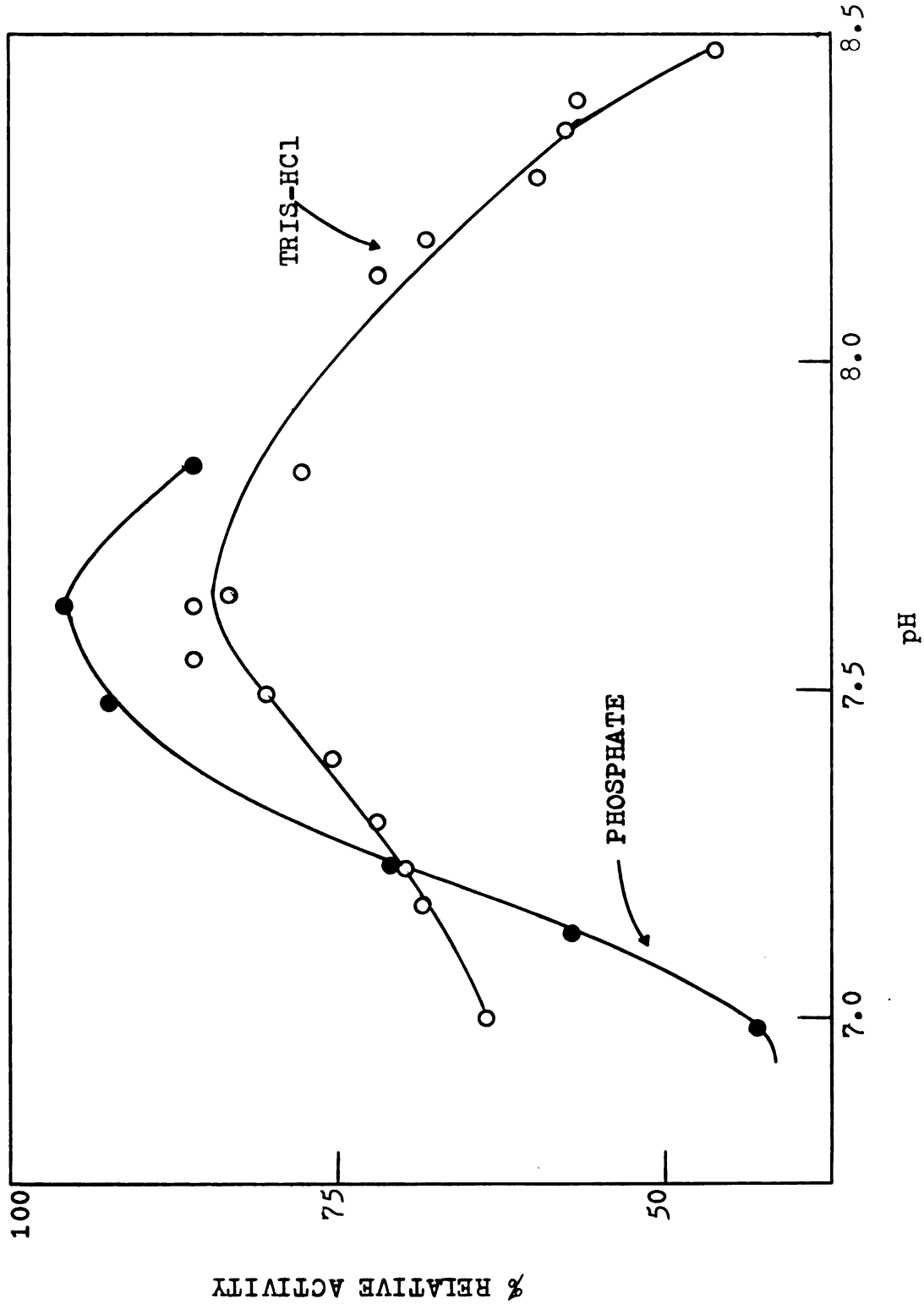


Table XXI. Substrate specificity of the lactonase. The rate of proton release was followed by automatic titration at pH 7.0. The assay consisted of cell extract (5 mg) and 40 μmoles of the respective lactone in a volume of 1.0 ml. All substrates were saturating at this concentration.

Lactone	Observed Rate (μmoles/5 min)	Non-Enzymatic Rate (μmoles/5 min)	Enzymatic Rate (μmoles/5 min)
D-Galactono-γ-lactone	4.13	0.625	3.50
D-Fucono-γ-lactone	3.40	0.055	3.34
D-Ribono-γ-lactone	2.08	0.301	1.78
L-Galactono-γ-lactone	0.68	0.70	0
D-glucono-δ-lactone	18.7	20.1	0

Figure 26. Lineweaver-Burk plot relating lactonase activity to D-galactono-γ-lactone concentration. The standard assay was employed with the exception that the lactone concentration was varied, as indicated, with the lactonase concentration constant. The Sephadex G-200 fraction with the highest specific activity was employed.

Figure 26

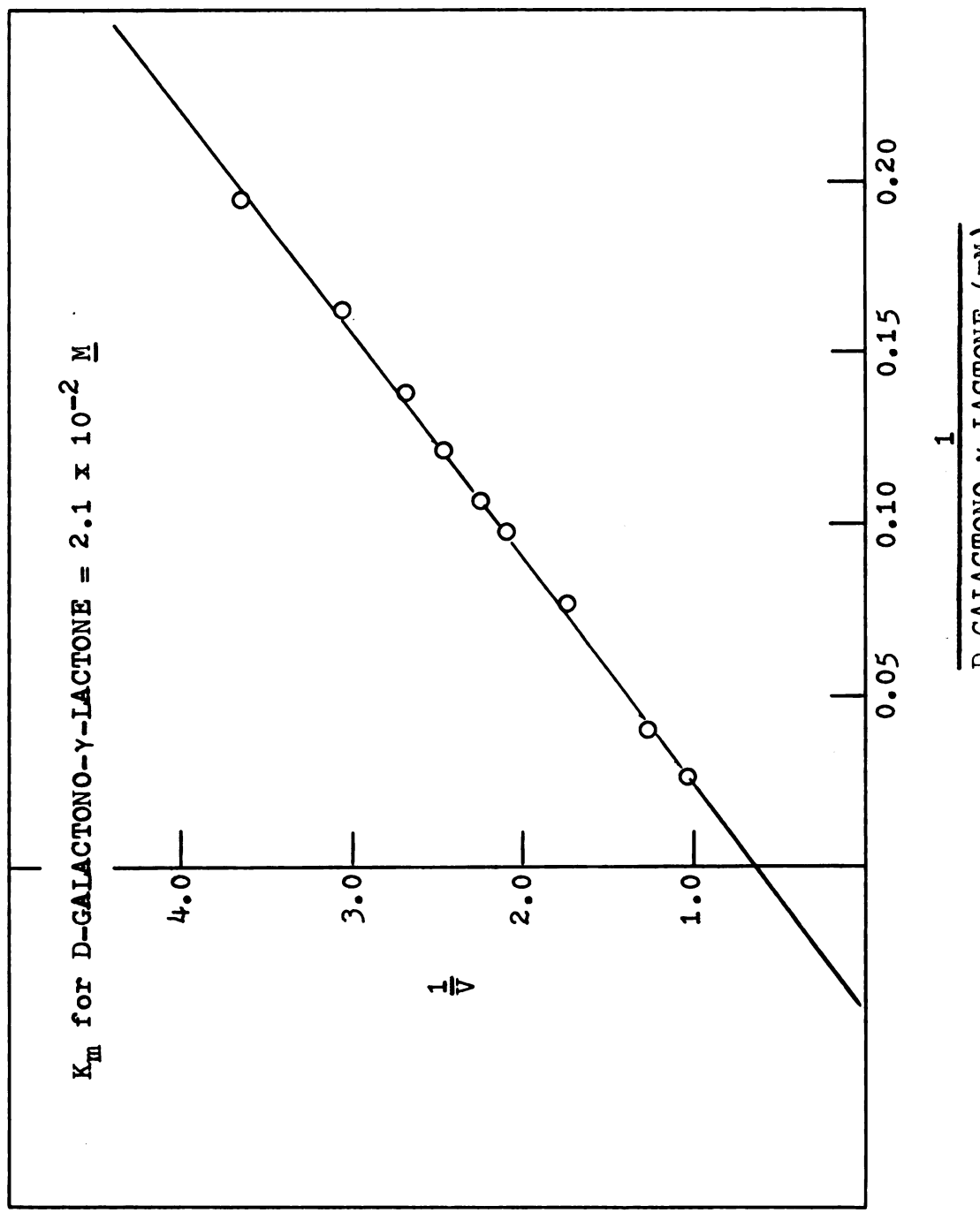

K_m for D-GALACTONO-γ-LACTONE = 2.1 x 10^{-2} M
$\frac{1}{V}$
4.0
3.0
2.0
1.0
0.05
0.10
0.15
0.20
$\frac{1}{\text{D-GALACTONO-γ-LACTONE (mM)}}$

Figure 27. Thermal denaturation of the lactonase at 60°C. The Sephadex G-200 fraction with the highest specific activity was used. The protein solution (1 ml, 1.7 mg per ml) was in 0.05 molar Bicine (pH 7.5). Aliquots were withdrawn at the indicated times and assayed with the standard assay.

Figure 27

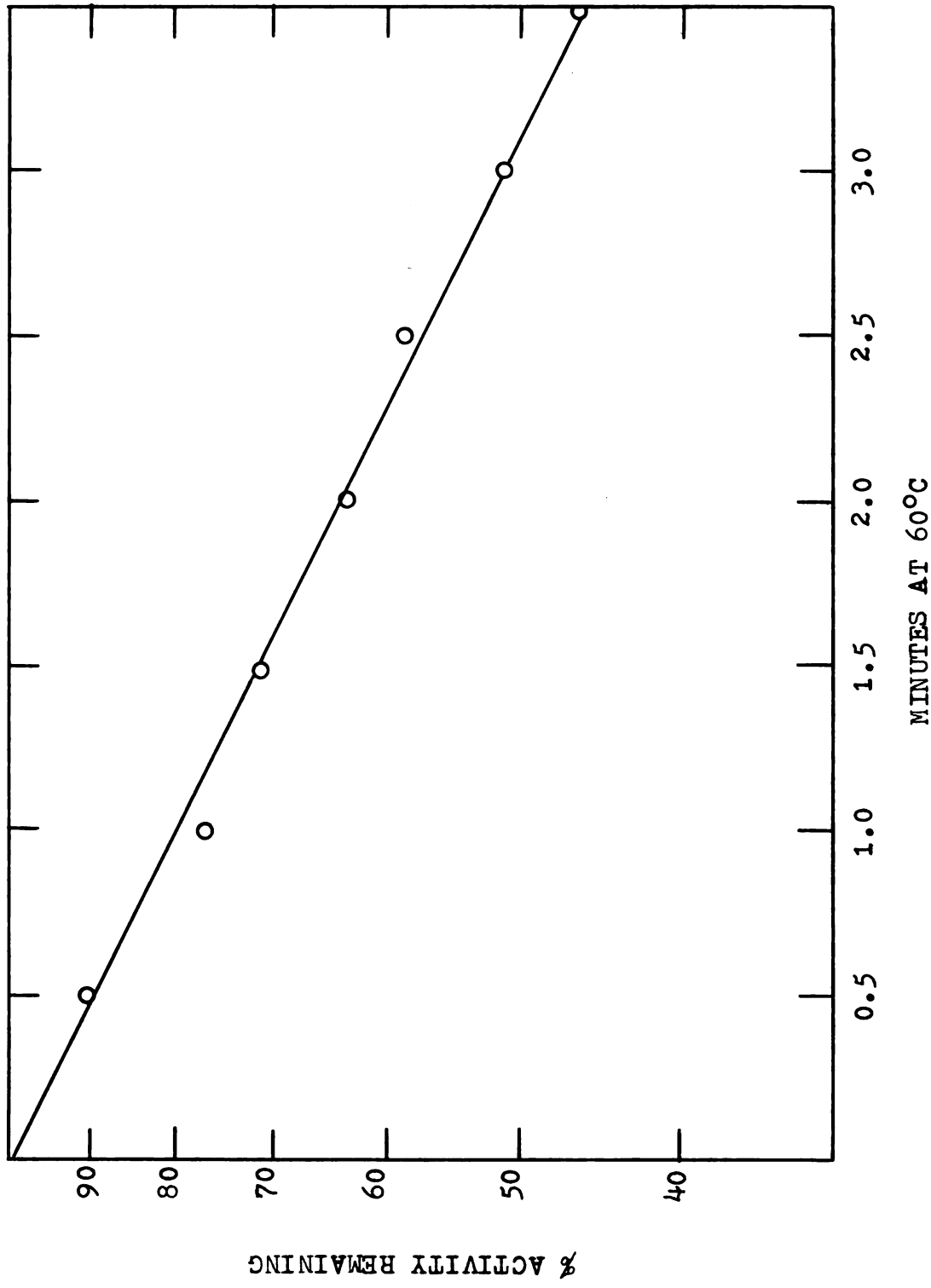

% ACTIVITY REMAINING
90
80
70
60
50
40
0.5
1.0
1.5
2.0
2.5
3.0
MINUTES AT 60°C

Table XXII. Effect of various reagents on lactonase activity. The standard assay was employed in which the enzyme was added to the cuvette containing the reagent. The Sephadex G-200 fraction with the highest specific activity was used. The pH of all reagents was 7.0.

Reagents	Concentration	% of Control Activity
p-Chloromercuribenzoate	1 mM	100
Iodoacetic acid	1 mM	100
2-Thioethanol	1 mM	100
Reduced glutathione	1 mM	100
Sodium sulfide	1 mM	100
EDTA	5 mM	100
$MgCl_2$	5 mM	100
$MnCl_2$	5 mM	100
$CoCl_2$	5 mM	100
NH_4Cl	5 mM	100
$FeSO_4$	5 mM	103
$NiCl_2$	5 mM	103
$CuSO_4$	5 mM	90
$CaCl_2$	5 mM	100
$ZnCl_2$	5 mM	97
Sodium phosphate buffer (pH 7.0)	50 mM	100
Boiled enzyme	--	0

Induction- The inducibility of the γ-lactonase was tested by growth on various substrates. The data (Table XXIII) indicate that D-galactose, D-fucose, L-arabinose, and 6-iodo-6-deoxy-D-galactose induced the enzyme 2-5 fold over the non-induced level present in nutrient broth-grown cells.

4. Product Identification

The reaction product resulting from γ-lactonase action of D-fucono-γ-lactone, i.e. D-fuconate, was identified by chromatography and derivatization.

Preparation of the Reaction Product- The reaction mixture consisted of 20 μmoles of D-fucono-γ-lactone and 0.5 mg of a Sephadex G-200 fraction in a total volume of 1.0 ml. The rate of proton release, i.e. the reaction rate, was followed by automatic titration at pH 7.0 using a Sargent recording pH stat and 0.01 molar NaOH. The reaction was complete within 20 minutes as indicated by the total absence of lactone and by the negligible rate of proton release. The non-enzymatic rate, as measured in the absence of protein, was less than 0.2 μmole/20 minutes. The reaction product was deproteinized by passage through a column of Dowex-50W-H^+ (0.5 x 1 cm) and was spotted on paper. The chromatograms were developed in solvent systems 1 and 5. The reaction product was found to co-chromatograph with authentic D-fuconate in both solvent systems.

Table XXIII. Inducibility of the γ-lactonase by growth on various substrates. The standard assay was employed. Cell extracts were prepared by sonic disruption in 0.01 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). Protein was estimated by the biuret assay.

Growth Substrates	Specific Activity (μmoles/hr/mg)
D-Fucose	19.5
L-Arabinose	20.6
D-Glucose	4.86
D-Galactose	20.9
Nutrient Broth	7.74
6-Iodo-6-deoxy-D-galactose	24.2

A portion of the Dowex-50W-H^+ eluate was relactonized and chromatographed on paper which was then developed in solvent systems 1 and 5. The relactionization product co-chromatographed with authentic D-fucono-γ-lactone. It was concluded that D-fuconate was the sole reaction product of γ-lactonase action on D-fucono-γ-lactone.

It is felt that, although the lactonase was not purified beyond Sephadex G-200 chromatography, the enzymatic activity observed was due to a single enzyme on the basis of the following: (i) the thermal denaturation profile was first order; (ii) only one symmetrical peak was observed when ammonium sulfate fractions of galactose, fucose, or arabinose cell extracts were chromatographed on Sephadex G-200; (iii) a single, symmetrical pH optimum was observed.

E. CHARACTERIZATION OF D-FUCONATE DEHYDRATASE

The preceeding three sections described the properties of the enzymes responsible for the formation of D-fuconate from D-fucose. The present section is concerned with the purification and properties of a specific dehydratase which catalyzes the irreversible dehydration of D-fuconate and the consequent formation of 3,6-dideoxy-D-*threo*-hexulosonic acid (2-keto-3-deoxy-D-fuconate, D-KDF). It will also be shown that the enzyme which dehydrates D-fuconate also dehydrates L-arabonate; L-arabonate dehydratase has been reported in the literature but was not characterizable due to its extreme lability (54-56).

Another dehydratase, D-galactonate dehydratase, similar to D-fuconate dehydratase, is also present in this pseudomonad. Data are presented in this section which distinguishes D-fuconate dehydratase from D-galactonate dehydratase. Due to the structural similarity between D-galactonate and D-fuconate, D-galactonate dehydratase might also be expected to dehydrate D-fuconate, however, data will be given in this section and later sections which support the conclusion that D-galactonate dehydratase does not dehydrate D-fuconate *in vivo*.

1. Preliminary Experiments

When cell extracts of D-fucose-grown cells were incubated with D-fuconate, followed by oxidation with periodate, a 2-thiobarbituric acid (TBA)-positive compound was formed. The physical properties of the TBA chromogen necessitated that the parent compound possess the structure of a 2-keto-3-deoxy sugar acid (see Product Identification, this section) which consequently required the postulation of a dehydration step. Thin-layer chromatography indicated that only one TBA-positive compound accumulated in the presence of D-fuconate and cell extract; analysis of fractions resulting from Sephadex G-200 chromatography of the protamine sulfate-treated cell extract indicated that probably one enzyme was responsible for D-fuconate dehydration. Preliminary studies also indicated that the enzyme possessed an absolute requirement for a divalent cation and was activated by thiols.

Due to the structural similarity between D-galactonate and D-fuconate (6-deoxy-D-galactonate), it was initially postulated that the enzyme which dehydrated D-fuconate was the same enzyme which dehydrated D-galactonate, D-galactonate dehydratase (E.C. 4.2.1.6). Evidence to the contrary was obtained in the preliminary studies which indicated that the two activities were distinguishable in the following analyses: (1) Sephadex

G-200 and DEAE-cellulose chromatography; (ii) pH optimum; (iii) thermal denaturation; (iv) sulfhydryl inhibitor responses; and (v) mixed substrate studies.

D-Galactonate dehydratase was later found to dehydrate D-fuconate; however, the K_m value was about 0.12 M. The specificity of the dehydratases can be summarized as (i) D-Fuconate dehydratase dehydrates L-arabonate and, at low concentrations, D-fuconate, but will not dehydrate D-galactonate; (ii) D-Galactonate dehydratase dehydrates D-galactonate and, at high concentrations, D-fuconate, but will not dehydrate L-arabonate. In the following experiments, L-arabonate was used as the substrate for D-fuconate dehydratase whenever D-galactonate dehydratase was present to prevent possible interference from D-galactonate dehydratase.

The results of the preliminary experiments basically indicated that an enzyme had been found which dehydrated D-fuconate at physiological concentrations, that the enzyme was not D-galactonate dehydratase, and that the resultant product was a 2-keto-3-deoxy sugar acid.

2. Purification

Cell extracts of L-arabinose-grown cells were prepared as in Experimental Procedures with the exception that the cells were disrupted in 0.10 molar Bicine buffer

(pH 7.4). Except where indicated otherwise, fractionation procedures were carried out at 0-4°C. A summary of the purification is given in Table XXIV.

Protamine Sulfate Fractionation- The protein concentration was adjusted to 14.5 mg per ml by dilution with 0.10 molar Bicine buffer (pH 7.4). Ammonium sulfate (10.0 g) was dissolved in 380 ml cell extract to a final concentration of 0.20 molar, and then 75 ml of a 2% protamine sulfate solution in 0.10 molar Bicine (pH 7.0) was added with stirring to a final concentration of 0.33%. After 30 minutes the suspension was centrifuged at 40,000 x g for 10 minutes, and the resulting precipitate was discarded.

Ammonium Sulfate Fractionation- The 40,000 x g supernatant (455 ml) was brought to 40% of saturation by the addition of 89.5 g of ammonium sulfate and the resulting precipitate was centrifuged down and discarded. The supernatant was then brought to 60% of saturation with 60.0 g of ammonium sulfate and centrifuged, and the resulting precipitate was collected by centrifugation and dissolved in 75 ml 0.10 molar Bicine buffer (pH 7.4). The protein concentration was 21 mg per ml.

Sephadex G-200 Chromatography- The above 40-60% fraction was placed on a column (6 x 60 cm) of Sephadex G-200 equilibrated with 0.10 molar Bicine buffer (pH 7.4). The enzyme was eluted with the same buffer. Fractions (15 ml) were collected, and those which contained the

Table XXIV. Purification of the D-Fuconate Dehydratase*

Fraction	Units	Specific Activity	Fold	280/260 nm
Cell extract	39,400	4.67	1	.68
Protamine sulfate	39,000	5.91	1.3	.84
Ammonium sulfate	29,500	18.7	4.0	1.22
Sephadex G-200	7,000	81.1	17.3	1.48
DEAE-cellulose	2,220	141	30.2	1.60

*L-Arabonate as substrate

most activity were pooled (75 ml total). A typical Sephadex G-200 elution profile for D-fuconate and D-galactonate dehydratases is presented in Figure 28.

DEAE-Cellulose Chromatography- DEAE-cellulose (Sigma, exchange capacity = 0.9 meq per g was pretreated as recommended by Sober et al. (50) and equilibrated with 0.01 molar sodium phosphate buffer (pH 7.0). The pooled Sephadex G-200 fractions were reduced in volume to 5 ml using a Diaflow ultrafiltration cell equipped with a 10,000 molecular weight cut-off ultrafiltration membrane. The Sephadex G-200 concentrate was applied to a DEAE-cellulose column (2 x 6 cm) which was then washed with 60 ml of the above buffer and then successively eluted with a stepwise gradient composed of 60 ml each of 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 molar sodium chloride in 0.01 molar phosphate buffer (pH 7.0). A typical elution profile for D-fuconate and D-galactonate dehydratase is presented in Figure 29. The DEAE-cellulose step must be performed as quickly as possible due to the denaturing characteristics of DEAE-cellulose towards D-fuconate dehydratase.

3. Properties

pH Optimum- D-Fuconate dehydratase activity as a function of pH was maximal at pH 7.1-7.4 in Bicine and Hepes buffers (Figure 30). The pH optimum using L-arabonate

Figure 28. Fractionation of D-galactonate and D-fuconate dehydratases on Sephadex G-200. The details are presented in the text. L-Arabonate was used as the substrate for D-fuconate dehydratase. Fractions were analyzed by the semicarbazide assay.

Figure 28

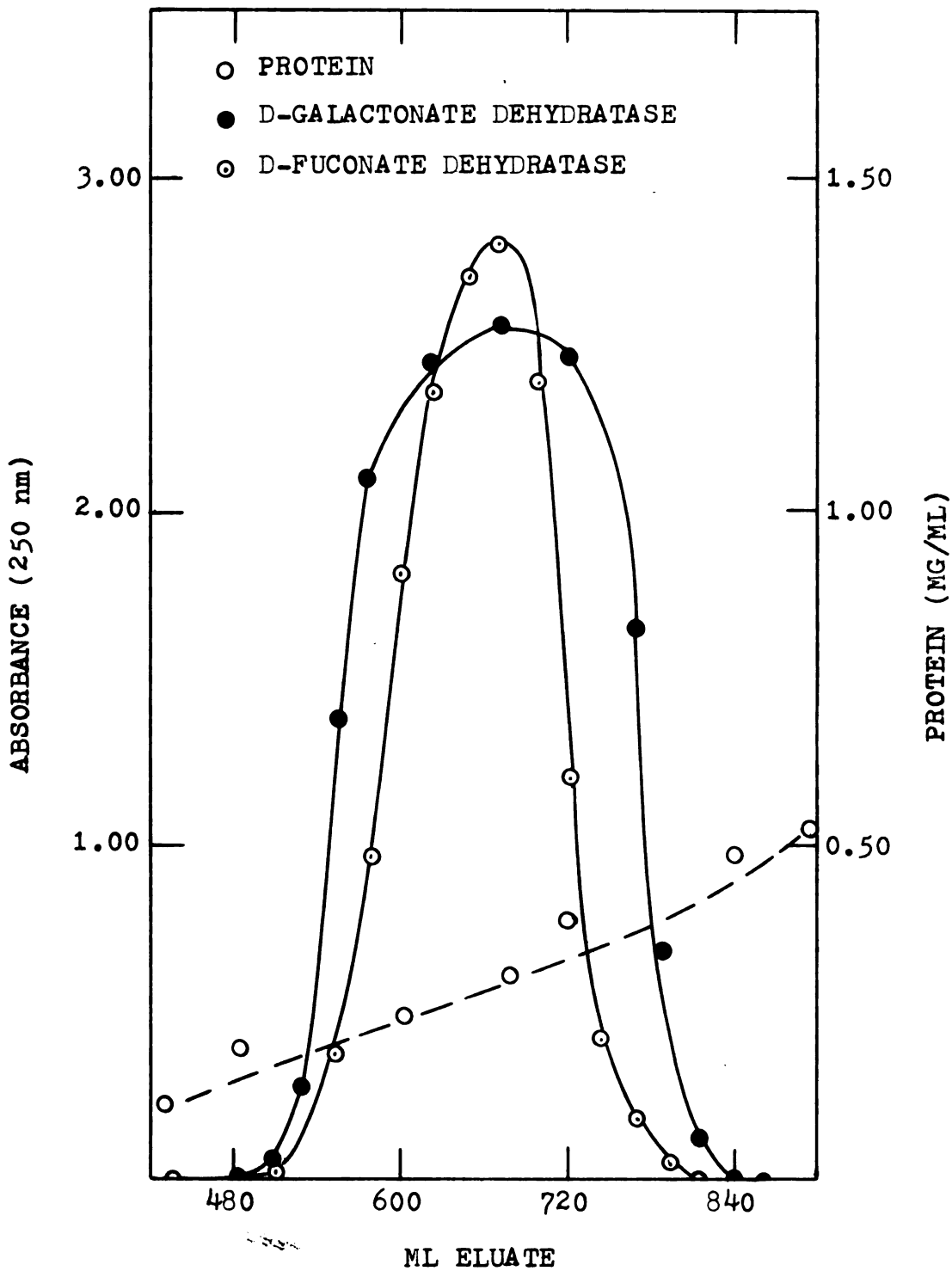

PROTEIN
D-GALACTONATE DEHYDRATASE
D-FUCONATE DEHYDRATASE
3.00
2.00
1.00
1.50
1.00
0.50
ABSORBANCE (250 nm)
PROTEIN (MG/ML)
480
600
720
840
ML ELUATE

Figure 29. Fractionation of dehydratases on DEAE-cellulose. Details are presented in the text. L-Arabonate was used as the substrate for D-fuconate dehydratase. ⊖-⊖, D-fuconate dehydratase; ●-●, D-galactonate dehydratase; O-O, protein. The semicarbazide assay for α-keto acids was used.

Figure 29

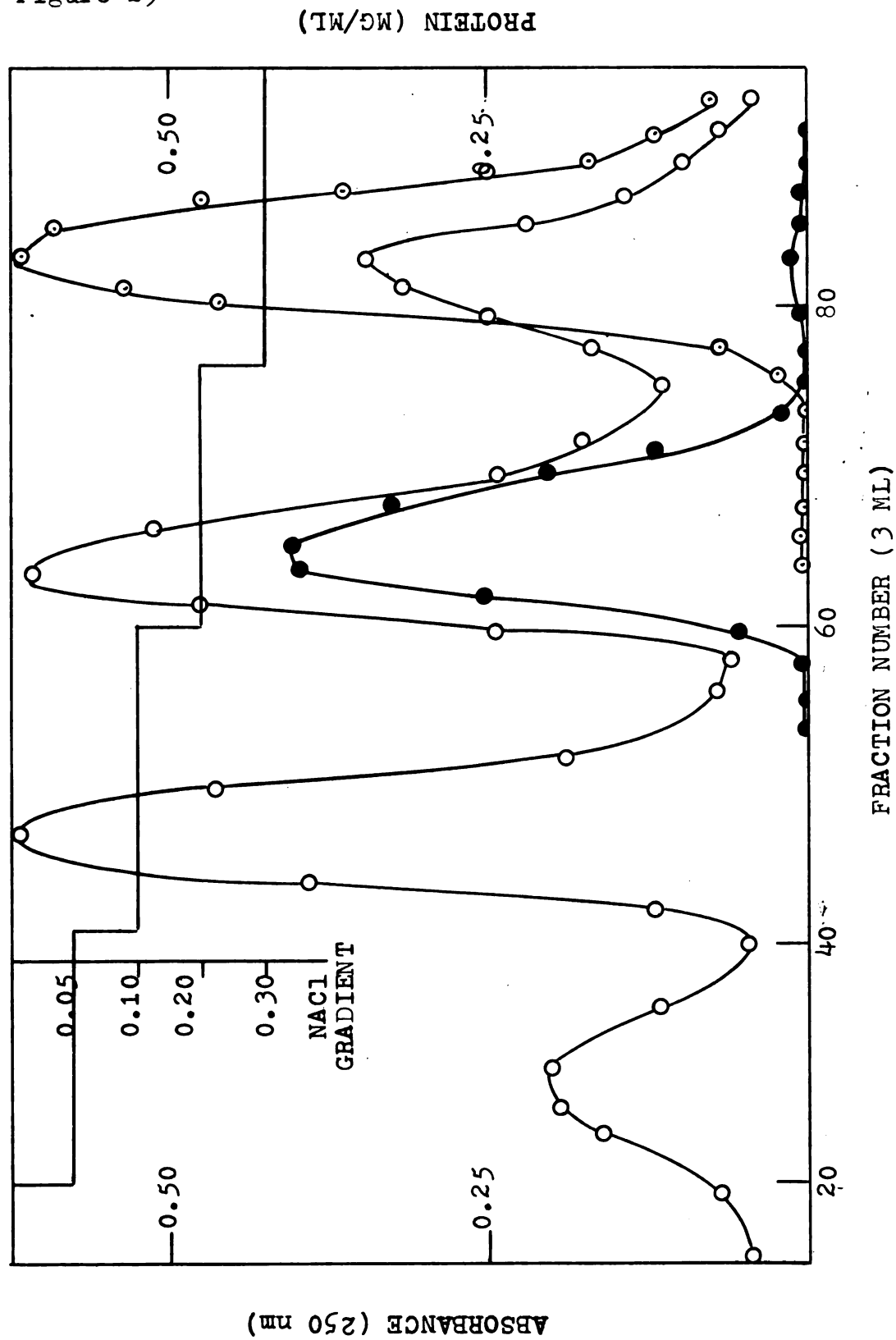
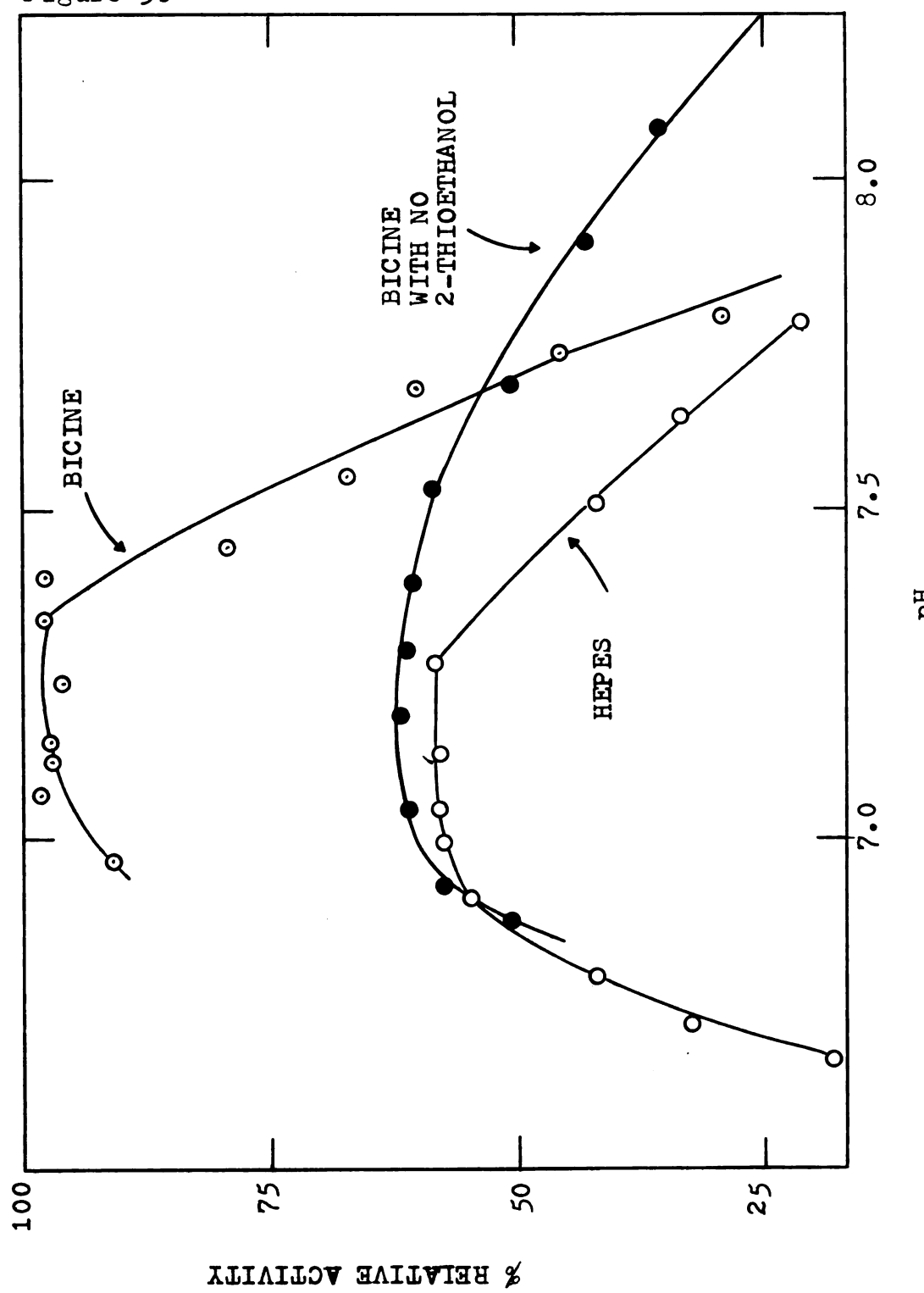

PROTEIN (MG/ML)
0.50
0.25
0.05
0.10
0.20
0.30
NACl
GRADIENT
0.50
0.25
20
40
60
80
FRACTION NUMBER (3 ML)
ABSORBANCE (250 nm)

Figure 30. pH Optimum of D-fuconate dehydratase. The standard assay was employed except that the pH and the buffer composition were varied, as indicated, with the dehydratase concentration constant. The DEAE-cellulose fraction with the highest specific activity was used. Each buffer was 0.20 molar. The pH measurements were determined on duplicate reaction mixtures. The pH did not change during the course of the 30 minute reaction period.

