MUTATIONAL ACQUISITION OF D-FUCONATE CATABOLISM IN *KLEBSIELLA PNEUMONIAE*: ELUCIDATION OF THE PATHWAY AND CHARACTERIZATION OF D-GALACTONATE (D-FUCONATE) DEHYDRATASE

By

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ABSTRACT

MUTATIONAL ACQUISITION OF D-FUCONATE CATABOLISM IN *KLEBSIELLA PNEUMONIAE*: ELUCIDATION OF THE PATHWAY AND CHARACTERIZATION OF D-GALACTONATE (D-FUCONATE) DEHYDRATASE

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The genetic and enzymatic basis for the acquisition of the ability of *Klebsiella pneumoniae* to use the xenobiotic D-fuconate as a sole carbon source was determined. A single mutation resulted in the constitutive production of D-galactonate (D-fuconate) dehydratase, a previously undescribed enzyme which is induced in the parental strain by D-galactonate but not by D-fuconate. The dehydratase product, 2-keto-3-deoxy-D-fuconate (KDF), then induces a new class II aldolase, which cleaves KDF to pyruvate and D-lactaldehyde. This aldolase has no other known inducer. A mutant missing KDF aldolase failed to grow only on D-fuconate (out of 24 compounds tested that supported parental-strain growth), whereas a mutant missing the dehydratase failed to grow on both D-fuconate and D-galactonate. D-Galactonate (D-fuconate) dehydratase (four percent of the cellular protein) was purified to electrophoretic homogeneity and characterized with respect to substrate specificity, kinetic constants, pH optimum, metal requirement, molecular weight, isoelectric point, and amino acid composition.

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This thesis is dedicated to my dear parents, Charles A. and Bernice D. Hauswald, who gave both understanding and encouragement over the years and who now embark on a new journey of life, as do I.

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LIST OF ABBREVIATIONS

MW	molecular weight
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
PEP	Phosphoenol pyruvate
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
g	acceleration of gravity
g	gram
mg	milligram
µg	microgram
l	liter
ml	milliliter
µl	microliter
M	molar
mM	millimolar
µM	micromolar
cm	centimeter
mm	millimeter
µm	micrometer
nm	nanometer

EDTA	ethylenediamine tetraacetic acid
DEAE-	diethylaminoethyl-
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
PIPES	1,4-piperazinediethane sulfonic acid
A	absorbance
S	sedimentation coefficient (Svedberg; 1×10^{-13} second)
SDS	sodium dodecyl sulfate
TBA	thiobarbituric acid
α-Keto	alpha-keto (2-keto)
β-formyl pyruvate	beta-formyl pyruvate (3-formyl pyruvate)
KDF	2-keto-3-deoxy-D-fuconic acid
KDQ	2-keto-3-deoxy-D-quinovonic acid
KDGal	2-keto-3-deoxy-D-galactonic acid
D,L-KDA	2-keto-3-deoxy-D,L-arabonic acid
KDPGal	2-keto-3-deoxy-6-phospho-D-galactonic acid

GENERAL INTRODUCTION

The purpose of this work was to determine the basis for the mutational acquisition of the ability of Klebsiella pneumoniae to use D-fuconate (6-deoxy-D-galactonate) as the sole carbon and energy source for growth. The fact that D-fuconic acid apparently does not exist in Nature outside of serving as an intermediate of D-fucose metabolism in a pseudomonad, coupled with the inability of Klebsiella species to use the aldose D-fucose as a growth substrate, would indicate that D-fuconate catabolism in Klebsiella could serve as a model for a study of the degradation of xenobiotics (compounds that are rarely, if ever, found in Nature). As will be pointed out in the Literature Review, the ability of a microorganism to utilize a given xenobiotic is often dependent upon mutation and selection. In most cases, the mutations are found not to result in the formation of new enzymes, but rather in alterations in the inducibility of an enzyme. In the present case, the mutation resulted in the constitutive production of a dehydratase normally induced by D-galactonate but not by D-fuconate. The product of the dehydratase reaction, 2-keto-3-deoxy-D-fuconate, induces the formation of an aldolase which catalyzes the cleavage of the intermedate to pyruvate and D-lactaldehyde. Curiously, the aldolase has no known inducer other than this intermediate of the xenobiotic catabolism.