Figure 30


BICINE
WITH NO
2-THIOETHANOL
BICINE
HEPES
100
75
50
25
% RELATIVE ACTIVITY
7.0
7.5
8.0
pH

as substrate was superimposable with the pH optimum using D-fuconate as substrate. The pH optimum in the absence of 2-thioethanol was also 7.1-7.4, but was broader than the pH profile obtained in the presence of 2-thioethanol.

Substrate Specificity- A relatively large number of carbohydrates were examined as possible substrates for the dehydratase. Of the following carbohydrates only D-fuconate and L-arabonate were found to serve as substrates at the 60 mM level: D-fuconate, L-arabonate, D-arabonate, L-fuconate, 6-iodo-6-deoxy-D-galactonate, D-galactonate, D-ribonate, D-xylonate, D-lyxonate, D-mannonate, L-rhamnonate, D-gluconate, L-galactonate, L-gluconate, D-galactarate, D-glucarate, cellobionate, D-glucuronate, D-galacturonate, 3-deoxy-D-xylo-hexonate, N-acetyl-D-glucosamine, and glucosamine.

From the Lineweaver-Burk plots presented in Figures 31 and 32 the K_m values for D-fuconate and L-arabonate were found to be 4.0 and 4.3 mM respectively. The relative V_{max} values were 0.47 and 1.00 respectively. 2-Thioethanol had no effect on the K_m value for D-fuconate but did increase the V_{max} value, as previously noted.

Metal Ion Activators- D-Fuconate dehydratase possesses an absolute requirement for a divalent cation as shown in Table XXV. Mg^{2+} was found to be the most effective activator with Mn^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} being partially effective. The effect of Mg^{2+} concentra-

Figure 31. Lineweaver-Burk plot relating D-fuconate dehydratase activity to D-fuconate concentration. The standard assay was employed except that the D-fuconate concentration was varied, as indicated, with the dehydratase concentration constant. The DEAE-cellulose fraction with the highest specific activity was utilized.

Figure 31

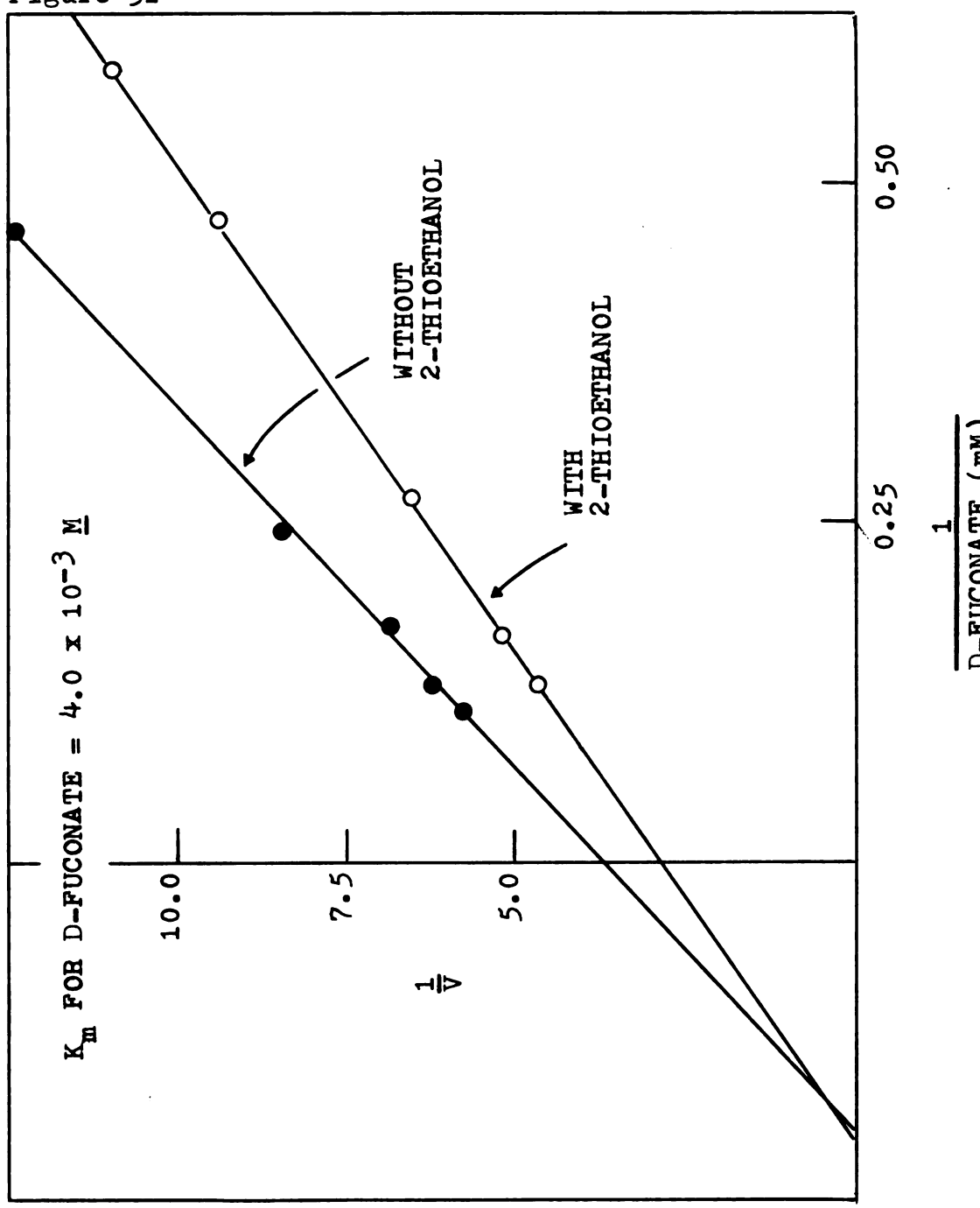

K_m FOR D-FUCONATE = 4.0 x 10^{-3} M
WITHOUT 2-THIOETHANOL
WITH 2-THIOETHANOL
$\frac{1}{V}$
10.0
7.5
5.0
0.25
0.50
$\frac{1}{\text{D-FUCONATE (mM)}}$

Figure 32. Lineweaver-Burk plot relating D-fuconate dehydratase activity to L-arabonate concentration. The standard assay was employed except that the L-arabonate concentration was varied, as indicated, with the dehydratase concentration constant. The DEAE-cellulose fraction with the highest specific activity was utilized.

Figure 32

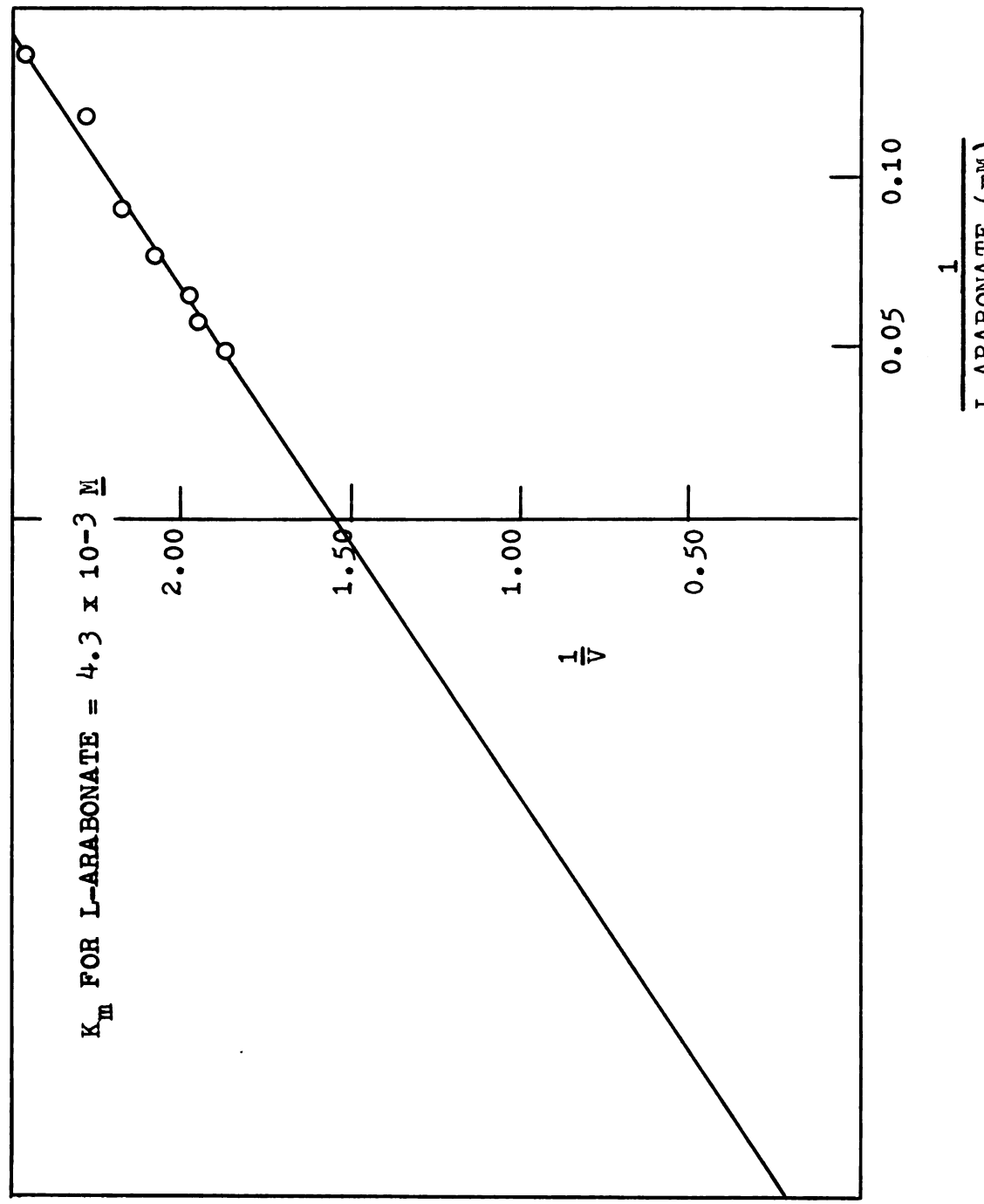

K_m FOR L-ARABONATE = 4.3 x 10-3 M
2.00
1.50
1.00
0.50
$\frac{1}{V}$
0.05
0.10
$\frac{1}{\text{L-ARABONATE (mM)}}$

Table XXV. Effect of metal ions on D-fuconate dehydratase. The standard assay was used with the exceptions that 2 μmoles of the metal salt and 0.5 μmole EDTA (pH 7.4) were added. The DEAE-cellulose fraction with the highest specific activity was used. L-Arabonate was used as the substrate. The pH of all reagents was 7.4.

Compound	Relative Rate
$MgCl_2$, $MgSO_4$	100%
$MgCl_2$ + NH_4Cl	88
$MnCl_2$	85
$FeSO_4$	37
$CuSO_4$	29
$CoCl_2$	18
$NiCl_2$	8
$CaCl_2$	0
$ZnCl_2$	0
$MgCl_2$ + boiled enzyme	0
No ion added	0

tion upon D-fuconate dehydratase activity was studied (Figure 33); the optimum Mg^{2+} concentration was determined to be about 20 mM. The effect of concentration of other ions on dehydratase activity was not tested. Activity with 3.3 mM EDTA in the absence of added metal ions was nil. Ammonium ion was 22% inhibitory at 3.3 mM.

Sulfhydryl Activation and Inhibition- Sulfhydryl compounds activated the dehydratase from 30-60% depending upon the stage of purification (Table XXVI). p-Chloromercuribenzoate (50 μM) produced 88% inhibition although iodoacetate was without effect at 2 mM.

Reversibility of the Dehydration- Incubation of 0.6 to 6 mM 2-keto-3-deoxy-D-fuconate (KDF) or 2-keto-3-deoxy-L-arabonate (KDA) in the otherwise standard assay, using Sephadex G-200 fractions containing the dehydratase but devoid of KDF aldolase, resulted in no detectable loss of TBA-positive material (< 0.004 μmole per hour per mg protein). In addition, enzymatic preparations of KDF or KDA were consistently obtained in yields amounting to 100% of the starting material. All attempts to demonstrate the reversibility of dehydration were repeatedly unsuccessful.

In addition to establishment of the equilibrium position of the dehydratase reaction, it was also determined by the same experiments that the dehydratase preparation did not cleave, or remove by other means, KDF or KDA.

Figure 33. Effect of Mg^{2+} concentration on D-fuconate dehydratase activity. The standard assay was employed with the exception that the Mg^{2+} concentration was varied as indicated. A DEAE-cellulose fraction devoid of D-galactonate dehydratase was used. D-Fuconate was used as the substrate.

Figure 33

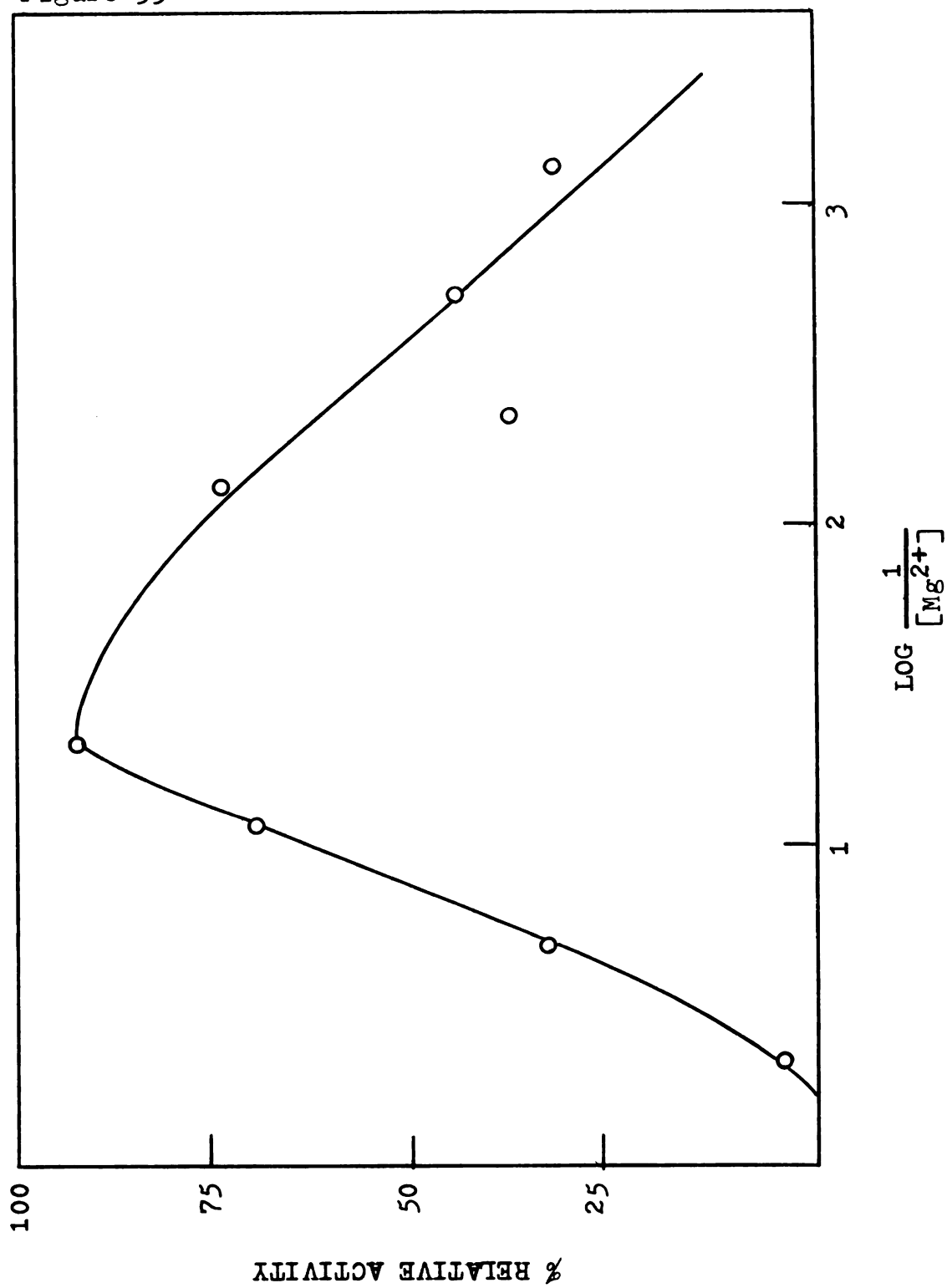


Table XXVI. Sulfhydryl activation and inhibition of D-fuconate dehydratase. The standard assay was employed with the exception of the variation in the thiol or the thiol inhibitor added. The DEAE-cellulose fraction with the highest specific activity was used. The pH of all reagents was 7.4. L-Arabonate was used as the substrate.

Compound	Concentration	Relative Rate
No thiol or thiol inhibitor added	-	100%
2-Thioethanol	2.85 mM	130
Glutathione (reduced)	2.68 mM	145
Dithiothreitol	2.68 mM	142
Iodoacetate	2.23 mM	104
p-Chloromercuribenzoate	0.05 mM	12

Stability- D-Fuconate dehydratase is not heat-stable relative to D-galactonate dehydratase as shown in Figure 34. The half-life at 53°C of the former, in a DEAE-cellulose fraction, is 80 seconds compared to over 1 hour for the latter. D-Fuconate dehydratase, present in Sephadex G-200 fractions, loses 50% activity upon freezing overnight in 0.10 molar Bicine buffer (pH 7.4) and is equally unstable towards lyophilization in the same buffer system. The enzyme, however, remains completely active after 24 hours at room temperature in 0.10 molar Bicine buffer (pH 7.4). D-Fuconate dehydratase, present in Sephadex G-200 fractions, was found to be inactivated by various buffers; the buffers (0.10 M) and the relative activities of the dehydratase at pH 7.5 are: Bicine (100%), sodium barbitol (60%), Hepes (45%), glycylglycine (41%), phosphate (30%), Tris-HCl (15%), Tes (9%), and Tricine (3%).

Induction- The inducibility of D-fuconate dehydratase was tested by growth on various substrates. The data (Table XXVII) indicate that D-fucose, D-galactose, and L-arabinose induced the enzyme 13-fold over the non-induced level as present in nutrient broth-grown cells. D-Galactose apparently acts as a gratuitous inducer of D-fuconate dehydratase since the enzyme is inoperative on D-galactonate.

Figure 34. Line A: Thermal denaturation of D-fuconate dehydratase at 53°C. A DEAE-cellulose fraction was used. The protein solution (3.1 mg per ml) was heated in 0.20 molar Bicine buffer (pH 7.4). Aliquots were withdrawn at the indicated times and were analyzed with the standard assay using both L-arabonate and D-fuconate, with no added thiol. Line B: Thermal denaturation of D-galactonate dehydratase at 53°C. A Sephadex G-200 fraction was used. The protein solution (2.1 mg per ml) was heated in 0.20 molar Bicine buffer (pH 7.4). Aliquots were withdrawn at the indicated times and were analyzed with the standard assay.

Figure 34

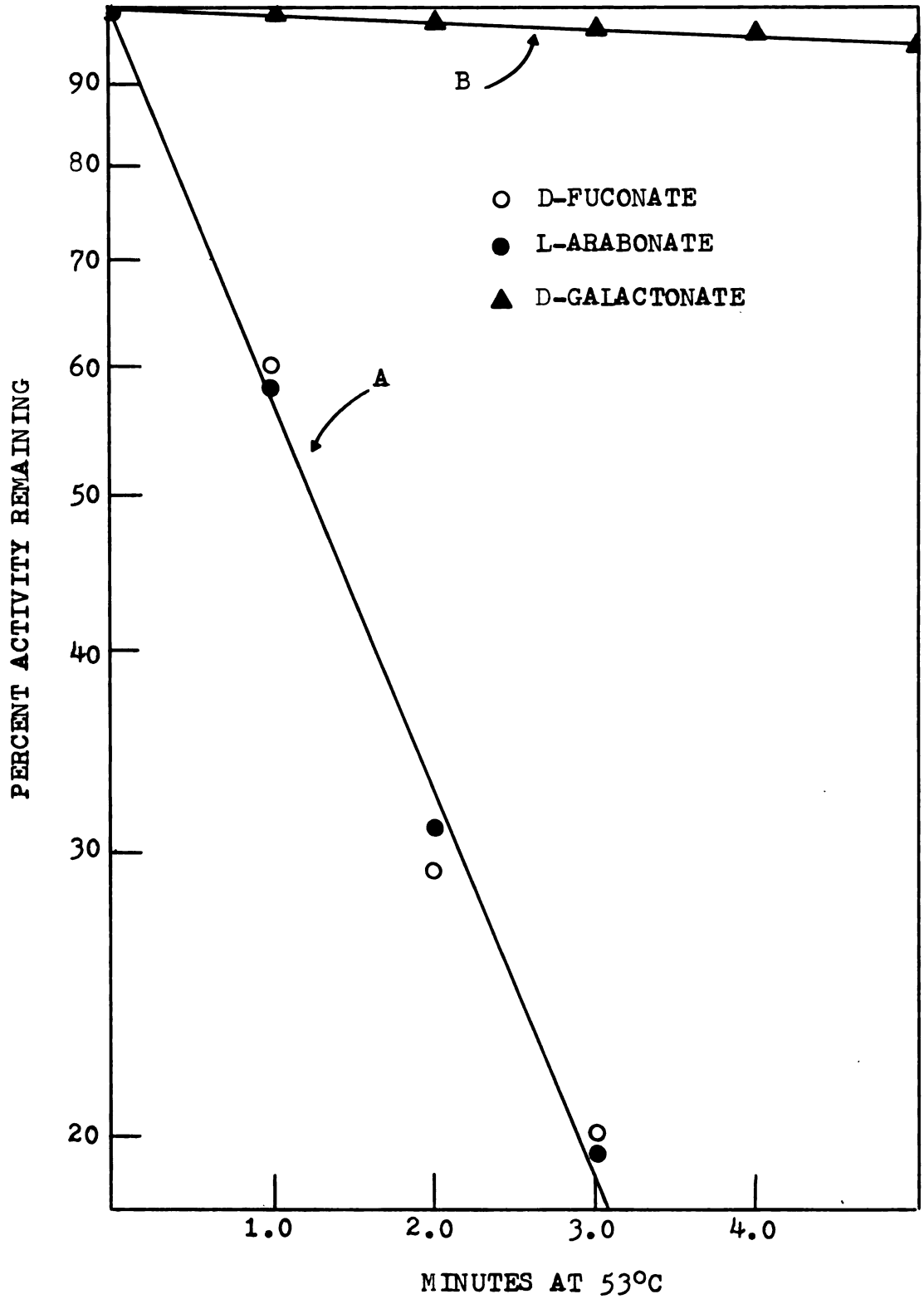

B
A
D-FUCONATE
L-ARABONATE
D-GALACTONATE
PERCENT ACTIVITY REMAINING
90
80
70
60
50
40
30
20
1.0
2.0
3.0
4.0
MINUTES AT 53°C

Table XXVII. Inducibility of D-fuconate dehydratase by growth on various substrates. The standard assay was employed with L-arabonate as the substrate. Cell extracts were prepared by sonic disruption in 0.01 molar Bicine buffer and 2-thioethanol (pH 7.4). Protein was estimated by the biuret assay.

Growth Substrates	Specific Activity (μmoles/hr/mg)
D-Fucose	4.67
L-Arabinose	4.27
D-Glucose	0.562
D-Galactose	4.25
Nutrient Broth	0.329
6-Iodo-6-deoxy-D-galactose	0.570

4. Identification of the Dehydration Product

Among other things, the initial experiments indicated that when cell extracts of D-galactose-, D-fucose- or L-arabinose-grown cells were incubated with D-fuconate, a TBA-positive product accumulated in amounts approaching theoretical limits. The product was proposed to be a 2-keto-3-deoxy aldonic acid solely on the basis of the physical properties of the TBA chromogen. This section will concern the enzymatic synthesis, the structural elucidation, and the chemical synthesis of the dehydration product.

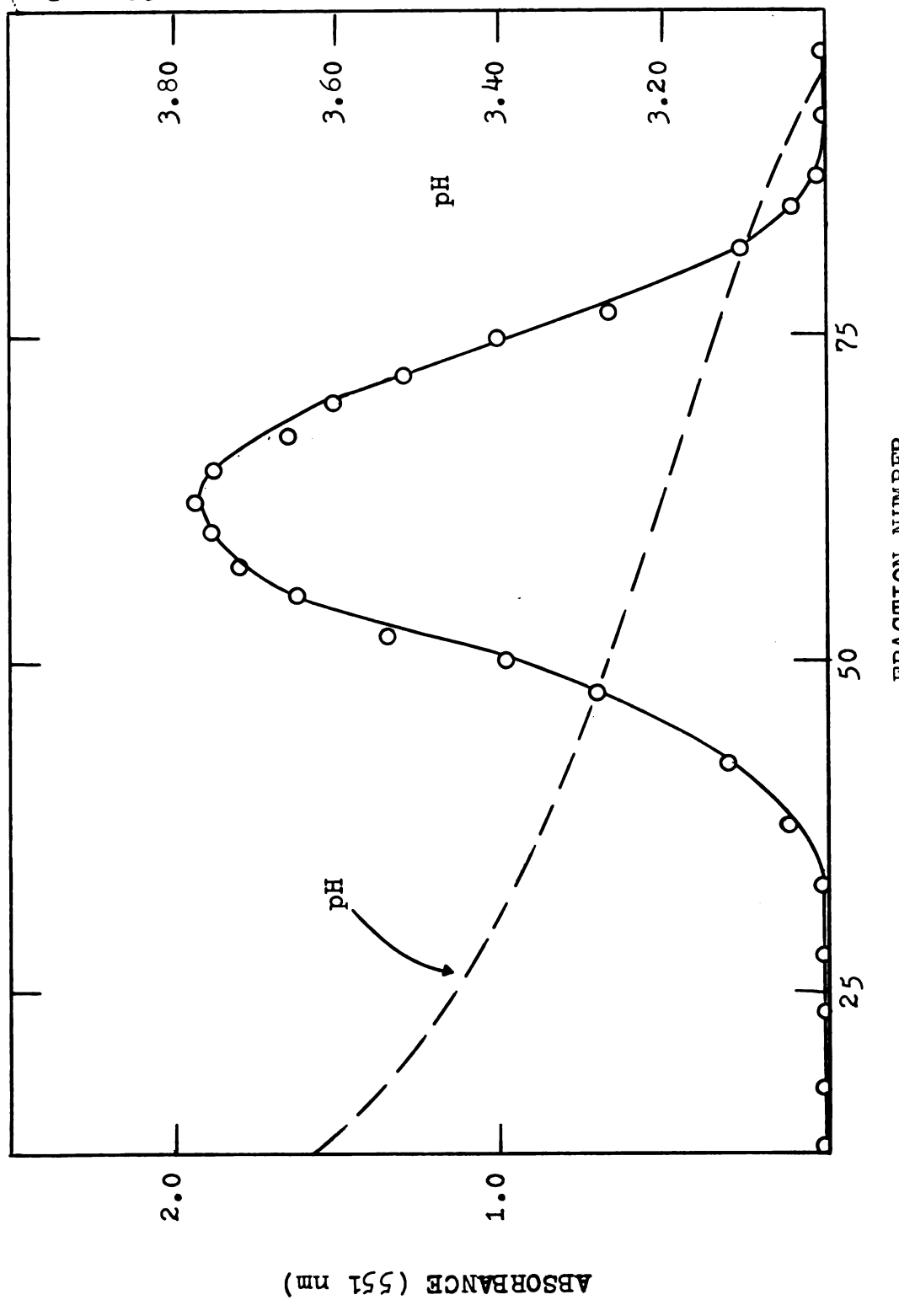
Enzymatic Synthesis of the Dehydration Product- To obtain sufficient material for characterization, the enzymatic preparation of the dehydration product was carried out on a large scale (0.5 mmole). The reaction mixture contained 50 μmoles $MgCl_2$, 500 μmoles potassium D-fuconate, and 20 mg of a Sephadex G-200 fraction containing the dehydratase, in a total volume of 10 ml. The pH was maintained at 7.4 with a Sargent recording pH stat using 0.01 molar sodium hydroxide as the titrant. Aliquots were withdrawn at various intervals and were assayed with TBA for 2-keto-3-deoxy aldonic acid formation. After the reaction was complete (2 hours), the reaction mixture was deproteinized by passage through a column of Dowex-50W-H^+ (0.5 x 35 cm). The column was washed free of non-cationic material with deionized water (750 ml), and the

eluate was passed through an AG1-X8 formate column (200-400 mesh, 0.5 x 35 cm) which was also washed with deionized water (750 ml). The formate column was eluted with a linear gradient of 0-0.08 molar formic acid using a Technicon Autograd gradient device. Fractions (14 ml) were collected and analyzed for KDF by the TBA assay (Figure 35). The pH of the eluate from the formate column was also monitored using a flow-through cell equipped with a micro-electrode. Fractions containing KDF were pooled and reduced under vacuum to a syrup to remove formic acid (bath temperature, 30°C). The free acid was converted to the potassium salt with Dowex-50W-K^+ and lyophilized to form a semicrystalline solid. The yield was 490 μmoles (98%). The solid was dissolved in water, and the pH was adjusted to 7.4 with KOH. The dehydration product was shown to be chromatographically homogeneous on Whatman #1 paper in solvent systems 2 and 6 and possessed R_f values of .29 and .78 respectively.

Absorption Spectrum of the TBA Chromogen- 2-Keto-3-deoxy aldonic acids and 2-deoxy sugars are cleaved by periodate to form β-formyl pyruvate and malondialdehyde, respectively. It was observed by Weissbach and Hurwitz (17) that a compound resulting from periodate oxidation of 2-keto-3-deoxy aldonic acids reacted with TBA to form an intense pink color which absorbed maximally at 549-551 nm which they concluded to be a TBA-β-formyl pyruvate com-

Figure 35. Dowex-1 formate chromatography of the enzymatically prepared dehydration product. The column was eluted with a 0-0.08 molar formic acid gradient. The fractions (14 ml) were assayed with 2-thiobarbituric acid.

Figure 35


3.80
3.60
3.40
3.20
pH
pH
2.0
1.0
ABSORBANCE (551 nm)
25
50
75
FRACTION NUMBER

plex. Malondialdehyde also reacts to form a TBA chromogen; however, the λ_{max} is 532 nm (6, 27, 57, 58). The malondialdehyde-TBA chromogen is alkali stable, in distinct contrast to the β-formyl pyruvate-TBA chromogen which is alkali unstable (14, 27). Both complexes exhibit shifts to longer wavelengths in the presence of alkali, and no other compounds tested except malondialdehyde and β-formyl pyruvate yielded chromogens in the 530-550 nm range.

The absorption spectrum of the TBA chromogen resulting from the periodate oxidation of the dehydration product is presented in Figure 36. The chromogen possessed a λ_{max} at 551 nm and was alkali unstable. The results indicate that the dehydration product possessed the structure of a 2-keto-3-deoxy aldonic acid. No over-oxidation of β-formyl pyruvate by periodate, which would result in concomitant loss of color formation (17, 59), was observed under the conditions of the assay (Figure 37).

Ceric Sulfate Decarboxylation- To substantiate the probable identity of the dehydration product as a 2-keto-3-deoxy aldonic acid, a ceric sulfate decarboxylation was performed. The dehydration product was first reduced with $NaBH_4$ and then oxidized by ceric sulfate to form, theoretically, a 2,5-dideoxy-pentose (Table XXVIII). The $NaBH_4$ reduction products were TBA-negative since β-formyl lactate resulting from periodate oxidation of the mixed metasaccharinic acids does not react with TBA to form a

Figure 36. A. Absorption spectrum of the TBA chromogen resulting from the periodate oxidation of the dehydration product.
B. Absorption spectra of the TBA chromogen resulting from the periodate oxidation of the dehydration product at various time intervals following adjustment of the pH to 11.2 with KOH. The uppermost spectrum is at t_o; each successive spectrum was taken at two minute intervals.

Figure 36

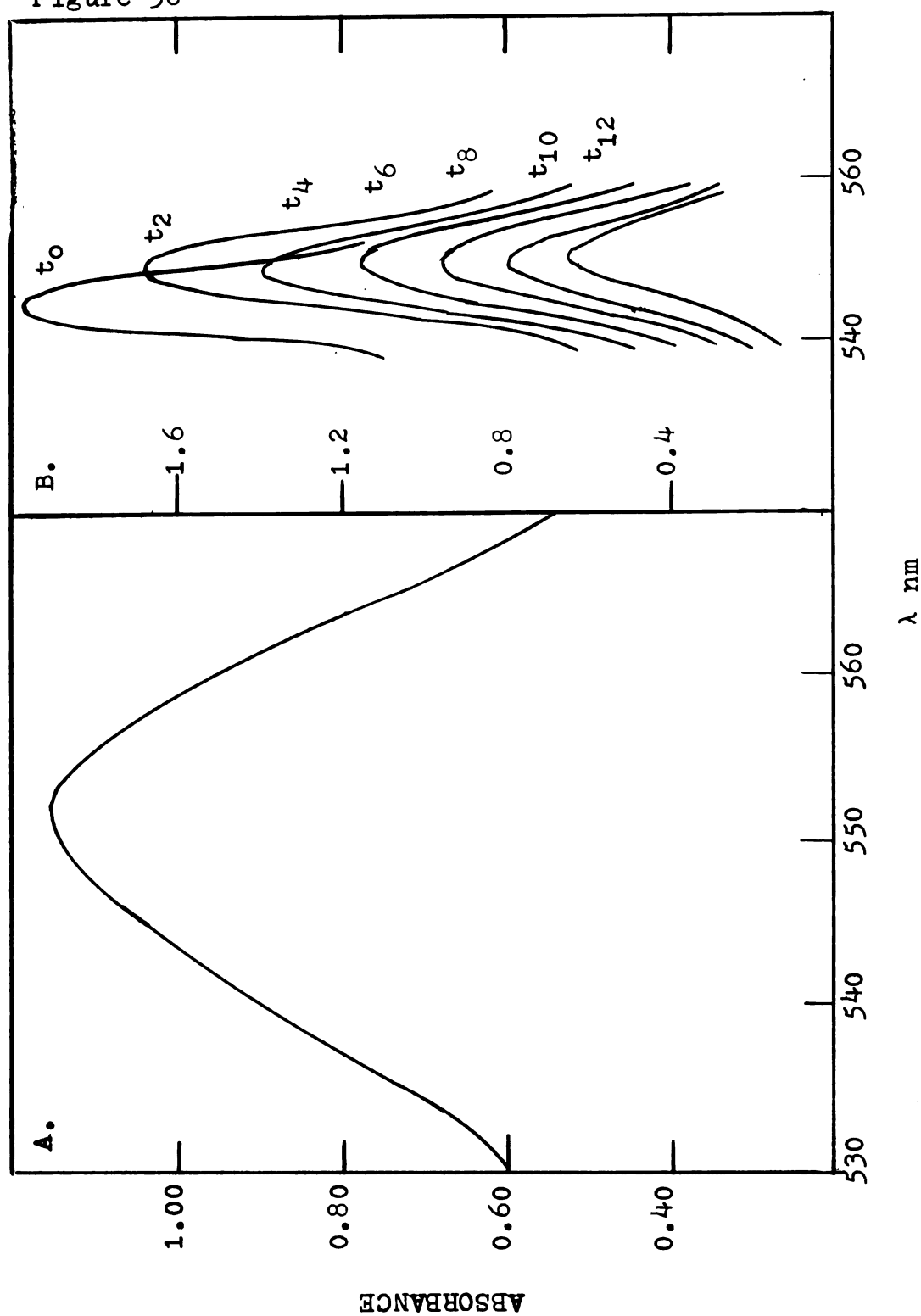

A.
B.
t_0
t_2
t_4
t_6
t_8
t_{10}
t_{12}
1.00
0.80
0.60
0.40
1.6
1.2
0.8
0.4
530
540
550
560
540
560
ABSORBANCE
λ nm

Figure 37. Stability of the β-formyl pyruvate-TBA complex under the conditions of the assay.

Figure 37

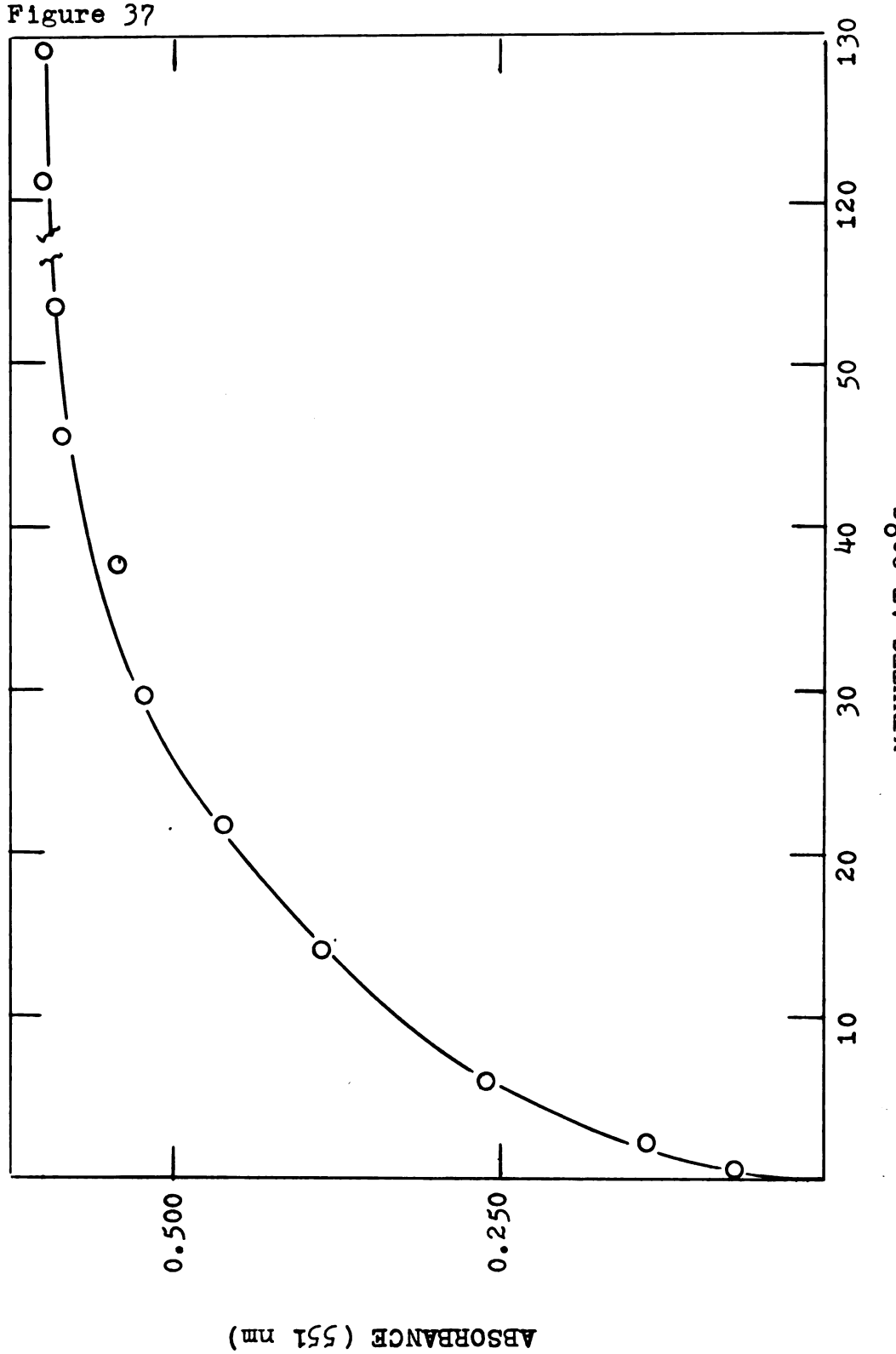
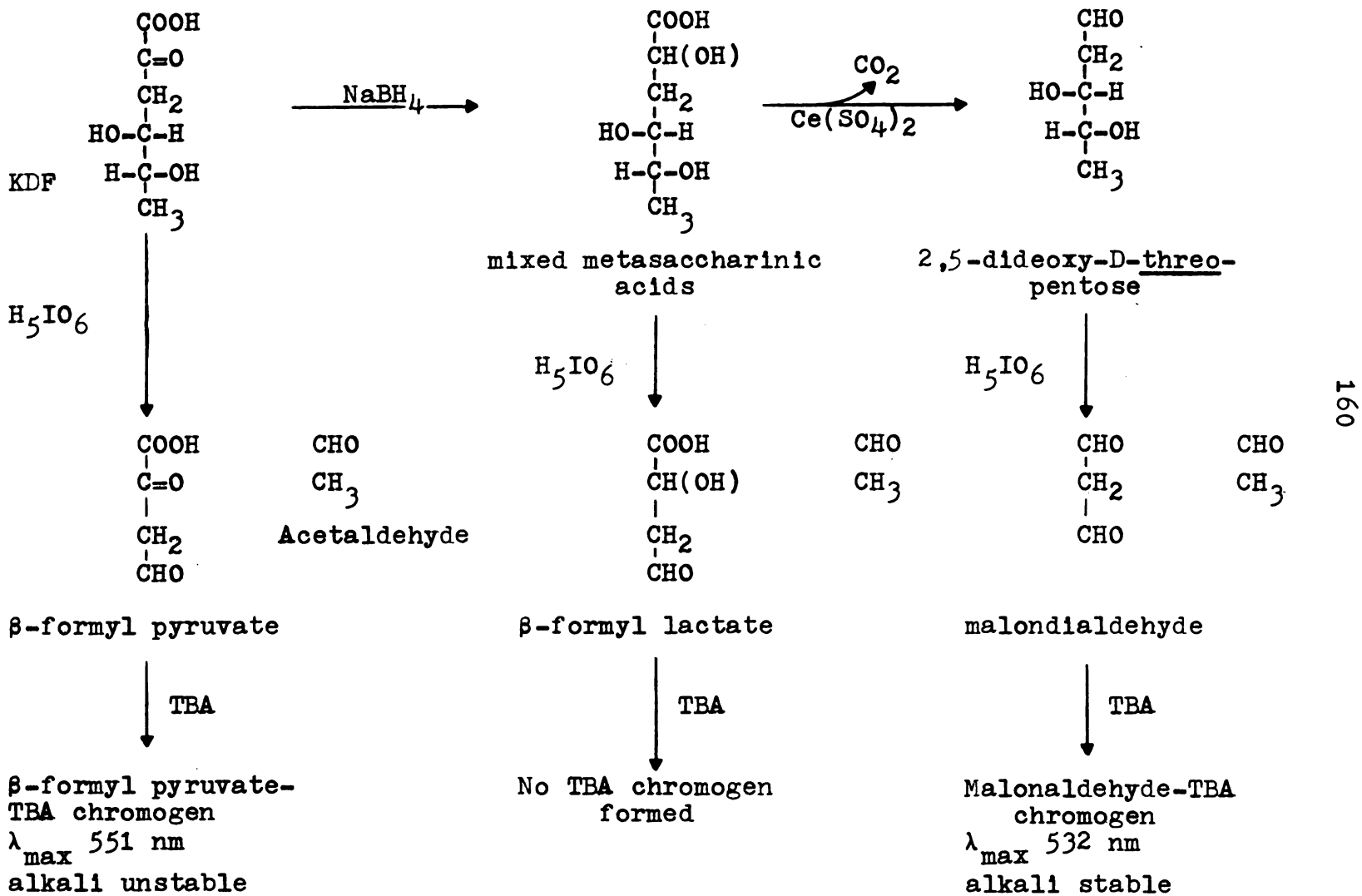

ABSORBANCE (551 nm)
0.500
0.250
10
20
30
40
50
120
130
MINUTES AT 30°C

Table XXVIII. Ceric sulfate decarboxylation of KDF



visible chromogen (57). However, after ceric sulfate decarboxylation of the mixed metasaccharinic acids, a TBA-positive compound was formed which absorbed maximally at 532 nm and was alkali stable and was thus characteristic of a 2-deoxy aldose (Figure 38). The data substantiated the presence in the dehydration product of keto group at C-2 and the methylene group at C-3. Since 2,5-dideoxy pentose standards were not available, the ceric sulfate oxidation product could not be rigorously identified; however, the results strongly support the contention that the dehydration product has the structure of a 2-keto-3-deoxy aldonic acid.

Quantitative recovery of CO_2 by the procedure of Meister (13) indicated that the dehydration product yielded 0.996 mole CO_2 per mole KDF.

α-Keto Acid Derivatives- The dehydration product reacts with 3-methyl-2-benzothiazolinone hydrazone to form an azine which absorbs maximally at 325 nm, characteristic of α-keto acids (20). The spectrum is presented in Figure 39. The dehydration product also forms a semicarbazone which absorbs maximally at 250 nm (Figure 40), also characteristic of α-keto acids (19).

o-Phenylenediamine will condense with 2-keto hexonic acids to form 2-hydroxyquinoxalines (60). The absorption spectra of quinoxalines are highly specific, the 330/360 nm absorbancy ratio being 1.51 ± 0.07 for

Figure 38. Absorption spectra of the TBA chromogen resulting from the periodate oxidation of the sodium borohydride-reduced and ceric sulfate-oxidized dehydration product. The alkaline stability of the malondialdehyde-TBA chromogen is indicated; the pH 11.2 spectrum was not changed after 2 hours. The pH of the TBA assay is 2.0 which was adjusted to pH 11.2 with 0.5 molar KOH.

Figure 38

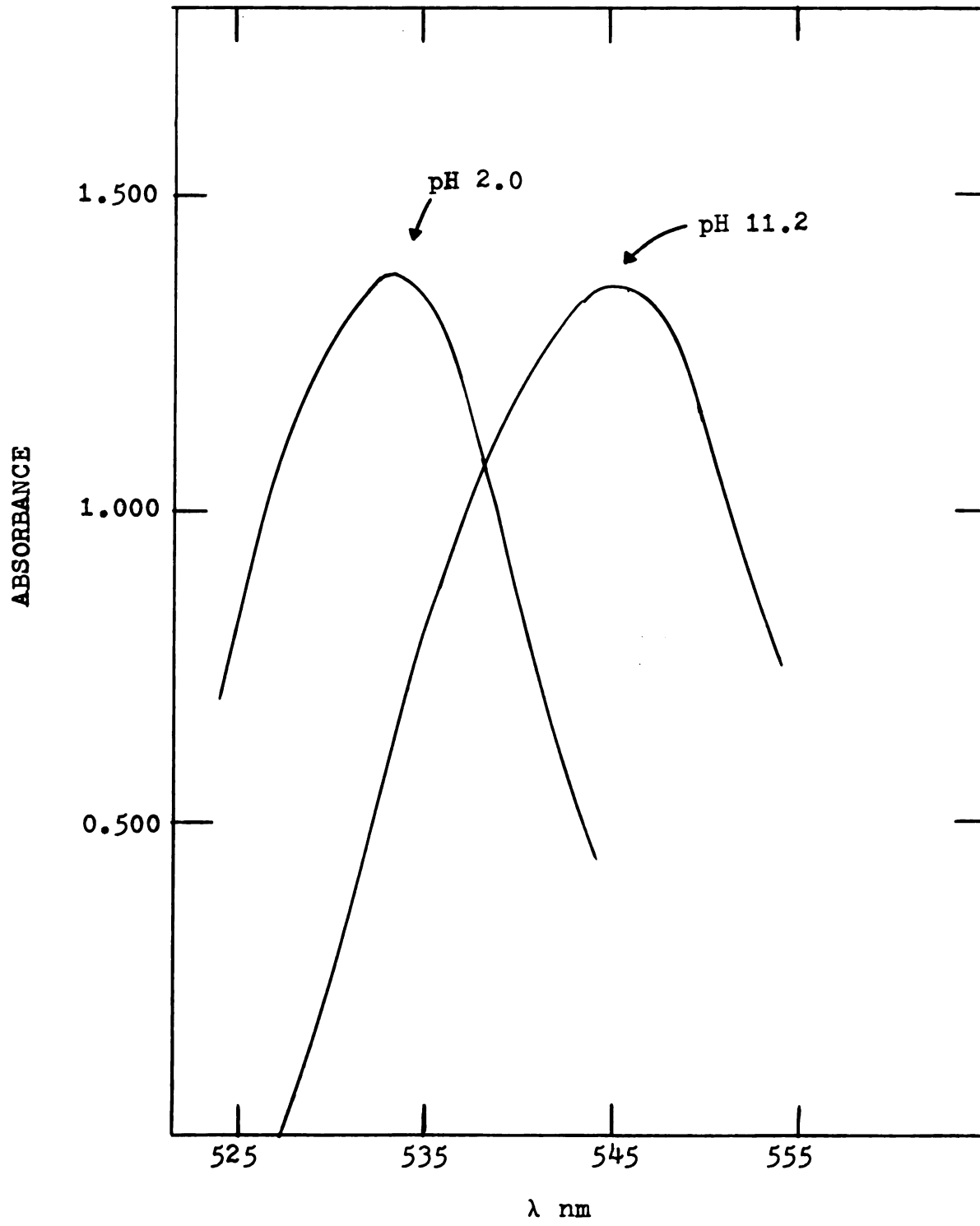


Figure 39. Absorption spectrum of the 3-methyl-2-benzothiazolinone hydrazone azine of the dehydration product.

Figure 39

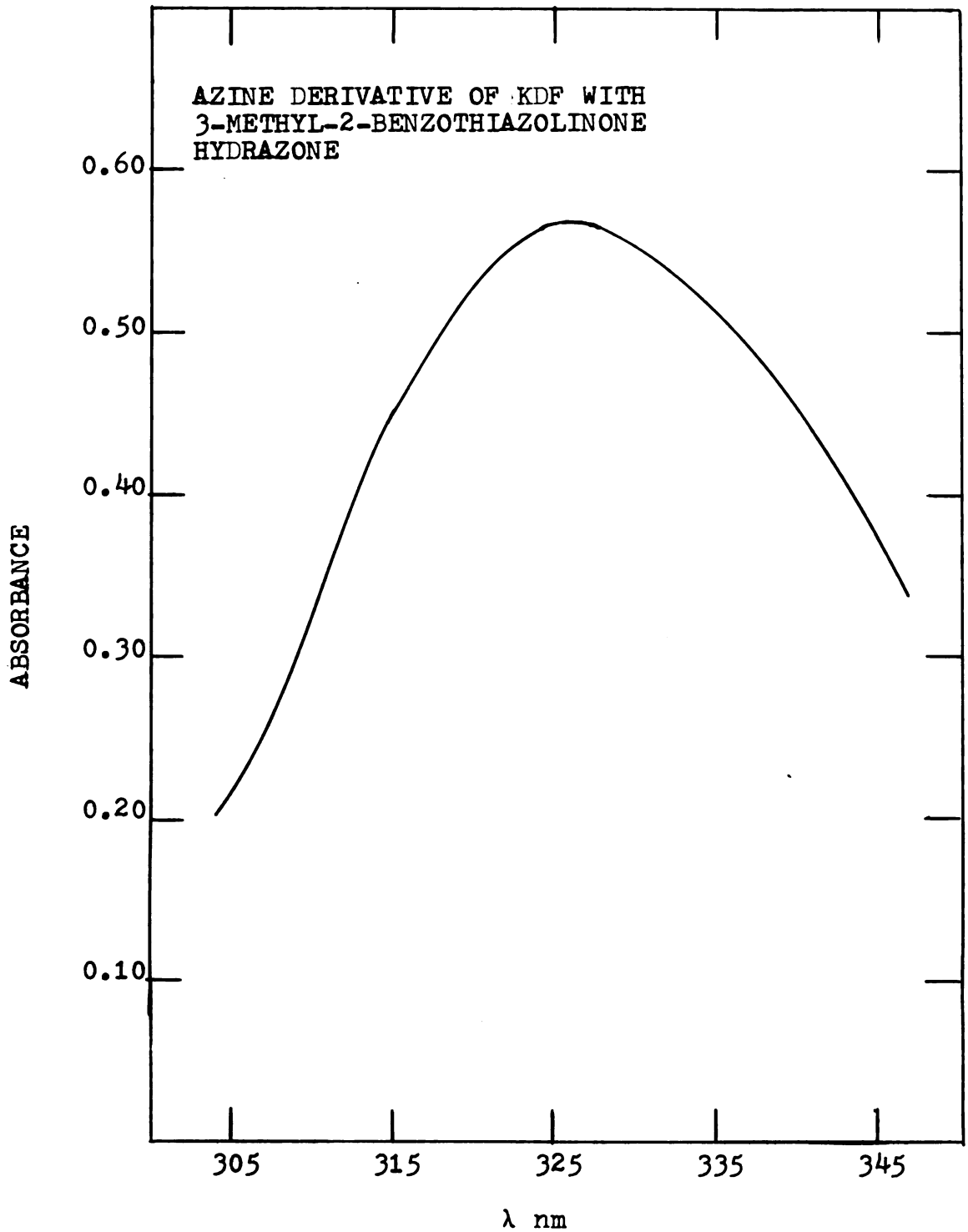
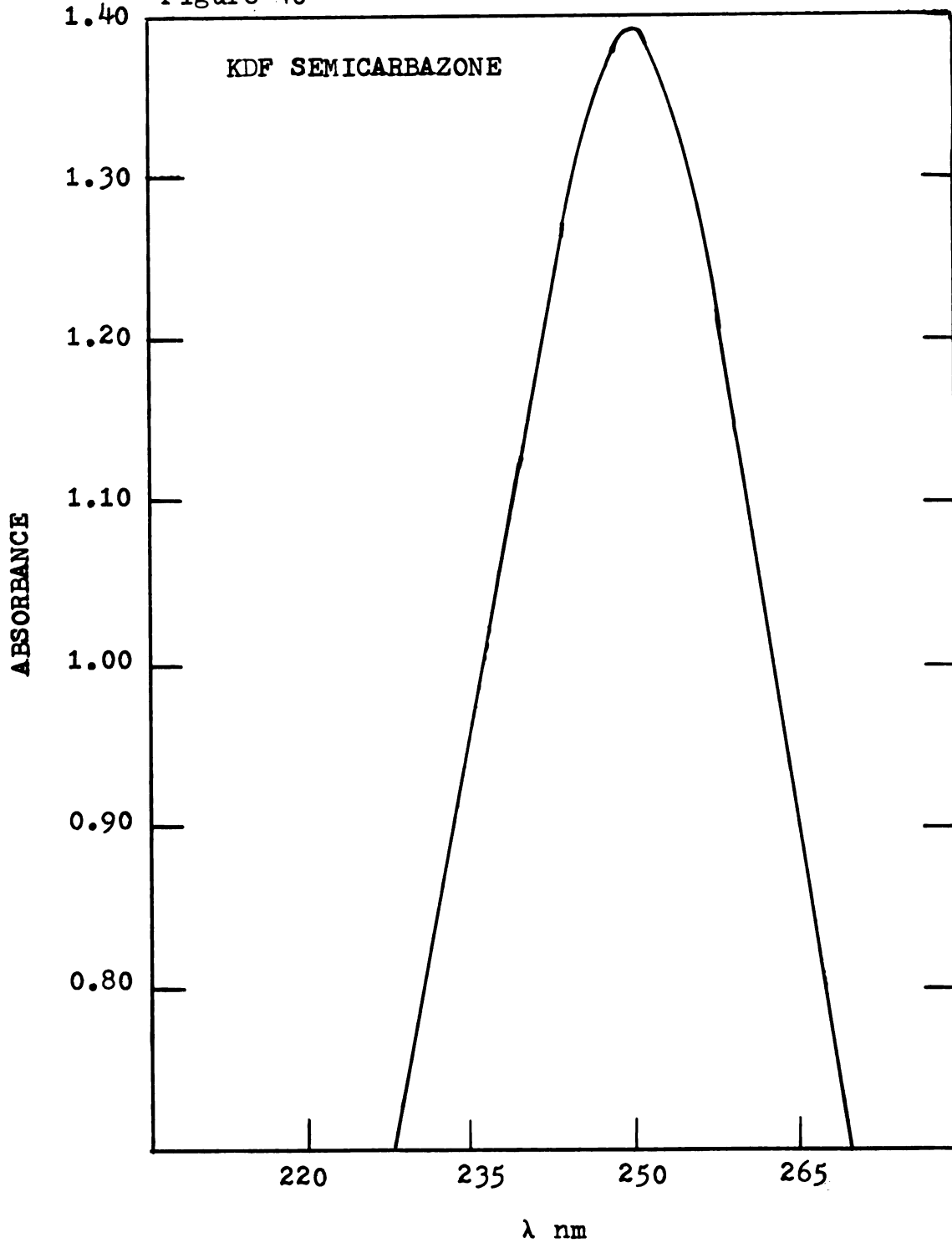

AZINE DERIVATIVE OF KDF WITH
3-METHYL-2-BENZOTHIAZOLINONE
HYDRAZONE
ABSORBANCE
0.60
0.50
0.40
0.30
0.20
0.10
305
315
325
335
345
λ nm

Figure 40. Absorption spectrum of the semicarbazone of the dehydration product.

Figure 40


KDF SEMICARBAZONE
1.40
1.30
1.20
1.10
1.00
0.90
0.80
ABSORBANCE
220
235
250
265
λ nm

2-keto hexonic acid derivatives. The quinoxaline spectrum for the dehydration product (Figure 41) possesses a 330/360 nm ratio of 1.51; repeated analyses yielded the average value, 1.51 ± 0.02.

The data obtained from these three derivatives are consistent with the previously obtained data which indicated that the dehydration product possessed the structure of a 2-keto-3-deoxy aldonic acid.

Periodate Oxidation- It cannot be assumed that the configuration of the hydroxyl groups remaining in the 2-keto-3-deoxy aldonic acid has not been changed, despite the absence of a precedent of such a reaction. The assignment of the configuration of the hydroxyl groups at C-4 and C-5 as threo was possible, however, as a result of studies of the different rates of release of β-formyl pyruvate in the periodate oxidation of 3-deoxy-D-threo- and 3-deoxy-D-erythro-hexulosonic acids. The rate of periodate oxidation of 3-deoxy hexulosonic acids is contigent upon the configuration of the hydroxyl groups on C-4 and C-5, and it has been found that the erythro configuration is oxidized at a more rapid rate than the threo configuration (14, 27, 61-65). The congruence of the reaction rates for the periodate oxidations of the dehydration product and 3-deoxy-D-threo-hexulosonic acid (2-keto-3-deoxy-D-galactonate) is indicated in Figure 42; the results of these experiments clearly indicate that

Figure 41. Absorption spectrum of the quinoxaline derivative of the dehydration product.

Figure 41

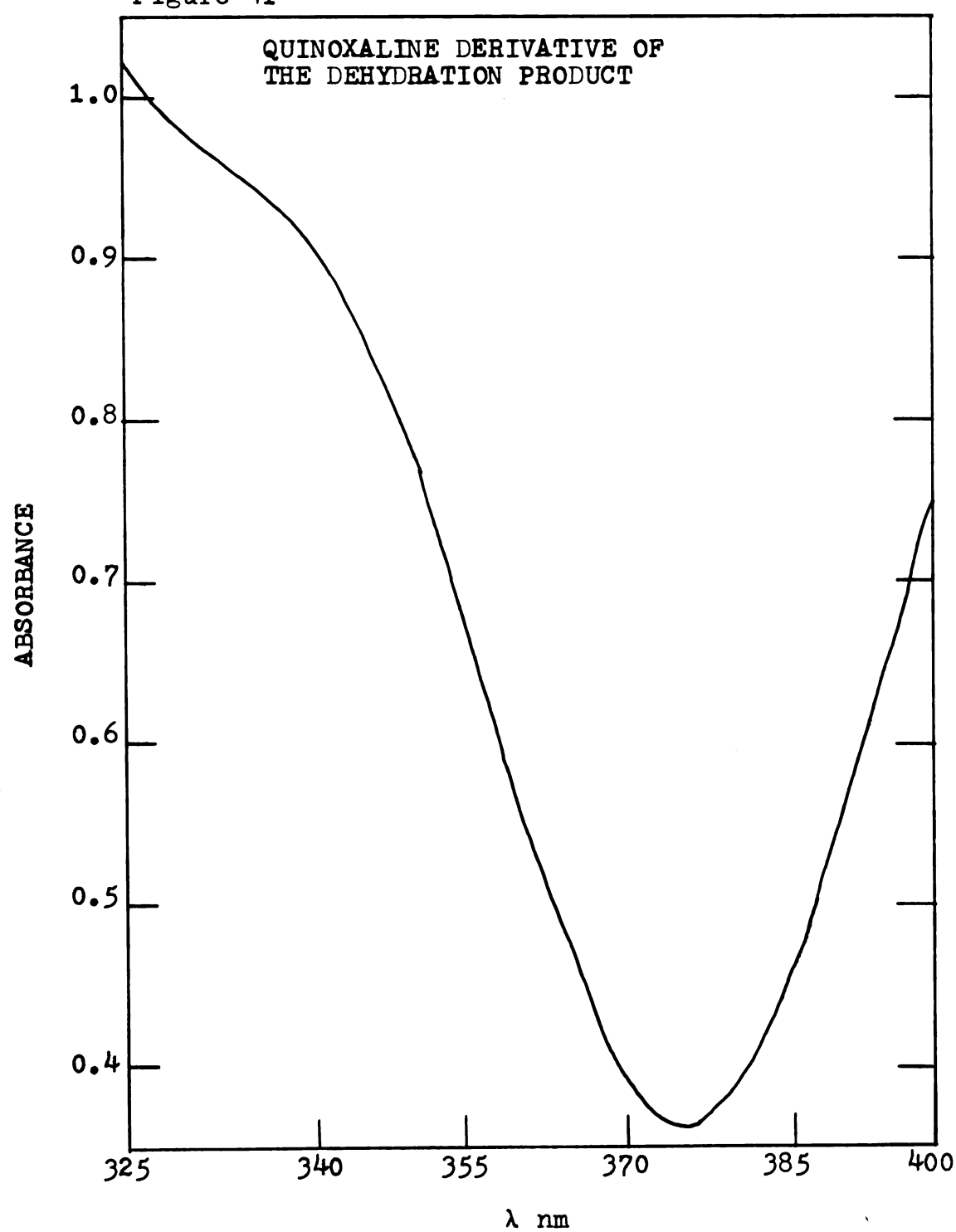
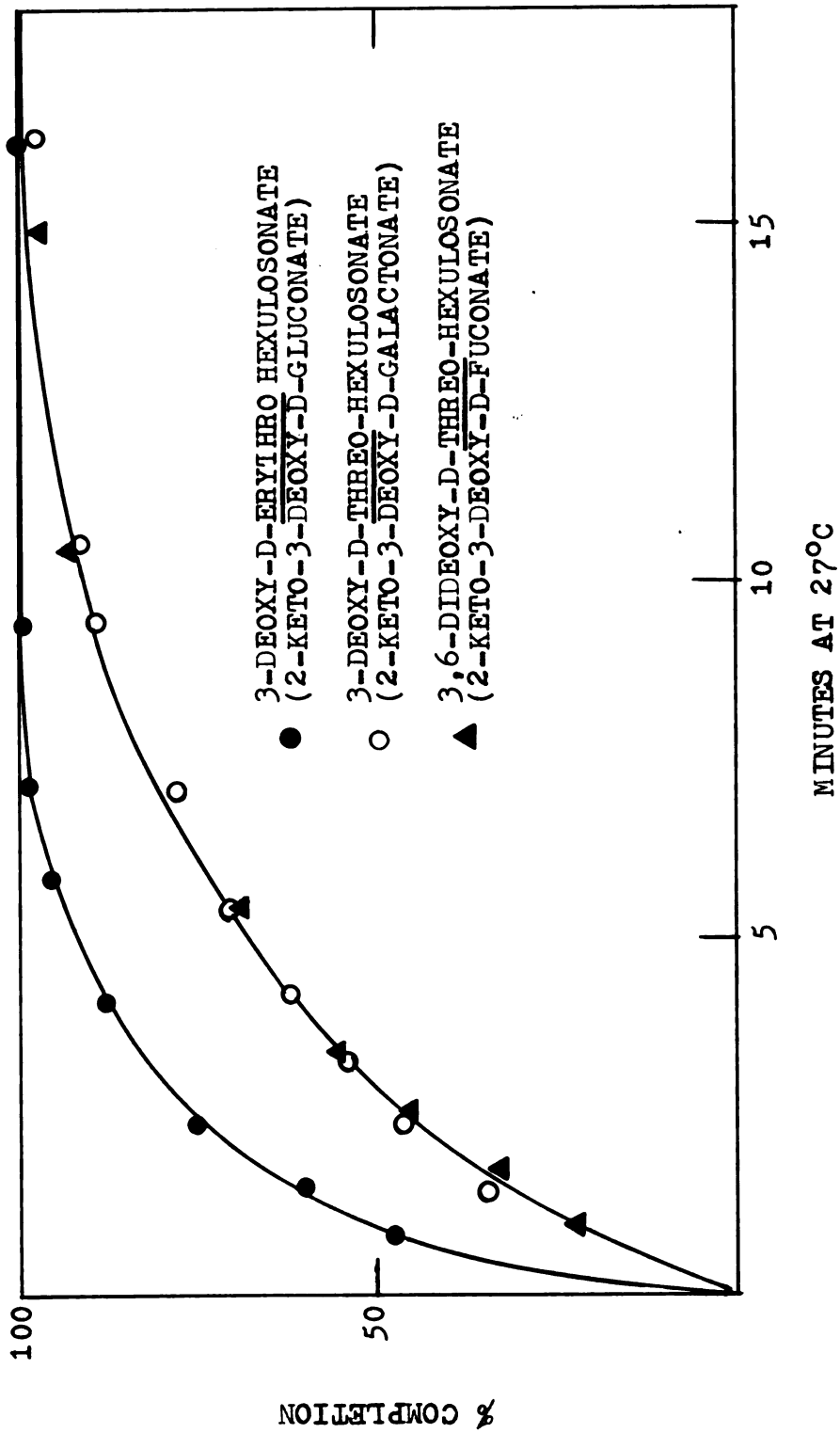

QUINOXALINE DERIVATIVE OF
THE DEHYDRATION PRODUCT
ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
325
340
355
370
385
400
λ nm

Figure 42. A comparison of the rates of release of β-formyl pyruvate in the periodate oxidations of the dehydration product, 3-deoxy-D-threo-hexulosonic acid, and 3-deoxy-D-erythro-hexulosonic acid.

Figure 42


3-DEOXY-D-ERYTHRO HEXULOSONATE
(2-KETO-3-DEOXY-D-GLUCONATE)
3-DEOXY-D-THREO-HEXULOSONATE
(2-KETO-3-DEOXY-D-GALACTONATE)
3,6-DIDEOXY-D-THREO-HEXULOSONATE
(2-KETO-3-DEOXY-D-FUCONATE)
100
50
% COMPLETION
5
10
15
MINUTES AT 27°C

the configuration of the hydroxyl groups at C-4 and C-5 has remained intact through dehydration at C-2 and C-3.

Quantitative determination of periodate indicated that 0.89 mole periodate was consumed per mole α-keto acid. Acetaldehyde, the co-product of the periodate oxidation, was also detected but in amounts of 0.2-0.3 mole per mole α-keto acid. Acetaldehyde, however, is the only known compound which interferes with the TBA assay (66) and probably condenses via an acid-catalyzed aldol condensation with the enol formed by the rearrangement of the active methylene carbanion of β-formyl pyruvate.

Cleavage by 2-Keto-3-deoxy-D-fuconate- The dehydration product is cleaved by KDF aldolase in an endpoint assay to yield 0.98 mole pyruvate per mole α-keto acid and 0.96 mole lactaldehyde per mole α-keto acid. These data provide additional structural proof of the dehydration product.

The previous data on the dehydration product are summarized on Table XXIX. It has been concluded that the structure of the dehydration product is 3,6-dideoxy-D-threo-hexulosonic acid (KDF). As a final proof of structure, the chemical synthesis of KDF was undertaken and is presented in the next subsection.

Table XXIX. Characterization of the dehydration product as KDF

Characterization Method		Expected Result	Observed
TBA chromogen	λ_{max}	549-551 nm	551 nm
	alkali stability	unstable	unstable
TBA chromogen after $NaBH_4$ reduction		none	none
TBA chromogen after $NaBH_4$ reduction and ceric sulfate oxidation			
	λ_{max}	532 nm	532 nm
	alkali stability	stable	stable
Semicarbazone	λ_{max}	250 nm	250 nm
o-Phenylenediamine 330/360 nm ratio (2-hydroxyquinoxaline)		1.51 ± 0.07	1.51 ± 0.02
3-Methyl-2-benzothiazolinone hydrazone azine			
	λ_{max}	316-345 nm	325 nm

Ceric sulfate decarboxylation	1.00 mole/mole	0.996 mole/mole
Hydroxyl configuration periodate rate study	threo	threo
Periodate consumption	1.00 mole/mole	0.89 mole/mole
Acetaldehyde detection after periodate oxidation	+	+
Pyruvate liberation after cleavage with KDF aldolase	1.00 mole/mole	0.98 mole/mole
Lactaldehyde liberation after cleavage with KDF aldolase	1.00 mole/mole	0.96 mole/mole

5. Chemical Synthesis of 2-Keto-3-deoxy-D-fuconate

This compound was synthesized via modifications of the method used for the synthesis of N-acetylneuraminic acid (67). The reaction involves an aldol-type condensation of lactaldehyde with a pyruvyl carbanion generated by alkaline β-decarboxylation of oxalacetate. DL-Lactaldehyde was prepared by the oxidative deamination of DL-threonine using the procedure of Abeles et al. (68). Chromatography of recrystallized DL-lactaldehyde on Dowex-1-HSO_3^- by the procedure of Huff (69) and analysis of the fractions with 3-methyl-2-benzothiazolinone hydrazone indicated the presence of at least 12 carbonyl compounds with lactaldehyde in excess of 99%. Thin-layer chromatography plates developed in solvent system (1) and visualized with 3-methyl-2-benzothiazolinone hydrazone also indicated at least 6 carbonyl compounds were present in minor amounts. Paper chromatograms developed in solvent system 3 indicated 5 ninhydrin positive compounds were present in the above recrystallized DL-lactaldehyde. Despite the above contaminants, the DL-lactaldehyde dimer melted at 103-05°C; reported 105°C (74). The 2,4-dinitrophenylhydrazone was also prepared, m.p. 156°C; reported 156.5-157°C (70). It should be pointed out that previous workers who have prepared lactaldehyde by the above procedure have assumed purity on the physical evidence of melting points (68, 70-73, 262); the possible effects of the

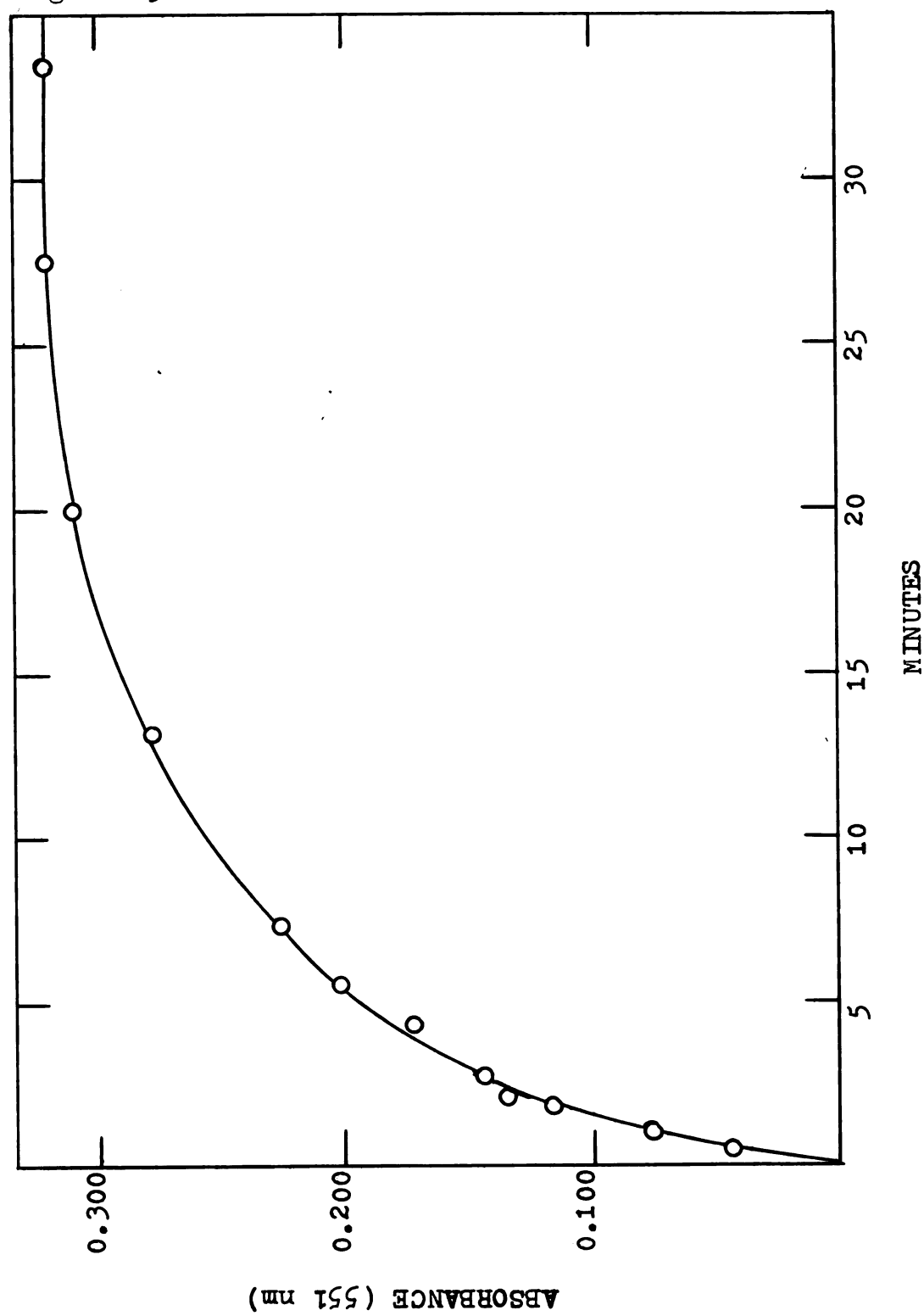
13-15 contaminants upon the enzymatic systems which were under investigation is a matter of conjecture. The recrystallized lactaldehyde was used without further purification for the synthesis of KDF.