This thesis is divided into two sections. Section I deals with the isolation of a D-fuconate-positive mutant of K. pneumoniae, elucidation of the pathway of D-fuconate catabolism, and determination of the genetic and enzymatic basis for the acquired function. Section II deals with the purification and characterization of a previously undescribed enzyme, D-galactonate (D-fuconate) dehydratase, which is the passkey in the gained ability to use D-fuconate.

LITERATURE REVIEW REGARDING GAIN MUTATIONS, SUGAR ACID CATABOLIC PATHWAYS IN MICROORGANISMS, AND STRATEGIES FOR SUGAR CATABOLISM IN THE ENTEROBACTERIACEAE

Gain mutations

The constitutive production of enzymes for which novel compounds serve as fortuitous substrates is one of several biochemical bases by which gain mutations augment the growth of a bacterium on the novel compounds. Examples of this are the utilization of L-arabitol via ribitiol dehydrogenase (1), D-arabinose and L-xylose via L-fucose isomerase (2), mannitol via D-arabitol dehydrogenase (3), and β-glycerol phosphate via alkaline phosphatase (4). A more complex situation involving this type of mutation is the xylitol pathway in Aerobacter aerogenes (1,5,6). A single mutation leading to the derepression of ribitol dehydrogenase enables this bacterium to use xylitol as a fortuitous substrate but not its inducer (1,5). Through further mutation, a derivative strain with an improved rate of growth on xylitol was obtained from the first mutant made possible by the production of an altered ribitol dehydrogenase (6). This type of structural-gene mutation has been shown to operate in other instances which augmented growth of a bacterium on a novel compound (9). In addition, a third mutant with an even faster growth rate on xylitol was obtained. This strain constitutively produced an active transport system that accepted xylitol as well as D-arabitol (6), and produced the same altered ribitol dehydrogenase as seen in the second mutant. Rigby et al. (7) have shown that continuous culturing of the

constitutive strain, using xylitol as the sole carbon source, evolves faster growing strains which show elevated levels of ribitol dehydrogenase activity. Ribitol dehydrogenase has been observed to comprise up to 25% of the cell protein. Through examination of these strains with respect to growth on xylitol, kinetic parameters of the purified ribitol dehydrogenase, and mutation frequency for ribitol dehydrogenase, these authors concluded that the increased activity was due to duplication or amplification of the rdh gene. Such amplification occurs at frequencies much higher than mutational alteration of substrate affinity, and this allows the bacterial population to adapt to short-term environmental stress without altering the genetic constitution (7,8).

The acquisition of a catabolic pathway for L-1,2-propanediol by mutant strains of Escherichia coli K-12 provides yet another instance in which constitutive production of an enzyme plays a role in the gain mutation. Mutants selected through repeated transfer to medium containing L-1,2-propanediol have been found to lose the fucose permease, fucose isomerase, fuculose kinase, and fuculose-1-phosphate aldolase, normally functional in the catabolism of L-fucose in the wild-type organism (10). However, production of the propanediol oxidoreductase and the L-lactaldehyde dehydrogenase (normally acting in the breakdown of L-lactaldehyde to pyruvate, aerobically, and of L-lactaldehyde to propanediol, anerobically, respectively) were found in this bacterial mutant. These changes work in concert with an enhanced facilitated diffusion across the cell membrane in the trapping of propanediol by hastening its rate of entry and conversion to lactate.

Other mutations, such as the formation of new enzymes (11) in a few instances, and the type of mutation leading to the use of L-mannose, a toxic and unnatural sugar, in A aerogenes (12) are uncommon. Considering the mutational acquisition of the ability of A. aerogenes to use L-mannose, the wild-type organism can not use this sugar despite the fact that there is an inducible metabolic pathway for its degredation. Apparently the natural breakdown of L-mannose involves the formation of a metabolite that inhibits cell growth. However, through a series of mutations a strain can be obtained which overcomes this innate toxicity of the metabolite through some unknown process (13).

Other examples of gain mutations in bacteria are cited in published reviews (9, 14).