DL-Lactaldehyde (0.34 g) was dissolved in 2.0 ml water, and the pH was adjusted to 7.2. Oxalacetate (0.226 g) was dissolved gradually in 2.0 ml water; the pH was maintained at 8.5 with a Sargent recording pH stat using 8.5 molar NaOH. The lactaldehyde solution was then added to the oxalacetate solution, and the pH was adjusted to and maintained at 11.0 at 25°C. The progress of the reaction was followed by withdrawing aliquots from the reaction mixture and analyzing for 2-keto-3-deoxy acids with TBA.

The reaction was judged to be complete within 20 minutes (Figure 43) after which the solution was neutralized with AG50W-X8 and filtered. The filtrate was treated with Norit (1.5 g) until it was colorless, and the resultant solution was applied to an AG1-X8 formate column (200-400 mesh, 0.5 x 15 cm) and eluted with one liter of a 0-0.6 molar formic acid gradient followed by one liter of a 0.6-5.5 molar formic acid gradient. The column was washed free of non-ionic substances with water (0.5 l) before initiating elution procedures. Fractions (15 ml) were collected and analyzed with the TBA and semicarbazide assays. One TBA-positive peak and

Figure 43. Reaction rate of the chemical synthesis of 3,6-dideoxy-hexulosonic acid. Aliquots (5 µl) were withdrawn from the reaction mixture and were assayed by the TBA assay. Details are given in the text.

Figure 43



two semicarbazide-positive peaks were observed in the elution profile (Figure 44). The fractions containing the TBA-positive peak were pooled and freed of formic acid by three evaporations under reduced pressure; yield 680 μmoles, 40%. The second semicarbazone peak was eluted at approximately 5 molar formic acid and thus resembled pyruvic acid (18); the fractions containing the second semicarbazone peak were pooled and treated as above and yielded a substance which reacted with lactic acid dehydrogenase and NADH.

The pooled fractions (TBA-positive peak) were rechromatographed on the AG1-X8 formate column with a 0-0.6 molar formic acid gradient; one slightly skewed peak was obtained (Figure 45). The compound co-chromatographed with enzymatically prepared KDF in solvent systems 2 and 6. Chemically prepared 3,6-dideoxy-DL-hexulosonic acid, DL-KDF, prepared above, was cleaved to 0.485 mole pyruvate per mole by KDF aldolase (presented in the next section). The rate of release of β-formyl pyruvate in the periodate oxidation of chemically synthesized DL-KDF was identical to that of enzymatically prepared D-KDF using the techniques described in Figure 42.

Chromatography of the pooled fractions containing DL-KDF on an AG1-X8 carbonate column (200-400 mesh, 0.5 x 15 cm) by the procedure of Hershberger, Davis and Binkley (75) or on an AG1-X8 borate column (200-400 mesh, 1 x 30

Figure 44. Elution of 3,6-dideoxy-DL-hexulosonic acid and pyruvic acid from Dowex-1 formate using a 0-5.5 molar formic acid gradient. Details are given in the text. Fractions were analyzed with the semicarbazide assay.

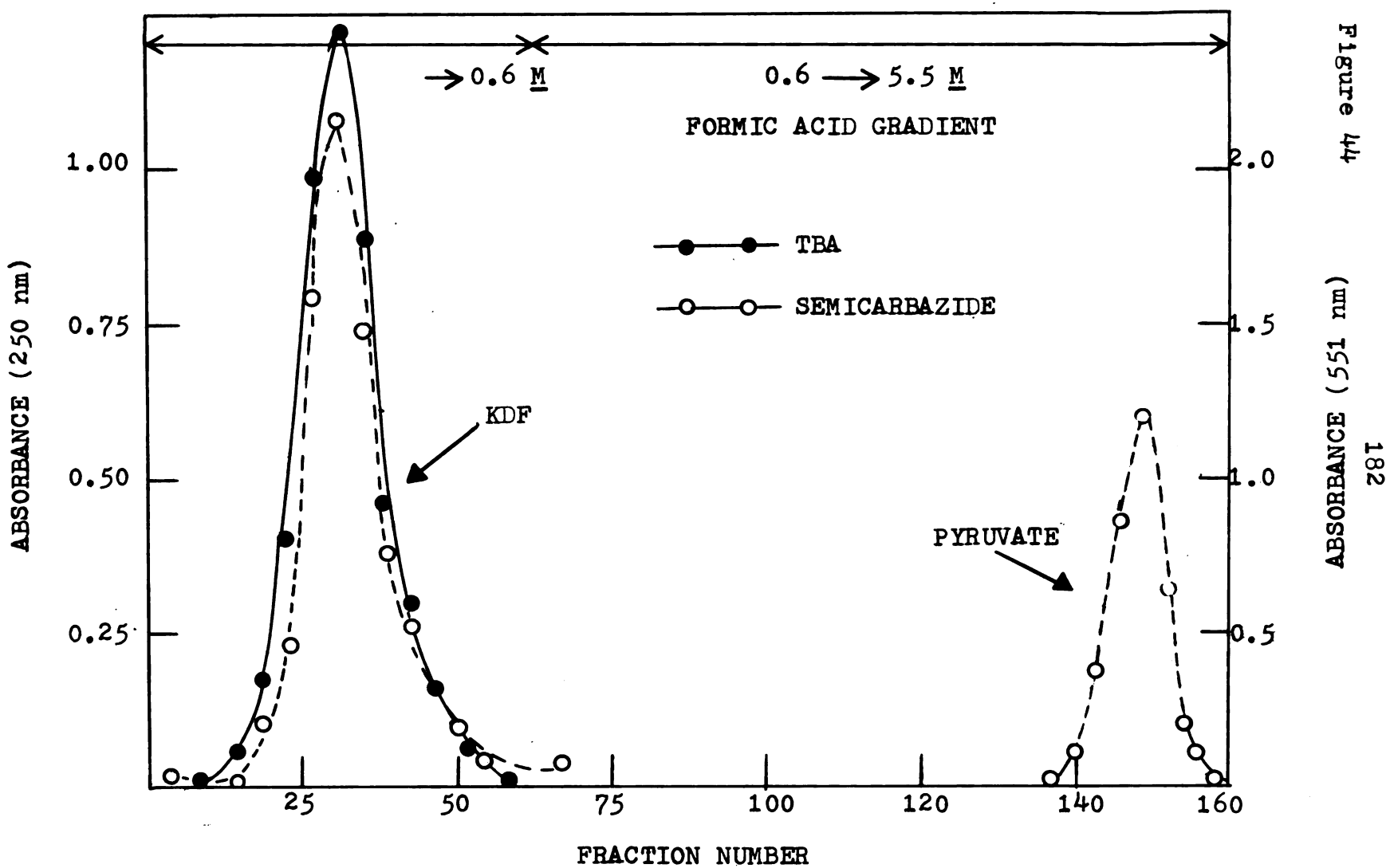
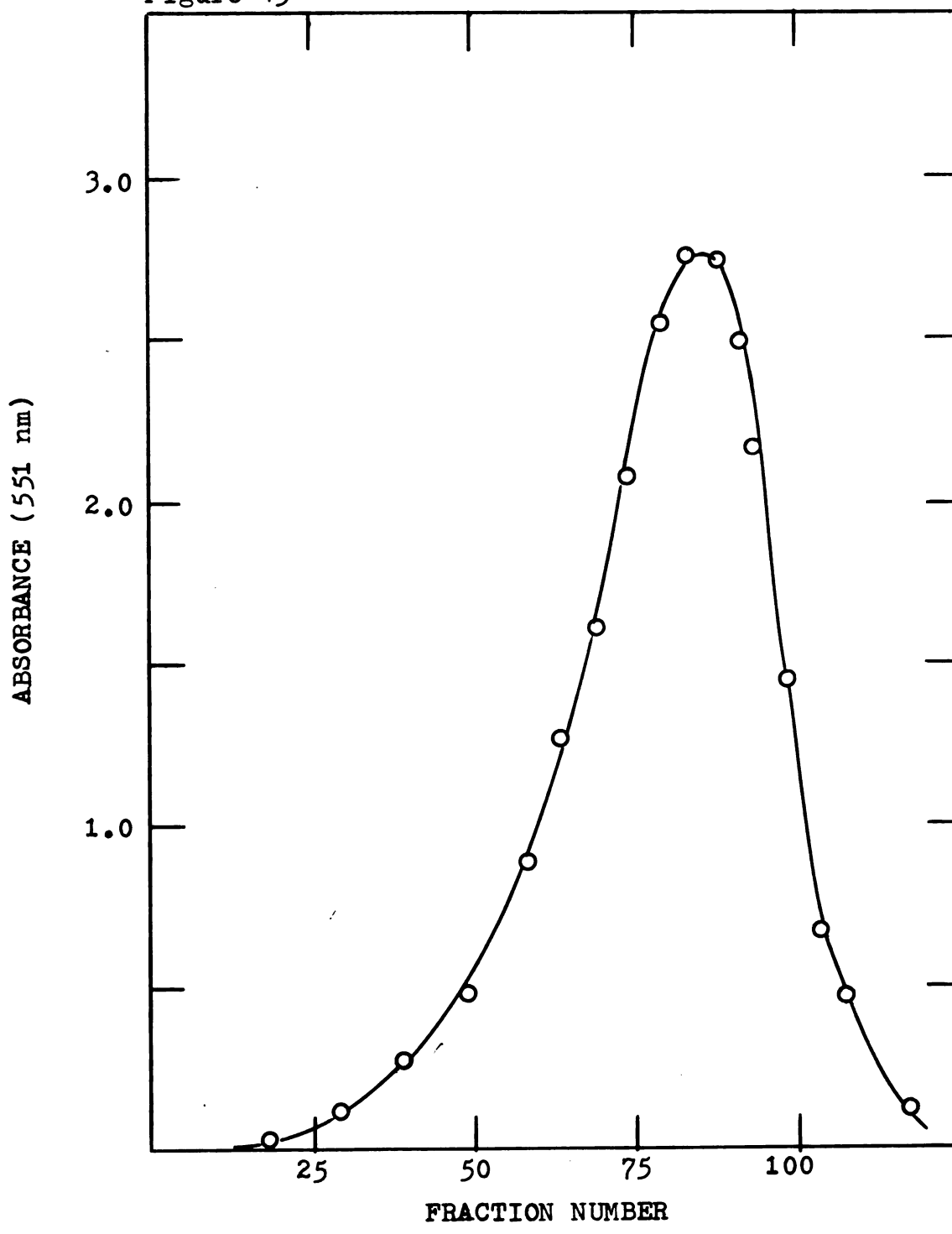

→0.6 M
0.6 → 5.5 M
FORMIC ACID GRADIENT
TBA
SEMICARBAZIDE
KDF
PYRUVATE
ABSORBANCE (250 nm)
ABSORBANCE (551 nm)
1.00
0.75
0.50
0.25
2.0
1.5
1.0
0.5
25
50
75
100
120
140
160
FRACTION NUMBER

Figure 44

Figure 45. Elution of 3,6-dideoxy-DL-hexulosonic acid from Dowex-1 formate using a 0-0.06 molar formic acid gradient. Details are given in the text.

Figure 45



cm) by the procedure of Samuelson, Ljungquist and Parck (76) also yielded one peak. It was concluded that the isomer of 3,6-dideoxy-DL-hexulosonic acid that was formed was the threo isomer on the basis of co-chromatography with enzymatically prepared 3,6-dideoxy-D-threo-hexulosonic acid (KDF), cleavage by KDF aldolase, and on the basis of the Dowex-1-borate, -formate and -carbonate chromatography in which one peak was consistantly obtained. It was also concluded that the condensation was stereospecific under the conditions employed or that the erythro isomer was less stable under the isolation conditions.

D-KDF was also synthesized using the same conditions employed for DL-KDF with D-lactaldehyde prepared from L-threonine. The product from the AG1-X8 formate column was identical to enzymatically prepared KDF and was cleaved to 0.98 mole pyruvate per mole KDF by KDF aldolase. It is a possibility that the erythro isomer was a contaminant to the extent of 1.5% and was not separable from the threo isomer utilizing the above chromatographic procedures. It is not known whether the erythro isomer is cleaved by KDF aldolase.

Synthetic KDF was found to be unstable under the solvent extraction procedure and partially degraded to a compound which reacts with lactic acid dehydrogenase and NADH and co-chromatographs with pyruvate. High pH

(>9) was found to be deleterious and resulted in orange colored solutions and large lossess of TBA-positive material. KDF forms a lactone which is easily hydrolyzed at pH 8.0. KDF may be stored indefinitely at -20°C and pH 7.4 without deterioration. KDF could also be isolated as the partially crystalline potassium salt by lyophilization.

The structure of the dehydration product of D-fuconate has been rigorously determined by degradation, derivatization, and, finally, by chemical synthesis. The enzymatically prepared compound has been found to be identical in all respects to the chemically prepared compound, and it has been concluded that the dehydration product of D-fuconate is 3,6-dideoxy-D-<u>threo</u>-hexulosonic acid.

F. CHARACTERIZATION OF 2-KETO-3-DEOXY-D-FUCONATE ALDOLASE

1. Preliminary Experiments

By analogy to known reactions of deoxy hexulosonic acids, the most likely degradative reaction for KDF would be cleavage by an aldolase. The pathways of galactose and glucose metabolism in some bacteria involve 2-keto-3-deoxy-D-galactonate and 2-keto-3-deoxy-D-gluconate respectively (78, 19), which undergo prerequisite phosphorylation by specific kinases prior to cleavage by specific aldolases (19, 77-79); however, KDF cannot be phosphorylated in a similar reaction due to the lack of an hydroxyl group on C-6 and thus would not be expected to be cleaved by these two aldolases. The pathway of 2-keto-3-deoxy-L-arabonate degradation in some bacteria involves dehydration to form α-keto glutarate semialdehyde (54-56); KDF cannot participate in a similar reaction, once again due to the lack of an hydroxyl group at C-6.

An enzyme has been described in a pseudomonad, however, which is instrumental in the degradation of the rare carbohydrate D-arabinose and which cleaves 2-keto-3-deoxy-D-arabonate to form pyruvic acid and glycolic acid (80). Consequently, a similar aldolase was sought which could cleave KDF, presumably to pyruvate and D-lactaldehyde. Spectrophotometric analysis for such an aldolase

using KDF, cell extract, lactic acid dehydrogenase, NADH and the appropriate controls indicated slow cleavage (< 0.3 μmole per hour per mg protein); further, incubation of KDF with cell extracts resulted in a decrease in TBA-positive material also amounting to 0.3 μmole per hour per mg protein. The enzyme was purified on Sephadex G-200, and activity was located with the lactic acid dehydrogenase and the TBA assays; the peaks were superimposable (Figure 46).

Further investigation indicated that the enzyme had an absolute requirement for a divalent cation, was protected by thiols, and also cleaved 2-keto-3-deoxy-L-arabonate (KDA), in addition to KDF. The products of the cleavage of KDF or KDA were tentatively characterized as pyruvate, by its reaction with lactic acid dehydrogenase and NADH, and as an aldehyde, by the formation of a tetraazopentamethine cyanine derivative with 3-methyl-2-benzothiazolinone hydrazone.

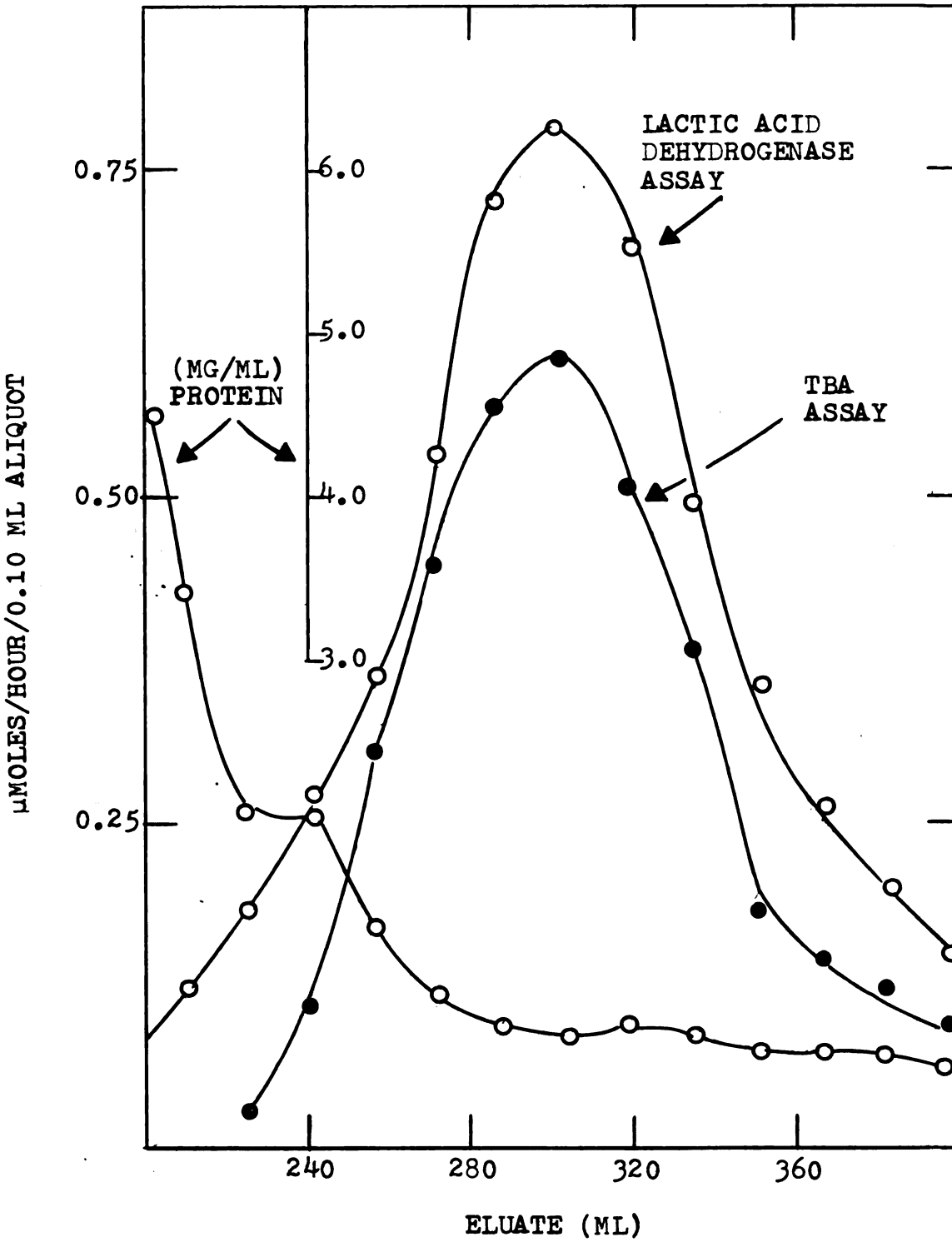
The observed cleavage of 2-keto-3-deoxy-L-arabonate (L-KDA) was totally unexpected since the only previously known pathway for L-KDA degradation, as elucidated by Weimberg and Doudoroff (54) and Weimberg (55), involves an entirely different sequence of reactions, as shown below.

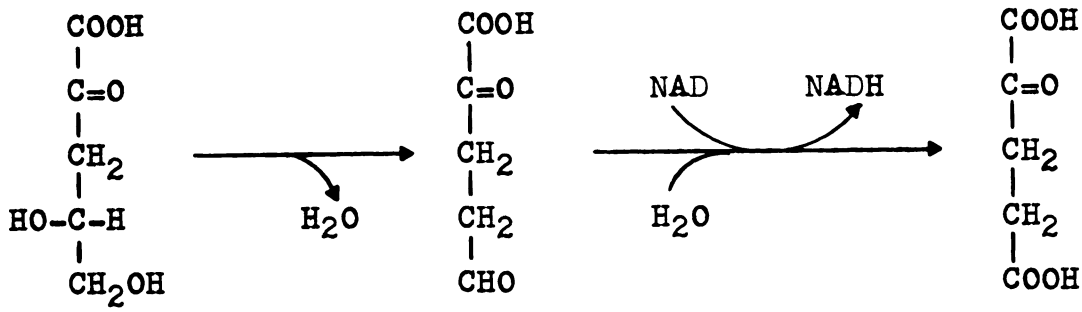
Figure 46. Sephadex G-200 profile of protamine sulfate-treated cell extract from D-fucose-grown cells. The fractions were analyzed for aldolase activity on KDF by two means: (i) the TBA assay; and (ii) the lactic acid dehydrogenase-coupled assay. The column (3 x 35 cm) was equilibrated with 0.01 molar sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer.

TBA endpoint assay: The assay consisted of 100 µl each fraction, 0.0849 µmole KDF, and 0.2 µmole sodium phosphate buffer (pH 7.0) in a total volume of 0.23 ml. The reaction was incubated at 27°C for 20 minutes before quenching with periodate.

Lactic acid dehydrogenase-coupled assay: The assay consisted of 30 µl of each fraction, 0.07 µmole NADH, 13 µg lactic acid dehydrogenase, 5 µmoles KDF (pH 7.0) and 5 µmoles sodium phosphate buffer in a total volume of 0.15 ml. The absorbancy change at 340 nm and 25°C was followed, and controls to correct for endogenous pyruvate and NADH oxidase were run.

Figure 46


LACTIC ACID
DEHYDROGENASE
ASSAY
TBA
ASSAY
(MG/ML)
PROTEIN
0.75
0.50
0.25
6.0
5.0
4.0
3.0
240
280
320
360
µMOLES/HOUR/0.10 ML ALIQUOT
ELUATE (ML)



L-KDA α-Keto glutarate semialdehyde α-Keto glutarate

The previously known pathway for L-KDA degradation involves the dehydration of L-KDA to form α-keto glutarate semialdehyde which is subsequently oxidized to α-keto glutarate. The L-KDA dehydratase of these workers was routinely assayed by following the rate of NAD^+ reduction or the rate of decrease of TBA-positive material (since α-keto glutarate semialdehyde will not yield β-formyl pyruvate upon periodate oxidation). Repeated attempts to demonstrate the presence of the above pathway in the pseudomonad under investigation were totally unsuccessful: (i) there was no NAD^+ or $NADP^+$ reduction when L-arabonate or L-KDA were added to reaction mixtures containing varying amounts of cell extracts, $NAD(P)^+$, metal ions, various buffers, and thiols; (ii) no reduction of α-keto glutarate with NADH or NADPH could be demonstrated; (iii) loss of TBA-positive material when KDA was incubated with Sephadex G-200 fractions of protamine sulfate-treated cell extracts corresponded to the aldolase peak as measured by KDF cleavage; (iv) the rate of decrease

of TBA-positive material when cell-free extracts were incubated with KDA corresponded to the rate of KDF cleavage measured in the same manner; and (v) nearly quantitative conversions (98%) of L-arabonate to KDA, as measured by the TBA assay, were carried out by cell-free extracts, indicating no dehydration of KDA occurred. Thus, it was concluded that KDA dehydratase was not present in this microorganism and that a new pathway for L-arabonate metabolism had been discovered.

The aldolase, hereafter referred to as KDF aldolase, was totally inactive on 2-keto-3-deoxy-D-galactonate, 2-keto-3-deoxy-D-gluconate, and their respective 6-phospho-derivatives. Keto deoxy galactonate formed by galactonate dehydratase was found to be phosphorylated by a kinase prior to cleavage, and is demonstrated in Figure 47; in addition, 2-keto-3-deoxy-6-phospho-D-gluconate was found to be cleaved by cell extracts. Thus, it was concluded that the pathways for glucose and galactose metabolism in the pseudomonad under investigation were identical to the previously described pathways (19, 77-79). Thus, despite the structural similarities between D-fucose and D-galactose, the two carbohydrates are degraded by entirely different routes; this will be discussed to a greater extent in the next section.

The results of the initial experiments indicated that an enzyme was present in D-fucose- and L-arabinose-grown cells which could cleave KDA or KDF to pyruvate

Figure 47. Sephadex G-200 profile of 40-60% ammonium sulfate fraction of cell extract from L-arabinose grown cells. Fractions were assayed for 2-keto-3-deoxy-D-galactonate kinase and 2-keto-3-deoxy-6-phospho-galactonate aldolase, and KDF aldolase activities. The column (4 x 60 cm) was equilibrated with 0.05 molar sodium phosphate buffer (pH 7.7), and the enzymes were eluted with the same buffer.

2-Keto-3-deoxy-D-galactonate kinase assay: The assay consisted of 0.5 µmole ATP, 1.0 µmole $MgCl_2$, 0.75 µmole 2-keto-3-deoxy-D-galactonate and 100 µl of each fraction. The fractions were then incubated for 30 minutes at 27°C and then assayed with 2-thiobarbituric acid. The product, 2-keto-3-deoxy-6-phospho-D-galactonate, is not oxidized by periodate, and thus, the decrease in TBA-positive material was followed.

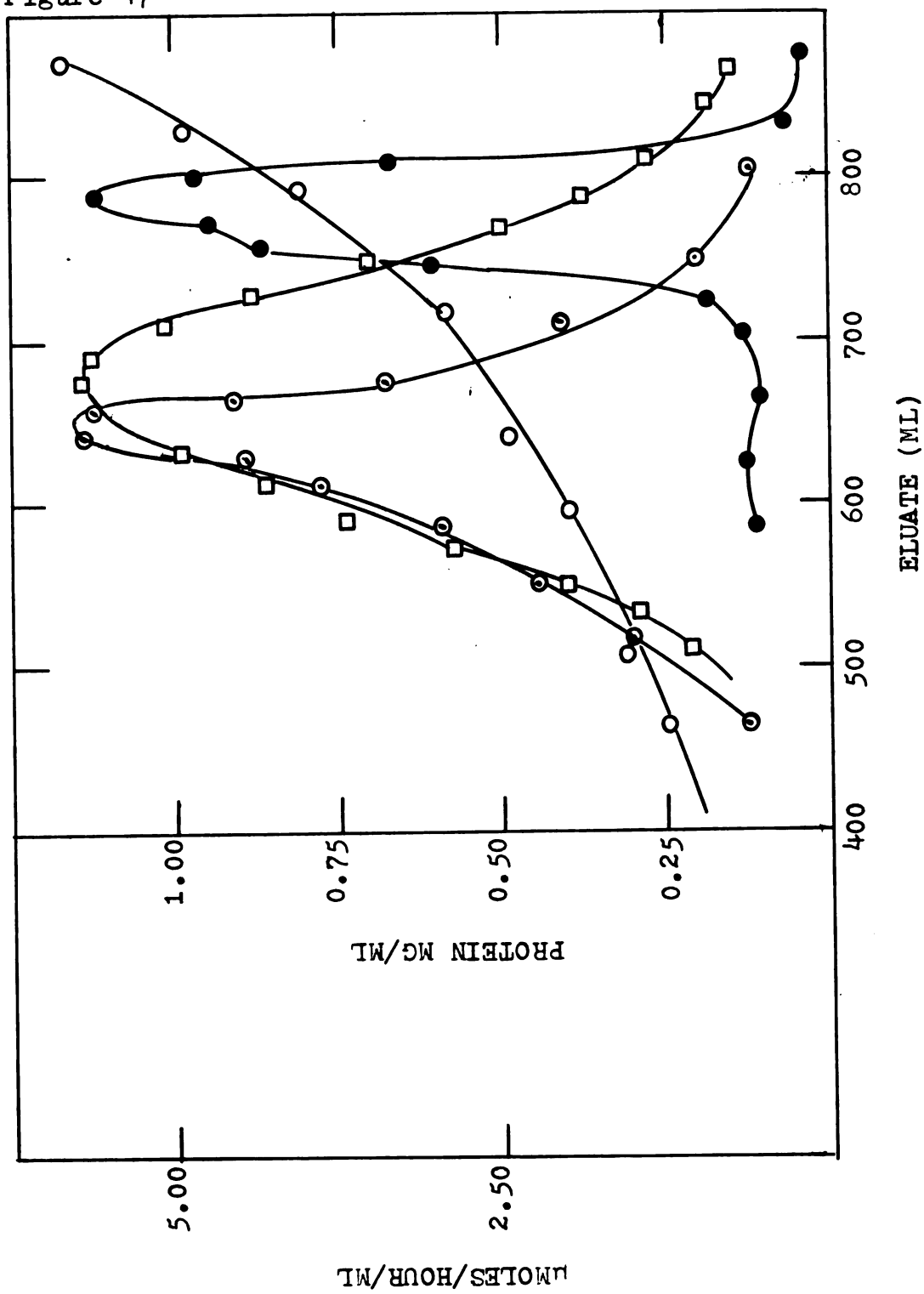
2-Keto-3-deoxy-D-galactonate aldolase assay: The assay consisted of 1.0 µmole 2-keto-3-deoxy-6-phospho-D-galactonate, 13 µg lactic acid dehydrogenase, 0.07 µmole NADH, and 100 µl of each fraction in a total volume of 0.15 ml. Activity was determined spectrophotometrically at 340 nm and 25°C. Controls to correct for endogenous NADH oxidase and pyruvate were also run.

KDF aldolase assay: The same spectrophotometric procedure was employed as described in Figure 46.

Legend:

⊙—⊙ KDF Aldolase
●—● 2-Keto-3-deoxy-6-phospho-D-galactonate Aldolase
□—□ 2-Keto-3-deoxy-D-galactonate Kinase
○—○ Protein

Figure 47


μMOLES/HOUR/ML
5.00
2.50
PROTEIN MG/ML
1.00
0.75
0.50
0.25
ELUATE (ML)
400
500
600
700
800

and an aldehyde. The enzyme required a divalent cation, was stabilized by thiols, was inactive on other deoxy hexulosonic acids, and was distinct from previously reported enzymes for D-galactose, D-glucose, and L-arabinose metabolism. In addition to the conclusion that the aldolase was the terminal enzyme instrumental in D-fucose metabolism, it was also concluded that a new L-arabinose pathway had been discovered.

2. Purification

Cell extracts of L-arabinose-grown cells were prepared as described in Experimental Procedures with the exception that the cells were disrupted in 0.10 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4) to eliminate NADH oxidase. Except where indicated otherwise, the fractionation procedures were carried out at 0-4°C. A summary of the purification is given in Table XXX.

Protamine Sulfate Fractionation- The protein concentration of the cell extract was adjusted to 16 mg per ml by dilution with 0.10 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). Ammonium sulfate (18.5 g) was dissolved in 700 ml cell extract to a final concentration of 0.20 molar, and then 140 ml of a 2% protamine sulfate solution in the above buffer was added with stirring to a final concentration of 0.33%. After 30 minutes the

Table XXX. Purification of KDF aldolase*.

Fraction	Units	Specific Activity	Fold	280/260
Cell extract	66,600	4.1	1	.62
Protamine sulfate	66,100	4.9	1.2	.87
Ammonium sulfate	57,400	16.5	4.1	1.20
Sephadex G-200	14,600	69.8	17.0	1.38
Heat Step	10,600	208	50.7	1.59

*KDA was used as substrate.

suspension was centrifuged at 40,000 x g for 10 minutes, and the resulting precipitate was discarded.

Ammonium Sulfate Fractionation- The 40,000 x g supernatant (840 ml) was brought to 40% of saturation by the addition of 165 g of ammonium sulfate, and the resulting precipitate was centrifuged down and discarded. The supernatant was then brought to 60% of saturation with 111 g of ammonium sulfate, and the resulting precipitate was collected by centrifugation and dissolved in 82 ml 0.10 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). The protein concentration at this stage was 42 mg per ml.

Sephadex G-200 Chromatography- The above 40-60% fraction was placed on a column (6 x 60 cm) of Sephadex G-200 equilibrated with 0.05 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). The enzyme was eluted with the same buffer. Fractions (15 ml) were collected, and those which contained the most activity were pooled (105 ml total).

Heat Step- The protein concentration of the pooled Sephadex G-200 fractions was adjusted to 2 mg per ml with 0.10 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). Sufficient 0.10 molar magnesium chloride was added to 10 ml of the pooled Sephadex G-200 fractions to bring the concentration to 0.6 mM. The protein solution was immersed in a 55°C bath for 8.5 minutes and then chilled

in ice. The flocculent precipitate was removed by centrifugation at 40,000 x g for 10 minutes. The supernatant was used as the purified aldolase fraction. The protein concentration was 0.5 mg per ml.

3. Properties

pH Optimum- KDF aldolase activity as a function of pH was maximal at pH 8.1 in Hepes and Tris-HCl buffers (Figure 48).

Substrate Specificity- KDF aldolase cleaved only KDF and KDA among the nine deoxy acids tested. The deoxy acids which were not cleaved by the aldolase were 2-keto-3-deoxy-D-galactonate, 2-keto-3-deoxy-D-gluconate, 2-keto-3-deoxy-6-phospho-D-galactonate, 2-keto-3-deoxy-6-phospho-D-gluconate, 2-keto-4-hydroxy glutarate, 3-deoxy-D-xylo-hexonate, and N-acetylneuraminic acid. The first four deoxy hexulosonic acids which did not serve as substrates also did not reduce the rate of cleavage of 20 mM KDF when added at equimolar concentrations.

From the Lineweaver-Burk plots presented in Figures 49 and 50, the K_m values for KDF and KDA were determined to be 2.9 mM and 1.8 mM respectively. KDF was cleaved at 47% of the rate of KDA cleavage under saturating conditions.

Metal Ion Activation- KDF aldolase activity has an absolute requirement for a divalent cation as indicated in Table XXXI. Mn^{2+} was found to be the most effective

Figure 48. pH Optimum of KDF aldolase. The standard assay was employed except that the pH and the buffer composition were varied, as indicated, with the aldolase concentration constant. The heat-treated Sephadex G-200 fraction with the highest specific activity was used. Each buffer was 0.20 molar. The pH measurements were determined on duplicate reaction mixtures. The pH did not change during the course of the reaction period. Hepes buffer was used with KDA as the substrate, and Tris-HCl was used with KDF as the substrate.

Figure 48

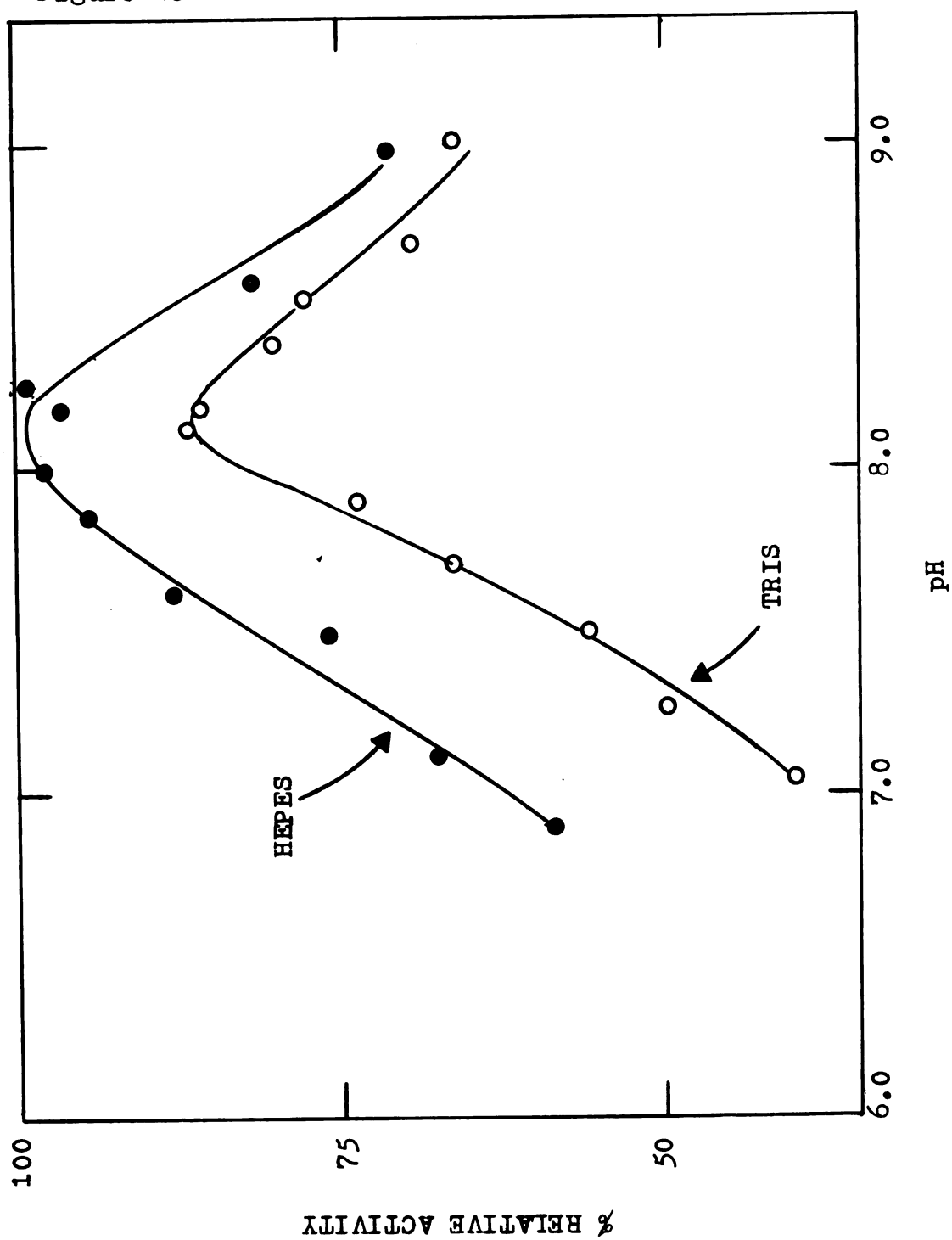


Figure 49. Lineweaver-Burk plot relating KDF concentration to KDF aldolase activity. The standard assay was employed except that the concentration of KDF was varied, as indicated, with the aldolase concentration constant. The heat-treated Sephadex G-200 fraction with the highest specific activity was used.

Figure 49

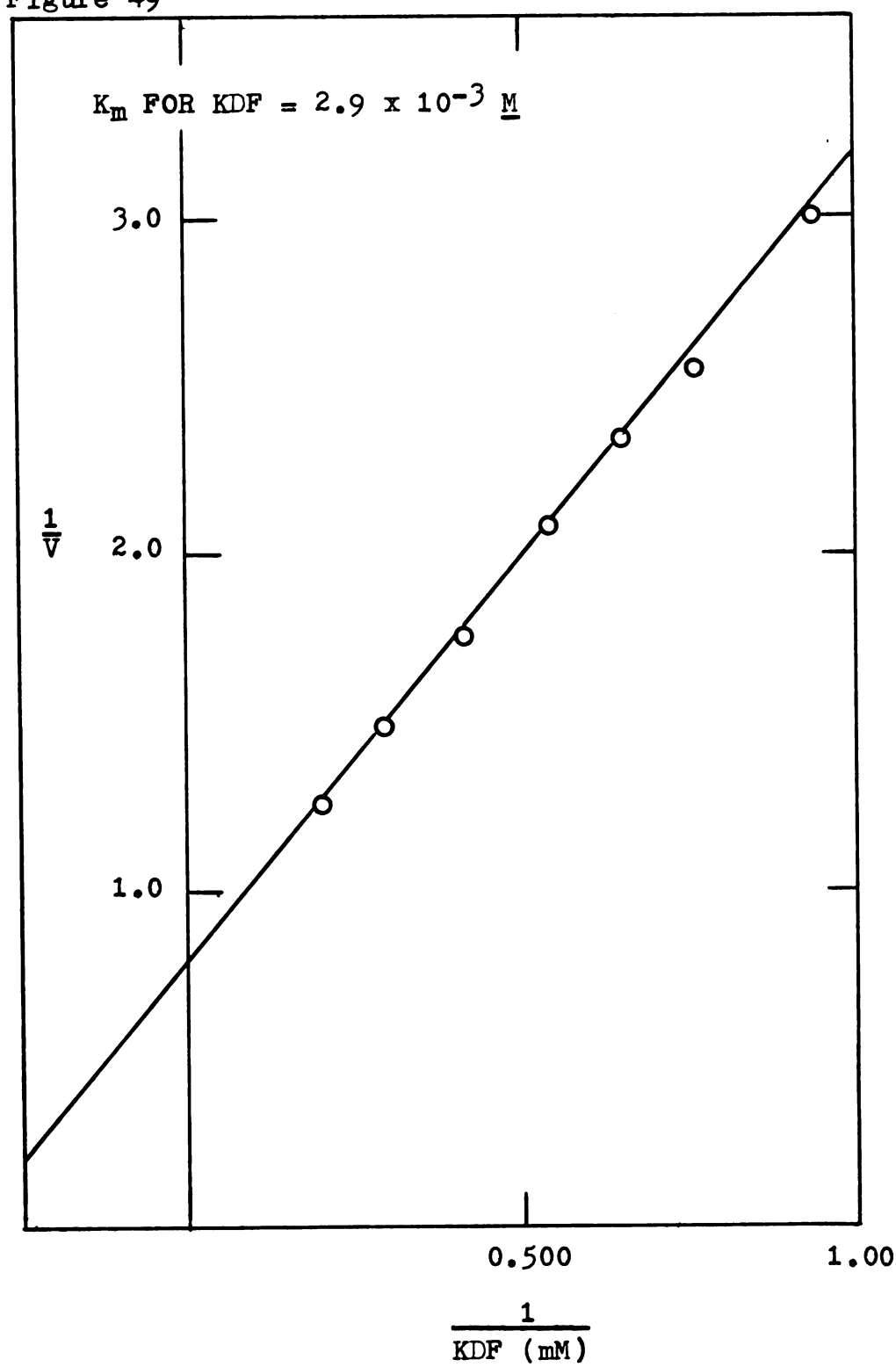


Figure 50. Lineweaver-Burk plot relating KDA concentration to KDF aldolase activity. The standard assay was employed except that the KDA concentration was varied as indicated. The heat-treated Sephadex G-200 fraction with the highest specific activity was utilized.

Figure 50

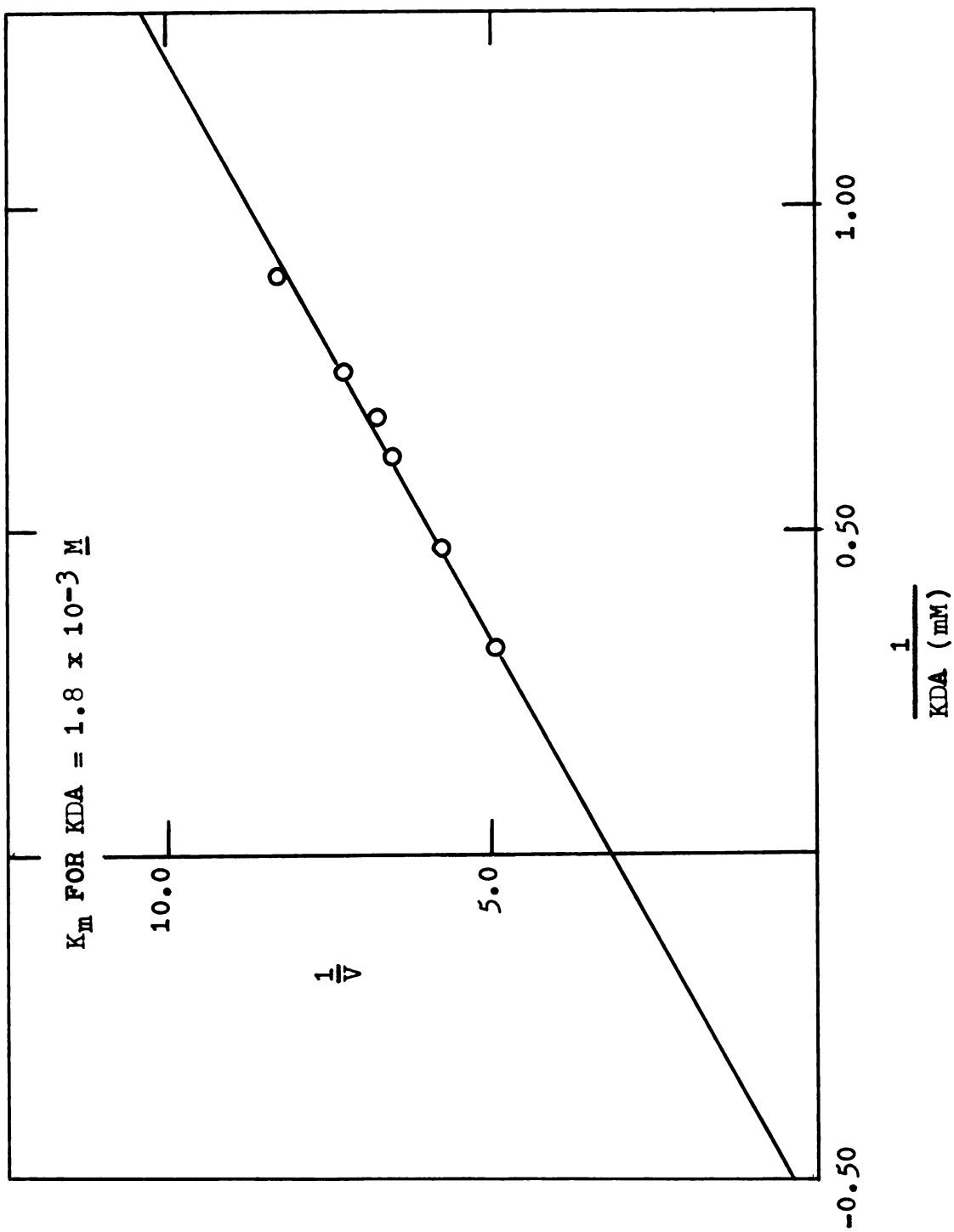


Table XXXI. Effect of metal ions on KDF aldolase. The standard assay was used with the exceptions that 0.2 μmole of the metal salt and 0.05 μmole EDTA (pH 7.0) were added. The heat-treated Sephadex G-200 fraction with the highest specific activity was used. KDF was used as the substrate.

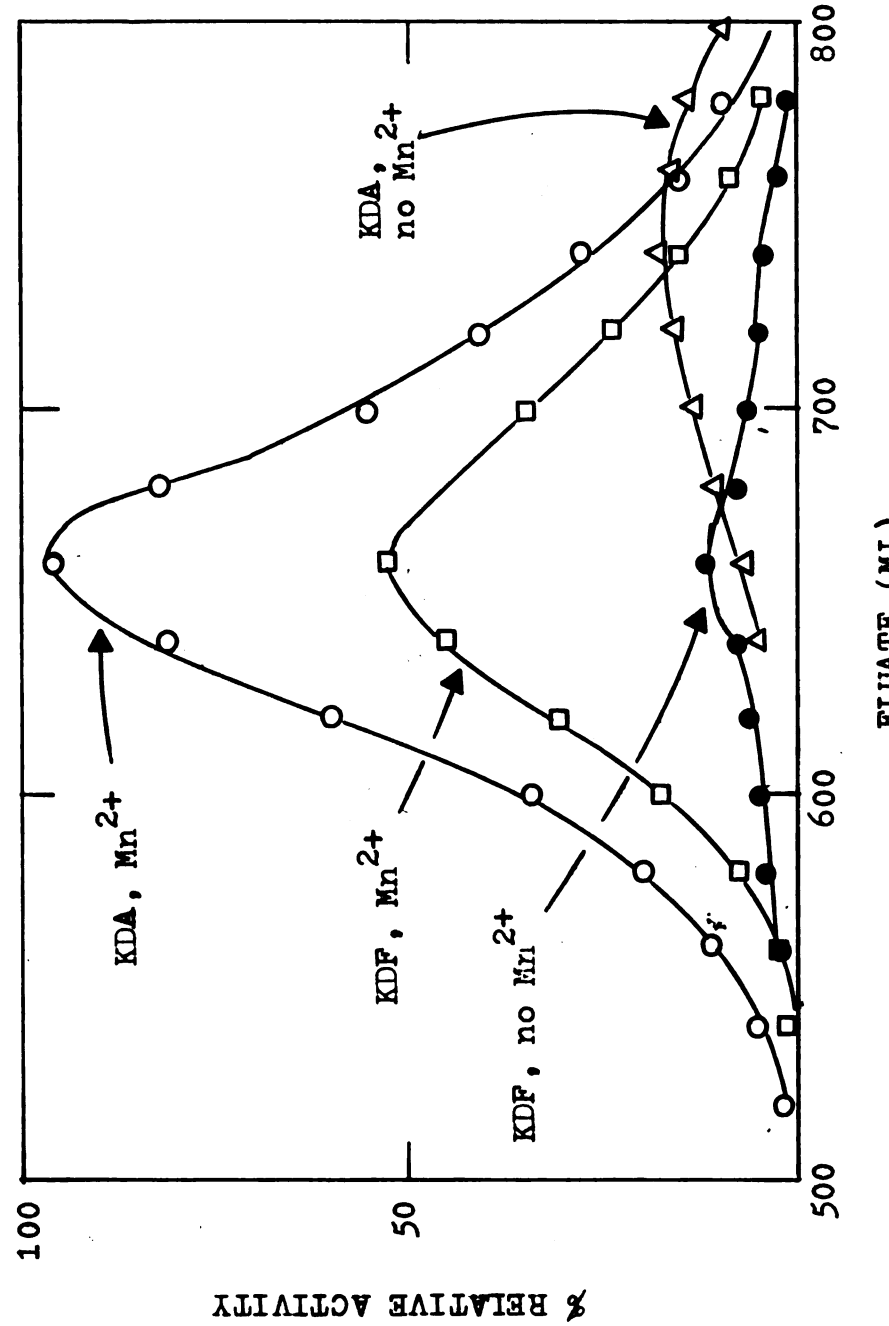
Compound	Relative Rate
$MnCl_2$	100%
$CoCl_2$	95
$MgCl_2$, $MgSO_4$	54
$NiCl_2$	45
$CuSO_4$	0
No ion added	2

activator at 3.3 mM with Co^{2+}, Mg^{2+}, and Ni^{2+} being partially effective. There was no variation in activity when assays were run in the presence of 3 mM to 60 mM $MnCl_2$. Aldolase activity with 0.33 mM EDTA in the absence of added metal ions was 2% of the control activity.

Analysis of Sephadex G-200 fractions for KDF and KDA cleavage in the presence and absence of added metal ion yielded some interesting results (Figure 51). Under conditions of saturating KDA and in absence of metal ion, one small aldolase peak was obtained at about 740 ml of the eluate (the 740 peak); activity in this peak was not diminished in the presence of 0.66 mM EDTA. Under conditions of saturating KDF another small aldolase peak was obtained at about 660 ml of the eluate (the 660 peak); the activity in this peak was completely abolished with 0.66 mM EDTA. When 3.0 mM $MnCl_2$ was incorporated into the assay, a new peak 10-fold higher in activity was observed with both KDA and KDF cleavage which was centered at the 660 peak. The 740 peak represents a lower molecular weight protein than the 660 peak, and it is tempting to suggest that the 740 peak represents a "monomer" fraction and that the 660 peak represents a "dimer" fraction. The "monomers," formed by dissociation of the "dimers," slowly cleave KDA but not KDF in the absence of added metal ion; the "dimers" slowly

Figure 51. Analysis of KDF and KDA cleavage by Sephadex G-200 fractions in the absence and in the presence of $MnCl_2$. No thiol was used in the otherwise standard assay.

Figure 51


KDA, Mn^{2+}
KDF, Mn^{2+}
KDF, no Mn^{2+}
KDA, no Mn^{2+}
100
50
% RELATIVE ACTIVITY
500
600
700
800
ELUATE (ML)

cleave KDF but not KDA, also in the absence of metal ion. The "dimers," and not the "monomers" cleave both KDF and KDA at a 10-fold rate in the presence of added metal ion. Attempts to reassociate the "monomers" in the 740 peak with 2-thioethanol into "dimers" were consistently unsuccessful. The 740 peak was not present in preparations in which 2-thioethanol had been incorporated. These results are of a preliminary nature and deserve further investigation.

Sulfhydryl Activation- The enzyme is insensitive to thiol inhibitors and thiols during assay (Table XXXII), but is stabilized by thiols during purification. A time-dependent renaturation process has been observed to occur when Sephadex G-200 fractions containing the aldolase were incubated with 2-thioethanol (Figure 52).

Equilibrium Constant- Equilibrium positions of the cleavage reaction with respect to both substrates were determined (Table XXXIII). The equilibrium could be approached from both directions, and the reactions were exothermic in the direction of synthesis at 1 mM; the equilibrium constants for KDF and KDA were determined to be 0.12 and 0.36 mM respectively.

Stability- KDF Aldolase possesses a half-life of about 12 minutes at 55°C (Figure 53). The thermal denaturation profiles for KDF and KDA are linear and superimposable, suggesting that the activity in the heat-treated Sephadex G-200 fraction was due to a single

Table XXXII. Effects of thiols and thiol group inhibitors on KDF aldolase during assay. The standard assay was employed with the exception of the variation in the thiol or the thiol inhibitor added. The Sephadex G-200 heat-treated fraction with the highest specific activity was used. The pH of all reagents was 7.0. KDF was used as the substrate.

Compound	Concentration	Relative Rate
2-Thioethanol	3 mM	100
Reduced glutathione	3 mM	95
Dithiothreitol	3 mM	101
Iodoacetate	2 mM	97
p-Chloromercuribenzoate	2 mM	98
No thiol or thiol inhibitor added	—	100

Figure 52. Time-dependent thiol renaturation of KDF aldolase present in Sephadex G-200 fractions not previously stabilized with thiols. Sephadex G-200 fractions were incubated with 0.0143 molar 2-thioethanol at 4°C for 11 hours in 0.10 molar Bicine buffer (pH 7.4). Each fraction was analyzed at 0, 2, 4, and 11 hour intervals. The aliquots were analyzed with the standard assay. A control with no thiol was also run but was assayed only at t_o and t_{11}.

Figure 52

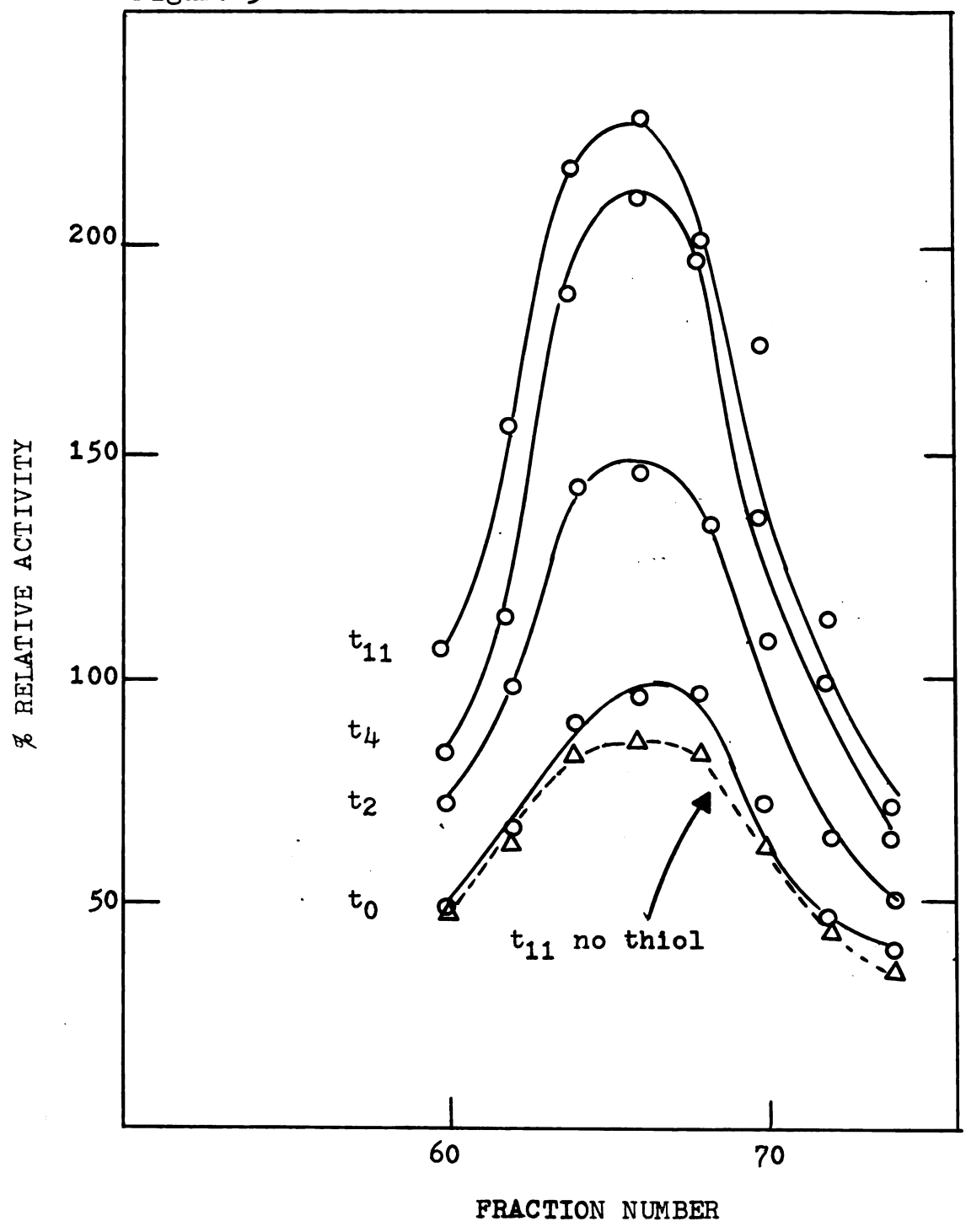
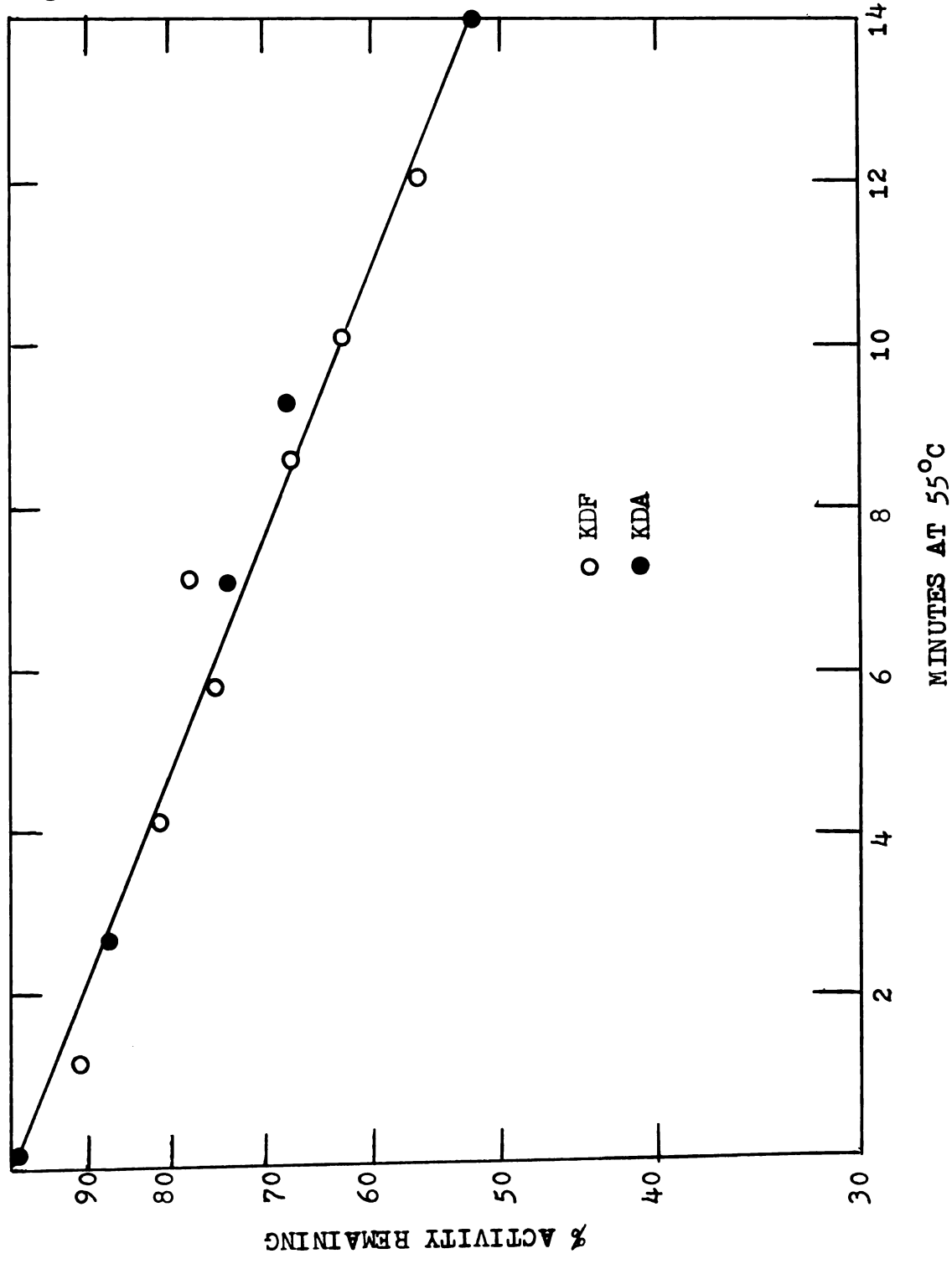

% RELATIVE ACTIVITY
200
150
100
50
t_{11}
t_4
t_2
t_0
t_{11} no thiol
60
70
FRACTION NUMBER

Table XXXIII. Equilibrium constants for the cleavage of KDF and KDA. Aliquots were withdrawn from an equilibrium solution and were assayed for 2-keto-3-deoxy acid, pyruvate, lactaldehyde, and glycolaldehyde.

Reaction Started With	Equilibrium Concentrations					
	pH	Pyruvate	Glycolaldehyde or Lactaldehyde	KDA	KDF	Keg
Glycolaldehyde + Pyruvate	7.40	1.85 mM	1.77 mM	8.45 mM	***	0.388 mM
KDA	7.40	1.23 mM	1.07 mM	3.81 mM	***	0.346 mM
KDF	7.50	0.633 mM	0.592 mM	***	2.62 mM	0.142 mM
Lactaldehyde + Pyruvate	7.50	1.13 mM	1.29 mM	***	13.8 mM	0.105 mM

Figure 53. Thermal denaturation of KDF aldolase at 55°C. The heat-treated Sephadex G-200 fraction was used. The protein solution (1 ml, 0.62 mg per ml) was heated in 0.10 molar Hepes and 0.143 mM 2-thioethanol (pH 7.4). Aliquots were withdrawn and assayed with the standard assay using both KDF and KDA.

Figure 53


% ACTIVITY REMAINING
90
80
70
60
50
40
30
2
4
6
8
10
12
14
MINUTES AT 55°C
KDF
KDA

enzyme. The enzyme, present in the heat-treated Sephadex G-200 fractions, lost 70% activity when stored at -20°C for three weeks in 0.10 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4), but was completely stable to lyophilization in the same buffer system. As mentioned previously, the aldolase appears to dissociate on Sephadex G-200; this could be prevented by incorporating 2-thioethanol into the eluant.

Induction- The inducibility of KDF aldolase was tested by growth on various substrates (Table XXXIV). D-Fucose and L-arabinose induced the aldolase to a level 3-4 fold over the non-induced level present in nutrient broth-grown cells.

4. Identification of Cleavage Products

The cleavage products of KDF and KDA in the aldolase reaction were initially characterized as a compound which reacted with lactic acid dehydrogenase and as a compound which reacted with 3-methyl-2-benzothiazolinone hydrazone to form a tetraazopentamethine cyanine dye. The cleavage products were suspected to be pyruvate and an aldehyde, either lactaldehyde from KDF or glycolaldehyde from KDA. More rigorous procedures were carried out to positively identify the products and are presented in this section.

A reaction mixture composed of 5 μmoles $MgCl_2$, 10 μmoles 2-keto-3-deoxy-D-fuconate, 7.35 μmoles 2-thioethanol, and 0.5 mg of a heat-treated Sephadex fraction

Table XXXIV. Induction of KDF aldolase by various growth substrates. The standard assay was employed with KDF as the substrate. Protein was estimated by the biuret assay. Cell extracts were prepared by sonic disruption in 0.10 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4).

Growth Substrate	Specific Activity (μmoles/hour/mg)
D-Fucose	3.91
L-Arabinose	4.23
D-Glucose	1.28
D-Galactose	1.34
Nutrient Broth	1.08

in a total volume of 1.0 ml was incubated at 27°C for 1 hour. The reaction was quenched by the addition of 1.5 ml of a saturated solution of 2,4-dinitrophenylhydrazine-HCl in 2 N HCl. The hydrazones were allowed to form for 20 minutes after which the mixture was extracted with three 10-ml portions of toluene. The toluene extracts were combined and reduced in volume under vacuum (bath temperature 20°C) to 0.5 ml. Thin-layer chromatography plates were spotted with 50 µl samples and were developed two-dimensionally in the following solvent systems (81): 1st dimension, n-butanol-ethanol-0.5 N NH_4OH (70:10:20); 2nd dimension, benzene-tetrahydrofuran-glacial acetic acid (57:35:8). The plates were sprayed with an aqueous 10% KOH solution after drying, and the R_f values and coloration of the 2,4-dinitrophenylhydrazones recorded. Two well-defined spots with distinct coloration were observed. The 2,4-dinitrophenylhydrazones were scraped from plates not sprayed with KOH and were eluted with 0.5 N NaOH and the absorption spectra of the alkaline solutions of the 2,4-dinitrophenylhydrazones recorded (82). Standard 2,4-dinitrophenylhydrazones of pyruvate and lactaldehyde were prepared by the method of Haway and Thompson (83) and Huff and Rudney (70), respectively, and were treated in the same manner as the extracted 2,4-dinitrophenylhydrazones with the exception that the solid standards were dissolved in toluene before spotting on thin-layer

plates. The results, in Table XXXV, indicate that pyruvate and lactaldehyde were formed from the cleavage of KDF. The absorption spectra of the standard 2,4-dinitrophenylhydrazones of pyruvate and lactaldehyde were also found to correspond to the absorption spectra of spots 1 and 2, respectively, and are presented in Figures 54 and 55. It was concluded that pyruvate and lactaldehyde were the cleavage products of KDF aldolase action upon KDF.

Cleavage Products of 2-Keto-3-deoxy-L-arabonate- The proceeding procedure was duplicated with KDA with the exception that the 2,4-dinitrophenylhydrazone of glycolaldehyde was prepared by the method of Powers, Tabakoglu, and Sable (84). The results are presented in Table XXXVI. It was likewise concluded that the products of KDA cleavage were pyruvate and glycolaldehyde.

The identification of the cleavage products of KDF and KDA were carried out by conversion of the cleavage products to the respective 2,4-dinitrophenylhydrazones and extraction with toluene, by two-dimensional thin-layer chromatography, and by comparison of the visible absorption spectra of the isolated 2,4-dinitrophenylhydrazones with standard 2,4-dinitrophenylhydrazones. It has been concluded that KDF aldolase cleaves KDF and KDA in an aldol-type cleavage reaction between C-3 and C-4.

Table XXXV. Identification of the products of KDF cleavage by KDF aldolase. Details are given in the text.

2,4-Dinitrophenylhydrazones	Color After KOH Spray	R_f Values	
		Dimension #1	Dimension #2
Pyruvate standard	Purple-brown	.37	.41
Spot #1 Cleavage reaction	Purple-brown	.36	.42
D-Lactaldehyde standard	Brown	.61	.67
Spot #2 Cleavage reaction	Brown	.61	.68

Figure 54. Absorption spectra of the 2,4-dinitrophenylhydrazones of pyruvic acid and spot #1 obtained from thin-layer chromatography of the KDF cleavage products. The solvent was 0.5 N NaOH. Details are given in the text.