Sugar Acid Catabolic Pathways in Microorganisms

Since the original report of MacGee and Doudoroff (15) on the identification of 2-keto-3-deoxy-6-phospho-D-gluconic acid as an intermediate in the metabolism of D-glucose in Pseudomonas saccharophila, there is growing evidence that the production of 2-keto-3-deoxy sugar acids represent a general trend in the bacterial and mammalian utilization of monosaccharides and their respective acid derivatives. Thus, the metabolism of such compounds as D-glucose (16), D-galactose (17-19), D-arabinose (20), D-mannose (21), L-arabinose (22-23), D-gluconate (24-27), D-galactonate (28-29), L-galactonate (30), D-fucose and D-fuconate (31-34), L-fucose and L-fuconate (35-38), D-glucarate and D-galactarate (39-41), D-glucosaminic acid (42), and D-glucuronic and D-galacturonic acids (43), have been found to involve formation of analogous keto-deoxy intermediates

possessing common structural features.

Review of the literature shows that the formation of such 2-keto-3-deoxy sugar acids and sugar-acid phosphates have generally been effected through the action of specific dehydratases some of which require a phosphorylated and some a non-phosphorylated substrate. Such enzymatic dehydrations are found to occur α-β to the carboxyl group of aldonic, aldaric, and uronic acids. Phosphorylation reactions leading to the formation of sugar-acid phosphate intermediates, as effected by specific kinases, can occur either before or after such dehydrations. As for the degredation of such keto-deoxy intermediates, reactions involving aldol-type cleavage reactions are most common; other cleavages such as decarboxylation or dehydrogenation reactions are less commonly found.

With these observations, four basic routes for the degredation of sugar acids can be enumerated: (i) phosphorylation of the sugar acid followed by dehydration and then cleavage; (ii) dehydration of the sugar acid followed by phosphorylation and then cleavage; (iii) dehydration of the sugar acid, without phosphorylation, followed by cleavage; and (iv) successive dehydration followed by decarboxylation or dehydrogenation type cleavages to give the end products.

Regarding the first case, most evidence has been gained through the study of D-glucose and D-gluconate metabolism in bacteria. Entner and Doudoroff (16) found that cell-free extracts of Pseudomonas saccharophila could convert D-glucose to pyruvate and triose phosphate through the following entirely new pathway involving a dehydratase and aldolase:

D-glucose ——→ D-glucose-6-phosphate ——→ 6-phospho-D-gluconate ——→

2-keto-3-deoxy-6-phospho-D-gluconate ——→ pyruvate + D-glyceraldehyde-3-phosphate. This so-called Entner-Doudoroff pathway is known to be widely distributed amoung species of the Enterobacteriaceae and pseudomonads (44); however, in members of the Enterobacteriaceae, the presence of an inducible kinase allows D-gluconate to be phosphorylated directly to 6-phospho-D-gluconate. In E. coli (45) and Salmonella typhimurium (46), D-gluconate is metabolized through the following pathway: D-gluconate ——→ 6-phospho-D-gluconate (6PG) ——→ 2-keto-3-deoxy-6-phospho-D-gluconate (KDPG) ——→ pyruvate + triose phosphate; an active hexose monophosphate shunt (HMP) pathway is also utilized by these bacteria to convert 6-phospho-D-gluconate to ribulose-5-phosphate and pentose intermediates. Pseudomonas fluorescens (putida), has been found to possess both the Entner-Doudoroff (ED) pathway, HMP pathway, and an inducible D-gluconate kinase (converting D-gluconate to 6-PG) leading to the formation of pyruvate and triose phosphate (26): D-glucose ——→ D-gluconate ——→ 6-phospho-D-gluconate ——→ 2-keto-3-deoxy-6-phospho-D-gluconate ——→ pyruvate + D-glyceraldehyde-3-phosphate. The presence of 2-keto-D-gluconate and 2-keto-6-phospho-D-gluconate intermediates in Aerobacter cloaceae and A. aerogenes (48,49), Klebsiella aerogenes (50), and a variety of pseudomonads when grown on either D-gluconate or 2-keto-D-gluconate has been well documented. In Pseudomonas fluorescens (47) the presence of a dehydrogenase, kinase, and reductase results in the following divergent pathway for D-glucose: D-glucose ——→ D-gluconate ——→ 2-keto-D-gluconate ——→ 2-keto-6-phospho-D-gluconate ——→ 6PG ——→ KDPG ——→ pyruvate + D-glyceraldehyde-3-phosphate.

Formation of 2-keto-3-deoxy compounds through dehydration of the sugar acid before phosphorylation is quite common in the metabolism of other sugars besides D-glucose and D-gluconate. In the metabolism of the uronic acids D-glucuronic and D-galacturonic acids in E. coli (43), D-mannonic and D-altronic acids are formed, respectively. Each are found to undergo dehydration by specific dehydratases to a common product, 2-keto-3-deoxy-D-gluconic acid (KDG), which is found to be further metabolized through a kinase and aldolase in the following manner: KDG ⟶ 2-keto-3-deoxy-6-phospho-D-gluconic acid ⟶ pyruvate + triose phosphate. In Rhodopseudomonas spheroides (21) mutant cells that are able to grow on D-glucose are found to acquire D-gluconate dehydratase and enhanced levels of KDG kinase and 6-phospho-D-gluconate dehydrogenase activities. These activities allow D-glucose to be metabolized by way of the ED pathway. In addition, the presence of an "aldose dehydrogenase" system allows D-mannose and D-glucose to be degraded through the following pathways: D-mannose ⟶ D-manno-γ-lactone ⟶ mannonic acid ⟶ KDG ⟶ KDPG ⟶ pyruvate + triose phosphate; D-glucose ⟶ D-glucono-γ-lactone ⟶ D-gluconic acid ⟶ KDG ⟶ KDPG ⟶ pyruvate + triose phosphate. Along these same lines, it has been found that growth of Clostridium aceticium (51, 25) and the Achromobacter-Alcaligenes 64-70% (G-C) subgroup (52) on D-gluconate induce the dehydratase responsible for the following series of reactions: D-gluconate ⟶ KDG ⟶ KDPG.

The third route, dehydration of the sugar acid without phosphorylation, mainly concerns the metabolism of the sugars D-arabinose, D-galactose, D-galactonate, D-glucarate, D-fucose, L-arabinose, and

L-fucose. In Pseudomonas saccharophila, the sugars D-arabinose and D-galactose are metabolized through two completely different routes than are generally found in the various bacterial classes. Mutant strains adapted to grow on D-arabinose (20) are found to possess the enzymes responsible for effecting the following reactions: D-arabinofuranose ⟶ D-arabino-γ-lactone ⟶ 2-keto-3-deoxy-D-arabonic acid ⟶ pyruvate + glycolaldehyde. Galactose adapted strains, which cannot utilize either D-glucose or D-fructose are found to degrade D-galactose as follows: D-galactofuranose ⟶ D-galactono-γ-lactone ⟶ D-galactonic acid ⟶ 2-keto-3-deoxy-D-galactonic acid ⟶ pyruvate + D-glyceraldehyde (19). This later scheme has also been found to function in the metabolism of D-galactose in Gluconobacter liquefaciens (18) although cleavage is believed to take place through a phosphorylated intermediate, 2-keto-3-deoxy-6-phospho-D-galactonate, to yield the products pyruvate and triose phosphate. Recently in the K-12 strain of E. coli (28,29) the direct catabolism of D-galactonic acid was found to precede as follows: D-galactonate ⟶ 2-keto-3-deoxy-D-galactonate ⟶ 2-keto-3-deoxy-6-phospho-D-galactonate ⟶ pyruvate + D-glyceraldehyde-3-phosphate. This strain was found to utilze D-galactonate as a sole carbon source for growth and to possess an inducible system for the catabolism of this aldonic acid, i.e., a D-galactonate dehydratase, a 2-keto-3-deoxy-D-galactonate kinase, and a 2-keto-3-deoxy-6-phospho-D-galactonate aldolase, when grown on the inducer D-galactonate.

The manner by which D-gluconate is catabolized in Aspergillus niger (54) is markedly different than described for pseudomonad and

Escherichia species in that no phosphorylation occurs: D-gluconate ——→KDG——→pyruvate + D-glyceraldehyde. Metabolism of the dicarboxylic sugar acid D-glucarate by E. coli (40) and possibly other members of the family Enterobacteriaceae, is quite different than that which will be discussed for pseudomonads. D-Glucarate is initially dehydrated at each end of the compound to give a mixture of 2-keto-3-deoxy-D-glucaric acid and 5-keto-4-deoxy-D-glucaric acid of which the latter predominates; both intermediates are degraded further by the presence of a 2-keto-3-deoxy-D-glucaric acid aldolase to pyruvate and tartronic semialdehyde. Tartronic semialdehyde can be further degraded to glyceric acid by a reductase. There is good evidence that D-glucarate is degraded in the same manner by the species Aerobacter aerogenes, Salmonella typhimurium, and Klebsiella pneumoniae (40).