Figure 54

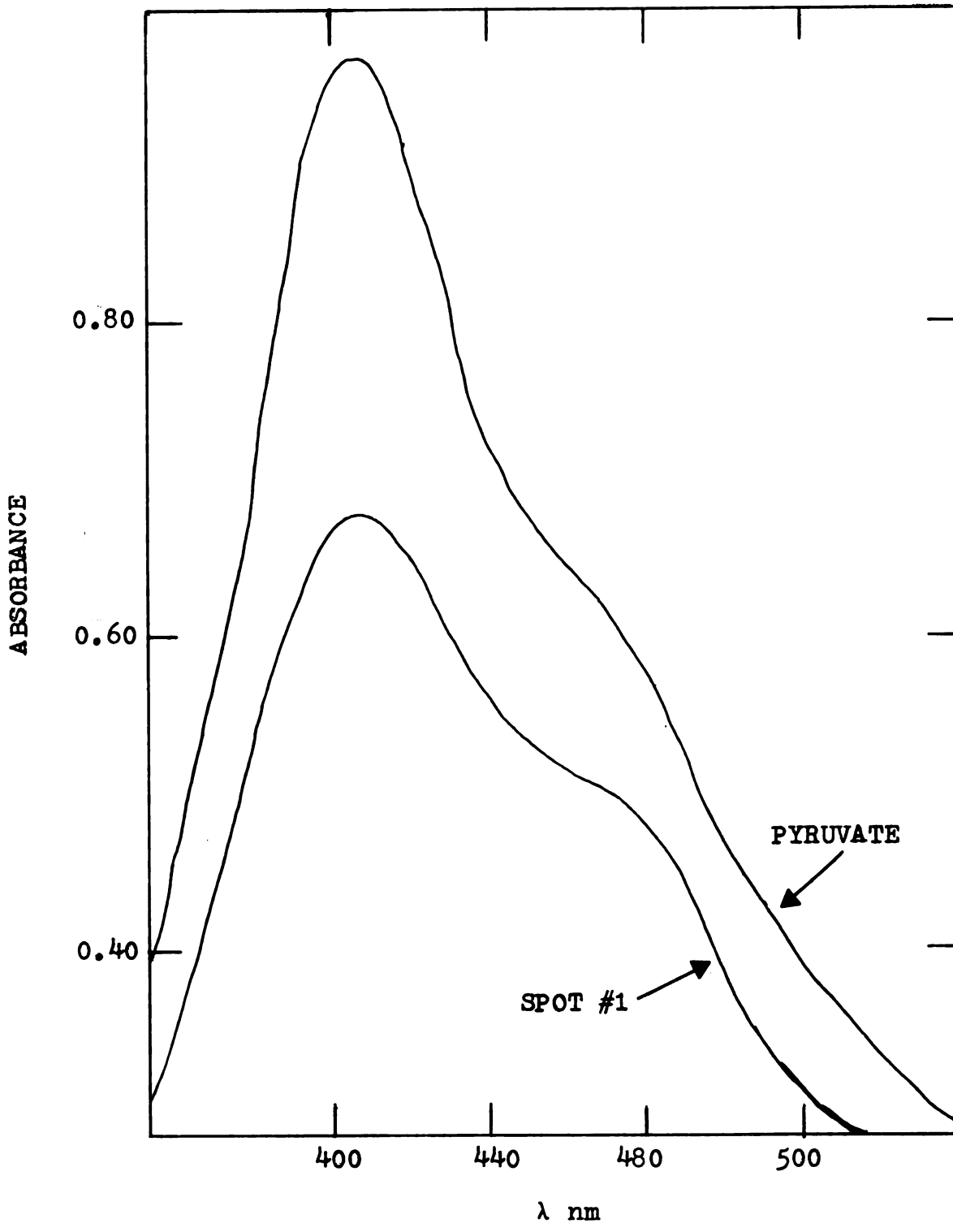


Figure 55. Absorption spectra of the 2,4-dinitrophenylhydrazones of D-lactaldehyde and spot #2 obtained from thin-layer chromatography of the KDF cleavage mixture. The solvent was 0.5 N NaOH. Details are given in the text.

Figure 55

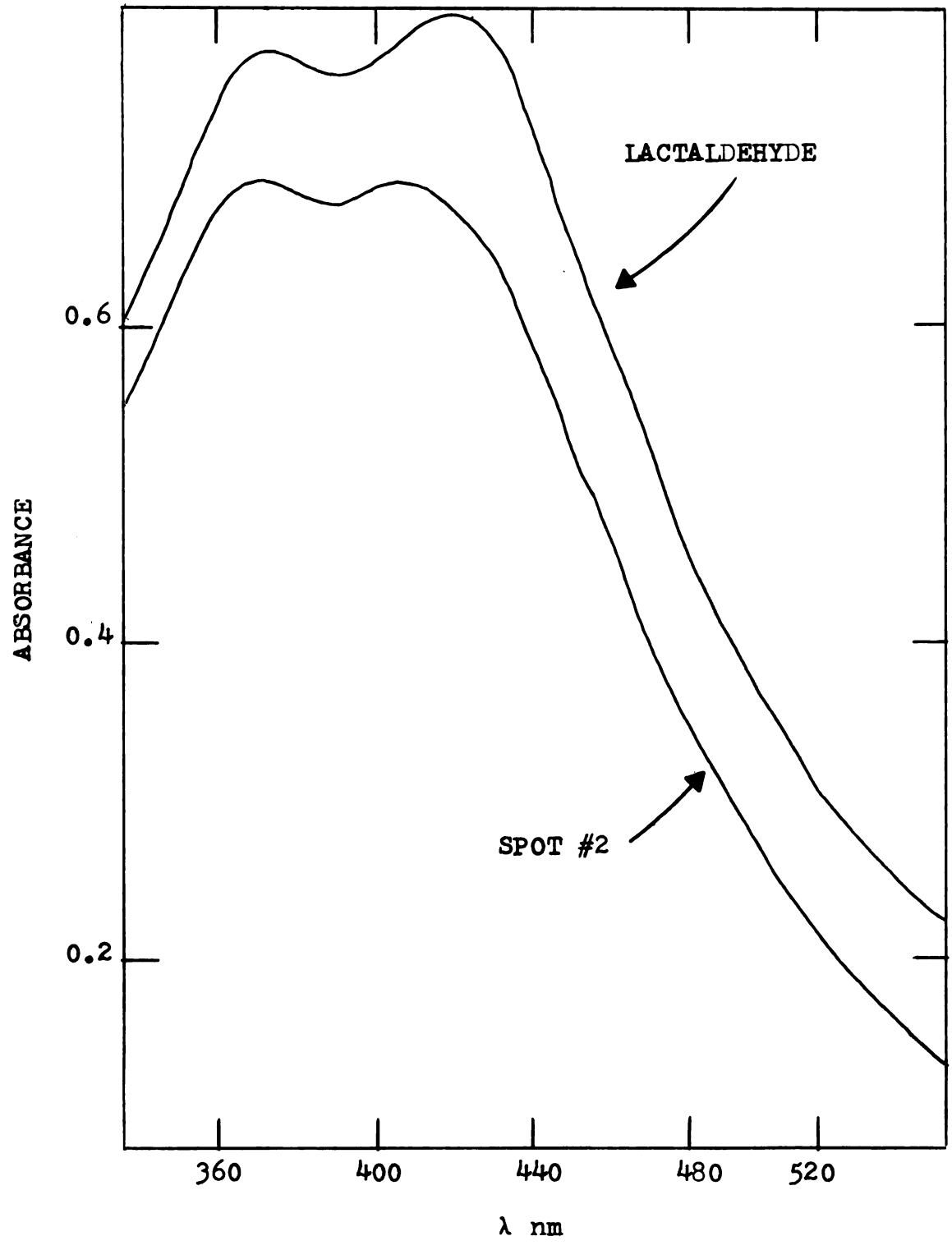

LACTALDEHYDE
SPOT #2
ABSORBANCE
0.6
0.4
0.2
360
400
440
480
520
λ nm

Table XXXVI. Identification of the products of KDA cleavage by KDF aldolase. Details are given in the text.

2,4-Dinitrophenylhydrazones	Color After KOH Spray	R_f Values	
		Dimension #1	Dimension #2
Pyruvate standard	Purple-brown	.38	.40
Spot #1 Cleavage reaction	Purple-brown	.37	.41
Glycolaldehyde standard	Yellow-brown	.59	.52
Spot #2 Cleavage reaction	Yellow-brown	.60	.52

G. ANALYSIS OF MUTANTS LACKING D-FUCONATE DEHYDRATASE AND 2-KETO-3-DEOXY-D-FUCONATE ALDOLASE, AND THE RELATIONSHIPS AMONG THE DEGRADATIVE PATHWAYS FOR D-FUCOSE, L-ARABINOSE, AND D-GALACTOSE

1. Mutant Strain 5-1-10-1 (D-Fuconate Dehydrataseless)

A mutant was isolated which was incapable of using L-arabinose or D-fucose as growth substrates but was identical to the parental strain in other respects (see Experimental Procedures for the methods of mutagenesis and isolation, and the Appendix for the properties of the parental strain). The mutant, which was designated strain 5-1-10-1, was found to be missing D-fuconate dehydratase, but possessed normal levels of the other enzymes of the D-fucose pathway (Table XXXVII). The level of D-galactonate dehydratase was also determined and was found to be normal. The growth rates of mutant strain 5-1-10-1 on D-glucose and D-galactose were identical to those of the wild type (Figures 56 and 57).

The above data thus support two conclusions: (i) D-fuconate dehydratase is absolutely functional in the metabolism of D-fuconate and L-arabonate; and (ii) D-galactonate dehydratase is not functional in the metabolism of D-fuconate. Previous evidence indicated that D-galactonate dehydratase could dehydrate D-fuconate; however, the K_m value was about 120 mM. The K_m value was of such a magnitude to warrant the assumption that the enzyme was not involved in the <u>in vivo</u> dehydration of

Table XXXVII. Enzyme levels in mutant strain 5-1-10-1, the parental strain and a revertant derived from the mutant. Cell extracts of D-galactose grown cells, unless otherwise indicated, were prepared by sonic disruption in 0.01 molar Bicine buffer and 0.143 mM 2-thioethanol (pH 7.4). Protein was estimated by the biuret assay. Aldolase assays were identical to the assay outlined in the Experimental Procedures with the exception that 1.05 µmole KDF was employed. Specific activities are reported as µmoles per hour per mg protein.

	Specific Activity		
Enzyme	Wild Type	Mutant	Revertant
D-Aldohexose dehydrogenase*	13.9	12.7	14.6
L-Arabino-aldose dehydrogenase	11.8	20.1	17.5
D-Fuconate dehydratase	4.25	< .0005	3.72
Aldolase	1.34	1.91	1.57
D-Galactonate dehydratase	6.50	7.11	6.79

*Cell extracts were prepared from D-glucose grown cells.

Figure 56. Growth of wild-type cells on D-fucose, L-arabinose, D-galactose, and D-glucose.

Figure 56

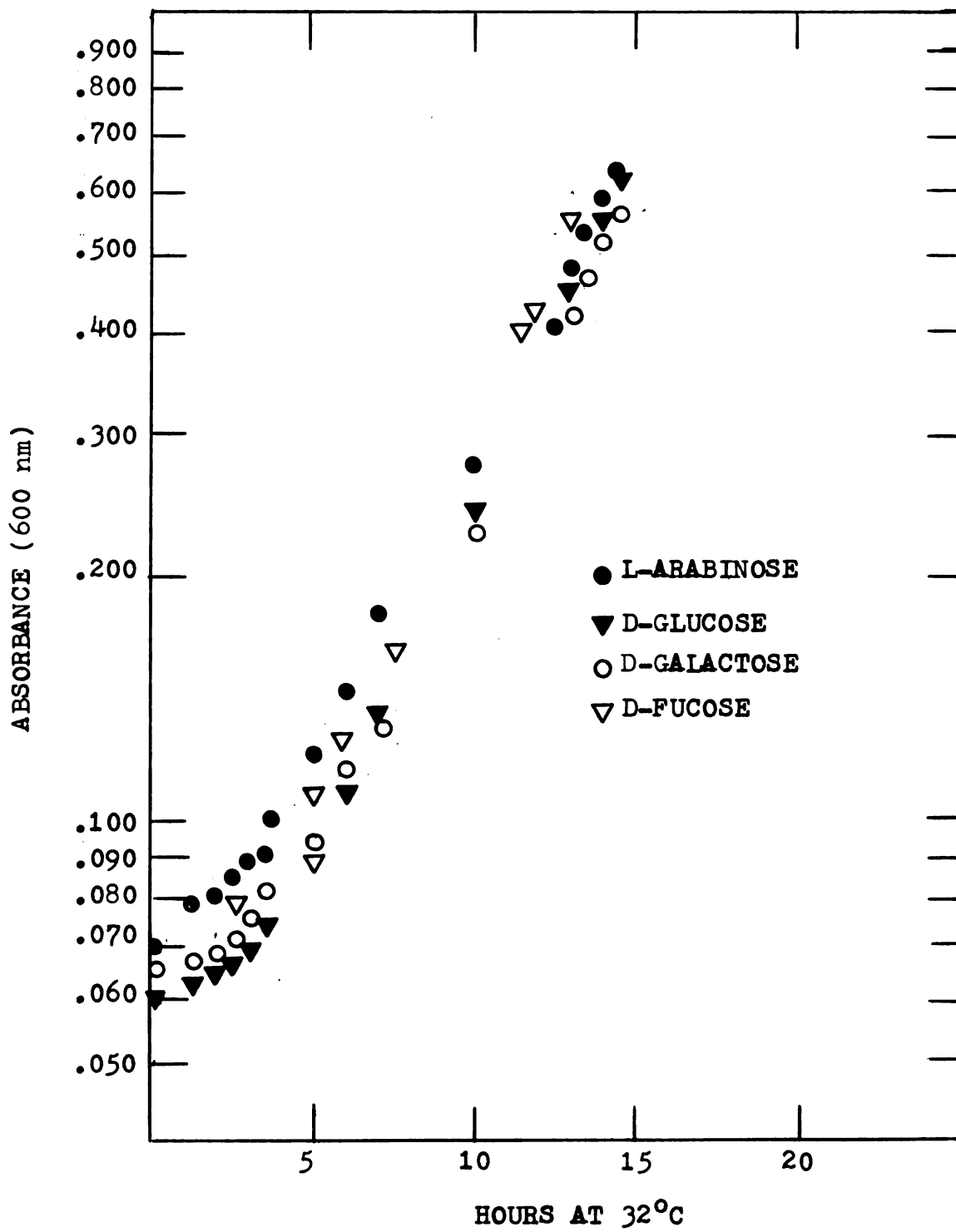
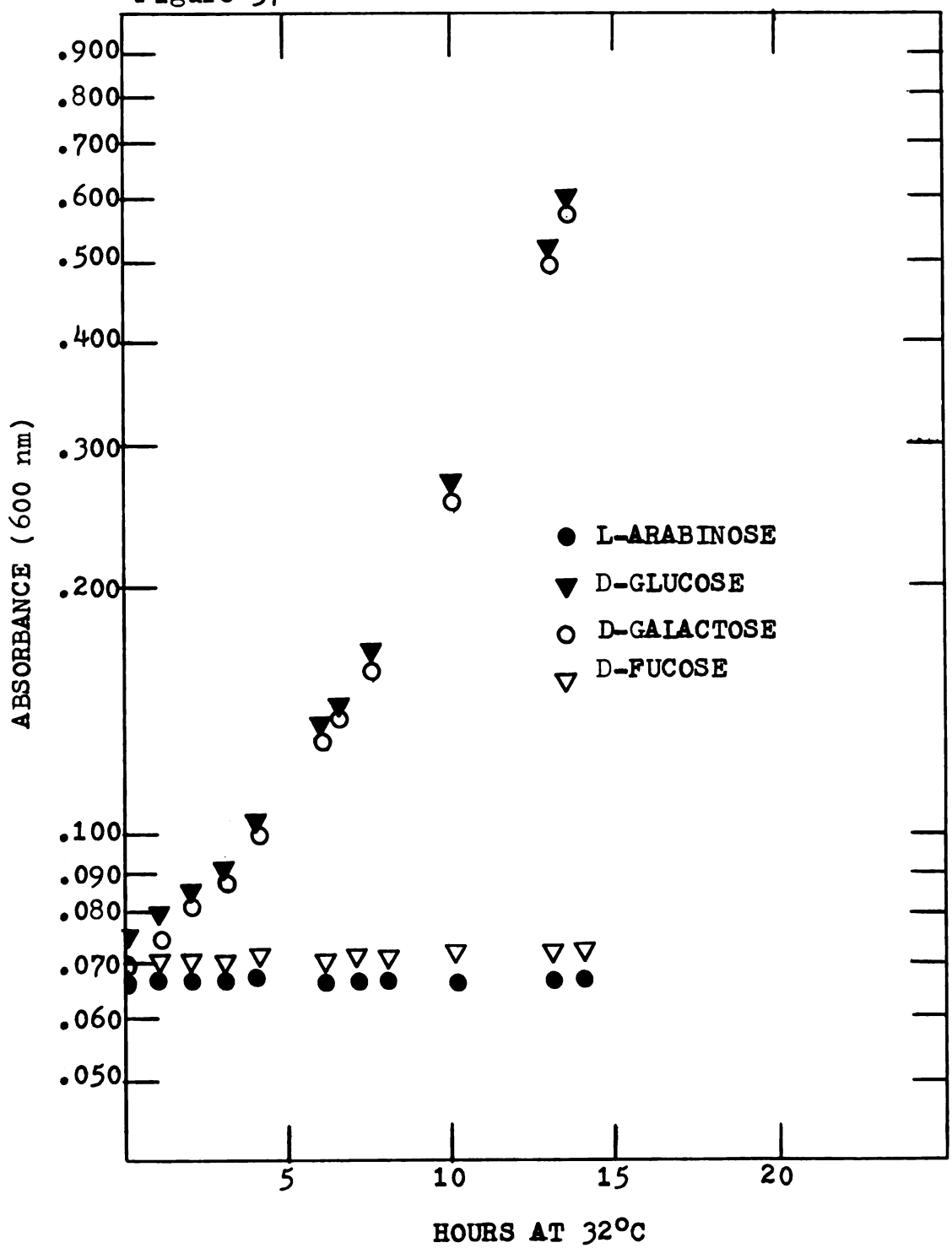

.900
.800
.700
.600
.500
.400
.300
.200
.100
.090
.080
.070
.060
.050
ABSORBANCE (600 nm)
5
10
15
20
HOURS AT 32°C
L-ARABINOSE
D-GLUCOSE
D-GALACTOSE
D-FUCOSE

Figure 57. Growth of mutant strain 5-1-10-1 on D-fucose, L-arabinose, D-galactose, and D-glucose.

Figure 57


.900
.800
.700
.600
.500
.400
.300
.200
.100
.090
.080
.070
.060
.050
ABSORBANCE (600 nm)
L-ARABINOSE
D-GLUCOSE
D-GALACTOSE
D-FUCOSE
5
10
15
20
HOURS AT 32°C

D-fuconate. The fact that the D-fuconate dehydrataseless mutant possessed D-galactonate dehydratase but failed to grow on D-fuconate corroborates this assumption.

Thus, not only can D-galactonate dehydratase activity be distinguished from D-fuconate dehydratase activity by means of Sephadex G-200 and DEAE-cellulose elution profiles, thermal denaturation profiles, differential metal ion requirements, differential sulfhydryl inhibitor responses, pH optima and mixed substrate studies, but a mutant has been obtained which is lacking D-fuconate dehydratase and yet contains normal levels of D-galactonate dehydratase. A comparison of some of the properties of the two dehydratases is presented in Table XXXVIII.

2. Mutant Strain 73-1-2 (Lacking 2-Keto-3-deoxy-D-fuconate Aldolase)

Another mutant which exhibited defective growth on D-fucose and L-arabinose was obtained by procedures identical to those used for the isolation of mutant strain 5-1-10-1. This mutant, designated 73-1-2, grew on L-arabinose and D-fucose at about 40% the wild-type rate but grew normally on D-glucose and D-galactose (Figures 56 and 58). This mutant when grown on L-arabinose or D-fucose was found to possess about one-fourth the normal level of 2-keto-3-deoxy-D-fuconate aldolase but possessed normal levels of the other enzymes of the D-fucose pathway (Table XXXIX). The slow-growing mutant strain is believed

Table XXXVIII. A comparison of the properties of D-fuconate and D-galactonate dehydratases

Property	D-Fuconate Dehydratase	D-Galactonate Dehydratase
K_m for D-fuconate	4.00 mM	120 mM
K_m for L-arabonate	4.25 mM	not a substrate
K_m for D-galactonate	not a substrate	12 mM
Half-life at 53°C	80 sec	> 1 hour
pH optimum	7.1-7.4	6.5-6.8
Metal ion requirement (EDTA ± excess cation)	absolute	2-3 fold activation
Sulfhydryl inhibitor response		
2 mM iodoacetate	no effect	no effect
50 μM p-chloromercuribenzoate	88% inhibition of D-fuconate dehydration	66% inhibition of D-fuconate dehydration
Mixed substrate studies	non-additive rates with L-arabonate and D-fuconate	non-additive rates with D-galactonate and D-fuconate
0-40% Ammonium sulfate fraction	10% total activity	90% total activity
40-60% Ammonium sulfate fraction	90% total activity	10% total activity
Calcium phosphate gel step (mixtures of equivalent amounts of each enzyme)	100% inactivation	9% inactivation

Figure 58. Growth of mutant strain 73-1-2 on D-fucose, L-arabinose, D-glucose, and D-galactose.

Figure 58

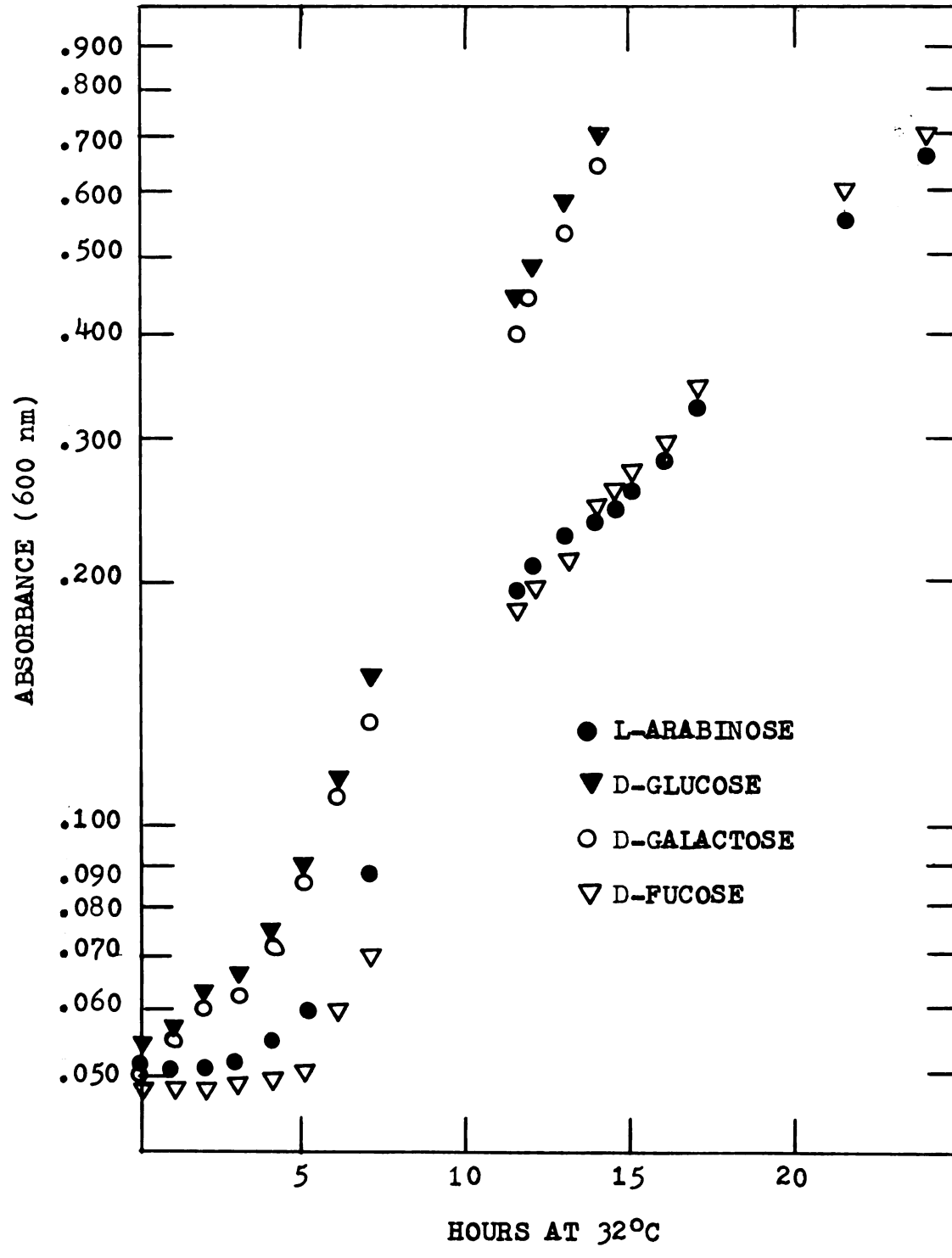

.900
.800
.700
.600
.500
.400
.300
.200
.100
.090
.080
.070
.060
.050
ABSORBANCE (600 nm)
5
10
15
20
HOURS AT 32°C
L-ARABINOSE
D-GLUCOSE
D-GALACTOSE
D-FUCOSE

Table XXXIX. Enzyme levels in mutant strain 73-1-2 and the parental strain. Cell extracts were prepared from L-arabinose grown cells unless otherwise noted and were prepared as in Table XXXVII. Protein was estimated by the biuret assay. Specific activities are reported as μmoles per hour per mg protein.

Enzyme	Specific Activity	
	Wild Type	Mutant
D-Aldohexose dehydrogenase*	16.3	14.1
L-Arabino-aldose dehydrogenase	15.7	11.8
D-Fuconate dehydratase	4.27	3.32
KDF Aldolase	4.23	0.91

*Cell extracts prepared from D-glucose grown cells.

to be a leaky mutant, in which a change in an amino acid residue in the aldolase has resulted in a less catalytically active protein.

The isolation of mutant strain 73-1-2, which simultaneously exhibited a reduced growth rate on L-arabinose and D-fucose and a correspondingly diminished level of KDF aldolase, supports the prior conclusions that KDF and KDA are degraded by the same enzyme and that L-KDA dehydratase, the only other enzyme reported to act upon L-KDA (54-56), is not present in this microorganism.

The fact that the two mutant strains, 5-1-10-1 and 73-1-2, possess corresponding defects in both the L-arabonate and D-fuconate pathways suggests the common identity of the two pathways and substantiates the previous conclusion that both D-fuconate and L-arabonate are dehydrated by the same enzyme and that both KDF and KDA are cleaved by the same enzyme. In summary, the enzymes of D-fuconate and L-arabonate metabolism are identical, but, despite the structural similarity between D-fuconate and D-galactonate, the enzymes responsible for D-galactonate degradation do not participate to a detectable extent in the degradation of either D-fuconate or L-arabonate.

DISCUSSION

The data presented in this thesis show that, in the pseudomonad under investigation, D-fucose is degraded by the pathway shown in Figure 59. D-Fucose is degraded through a modified Entner-Douderoff pathway by oxidation to the lactones, hydrolysis to the free acid, dehydration to the keto deoxy acid, and cleavage to form pyruvate and D-lactaldehyde. The data also indicate that the enzymes of the D-fucose pathway function in a new pathway for the degradation of L-arabinose (Figure 60A); instead of being dehydrated to form α-keto glutarate semialdehyde, 2-keto-3-deoxy-L-arabonate is cleaved to form pyruvate and glycolaldehyde. All attempts to demonstrate the previously known pathway (Figure 60B) were unsuccessful.

In this section, the major conclusions, and the data on which they are based, will be discussed, and an attempt will be made to relate the results obtained from this work to those from related studies in the literature.

The initial experiments indicated the presence of at least two soluble, pyridine nucleotide-linked dehydrogenases which were capable of oxidizing D-fucose and which could be differentiated on the basis of nucleotide and substrate specificity and Sephadex G-200 elution pro-

Figure 59. Pathway of D-fucose degradation

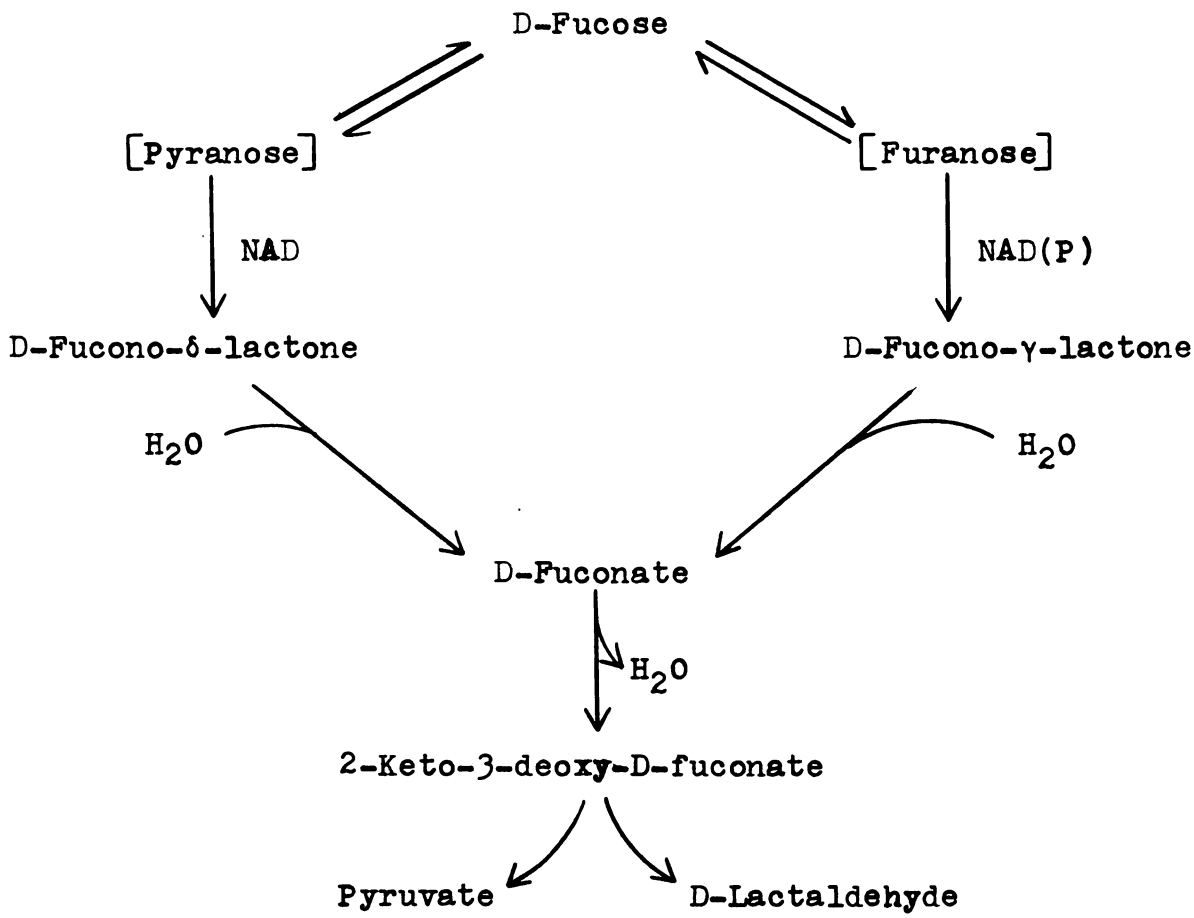
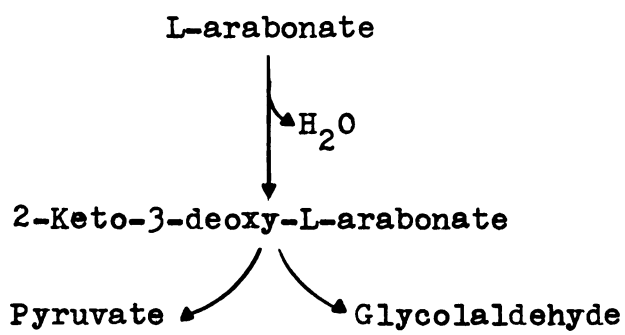
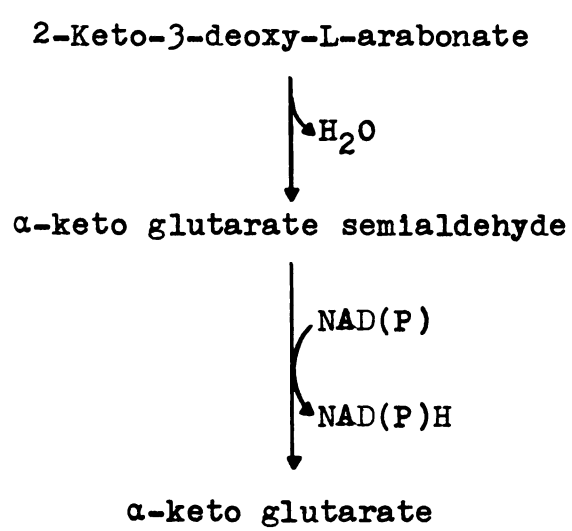


Figure 60. L-Arabinose degradation in pseudomonads.

A. New pathway



B. Previously known pathway (54-56)



files. No activity which modified D-fucose by means of isomerization, phosphorylation, epimerization, or reduction was detected in crude cell extracts. Thus, it was concluded that pathways for D-fucose metabolism other than the oxidative pathway described above did not exist in this pseudomonad; it was also concluded that D-fucose was not degraded by a pathway analogous to those which occur in other organisms for the degradation of L-fucose or L-rhamnose, which are the two other common 6-deoxy aldohexoses (263-265).

Based on substrate specificities of the two fucose dehydrogenases, the NAD-dependent enzyme may be designated a D-aldohexose dehydrogenase and the NAD(P)-dependent enzyme may be designated an L-arabino-aldose dehydrogenase. Substrate specificity cannot, however, be used as the sole criterion in ascertaining the participation of a particular enzyme in a metabolic process. The induction by a specific carbohydrate of an enzyme which, in turn, is operative on the carbohydrate suggests that the enzyme may be functional in the metabolism of the carbohydrate. Both dehydrogenases were induced by growth on D-fucose and, in the absence of any mutational evidence to indicate the contrary, may be considered to be functional in the degradation of D-fucose. The D-aldohexose dehydrogenase is also induced by growth on D-glucose whereas the L-arabino-aldose dehydrogenase is also induced

by growth on D-galactose, L-arabinose, and 6-iodo-6-deoxy-D-galactose. The last carbohydrate is readily metabolized by the pseudomonad and is probably oxidized by the latter enzyme *in vivo*. For the sake of clarity, the two dehydrogenases will be distinguished in the remainder of this section on the basis of substrate specificity rather than nucleotide specificity.

Evidence was obtained which establishes the products of carbohydrate oxidation to be a δ-lactone with the D-aldohexose dehydrogenase and a γ-lactone with the L-*arabino*-aldose dehydrogenase. The ring sizes of the lactones were determined by chromatography and by the ability of the respective dehydrogenases to convert δ- and γ-lactones to the corresponding aldose (Tables X and XVII).

The K_m values at pH 8.1 for the physiologically important carbohydrates ranged from 0.86 mM to 5.8 mM for the D-aldohexose dehydrogenase and from 0.14 mM to 0.50 mM for the L-*arabino*-aldose dehydrogenase. The K_m values for the latter were also run at the pH optimum, pH 9.4, and were found to be 5 to 14-fold higher than the K_m values at pH 8.1. The observed increase in K_m and V_{max} values at pH 9.4 provokes thought about the implications inherent in the determination of a K_m value at only one hydrogen ion concentration, as is the case for most enzymes reported in the literature, except those enzymes which, due to an abnormally high K_m value, were investigated to determine the lowest and yet most meaningful K_m

value (85, 86).