Concerning the metabolism of D-fucose in bacteria, a rare carbohydrate, Dahms and Anderson (31) were able to isolate a pseudomonad which readily utilized the sugar as sole carbon source for growth. Elucidation of the pathway revealed that D-fucose is metabolized as follows: D-fucose——→D-fucono-δ-lactone and D-fucono-γ-lactone——→2-keto-3-deoxy-D-fuconate——→pyruvate + D-lactaldehyde (31-34). The enzymes involved in the pathway were also found to be effective in the metabolism of the common sugar L-arabinose by a similar series of reactions: L-arabinose——→L-arabono-γ-lactone——→L-arabonic acid——→2-keto-3-deoxy-L-arabonic acid (KDA)——→pyruvate + glycolaldehyde (23). Thus a new pathway for the metabolism of L-arabinose has been shown to occur in a pseudomonad involving an aldolase type cleavage at the level of KDA rather than dehydration followed by dehydrogenation. This latter

pathway has subsequently been found to be effective in the metabolism of L-arabinose in Rhizobium japonicium which use this sugar as a prefered carbon source (55). Along these same lines, the metabolism of L-fucose in pork liver (35-38) has been found to procede as follows: L-fucose ⟶ L-fucono-δ-lactone ⟶ L-fuconate ⟶ 2-keto-3-deoxy-L-fuconate ⟶ 2,4(or 5)-diketo-5(or 4)-monohydroxyhexonate ⟶ two moles of lactate.

Concerning the final reaction group, namely those pathways showing successive dehydration followed by either dehydrogenastion or decarboxylation, the following sugars as found in pseudomonads are considered: D-glucarate, D-galactarate, and L-arabinose. Both D-glucarate and D-galactarate are found to be initially dehydrated to 4-deoxy-5-keto-D-glucarate which is further dehydrated and decarboxylated to 2-keto-glutaric acid semialdehyde followed by dehydrogenation to 2-keto-glutaric acid in most pseudomonads studied (39,56). L-Arabinose, as exemplified by Pseudomonas saccharophila (57), is metabolized through the following pathway: L-arabinose ⟶ L-arabino-δ-lactone ⟶ 2-keto-3-deoxy-L-arabonic acid ⟶ 2-ketoglutaric acid semialdehyde ⟶ 2-ketoglutaric acid.

Strategies for Sugar Catabolism in the Enterobacteriaceae

Klebsiella pneumoniae, being a member of the family Enterobacteriaceae, should show characteristic pathways of the class for common sugars. The following carbohydrates and their pathways are considered for future reference when considering growth studies of this bacterium: D-arabinose, L-arabinose, L-fucose, D-galactose, L-lyxose, L-xylose, L-arabitol,

xylitol, glycerol, D-lyxose, D-allose, L-mannose, D-xylose, D-fructose, D-mannose, sorbitol, mannitol, galactitol, cellobiose, gentiobiose, sucrose, and L-sorbose.

As described by Mortlock et al. (1), metabolism of various pentitiols and pentoses in Aerobacter aerogenes (presently classified as Klebsiella pneumoniae) were found to procede through the HMP pathway intermediates D-ribose-5-phosphate, D-ribulose-5-phosphate, and D-xylulose-5-phosphate, and two rare intermediates L-xylulose-5-phosphate and L-ribulose-5-phosphate. Investigations showed that: (i) D-ribose can be converted to D-ribulose-5-phosphate; (ii) D-arabinose and ribitol to D-ribulose then to D-ribulose-5-phosphate; (iii) D-xylose, D-lyxose, D-arabitol, xylitol to D-xylulose then to D-xylulose-5-phosphate; (iv) L-arabinose to L-ribulose then to L-ribulose-5-phosphate; (v) and L-xylose, L-lyxose, and L-arabitol to L-xylulose then to L-xylulose-5-phosphate.

The metabolism of D-arabinose, as has been deduced from studies with E. coli (59,60) and A. aerogenes (1), suggest two pathways. In A. aerogenes and strain B/R of E. coli (61), which utilize D-arabinose but not L-fucose, the pathway is as follows: D-arabinose——→D-ribulose ——→D-ribulose-5-phosphate——→D-xylulose-5-phosphate. In the K-12 strain of E. coli (60), which does use L-fucose, the following reactions are found: D-arabinose——→D-ribulose——→D-ribulose-1-phosphate ——→ dihydroxyacetone phosphate (DHAP) + D-glyceraldehyde. It is believed that the K-12 strain of E. coli makes use of the L-fucose enzymes in order to effect the isomerase, kinase, and aldolase cleavage reactions (1,60).

L-Arabinose metabolism in A. aerogenes is as follows: L-arabinose ——→ L-ribulose ——→ L-ribulose-5-phosphate ——→ D-xylulose-5-phosphate (62). L-Fucose metabolism in E. coli (63-65) has been studied and is believed to be similar in A. aerogenes, Salmonella enterdidis, and several Shigella species: L-fucose ——→ L-fuculose ——→ L-fuculose-1-phosphate ——→ DHAP + L-lactaldehyde. D-Galactose metabolism in these bacteria is believed to take place strictly through the Le Loir pathway: D-galactose ——→ D-galactose-1-phosphate ——→ UDP-glucose ——→ D-glucose-1-phosphate (78).

In addition, the metabolism of L-mannose, D-fructose, sorbitol and mannitol, galactitol, and L-sorbose in A. aerogenes are as follows: L-mannose ——→ L-fructose ——→ L-fructose-1-phosphate ——→ DHAP + L-glyceraldehyde (11,12); D-fructose ——→ D-fructose-1-phosphate ——→ D-fructose-1,6-diphosphate (66-68); mannitol and sorbitol ——→ ——→ mannitol-1-phosphate and sorbitiol-1-phosphate ——→ D-fructose-6-phosphate (69,70); galactitol ——→ D-galactitol-6-phosphate ——→ D-tagatose-6-phosphate ——→ D-tagatose-1,6-diphosphate ——→ DHAP + D-glyceraldehyde-3-phosphate (71); L-sorbose ——→ L-sorbose-1-phosphate ——→ D-glucitol-6-phosphate ——→ D-fructose-6-phosphate (72).

The dissacharides, gentiobiose, cellobiose, and sucrose are all found to be cleaved to give characteristic glycolytic intermediates in K. pneumoniae. Gentiobiose is metabolized through an ATP-dependent phosphorylation of the dissacharide to give gentiobiose monophosphate followed by hydrolysis to give D-glucose and D-glucose-6-phosphate (73). Cellobiose is similarly modified to give D-glucose and D-glucose-6-phosphate as hydrolysis products (74-76). Sucrose is not phosphorylated,

but hydrolyzed directly to give D-glucose and D-fructose (77).

SECTION I

Elucidation of the Metabolic Fate of D-Fuconate

INTRODUCTION

The pathway for the catabolism of D-fuconate in Klebsiella pneumoniae has not been previously determined. D-Fuconate is known to be an intermediate in the metabolism of D-fucose in a pseudomonad, but K. pneumoniae seems not able to be forced to use D-fucose as a sole carbon and energy source for growth. However, K. pneumoniae will grow on D-fuconate after a lag of several days. The experimental results presented in this section will provide chemical, enzymatic, and genetic evidence for a pathway for D-fuconate in this bacterium as follows: D-fuconate ⟶ 2-keto-3-deoxy-D-fuconate ⟶ pyruvate + D-lactaldehyde. The gain mutation which facilitates growth of this bacterium on D-fuconate has been determined to be the constitutive production of a dehydratase that is normally induced by D-galactonate but not by D-fuconate. The aldolase which cleaves 2-keto-3-deoxy-D-fuconate is induced by this substrate, but not by D-galactonate or its metabolites. Thus, whereas D-fuconate is apparently a fortuitous substrate for D-galactonate dehydratase, the normal function for the aldolase is difficult to conceive.

MATERIALS AND METHODS

Bacterial Strain, Cell Growth, and Preparation of Cell-Free Extracts

Bacterial Strain. Klebsiella pneumoniae PRL-R3,U^-, an auxotroph requiring uracil for growth, was used as the parental strain for the D-fuconate-positive mutants.