The dehydrogenases were easily distinguished on the basis of thermal denaturation. The half-lives at 55°C were found to be 42 seconds and 13 minutes for the D-aldohexose and L-arabino-aldose dehydrogenases, respectively (Figure 23). These properties were used in the heat step of the purification of the latter enzyme. Both enzymes were insensitive to thiols, thiol group inhibitors, metal ion activators, and EDTA.

Due to the fact that pseudomonads are noted for possession of multiple carbohydrate dehydrogenases, it was of interest to determine whether the pseudomonad under study possessed only two dehydrogenases, or if the activities on these various sugars could be resolved further. This was investigated by measuring the ratios of activities on various substrates when the enzymes were subjected to the following: (i) chromatography on Sephadex G-200 and DEAE-cellulose; (ii) adsorption on and elution from calcium phosphate gel; (iii) thermal inactivation; (iv) assaying at different pH values; (v) mixing substrates for possible additive rates. In all cases, the ratios remained constant, indicating that the L-arabino-aldose dehydrogenase and the D-aldohexose dehydrogenase are single enzymes and that there is, in the pseudomonad under study, no other dehydrogenase active on D-glucose, D-galactose, L-arabinose, or D-fucose when the pseudomonad is grown on any of these

carbohydrates. An additional dehydrogenase has been observed in D-xylose grown cells. It appears to be a D-xylose specific dehydrogenase which does not oxidize any of the above four carbohydrates and D-arabinose, D-glucose, L-fucose, and D-lyxose; this enzyme is not detectable in cell extracts of the pseudomonad when grown on carbohydrates other than D-xylose.

The presence of bacterial enzymes which oxidize carbohydrates to the corresponding lactone is well established. Many of these enzymes are soluble and specific for non-phosphorylated aldoses. Table XL lists the specificities of various soluble aldose dehydrogenase that have been found in other bacteria by other workers.

The only enzymes closely related to the unique D-aldohexose dehydrogenase, herein described, have been characterized by Cline and Hu (87) and Avigad et al. (42). The NAD-dependent aldose dehydrogenase of Cline and Hu exhibited entirely different substrate requirements, was totally inactive on D-mannose, and was active on D-xylose. The NADP-dependent aldohexose dehydrogenase of Avigad et al. was active only on D-glucose, D-mannose, 2-deoxy-D-glucose, and D-mannosamine whereas the NAD-dependent D-aldohexose dehydrogenase described in this thesis was active on all the D-aldohexoses tested and possesses the broadest substrate specificity of any soluble dehydrogenase previously described. Microbial dehydrogenases operative on D-mannose are a rarity, there being only

Table XL. A comparison of various soluble bacterial dehydrogenases specific for nonphosphorylated monosaccharides: RCHO ⟶ (lactone) ⟶ RCOOH

Enzyme	Source	Electron Acceptor Specificity	Substrate Specificity	Ring Form of Lactone	Anomer	Ref.
L-Arabino-aldose dehydrogenase	Pseudomonad	NAD^+, $NADP^+$	D-fuc, D-gal, L-arab	γ	β-D	
D-Aldohexose dehydrogenase	Pseudomonad	NAD^+	D-aldohexose	δ	β-D	
Aldose dehydrogenase	Pseudomonad	NAD^+	D-gal, D-glu, D-xyl, D-fuc, L-arab		β-D-	(87)
Aldohexose dehydrogenase	Gluconobacter cerinus	$NADP^+$	D-glu, D-man, D-dglu, D-dman	δ		(42)
D-Arabinose dehydrogenase	Pseudomonas saccharophila	NAD^+		γ		(80)
D-Arabinose dehydrogenase	Pseudomonad	NAD^+, $NADP^+$	D-arab, L-gal, L-fuc		α-D-	(87)
D-Arabinose dehydrogenase	Pseudomonas fragi	NAD^+, $NADP^+$				(88)
L-Arabinose dehydrogenase	Pseudomonas saccharophila	NAD^+	D-gal, L-arab	γ		(54, 40)
L-Arabinose dehydrogenase	Pseudomonas saccharophila	NAD^+	D-gal, L-arab	γ		(40)

D-Galactose dehydrogenase	Pseudomonad	NAD^+	D-gal, L-arab	γ		(89)
D-Galactose dehydrogenase	*Pseudomonas saccharophila*	NAD^+	D-gal, L-arab	γ		(78, 40)
D-Galactose dehydrogenase	Pseudomonad	NAD^+				(90)
D-Galactose dehydrogenase	Pseudomonad	$NADP^+$	D-gal, D-fuc, D-dgal, L-arab		β-D-	(87)
D-Glucose dehydrogenase	Pseudomonad	NAD^+				(19)
D-Glucose dehydrogenase	*Bacillus cereus*	NAD^+	D-glu			(91)
D-Glucose dehydrogenase	*Bacillus cereus*	NAD^+	D-glu			(92)
D-Glucose dehydrogenase	*Acetobacter suboxydens*	NAD^+	D-glu, D-dglu	δ		(44)
D-Glucose dehydrogenase	*Acetobacter suboxydens*	$NADP^+$	D-glu, D-man	δ		(43)
D-Glucose dehydrogenase	*Pediococcus pentosaceus*	$NADP^+$	D-glu, D-dglu	δ		(39)
D-Glucose dehydrogenase	*Bacterium anitratum*	unknown	D-glu, D-xyl, malt, lact, cello	δ	β-D-	(93)

Abbreviations used: D-glu, D-glucose; D-gal, D-galactose; D-dglu, 2-deoxy-D-glucose; D-dgal, 2-deoxy-galactose; D-xyl, D-xylose; D-fuc, D-fucose; D-man, D-mannose; L-arab, L-arabinose; D-dman, 2-amino-2-deoxy-D-mannose; D-arab, D-arabinose; L-fuc, L-fucose; L-gal, L-galactose; malt, maltose; lact, lactose; cello, cellobiose.

two recorded instances in the literature (42, 43); the D-aldohexose dehydrogenase herein described constitutes the third instance.

The ring form of the lactone resulting from the oxidation of D-glucose by the NADP-dependent aldohexose of Avigad et al. was determined to be a δ-lactone on the basis of reversibility of the reaction only with D-glucono-δ-lactone. The ring form of the product was not determined by Cline and Hu. All soluble dehydrogenase operative on D-glucose, for which the lactone ring size has been determined, yield D-glucono-δ-lactone (39, 42-44, 93). This is in agreement with the results obtained in the current study. However, all previously reported studies of soluble dehydrogenases operative on D-galactose, in which the lactone ring size has been determined, have indicated the product to be a γ-lactone. Thus, the D-aldohexose dehydrogenase isolated from the pseudomonad under investigation is the first reported instance of D-fucose or D-galactose oxidation in which the pyranose form of the carbohydrate is oxidized resulting in formation of the δ-lactone. D-Fucono-δ-lactone and D-galactono-δ-lactone have never been isolated due to their instability (46-48).

A substantial number of carbohydrate dehydrogenases have been characterized as to whether the configuration of the first carbon atom of the substrate is important in the oxidation of the sugar (38, 85, 87, 93, 94).

In all cases examined which are valid under a set of conditions discussed below, the β-D or the corresponding α-L form of the carbohydrate was determined to be the more reactive anomer. It is felt that this type of analysis must be restricted to those carbohydrates which exhibit simple mutarotation (95), that is a single slow mutarotative step solely involving pyranose interconversions; included in this series would be D-glucose, D-mannose, D-xylose, D-glucose and the disaccharides containing these carbohydrates in the reducing moiety. The carbohydrates which exhibit complex mutarotation, that is those which form substantial amounts of furanose forms in solution, cannot be used in an anomeric prefence analysis with certainty since other unknown rate parameters are introduced into the analysis; these carbohydrates are D-galactose, D-fucose, L-arabinose, and D-talose (95). A further obvious restriction must necessarily involve the utilization of only those dehydrogenases which are operative on the pyranose form of the carbohydrate.

In compliance with the previous restrictions, the investigation of the steric requirements of the D-aldohexose dehydrogenase with α-D-glucose and α,β-D-glucose, showed that the β-D-pyranose form was preferred over the α-D-pyranose form. This is in agreement with the results obtained with other soluble dehydrogenases. The L-arabinoaldose dehydrogenase was not similarly investigated due

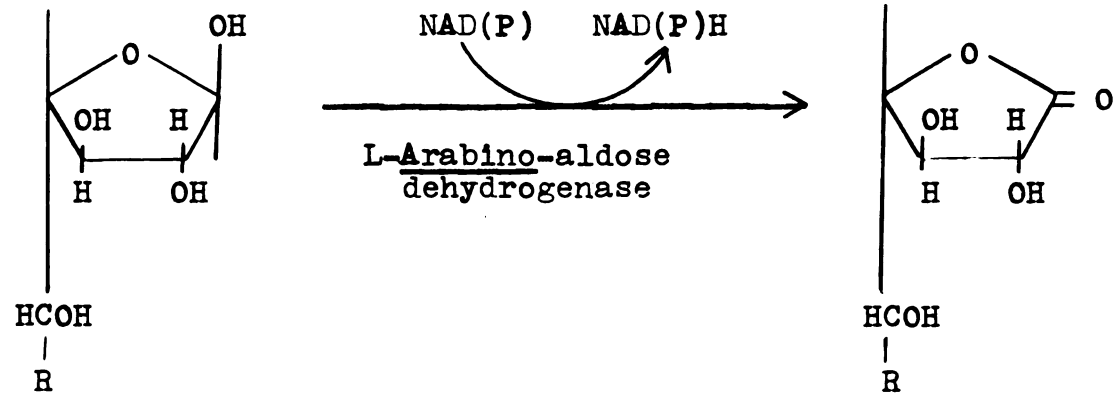
to the fact that the enzyme oxidizes only those carbohydrates which exhibit complex mutarotation and also due to the fact that enzyme appears to require the furanose form for catalysis.

The L-arabino-aldose dehydrogenase is also a unique enzyme and is not easily insertable into any previously established class of soluble carbohydrate dehydrogenases due to its substrate and nucleotide requirements. It is one of the rarer carbohydrate dehydrogenases operative with both NAD and NADP; it also possesses stringent substrate requirements and is operative on only those carbohydrates possessing the L-arabino-configuration. The product of this dehydrogenase has been identified as the γ-lactone, which is in agreement with results obtained with other soluble dehydrogenases operative on D-galactose. Since the γ-lactone is the product, it can be assumed that the enzyme is operative on the furanose form of the carbohydrate.

The relatively high content of furanoses in solution of some carbohydrates has been documented. The rate of mutarotation in aqueous solutions of galactose, arabinose and, presumably, fucose is substantially faster than the rate of mutarotation of mannose or glucose as was pointed out earlier; this has been interpreted as an indication that substantial amounts of the furanose forms of the former series are present at equilibrium (96).

The proportion of furanoses in an equilibrium solution depends upon the relative stabilities of the pyranose and the furanose forms. The stability of the furanose forms, likewise, depends on whether the substituents are cis or trans to each other; when the three substituents of the O-2, O-3, and C-5 are in a trans-trans arrangement, the furanose form is quite favorable, and its presence is indicated by fast mutarotation. From a detailed mathematical treatment of the mutarotation of D-galactose, Nelson and Beegle (53) postulated that an equilibrium of galactose contains 12% furanose at 20°C. A more recent study of the equilibrium composition of L-arabinose and D-galactose solutions at 25°C by gas chromatography of the trimethylsilyl ethers indicated that the β-furanose form amounts to 5.3 and 4.9% respectively; the β-pyranose to β-furanose conversion of galactose was noted to be more rapid than the β-pyranose to the α-pyranose, substantiating in part, the previous interpretation of fast versus slow mutarotation rates (97). Wells, Sweeley, Bentley, and coworkers have demonstrated also by gas chromatography of the trimethylsilyl ethers that equilibrium solutions of D-galactose contained 5.4% furanose (98). Since structures can be drawn for D-galactofuranose, D-fucofuranose, and L-arabinofuranose which exhibit the all-trans configuration (Figure 61), it is probable that the L-arabino-aldose dehydrogenase is

Figure 61. All-trans configuration of β-furanose forms of D-fucose, D-galactose, and L-arabinose.



R=H	:	α-L-Arabinofuranose	L-Arabono-γ-lactone
$R=CH_2OH$	:	β-D-Galactofuranose	D-Galactono-γ-lactone
$R=CH_3$	:	β-D-Fucofuranose	D-Fucono-γ-lactone

also operative on the β-anomer. It is not evident, however, why enzymes would be evolved which are operative on β-D-galactofuranose rather than β-D-galactopyranose, present in 15-fold excess or α-D-galactopyranose, present in a 6-fold excess. There exists the possibility that the L-arabino-aldose dehydrogenase possesses the capability of converting β-pyranose, present in 15-fold excess, to a β-furanose in a manner similar to a mutarotase.

Since D-fucono-γ-lactone is very stable and hydrolyzes slowly even at pH 9 (48) and since 20% of the γ-lactone formed from oxidation of D-fucose by the L-arabino-aldose dehydrogenase (Table V) was converted to the free acid by hydrolysis, it was evident in the preliminary experiments that an aldonolactonase was present. Aldonolactonases are a poorly characterized group of esterases which are operative on internal esters of aldonic acids. They are a necessary group of enzymes for the hydrolysis of those lactones which are stable towards non-enzymatic hydrolysis in the 6-8 pH range. Weimberg (88) has observed that Pseudomonas fragi is incapable of efficiently utilizing L-arabinose, D-xylose, and D-ribose because of the absence of a lactonase; the γ-lactones of the respective aldoses were found to accumulate in the media due to their stability at a neutral pH. Due to the stability of D-fucono-γ-lactone, the growth rate of the pseudomonad under study would probably be diminished in the absence of a lactonase.

A literature survey was undertaken to correlate the properties of various delactonizing enzymes with the properties of the lactonase isolated from the pseudomonad under study. The results of the survey are presented in Table XLI.

The general properties of aldonolactonases can be summarized as the following: (i) they are usually specific for δ- or γ-lactones; (ii) they require a metal ion and a thiol for maximum activity; (iii) the pH optima are in the range 6-7.5; and (iv) they all possess high Michaelis constants, as noted also by Yamada (103).

The enzyme partially characterized in this study was not activated by thiols or metal ions, nor was it inhibited by high concentrations of thiol group inhibitors. The K_m value and the pH optimum were found to be 21 mM and pH 7.6 respectively. There appear to be no striking differences between the properties of this lactonase and the properties of the previously described lactonases.

The degradation of D-fucose to the keto deoxy acid provides another example of a widespread metabolic mechanism in bacteria whereby utilization of carbohydrates and polysaccharides involve formation of deoxy sugar acid intermediates. 2-Keto-3-deoxy sugar acid intermediates are involved in the degradation of glucose (19, 125), galactose (78), D-altronic and mannonic acids (106), D-arabinose (117), L-arabinose (54-56), D-xylose and

Table XLI. A comparison of various aldonolactonases.

Enzyme (Hydrolases)	Source	Specificity	K_m	Metal Ion Requirement	Thiol	pH Opt.	Ref.
γ-Lactonase*	Pseudomonad	D-γ-Gal, D-γ-Rib D-γ-Fuc	20 mM	None			
D-γ-Gal** Lactonase	P. saccharophila						(78)
D-γ-Arab Lactonase	P. saccharophila						(80)
D-γ-Arab Lactonase	P. fragi						(88)
D-γ-Gal Lactonase	Rat liver						(41)
L-γ-Arab Lactonase	P. saccharophila						(54)
D-δ-Glu Lactonase	Bovine liver	D-δ-Glu		Mg^{2+}, Mn^{2+} Co^{2+}		6.0	(100)
D-δ-Glu Lactonase	Baker's yeast	D-δ-Glu, D-6-P-δ-Glu, δ-Lact	8.5 mM (D-δ-Glu)	Mg^{2+}, Mn^{2+} Co^{2+}	Yes		(99)
Lact-δ-lactone	P. graveolens	D-γ-Rib, D-γ-Gal D-γ-Glu	20 mM (Lact-δ)	None		6.5-6.7	(101)

Aldono-lactonase	Mammalian liver and kidney	D-γ-Man, D-γ-Gul, D-γ-Rib, D-γ-Gal, D-γ-Arab, D-γ-Glu	10-60 mM	Co^{2+}, Mn^{2+} Zn^{2+}	Yes		(102, 104)
Lactonase I	Mammalian liver and avian kidney	L-γ-Gul, D-γ-Gul, L-γ-Gal, D-γ-Gal, L-γ-Glu, D-γ-Glu, D-γ-Man	1-20 mM	Mg^{2+}, Mn^{2+}	Yes	6.8-7.0	(103)
Lactonase II	Mammalian liver	D-γ-Gal, D-γ-Glu, L-γ-Glu					(103)
γ-Carboxy-methyl-δ-butenolide hydrolase	P. fluorescens		78 μM	None		7.5	(105)

Abbreviations:

* Hydrolase

**Gal, Galactonolactone; Arab, Arabonolactone; Glu, Gluconolactone; Lact, Lactobionic acid; 6-P-Glu, 6-Phospho-gluconolactone; Rib, Ribonolactone; Man, Mannonolactone; Gul, Gulonolactone; Fuc, Fuconolactone.

D-ribose (88, 119), D-glucosaminic acid (107), alginic acid (61), polygalacturonic acid (63, 116), hyaluronic acid (108), chondroitan sulfate (115), pectin (109, 110), glucaric and galactaric acids (18, 26, 111, 112, 119), myo-inositol (113), and γ-hydroxy glutamate (114). 2-Keto-3-deoxy-D-arabino-heptonic acid-7-phosphate (17, 59, 121), 2-keto-3-deoxy-D-manno-octonic acid-8-phosphate (14, 75, 118, 120), N-acetyl-neuraminic acid-9-phosphate (122), and N-acetyl-neuraminic acid (122, 123), also belong to the group of keto deoxy sugar acids but are involved in biosynthetic reactions and are formed by aldol condensation of two smaller moieties. The 2-keto-3-deoxy sugar acids which contain 5 and 6 carbon atoms are formed from their respective aldonic acid by the action of a specific dehydratase.

The quantitative conversion of D-fuconate by cell-free extracts to a substance which after periodate oxidation yielded a TBA chromogen with λ_{max} 551 nm suggested that the substance was a 2-keto-3-deoxy aldonic acid and, thus, directly implicated the intervention of a dehydratase. The dehydration product was shown to possess the structure 3,6-dideoxy-D-threo-hexulosonic acid (KDF) by analysis of the condensation products of the dehydration product with semicarbazide-HCl, o-phenylenediamine, and 3-methyl-2-benzothiazolinone hydrazone, by ceric sulfate decarboxylation, by periodate rate and total consumption

studies, by analysis of the KDF aldolase cleavage products, by identification of the periodate cleavage products, and by chemical synthesis. Dehydration was found to occur at C-2 and C-3 leaving the C-4 and C-5 hydroxyl groups intact.

L-Arabonate was also dehydrated by the same enzyme to a substance which, after periodate oxidation, yielded an alkalai-unstable TBA chromogen with λ_{max} 551 nm. The common identity of the enzymes which dehydrated L-arabonate and D-fuconate was indicated by a variety of means, among which were the following: (i) Sephadex G-200 and DEAE-cellulose elution profiles and the pH optima of the enzyme were superimposable; (ii) rates from studies utilizing mixed substrates were not additive; (iii) thermal inactivation profiles of the enzyme with both substrates were superimposable and exhibited first order kinetics; and (iv) a mutant strain was isolated which lacked dehydratase activity on L-arabonate and D-fuconate, and a revertant was obtained from the mutant strain which regained the ability to utilize D-fucose and L-arabinose and possessed normal dehydratase activity on L-arabonate and D-fuconate. It was concluded that the observed activities were due to a single enzyme.

An enzyme reported to dehydrate L-arabonate has been found to occur in Pseudomonas saccharophila by Weimberg and Doudoroff (54) and Weimberg (55), and in Pseudomonas fragi by Weimberg (88). Weimberg and

Doudoroff were unable to purify and characterize the P. saccharophila enzyme due to its extreme lability; in contrast, the dehydratase isolated from the pseudomonad under study is very stable and retains 100% activity after 24 hours at 30°C, whereas the P. saccharophila enzyme is totally denatured after 5 minutes under the same conditions (54). Thus, this is the first reported purification and characterization of a dehydratase active on L-arabonate and D-fuconate. In P. fragi, Weimberg only reported the appearance of TBA-positive material when crude extracts were incubated with L-arabonate.

A literature survey was undertaken to correlate the properties of other aldonic acid dehydratases and the results of this survey are presented in Table XLII. The properties exhibited by most of the dehydratases can be listed as the following: (i) dehydration occurs at a position α,β to the terminal carboxyl group; (ii) the configuration of the hydroxyl groups at the dehydration site is usually threo (the exceptions are D-mannonic dehydratase and transeliminase which dehydrate erythro hydroxyl groups); (iii) a metal ion is usually required for maximal activity (the exception is D-galactarate dehydratase); (iv) most dehydratases requiring metal ions also require thiols (the exception is D-mannonic dehydratase); (v) the pH optimum ranges from 7-8; (vi) the K_m's are 0.1-8 mM; and (vii) the dehydratase reaction appears to be irreversible.

Table XLII. Comparison of various dehydratases

Enzymes	Substrate	Configuration of Hydroxyl Groups at the Dehydration Site	Metal Ion Activators	pH Opt.	Thiol Req.	K_m	Keg	Ref.
D-Fuconate dehydratase	D-Fuconate L-Arabonate	threo	$Mg^{2+} > Mn^{2+} > Fe^{2+}$	7.1-7.4	yes	4 mM	Irrev	(126)
Dihydroxy acid dehydratase (spinach)	α,β-dihydroxy isovaleric acid, acetolactate	threo	Mg>Mn>Co	8.0-8.2	no			(126)
Dihydroxy acid dehydratase (*E. coli*)	", α,β-dihydroxy-methyl valeric acid		$Fe^{2+} > Mg^{2+} > Mn^{2+}$	7.8-7.9	yes			(127)
Dihydroxy acid dehydratase (*S. cerevisiae*)	"		Mg^{2+}	7.2-7.4				(128)
6-Phospho-gluconate dehydratase (*P. fluorescens*)	6-Phospho-gluconate	threo	$Fe^{2+} > Mn^{2+} > Mg^{2+} > Mn^{2+} > Fe^{2+} > Mg^{2+}$	7.5-8.0	yes	6 mM	Irrev	(129, 130)
D-Altronate dehydratase (*E. coli*)	D-altronic acid	threo	Fe^{2+}	7.8	yes	5 mM	Irrev	(106)
D-Mannonate dehydratase (*E. coli*)	D-mannonic acid	erythro	$Co^{2+} > Mn^{2+}$	5.5	no	6 mM		(106)

KDA dehydratase (*P. saccharophila*)	KDA			7.3	yes	70 μM	(56)
D-Glucarate dehydratase (*E. coli*)	D-glucarate, D-idarate	threo	$Mg^{2+} > Mn^{2+} > Co^{2+}$	7.2-8.5	yes	.8 mM Irrev	(111)
D-Galactarate dehydratase (*E. coli*)	D-galactaric acid	threo	none	8.0	no	.4 mM	(112)
Tartarate dehydratase (*P. putida*)	Tartaric acid	threo	Fe^{2+}	8.5	yes	8 mM	(131)
Alginase (Pseudomonad)	Alginic acid			7.5			(61)
Trans eliminase (Pseudomonad)	Polygalacturonic acid	erythro	Mn^{2+} Ca^{2+}	8.3-8.5			(63)
Keto inositol dehydratase (*Aerobacter aerogenes*)	2-Keto myo-inositol, 2-keto epi-inositol	threo	$Co^{2+} > Mn^{2+}$	6.0-6.2	yes	.15 mM	(113)
D-Arabonate dehydratase (*P. saccharophila*)	D-arabonic acid	threo					(117)
L-Arabonate dehydratase (*P. saccharophila*)	L-Arabonic acid	threo					(54-56)

In comparison to the previously characterized dehydratases, D-fuconate dehydratase does not exhibit any unusual properties. Dehydration occurs at threo hydroxyl groups at C-2 and C-3, and a divalent cation and thiol are required for maximal activity. The pH optimum is 7.1-7.4, and the dehydration is an essentially irreversible reaction.

D-Fuconate dehydratase activity is influenced by the buffers used in the assay. The enzyme is particularly susceptible to Tris and the Tris derivatives prepared by Good et al. (124). It is interesting to note that the crude labile enzyme from P. saccharophila also exhibited denaturation by Tris (54); buffer effects on dehydratases have not been described except for the previous report. Inhibition of D-fuconate dehydratase by the buffers presented in the text is not believed to occur by chelation of ionic cofactors since no correlation exists between the $pK_M 2+$ (metal ion binding constants) and the observed activities in the various buffers.

D-Fuconate dehydratase is induced by D-galactose (Table XXVII), but is totally inactive on D-galactonate. D-Galactose may thus be considered to be a gratuitous inducer of D-fuconate dehydratase. This presents an interesting situation in that D-fucose is a gratuitous inducer of the galactose operon in other organisms (132, 133), whereas in this instance, galactose is a gratuitous

inducer of an enzyme instrumental in D-fucose degradation.

A mutant, strain 5-1-10-1, was isolated which was unable to utilize D-fucose or L-arabinose and was shown to lack D-fuconate dehydratase but possessed normal levels of D-galactonate dehydratase. This evidence justifies the conclusions that D-fuconate dehydratase is absolutely functional in the metabolism of D-fuconate and L-arabonate, that D-galactonate dehydratase is not functional in the metabolism of D-fuconate, and that there is little likelihood that this organism possess an alternative route for D-fucose degradation, a conclusion reached previously after the inability to detect other enzymes operative on D-fucose.

The further degradation of deoxy hexulosonic acids is characterized by one of three routes: (i) by dehydration (56); (ii) by direct cleavage (18, 117, 119, 122, 123, 134, 135); or (ii) by cleavage of a phosphorylated intermediate (19, 77-79). An aldolase was discovered which was induced by growth on L-arabinose or D-fucose and which catalyzed the cleavage of KDF and KDA. KDF aldolase was purified over 50-fold and some of its properties determined.

The cleavage products of KDF and KDA were determined to be pyruvate and lactaldehyde and pyruvate and glycolaldehyde, respectively. Equivalent amounts of each product were formed from the respective substrate.

Pyruvate, and not hydroxypyruvate, was shown to be the product of the KDF aldolase-catalyzed cleavage of KDF and KDA by chromatography and absorption spectra of the 2,4-dinitrophenylhydrazones.

A literature survey was undertaken to correlate the sulfhydryl requirements, the pH optima, and the metal ion requirements of aldolases which cleave deoxy hexulosonic acids and is presented in Table XLIII.

In contrast to the other aldolases in Table XLIII KDF aldolase has an absolute requirement for a divalent cation. The only other aldolases operative on a deoxy hexulosonic acid which possess a metal ion requirement are 2-keto-3-deoxy-D-glucarate aldolase (18) and 2-keto-3-deoxy-D-arabino-heptonate-7-phosphate synthetase from sweet potato (121).

The pH optimum for KDF aldolase was found to be 8-8.2 in Tris and Hepes buffers with each substrate. All similar aldolases possess pH optima in this range where the substrates are completely ionized. The requirement for metal ions in the presence of ionized substrates suggest the formation of an enzyme-M^{2+}-substrate chelate is necessary for catalysis to occur.

KDF aldolase in the assay reaction has no sulfhydryl requirement as is evident from the absence of stimulation in the presence of thiols or from the absence of inhibition in the presence of thiol group inhibitors.

Table XLIII. Comparison of various aldolases which cleave keto deoxy acids.

Enzyme	Source	Inhib. by Chelating Agents	Activ. by Divalent Metals	Reducing Compound Required	pH Opt.	Ref.
2-Keto-3-deoxy-D-fuconate aldolase	Pseudomonad	yes	yes	no	8.0-8.2	
γ-Hydroxy-α-keto-glutarate aldolase	Rat liver	no	no	yes	7.5-8.0	(134)
γ-Hydroxy-α-keto-glutarate aldolase	Rat liver		slight	yes	8.8	(135)
2-Keto-3-deoxy-6-phosphogluconate aldolase	*P. fluorescens*	no	no	no	7.0-8.0	(129, 130)
2-Keto-3-deoxy-6-phosphogluconate aldolase	*P. saccharophila*				6.8	(77)
2-Keto-3-deoxy-6-phosphogalactonate aldolase	*P. saccharophila*				6.8	(77)
2-Keto-3-deoxy-D-*arabino*-heptonate-7-phosphate synthetase	*E. coli*	no	no	no	6.4-7.4	(59)
2-Keto-3-deoxy-D-*arabino*-heptonate-7-phosphate synthetase	Sweet potato		yes	no	7.2	(121)

2-Keto-3-deoxy-D-*manno*-octonic acid-8-phosphate synthetase	*P. aeruginosa*	slight			7.2	(120)
N-Acetylneuraminic acid aldolase	*Cl. perfringens*				7.2	(123)
N-Acetylneuraminic acid aldolase	Hog kidney				7.2	(122)
N-Acetylneuraminic acid 9-phosphate synthetase	Rat liver		yes	yes		(138)
2-Keto-3-deoxy-D-glucarate aldolase	*E. coli*	yes	yes	no	7.4-8.6	(18)

However, when extracts were prepared in the absence of thiols and fractionated on Sephadex G-200, a time- and thiol-dependent renaturation process was observed to occur. It is suggested that thiols prevent the dissociation or denaturation of KDF aldolase by reducing sulfhydryl groups necessary for the maintenance of conformational integrity.

The equilibrium constants for the KDF aldolase-catalyzed cleavage of KDF and KDA were determined to be 0.12 and 0.38 mM respectively. The position of equilibrium favors the synthesis of KDF or KDA at 10^{-3} M, and thus is not in agreement with the findings of other workers on aldolases of non-phosphorylated deoxy hexulsonic acids. Aldolases operative on non-phosphorylated deoxy hexulosonic acids possess equilibrium constants down to 10^{-2} M (111, 112, 118, 122, 123, 134, 135); aldolases operative on phosphorylated deoxy hexulosonic acids possess equilibrium constants in the range 10^{-3}-10^{-5} M (77, 129, 130) which are similar to the aldolases which cleave vicinal hydroxyl groups (136, 137) and to KDF aldolase. The results obtained in this study provide an exception to the conclusion of Fish (18) that all aldolases which cleave non-phosphorylated deoxy hexulosonic possess equilibrium constants that favor cleavage.

The aldolases listed in Table XLIII also possess

strict substrate requirements. This high degree of specificity has also been found for KDF aldolase which cleaves only KDF and KDA. Neither 2-keto-3-deoxy-D-galactonate nor 2-keto-3-deoxy-D-gluconate or the phosphorylated derivatives served as an inhibitor of the cleavage reaction.

KDF aldolase has been shown to be distinct from previously reported enzymes for the degradation of D-galactose, D-glucose, and L-arabinose. In addition to the conclusion that the aldolase was the terminal enzyme in the first known pathway for D-fucose metabolism, it was concluded, on the basis of mutant strain 73-1-2, which simultaneously possessed a reduced growth rate on L-arabinose and D-fucose and a correspondingly diminished level of KDF aldolase, that KDF aldolase was instrumental in the degradation of L-KDA. The only previously known pathway for L-KDA degradation involved its dehydration by L-KDA dehydratase to form α-keto glutarate semialdehyde (56). L-KDA dehydratase could not be demonstrated in the microorganism under investigation, and it has been concluded that a new L-arabinose pathway has been discovered.

The fact that the two mutant strains, 5-1-10-1 and 73-1-2, possess corresponding defects in both the L-arabonate and D-fuconate pathways suggests the common identity of the two pathways and substantiates the pre-

vious conclusion that both D-fuconate and L-arabonate are dehydrated by the same enzyme and that both KDF and KDA are cleaved by the same enzyme. In summary, the enzymes of D-fuconate and L-arabonate metabolism are identical, but, despite the structural similarity between D-fuconate and D-galactonate, the enzymes responsible for D-galactonate degradation do not participate to a detectable extent in the degradation of either D-fuconate or L-arabonate.

SUMMARY

The first known biodegradative pathway for D-fucose has been elucidated. The pathway was determined to be: D-fucose ⟶ D-fucono-γ-lactone + D-fucono-δ-lactone ⟶ D-fuconate ⟶ 2-keto-3-deoxy-D-fuconate ⟶ pyruvate + lactaldehyde. The enzymes were purified and some of their properties determined. The metabolic intermediates of D-fucose degradation were isolated and identified by derivatization and chemical synthesis. The enzymes of the D-fucose pathway were found to act upon L-arabinose and were found also to function in the biodegradation of L-arabinose as demonstrated by mutant strains which possessed simultaneous defects in both L-arabinose and D-fucose metabolism. A new pathway for the degradation of L-KDA was demonstrated through which L-KDA is cleaved by an aldolase to form pyruvate and glycolaldehyde.

BIBLIOGRAPHY

(1) Dahms, A. S., and Anderson, R. L., Fed. Proc., 28, 540 (1969).

(1A) Sumner, J. B., and Howell, S. F., J. Biol. Chem., 108, 51 (1935).

(2) Hestrin, S., J. Biol. Chem., 180, 249 (1949).

(3) Bean, R. C., and Porter, G. G., Anal. Chem., 31, 1929 (1959).

(4) Koch, R. B., Geddes, W. F., and Smith, F., Cereal Chem., 28, 424 (1951).

(5) Travelyan, W. E., Proctor, D. P., and Harrison, J. A., Nature, 166, 444 (1950).

(6) Warren, L., Nature, 186, 237 (1960).

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

(8) Gornall, A. G., Bardawill, C. J., and David, M. M., J. Biol. Chem., 177, 751 (1949).

(9) Dische, Z., and Borenfreund, E., J. Biol. Chem., 192, 583 (1951).

(10) Friedemann, T. E., and Haugen, G. E., J. Biol. Chem., 147, 415 (1943).

(11) Sayne, P. W., and Greenberg, D. M., J. Biol. Chem., 220, 787 (1956).

(12) Itagaki, E., and Suzuki, S., J. Biochem. (Tokyo), 56, 77 (1964).

(13) Meister, A., J. Biol. Chem., 197, 309 (1952).

(14) Ghalambor, M. A., Levine, E. M., and Heath, E. C., J. Biol. Chem., 241, 3207 (1966).

(15) Paz, M. A., Blumenfeld, O. O., Rojkind, M., Henson, E., Furfine, C., and Gallop, P. M., Arch. Biochem. Biophys., 109, 548 (1965).

(16) Sugimoto, K., and Okazaki, R., J. Biochem. (Tokyo), 62, 373 (1967).

(17) Weissbach, A., and Hurwitz, J., J. Biol. Chem., 234, 705 (1959).

(18) Fish, D. C., Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan, 1964.

(19) Mac Gee, J., and Doudoroff, M., J. Biol. Chem., 210, 617 (1954).

(20) Soda, K., Agr. Biol. Chem. (Tokyo), 31, 1054 (1967).

(21) Freundenberg, K., and Hixon, R. M., Chem. Ber., 56, 2119 (1923).

(22) Schmid, H., and Karrer, P., Helv. Chim. Acta, 32, 1371 (1949).

(23) Raymond, A. L., and Schroeder, E. F., J. Amer. Chem. Soc., 70, 2785 (1948).

(24) Moore, S., and Link, K. P., J. Biol. Chem., 133, 293 (1940).

(25) Evans, W. L., Edgar, R. H., and Hoff, G. P., J. Amer. Chem. Soc., 48, 2668 (1926).

(26) Fish, D. C., and Blumenthal, H. J., Methods Enzymol., 9, 53 (1966).

(27) Waravdekar, U. S., and Saslaw, L. D., J. Biol. Chem., 234, 1945 (1959).

(28) Wolfrom, L., and Thompson, A., Methods Carbohydrate Chem., 1, 121 (1962).

(29) Mayer, W., and Tollens, B., Chem. Ber., 38, 3021 (1905).

(30) Votocek, E., Z. Chem., 1, 803 (1900).

(31) Tollens, B., and Muther, E., Chem. Ber., 37, 306 (1904).

(32) Levvy, G. A., and Mc Allan, A., Biochem. J., 80, 433 (1961).

(33) Clark, E., J. Biol. Chem., 54, 65 (1926).

(34) Dimler, R., and Link, K. P., J. Biol. Chem., 150, 345 (1943).

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

(36) Mayo, J. W., Ph.D. Thesis, Michigan State University, East Lansing, Michigan, 1968.

(37) Simpson, F. J., Wolin, M. J., and Wood, W. A., J. Biol. Chem., 230, 457 (1958).

(38) Strecker, H., and Korkes, S., J. Biol. Chem., 196, 769 (1952).

(39) Lee, C. K., and Dobrogosz, W. J., J. Bacteriol., 90, 653 (1965).

(40) Doudoroff, M., Contopoulou, C. R., and Burns, S., Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957, Academic Press, N. Y., 1958, p. 313.

(41) Cuatrecasas, P., and Segal, S., J. Biol. Chem., 241, 5904 (1966).

(42) Avigad, G. A., Alroy, Y., and Englard, S., J. Biol. Chem., 243, 1936 (1958).

(43) Okamoto, K., J. Biochem. (Tokyo), 53, 348 (1963).

(44) King, T. E., and Cheldelin, V. H., Biochem. J., 68, 31 p (1958).

(45) Weimberg, R., Biochim. Biophys. Acta, 67, 359 (1963).

(46) Jermyn, M. A., Biochim. Biophys. Acta, 37, 78 (1962).

(47) Levene, P. A., and Simms, H. S., J. Biol. Chem., 65, 31 (1925).

(48) Levoy, G. A., McAllan, A., and Hay, A. J., Biochem. J., 82, 225 (1962).

(49) Wood, W. A., Methods Enzymol., 2, 212 (1955).

(50) Sober, H. A., Gutter, F. J., Wyckoff, M., J. Amer. Chem. Soc., 78, 756 (1965).

(51) Cori, O., and Lipmann, F., J. Biol. Chem., 195, 417 (1951).

(52) Lien, O. G., Anal. Chem., 31, 1363 (1959).

(53) Nelson, J. M., and Beegle, F. M., J. Amer. Chem. Soc., 41, 559 (1919).

(54) Weimberg, R., and Doudoroff, M., J. Biol. Chem., 217, 607 (1955).

(55) Weimberg, R., J. Biol. Chem., 234, 727 (1959).

(56) Stoolmiller, A. C., and Abeles, R. H., J. Biol. Chem., 241, 5764 (1966).

(57) Patton, S., and Kurtz, G. W., J. Dairy Sci., 34, 669 (1951).

(58) Jennings, W. G., Dunkley, W. L., and Reiber, H. G., Food Res., 20, 13 (1955).

(59) Srinivasan, P. R., and Sprinson, D. B., J. Biol. Chem., 234, 716 (1959).

(60) Lanning, M. C. and Cohen, S., J. Biol. Chem., 189, 189 (1951).

(61) Preiss, J. and Ashwell, G., J. Biol. Chem., 237, 309 (1962).

(62) Preiss, J. and Ashwell, G., J. Biol. Chem., 237, 317 (1962).

(63) Preiss, J. and Ashwell, G., J. Biol. Chem., 238, 1571 (1963).

(64) Preiss, J. and Ashwell, G., J. Biol. Chem., 238, 1577 (1963).

(65) Claus, D., Biochem. Biophys. Res. Commun., 20, 745 (1965).

(66) Warren, L., J. Biol. Chem., 234, 1971 (1959).

(67) Cornforth, J. W., Firth, M. E., and Gottschald, A., Biochem. J., 68, 57 (1958).

(68) Zagalak, B., Frey, P. A., Karabotsos, G. L., and Abeles, R. H., J. Biol. Chem., 241, 3028 (1966).

(69) Huff, E., Anal. Chem., 31, 1626 (1959).

(70) Huff, E., Rudney, H., J. Biol. Chem., 234, 1060 (1959).

(71) Huff, E., Biochim. Biophys. Acta, 48, 506 (1961).

(72) Van Eys, Jan, J. Biol. Chem., 236, 1531 (1961).

(73) Gupta, N. K., and Robinson, W. G., J. Biol. Chem., 235, 1609 (1960).

(74) Wohl, A., Chem. Ber., 41, 3599 (1908).

(75) Herschberger, C., Davis, M., and Binkley, S. B., J. Biol. Chem., 243, 1585 (1968).

(76) Samuelson, O., Ljungquist, K. T., and Parck, C., Svensk Papperstidn., 61, 1043 (1958).

(77) Shuster, C. W., and Doudoroff, M., Arch. Mikrobiol., 59, 279 (1967).

(78) De Ley, J. and Doudoroff, M., J. Biol. Chem., 221, 745 (1957).

(79) Kovachevich, R. and Wood, W. A., J. Biol. Chem., 213, 757 (1955).

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

(81) Berlet, H. H., Anal. Biochem., 22, 525 (1968).

(82) Dancis, J., Hutzler, J., and Levitz, M., Biochim. Biophys. Acta, 78, 85 (1963).

(83) Hawary, M. F. S., and Thompson, R. H. S., Biochem. J., 53, 340 (1953).

(84) Powers, H. H., Tabakoglu, G., and Sable, H. Z., Biochem. Prep., 4, 56 (1955).

(85) Metzger, R. P., Wilcox, S. S., Wick, A., J. Biol. Chem., 239, 1769 (1964).

(86) Metzger, R. P., Wilcox, S. S., Wick, A. N., J. Biol. Chem., 240, 2767 (1965).

(87) Cline, A. L., and Hu, A. S. L., J. Biol. Chem., 240, 4493 (1965).

(88) Weimberg, R., J. Biol. Chem., 236, 629 (1961).

(89) Wallenfels, K., and Kurz, G., Biochem. Z., 335, 559 (1962).

(90) Cline, A. L., and Hu, A. S. L., Biochim. Biophys. Acta, 93, 237 (1964).

(91) Bach, J., and Sadoff, H. L., J. Bacteriol., 83, 699 (1962).

(92) Doi, R., Halvorson, H., and Church, B., J. Bacteriol., 77, 43 (1959).

(93) Hauge, J. G., J. Biol. Chem., 239, 3630 (1964).

(94) Bean, R. C., and Hassid, W. Z., J. Biol. Chem., 213, 425 (1955).

(95) Bentley, R., and Bhate, D. S., J. Biol. Chem., 235, 1225 (1960).

(96) Eliel, E. L., Allinger, N. L., Angyal, S. J., and Morrison, G. A., Conformational Analysis, John Wiley and Sons, New York, 1967, p. 404.

(97) Conner, A. H., and Anderson, L., 157th National Meeting of the American Chemical Society, Minneapolis, Minn., April 1969, No. D 12.

(98) Sweeley, C. C., Bentley, R., Makita, M., and Wells, W. W., J. Amer. Chem. Soc., 85, 2497 (1963).

(99) Brodie, A., and Lipmann, F., J. Biol. Chem., 212, 677 (1954).

(100) Suzuki, K., Kawada, M., and Shimazono, N., J. Biochem. (Tokyo), 49, 448 (1961).

(101) Nishizuka, Y., and Hayaishi, O., J. Biol. Chem., 237, 2721 (1962).

(102) Winkelman, J., and Lehninger, A. L., J. Biol. Chem., 233, 794 (1958).

(103) Yamada, K., Ishikawa, S., and Shimazono, N., Biochim. Biophys. Acta, 32, 255 (1959).

(104) Bublitz, C., and Lehninger, A. L., Biochim. Biophys. Acta, 47, 288 (1961).

(105) Sistrom, W. R., and Stanier, R. Y., J. Biol. Chem., 210, 821 (1954).

(106) Smiley, J. D., and Ashwell, G., J. Biol. Chem., 235, 1571 (1960).

(107) Merrick, J. M., and Roseman, S., J. Biol. Chem., 235, 1274 (1960).

(108) Linker, A., Hoffmann, P., Meyer, K., Sampson, P., and Korn, E. D., J. Biol. Chem., 235, 3061 (1960).

(109) Albersheim, P., and Killias, U., Arch. Biochem. Biophys., 97, 107 (1962).

(110) Starr, M. P., and Moran, F., Science, 135, 920 (1962).

(111) Blumenthal, H. J., Methods Enzymol., 9, 660 (1966).

(112) Blumenthal, H. J., and Jepson, T., Methods Enzymol., 9, 665 (1966).

(113) Berman, T., and Magasanik, B., J. Biol. Chem., 241, 800 (1966).

(114) Maitra, U., and Dekker, E. E., J. Biol. Chem., 238, 3660 (1963).

(115) Hoffman, P., Linker, A., Lippman, V., and Meyer, K., J. Biol. Chem., 235, 3066 (1960).

(116) Preiss, J., and Ashwell, G., Biochem. Biophys. Res. Commun., 8, 357 (1962).

(117) Palleroni, N., and Doudoroff, M., J. Biol. Chem., 223, 499 (1956).

(118) Ghalambor, M., and Heath, E. C., Biochem. Biophys. Res. Commun., 11, 288 (1963).

(119) Dagley, S., and Trudgill, P. W., Biochem. J., 95, 48 (1965).

(120) Levine, D. H., and Racker, E., J. Biol. Chem., 234, 2532 (1959).

(121) Minamikawa, T., and Uritani, I., J. Biochem. (Tokyo), 61, 367 (1967).

(122) Roseman, S., Jourdian, G. W., Watson, D., and Rood, R., Proc. Natl. Acad. Sci. U. S., 47, 958 (1961).

(123) Comb, D. G., and Roseman S., J. Biol. Chem., 235, 2529 (1960).

(124) Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, M. M., Biochemistry, 5, 467 (1966).

(125) Entner, N., and Doudoroff, M., J. Biol. Chem., 196, 853 (1952).

(126) Kanamori, M., and Wixom, R. L., J. Biol. Chem., 238, 1004 (1963).

(127) Myers, J. W., J. Biol. Chem., 236, 1416 (1961).

(128) Wixom, R. L., Shatton, J. B., Strassan, M., J. Biol. Chem., 235, 128 (1960).

(129) Kovachevich, K., and Wood, W. A., J. Biol. Chem., 213, 745 (1954).

(130) Meloche, H. P., and Wood, W. A., Methods Enzymol., 9, 653 (1966).

(131) Hurlbert, R. E., and Jawby, W. B., Methods Enzymol., 9, 680 (1966).

(132) Buttin, G., Cold Spring Harbor, 26, 213 (1962).

(133) Buttin, G., J. Mol. Biol., 7, 164 (1963).

(134) Maitra, U., and Dekker, E. U., J. Biol. Chem., 239, 1485 (1964).

(135) Kuratomi, K., and Fukunga, K., Biochim. Biophys. Acta, 78, 617 (1963).

(136) Ghalambor, M. A., and Heath, E. C., J. Biol. Chem., 237, 2427 (1962).

(137) Charalampous, F. C., Methods Enzymol., 5, 283 (1962).

(138) Warren, L., and Felsenfeld, H., J. Biol. Chem., 237, 1421 (1962).

(139) Okano, A., Chem. Pharm. Bull. (Tokyo), 5, 176 (1957).

(140) Crumpton, M., and Davies, D. A., Biochem. J., 70, 729 (1958).

(141) MacPhillamy, A., and Elderfeld, J., J. Org. Chem., 4, 150 (1939).

(142) Khare, M. P., Helv. Chim. Acta, 45, 1515 (1962).

(143) Khare, M. P., Helv. Chim. Acta, 45, 1534 (1962).

(144) Khare, M. P., Helv. Chim. Acta, 37, 155 (1954).

(145) Tshesche, R., and Grimmer, G., Chem. Ber., 88, 1569 (1955).

(146) Satoh, D., and Ishii, H., Chem. Pharm. Bull. (Tokyo), 4, 284 (1956).

(147) Lichti, H., Tamm, C., and Reichstein, T., Helv. Chim. Acta, 39, 1914 (1956).

(148) Zelnick, R., and Schindler, O., Helv. Chim. Acta, 40, 2110 (1957).

(149) Power, F. B., and Rogerson, H., J. Chem. Soc., 101, (1912).

(150) Pigman, W., Chemistry of Carbohydrates, 2nd ed., Academic Press, New York, N. Y., 1948, p. 107.

(151) Votocek, E., Collect. Czech. Chem. Commun., 1, 46 (1929).

(152) Votocek, E., Collect. Czech. Chem. Commun., 1, 606 (1929).

(153) Votocek, E., J. Amer. Chem. Soc., 50, 1749 (1928).

(154) Kiliani, H., Chem. Ber., 25, 2116 (1892).

(155) Mannich, C., Arch. Pharm. (Weinheim), 276, 211 (1938).

(156) Reber, T., and Reichstein, T., Helv. Chim. Acta, 29, 343 (1946).

(157) Schmidt, O., Ann., 558, 70 (1947).

(158) Jacob, W., J. Biol. Chem., 96, 355 (1932).

(159) Lamb, I., and Smith, S., J. Chem. Soc., 442 (1936).

(160) Young, W. T., J. Org. Chem., 7, 241 (1942).

(161) Shah, N. M., Meyer, W., and Reichstein, T., Pharm. Acta Helv., 24, 113 (1949).

(162) Euw, J., and Gurtler, J., Helv. Chim. Acta, 40, 2079 (1959).

(163) Votocek, E., Chem. Zentra., I, 1042 (1901).

(164) Votocek, E., Chem. Zentra., II, 1361 (1902).

(165) Votocek, E., Chem. Zentra., II, 1527 (1905).

(166) Goldberg, M. L., and Sternbach, S. H., J. Amer. Chem. Soc., 80, 1639 (1958).

(167) Berger, J., Sternbach, L., and Calhoun, K. M., J. Amer. Chem. Soc., 80, 1636 (1958).

(168) Leach, B. E., J. Amer. Chem. Soc., 75, 401 (1953).

(169) Votocek, E., Chem. Zentra., I, 1818 (1906).

(170) Schmidt, O., and Mayer, W., Naturwissenschaften, 31, 247 (1943).

(171) Schmidt, O., and Mayer, W., Ann., 555, 26 (1944).

(172) Schmidt, O., Naturwissenschaften, 43, 130 (1965).

(173) Rosselet, J. P., and Hunger, A., Helv. Chim. Acta, 34, 2143 (1951).

(174) Stoll, A., and Junker, E., Modern Methods of Plant Analysis, Vol. 3, Paech, K., Ed., Springer Verlag, Berlin, p. 208.

(175) Leach, B. E., J. Amer. Chem. Soc., 75, 4011 (1953).

(176) Calhoun, K. M., and Johnson, J. E., Antibiotics and Chemotherapy, 6, 294 (1956).

(177) Springer, G., and Williamson, W., Fed. Proc., 18, 2348 (1959).

(178) Springer, G., and Williamson, W., Biochem. J., 85, 282 (1962).

(179) Kubat, E., Biochem. J., 85, 291 (1961).

(180) McMahon, T., and Nolan, M. O., J. Cell. Comp. Physiol., 50, 219 (1957).

(181) Sharon, N., and Shif, I., Biochem. J., 93, 210 (1964).

(182) Letherwood, E., Fed. Proc., 22, 239 (1963).

(183) Levvy, G., and McAllen, A., Biochem. J., 87, 206 (1963).

(184) Levvy, G., and McAllen, A., Biochem. J., 87, 361 (1963).

(185) Levvy, G., Nature, 187, 1027 (1960).

(186) Marney, A., and Got, O., Experienta, 20, 441 (1964).

(187) Remmer, H., Ann. Rev. Pharm., 5, 411 (1965).

(188) Braunvald, E., and Kloche, F., Ann. Rev. Med., 16, 371 (1965).

(189) MacLennon, A. P., and Randall, H. M., Biochem. J., 80, 309 (1961).

(190) Baker, J. R., Biochem. J., 82, 352 (1962).

(191) Spencer, J., and Giori, P., Can. J. Microbiol., 7, 185 (1961).

(192) Frommhagen, L. H., Virology, 2, 430 (1956).

(193) Itaki, O., Bull. Agric. Chem. Soc. Japan, 22, 436 (1958).

(194) Hijama, W., Tohoku J. Exptl. Med., 55, 333 (1952).

(195) Hirst, E. L., J. Chem. Soc., 323 (1951).

(196) Gefretin, T., Compt. Rend. Soc. Biol., 145, 115 (1951).

(197) Dillon, T., Proc. Royal Irish Acad. Sci., 55B, 189 (1953).

(198) Glegg, R. E., Science, 118, 614 (1953).

(199) Masamune, H., Tohoku J. Exptl. Med., 56, 37 (1952).

(200) Bertalanffy, F., Can. Med. Assoc., 70, 196 (1954).

(201) Evans, A., J. Fermen. Technol., 32, 147 (1954).

(202) Hosimoto, H., Bull. Japan Soc. Sci. Fishery, 20, 406 (1954).

(203) Emiliani, F., Rev. Fac. Ing. Quim., 21-22, 11 (1952-53).

(204) Kent, P. W., Brit. J. Exptl. Pathol., 36, 49 (1955).

(205) Dohlunan, C. H., Arch. Biochem. Biophys., 57, 445 (1955).

(206) Kirby, K. S., Biochem. J., 60, 582 (1955).

(207) Minganti, A., Exptl. Cell. Res. Suppl., 3, 248 (1955).

(208) Eagon, J., Compt. Rend., 241, 527 (1955).

(209) Glegg, R. E., Exp. Cell Res., 8, 453 (1955).

(210) Odin, L., Acta Chem. Scand., 9, 1235 (1955).

(211) Butt, H. R., J. Endocrin., 13, 167 (1956).

(212) Black, W. A. P., International Peat Symposium, Section B3 (1964).

(213) Quirk, J. P., Tappi, 39, 357 (1956).

(214) Ejirma, T., Igaku to Serbutsugaka, 33, 85 (1854).

(215) Vasseur, E., Arkiv Kemi, 1, 39 (1949).

(216) Paigen, K., J. Bacteriol., 92, 1397 (1966).

(217) Beggs, W. H., and Rogers, Palmer, J. Bacteriol., 91, 1869 (1966).

(218) Paigen, K., J. Bacteriol., 91, 1201 (1966).

(219) Ganeson, A. K., J. Mol. Biol., 16, 42 (1965).

(220) McBrain, D. C. H., J. Bacteriol., 91, 1391 (1966).

(221) Williams, B., and Paigen, K., Fed. Proc., 24, 417 (165).

(222) Williams, B., Biochem. Biophys. Res. Commun., 24, 143 (1966).

(223) Jayararnan, K., J. Mol. Biol., 18, 339 (1966).

(224) Echols, E., and Adkger, S., J. Bacteriol., 92, 601 (1966).

(225) Bertland, A., and Bugge, B., Arch. Biochem. Biophys. 116, 280 (1966).

(226) Alvardo, F., Biochi n. Biophys. Acta, 41, 233 (1960).

(227) Hu, A. S. L., and Cline, A. L., J. Biol. Chem., 240, 4493 (1965).

(228) Cuatrecasas, P., and Segal, S., J. Biol. Chem., 241, 5910 (1966).

(229) Kiliani, H., Chem. Ber., 53, 2866 (1930).

(230) Rheiner, A., and Reichstein, T., Helv. Chim. Acta, 35, 687 (1952).

(231) Ribas, I., Helv. Chim. Acta, 34, 1477 (1951).

(232) Reichstein, T., Helv. Chim. Acta, 35, 422 (1952).

(233) Reichstein, T., Helv. Chim. Acta, 36, 370 (1953).

(234) Richter, R., and Reichstein, T., Helv. Chim. Acta, 37, 76 (1954).

(235) Hegediis, H., Helv. Chim. Acta, 38, 1133 (1955).

(236) Katz, A., and Reichstein, T., Pharm. Acta Helv., 19, 231 (1944).

(237) Rittel, W., Hunger, A., and Reichstein, T., Helv. Chim. Acta, 36, 434 (1953).

(238) Jager, R. H., Helv. Chim. Acta, 42, 977 (1959).

(239) Kiliani, H., Arch. Pharm., 230, 250 (1892).

(240) Kiliani, H., Chem. Ber., 31, 2460 (1898).

(241) Kiliani, H., Chem. Ber., 51, 1633 (1918).

(242) Okada, S., J. Pharm. Soc. Japan, 73, 118 (1953).

(243) Sakasawa, P., Planta Med., 4, 20 (1956).

(244) Okano, A., Pharm. Bull. (Tokyo), 6, 173 (1957).

(245) Tschesche, R., Ann., 606, 160 (1957).

(246) Hoji, K., Pharm. Bull. (Tokyo), 9, 289 (1961).

(247) Kundig, W., Kundig, F. D., Anderson, B., and Roseman, S., J. Biol. Chem., 241, 3243 (1966).

(248) Luderitz, O., Staub, A. U., and Westphal, O., Bacteriol. Rev., 30, 192 (1966).

(249) Blumberg, W., Levine, W., and Persack, J., J. Biol. Chem., 242, 2850 (1967).

(250) Ashwell, G., and Volk, P., J. Biol. Chem., 240, 4549 (1965).

(251) Adye, J., and Powelson, D., J. Bacteriol., 81, 780 (1961).

(252) Bailey, J. M., and Pentchev, P. G., Amer. J. Physiol., 208, 385 (1965).

(253) Hindmarsch, G., J. Physiol. (London), 186,166 (1966.

(254) Levvy, G., and Conchie, J., Methods Enzymol., 9, 571 (1966).

(255) Hayaishi, D., Methods Enzymol., 9, 731 (1966).

(256) Esterly, J., Standen, A. C., and Pearson, B., J. Histochem. Cytochem., 15, 470 (1967).

(257) MacLennon, A. P., and Smith, D. W., Biochem. J., 74, 3 p (1960).

(258) Wheat, R., Rollins, E. L., and Leatherwood, J., Nature, 202, 492 (1958).

(259) Wheat, R., Rollins, E. L., Leatherwood, J. M., and Barnes, R. L., J. Biol. Chem., 238, 26 (1963).

(260) Bailey, J. M., Fishman, P. H., and Pentchev, P. G., J. Biol. Chem., 244, 781 (1969).

(261) Suzuki, N., Biochim. Biophys. Acta, 177, 371 (1969).

(262) Chiu, T., and Feingold, D. S., Biochemistry, 8, 98 (1969).

(263) Wilson, D. M., and Ajl, S., J. Bacteriol., 73, 410 (1957).

(264) Englesberg, E., Arch. Biochem. Biophys., 71, 179 (1957).

(265) Heath, E. C., Ghalambor, M. A., J. Biol. Chem., 237, 2423 (1962).

(266) Shah, N. M., Pharm. Acta Helv., 28, 120 (1953).

(267) Busch, H., Introduction to Biochemistry of the Cancer Cell, Academic Press, N. Y., p 265, 280.

APPENDIX A

OCCURRENCE AND METABOLISM OF D-FUCOSE AND ITS DERIVATIVES

D-Fucose (6-deoxy-D-galactose) is a constituent of cell walls, cardiac glycosides, mucoproteins, mucopolysaccharides and numerous other glycosides. This sugar and its 2-O-methyl-, 2,3-di-O-methyl-, 3-O-methyl-, and 2-amino-2-deoxy-, 3-amino-3-deoxy-, and N-acetyl-3-amino-3-deoxy-derivatives have been isolated from the hydrolysis products of heteroglycosides obtained from plants, fresh-water gastropods, and bacteria (139-176, 180-182, 229-246).

D-Fucose and D-digitalose (3-methoxy D-fucose) are liberated upon acid hydrolysis of chartreusin, an antibiotic active against Gram-positive organisms and mycobacteria; it is produced by Streptomyces chartreusis from African soil and by Streptomyces sp. from North American soil (166-168, 175-176).

D-Fucose has been isolated from cardiac glycosides obtained from seeds of Strophanthus sarmentosis and S. eminii (159, 162), and from leaves of Digitalis purpurea and D. lanata (145, 146). D-Digitalose has been isolated from the seeds of S. gerardii, S. commontii, S. armentosis, (173), and Digitalis purpurea (172). Strophanthus and Digitalis belong to the family Apocynaceae, which provides

numerous D-fucose-containing drugs widely used as heart stimulants. D-Fucose is a carbohydrate component of a cuproprotein, Stellacyanin (249) and has been isolated from the root glycosides of the morning glory, Ipomoe orizanesis (174), and from the family Cruciferae, which comprises the cabbages, cauliflowers, brussel sprouts, and mustard seed (174). 3-O-Methyl ethers of D-fucose have been obtained from the naturally occurring pure cardiac glycosides, strospesid and panstrosid (173). Other naturally occurring methoxy derivatives and methoxy glycosides of D-fucose are also documented (146-148, 177-179, 229-246).

The roots of various plants of the Convolvulaceae contain glycosides of D-fucose and have been used medicinally as purgatives for many centuries. The plants are cultivated in Mexico, Jamaica, and South America (161). In addition, four common species of aquatic gastropods have been found to possess specific polysaccharides containing D-fucose (180).

D-Fucosamine has been found to be present in bacteria. This amino sugar was first located in a specific lipopolysaccharide of the Gram-negative bacterium, Chromobacterium violaceum (140). Subsequently, it has been isolated from Bacillus lichenformis (181), B. subtilis (181), and B. cereus (182, 258, 259), Salmonella (248), Xanthomonas campestris (250), and Pseudomonas aeruginosa (261). Thus, D-fucose and its derivatives are quite

widespread; the cases cited represent but a few of the many sources that are documented (139-171, 174-176, 180).

The literature contains many instances in which fucose was detected and identified by co-chromatography with L-fucose, but in which no specific rotation studies were performed. Thus, D-fucose could conceivably be present in any of the following instances: O-methyl fucose in Myobacterium bovis (189) and M. tuberculosis (257), the organic material if egg shell matrix (190), the fucose polysaccharides produced in Corynebacterium insidiosium and C. sepedonicum (191), the polysaccharide found in the PR8 and the Lee strains of influenza virus (192), hemicellulose from bean seeds (193), fucose in the gastric mucosa (194), exudate from the bark of Ulmus fulva (195), mucoprotein from sedentary polychete worms (196), saponins hydrolyzate from Gypsophila (266), seed exudate of Ascophyllum nodosum (197), carbohydrate components of reticular fibers from lymph, lung, testis, and adipose tissue (198), placental carbohydrates (199), carbohydrate content of tissues of cancerous individuals (267), lung reticulin of cattle (200), polysaccharide produced by a strain of B. polymyxa (201), carbohydrates of mollusk style (202), fucose polysaccharide from cortical fibers (203), fucose content of human renal reticulin (204), Descement membrane of bovine cornea (205), quebracho tannin extract (206), amphibian egg jelly (207), capsular polysaccharide of Pseudomonas fluorescens (208),

guinea pig spermatozoa (209), human cervical mucus (210), urinary gonadotropins (211), sphagnum moss and peat (212), aspen wood (213), cerebrospinal fluids from patients with mental disorders (214), sea urchin eggs (215), microcysts of Myxococcus xanthus (251). The presence of L-fucose was inferred in each of the cases mentioned. However, because of the current realization of the prevalence of the D-isomer, it would be anticipated that in at least some of these cases D-fucose would be present instead of, or in addition to, L-fucose. This observation has also been made by Levvy (185), who stated, "Although L-fucose has been positively identified in mammalian tissue in at least two instances, in many cases, its presence has been inferred from evidence that does not distinguish it from its D-enantiomorph."

Thus, the literature substantiates the ubiquity of D-fucose. Although D-fucose was once thought to be a rarity compared to the supposed relative abundance of the L-isomer, it is felt that the widespread presence of D-fucose must now be recognized and that numerous new sources will continue to be unveiled.

Specific β-D-fucosidases have been reported in organisms on varying positions of the evolutionary scale. The visceral hump of the limpet, Patella vulgata, has been described as providing a good source of glycosides (183). It has been reported also that the limpet possesses an enzyme that hydrolyzes β-D-fucosides and that is dis-

tinguishable from β-D-galactosidase (183). In addition, Levvy et al. have reported a β-D-fucosidase from ox liver (184). Since then, specific β-D-fucosidases have been discovered in liver, kidney, and epididymis tissues of rat, ox, and pig (185). There are also documentations for specific β-D-fucosidases in cow ovary and uterus (148), in the digestive joice of the snail, Helix pomatia (186), and in the jejunum of rat, rabbit, mouse, and guinea pig (256). D-Fucose-containing cardiac glycosides have been shown to be cleaved in rat liver by a reportedly unknown glycosidase (187); presumably this enzyme is identical to the β-D-fucosidase isolated from rat liver by Levvy (185). It has also been shown that 92% of 6-deoxyhexose-glycosides from Digitalis sp. are metabolized by human subjects (188), and it is conceivable that man also possess a specific β-D-fucosidase. D-Fucono-γ-lactone has been found to be a competitive inhibitor of mammalian β-fucosidases (254).

No pathway for the metabolism of D-fucose has been elucidated in spite of its ubiquity. In fact, very little data concerning any aspect of D-fucose metabolism is available in the literature. Kundig et al. (247) have reported that in E. coli D-fucose induces a phosphotransferase which catalyzes a phosphoryl transfer from a phosphoprotein to galactose, thereby forming galactose-6-phosphate. Buttin (132-133) has reported D-fucose induction of galactokinase, galactose-1-P-

uridylyltransferase, and UDP-galactose-4-epimerase in E. coli. He also specifically reports that E. coli does not use D-fucose as a carbon source. Various other researchers have used D-fucose as a gratuitous inducer of the galactose operon on the assumption that the carbohydrate was not metabolized (216-224).

D-Fucose has also been reported to be a most efficient repressor of the lac operon (217, 218, 221) and to markedly enhance the fluorescence of UDP-galactose-4-epimerase (225). Alvardo (226) has shown that galactokinase isolated from Saccharomyces fragilis did not phosphorylate D-fucose using ATP as the phosphoryl donor.

Hu and Cline (227) and Wallenfels and Kurz (89) have isolated from D-galactose-grown Psuedomonas sp. a D-galactose dehydrogenase which oxidized D-fucose at the same rate as D-galactose. Cuatrecasas and Segal (228) have recently reported on a NAD-specific mammalian galactose dehydrogenase from rat liver which exhibited low activity on D-fucose. Hayaishi has also reported on a particulate lactose dehydrogenase of Pseudomonas graveolens which was highly active on D-fucose (255). There is no report in the literature that any of the above organisms which possess D-fucose dehydrogenases are capable of growing on D-fucose.

Echols et al. (224) have shown through inducer uptake experiments that D-galactose and D-fucose enter the cell via different transport systems. This is quite

interesting in light of the fact that specific β-glycosidases for D-fucosides and D-galactosides have been discovered (183-186); these findings suggest, perhaps, that two distinct operons are present in some bacteria for D-galactose and D-fucose metabolism. D-fucose has also been reported to be actively transported by rat and hamster small intestine and rat kidney (252, 253). A mutarotase from bovine kidney cortex has been shown to be very active on D-fucose (260) whereas mutarotase from rat kidney has been demonstrated to be inhibited by D-fucose (253). Despite the plethora of information concerning D-fucose, its biodegradative pathway has not been previously described in any organism.

APPENDIX B

ISOLATION AND CHARACTERIZATION OF THE PSEUDOMONAD USED IN THE PRESENT INVESTIGATION

The organism used in this study was isolated from commercial D-fucose. Solid, non-sterile D-fucose (50 mg) was added to a culture tube containing the mineral medium (10 ml) described in Experimental Procedures. Turbidity was noticeable after 5 days on a reciprocal shaker at 32°C. The culture was streaked on D-glucose-mineral agar; a colony was selected from the plate and was grown on D-fucose. This culture was streaked again on D-glucose-mineral agar; the above procedure was repeated twice again until purity was assured.

The organism thus selected was tested for classification by the Michigan State Veterinary Bacteriological Laboratory and the Michigan State Agricultural Pathological Laboratory. The characteristics of this organism, as determined by these two laboratories are as follows:

Gram stain: coccobacilli, Gram-negative

Bloodplate: sharp odor, similar to Pasteurella

Tryptone agar: slightly yellow, no pigment, sticky colonies

MacConkey agar: small, fine colonies

Kligler's agar: negative, growth light

Seller's agar: no gas butt, no fluorescence

Oxidase: positive

Catalase: positive, but slow

Nitrate reductase: positive

Citrate: positive

Urea: positive

Fermentation: non-fermentative, aerobic

Voges-Proskauer: positive

Indole: negative

Lysine: no growth

Motility: negative

Liquid Culture:

D-Glucose: positive

Lactose: negative

Starch: negative

L-sorbose: negative

Adonitol: negative

Dulcitol: negative

On the basis of the above data, the organism may be classified as a pseudomonad.

The organism was further tested for growth substrate specificity. D-Glucose log-phase cells were inoculated into mineral medium containing 0.5% carbohydrate. The carbohydrate and the time required to reach the midpoint of log-phase are listed below.

Carbohydrate	Time
D-Glucose	14 hours
D-Galactose	"
D-Galactarate	"
D-Galactonate	"
D-Glucarate	"
D-Fucose	"
L-Arabinose	"
Sucrose, Lactic Acid	24 hours
6-Iodo-6-deoxy-D-galactose	"
D-Gluconate	48 hours
Glycerol	"
D-Fructose	4 days
D-Glucosamine	"
D-Xylose	"

There was no growth after two weeks on D-arabinose, L-rhamnose, cellobiose, mannitol, sorbitol, lactose, propylene glycol, D-mannose, L-sorbose, L-mannose, L-galactose, L-glucose, or L-gluconate. After 2.5 weeks, slight growth was noticed on D-mannose, and full growth was noticed on propylene glycol.

Electron microscopy of this organism was performed by Richard F. Hamman on a RCA EMU-2E electron microscope. Specimens to be assayed were appropriately diluted and a 1 µl sample was placed on a 200 mesh copper grid (No. 2200, Ernest F. Fullam Assoc.) covered with 0.5% Formvar, air dried, and shadowed with tungsten oxide in a Kinney High

Vacuum Evaporator (New York Air Brake Co.) at an angle of 18°-20°. The tungsten oxide was generated by heating in air the 0.025 in. tungsten wire (Ladd Research Industries, Inc.). Polystyrene latex spheres of 0.26 μ, standard deviation 0.006 μ, (Dow Chemical Physical Research Lab, Midland, Michigan) were placed on the grids as a sizing reference prior to sample application.

Carbon stabilized grids were prepared by grinding a 1/16" dia. tip, 3/16" long on a carbon rod 1/8" in dia. and butting it against a flat-ended 1/8" rod. The evaporator "filament glow" rheostat was advanced to 100% (45-50 amps) and the "filament glow" switch was flashed on and off as rapidly as possible. This deposited an even layer of carbon over the entire grid surface. The use of carbon stabilized grids was necessary due to the failure of the Formvar film under conditions of negative staining with PTA. Samples to be negatively stained were mixed 1:1 with 2% PTA, pH 6.8 and placed on the grid to dry. If the staining was too heavy, appropriate dilutions were made. Samples were viewed at 50 KV accelerating voltage without an objective aperature in the EMU-2E. Electron micrographs were exposed on 2" x 10" projector slide plates, developed in D-19 for 1-2 minutes, fixed, and dried. Two electron micrographs of the pseudomonad are presented in Figure B-1. The microorganism is about 1.25 μ in length and is not flagellated.

Figure B-1