Media. Broth cultures of cells were grown in a mineral medium containing 0.15% KH_2PO_4, 0.71% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, 0.01% $MgSO_2$, and 0.0005% $FeSO_4 \cdot 7H_2O$, supplemented with 0.5% carbohydrate and 0.005% uracil, or in nutrient broth (Difco), prepared by mixing 8.0 g dehydrated powder with 1.0 liter distilled water to which 0.005% uracil was added. Mineral-medium-agar plates were prepared by mixing sterile mineral salts and agar to give the same concentration of salts as in the broth, but with an agar content of 1.5%. All mediums were adjusted to pH 7.0 before used.

Cell Growth. All broth and agar plate cultures were grown in the dark at 30°C in a thermostatically controlled incubation room. Broth cultures in 18 X 150 mm culture tubes were equipped with plastic closure caps and contained 7.0 ml of media; cultures of 100-ml volume were grown in 250-ml erylenmeyer flasks equipped with cotton plugs. Aeration was accomplished by constant motion on either reciprocal or rotary shakers of the New Brunswick Scientific Company. Broth cultures were inoculated from fully grown (7.0 ml) cultures having an absorbance at 600 nm of 0.60 or better.

Monitoring Cell Growth. Cell growth was monitored with either a Coleman Junior Spectrophotometer, Model 6A, or a Gilford, Model 2400, Spectrophotometer at a wavelength of 600 nm. Cultures grown in 18 X 150 mm culture tubes could be directly measured in the Coleamn Jr. using an uninoculated culture tube as a blank.

A plot of corrected absorbance versus the percent of maximal growth was used when reporting turbidity measurements. This plot, prepared by correlating absorbance values from undiluted samples to absorbance values from diluted samples, permitted one to correct for deviations from Beers law at the higher cell concentrations. The Gilford spectrophotometer was used when sampling growing cultures directly with the aid of sterile pasteur pipets; measurements were made in microcuvettes (0.2-ml volume).

Harvesting of Cells. Cell cultures were incubated at 30°C on appropriate shakers until an uncorrected optical density of 0.60 at 600 nm was reached on the Coleman Jr. or an uncorrected value of 2.0 at 600 nm with the Gilford spectrophotometer. The cells were suspended in 0.85% NaCl and centrifuged again at 12,000 X g for 10 min. The cell pellets could then be used within the hour or frozen at -20°C until needed.

Centrifugation. All centrifugations were done in a Sorval refrigerated centrifuge, model RC-2B, at 0-4°C. The rotor radius was either 4.34 or 5.75 inches.

Preparation of Cell-Free Extracts. Cell extracts were prepared by suspending the harvested, washed cells in 0.05 M potassium phosphate

buffer (pH 7.0) and exposing the solution to sonic vibration (10,000 Hz) in a Raytheon sonic oscillator, model DF-101, for 30 min. Prior to sonication, cell pellets derived from 7.0-ml cultures were resuspended in 0.5 ml of the sonication buffer and placed in 0.5 X 3.0 inch cellulose nitrate-centrifuge tubes equipped with rubber stoppers. These tubes were then placed in the chamber of the apparatus and cold water was added to the level of the solution inside of the tubes. Larger cell-suspensions were sonicated directly in the chamber. All sonications were done in the presence of glass beads and having the chamber temperature near $4^{o}C$ with the aid of a circulating water-ice bath. Following sonic disruption of the cells, the suspension was centrifuged at 12,000 X g for 10 min to remove whole cells and debris. The resultant supernatant was decanted as the crude extract.

Protein Determination

Protein concentration was determined using the procedure of Lowry et al. (79). Bovine serum albumin was used as the protein standard.

Gas-Liquid Chromatography

Trimethylsilylated derivatives of aldoses and their respective aldonic acids and lactones were prepared by incubating 0.1 mg of the carbohydrate with 0.2 ml dry pyridine and 0.2 ml N,O-Bis-(Trimethylsilyl) trifluoroacetamide (BSTFA) for 30 min at $50^{o}C$ in a teflon-sealed 0.5-dram vial. Gas-liquid chromatography was performed on a Beckman Varian Aerograph, model 210 , employing a 3% OV-17 column and temperature program from 100-$230^{o}C$. Traces were recorded on unmarked chart

paper but calibrated in terms of temperature knowing the rate of temperature change during the trace. Retention times were normalized to the internal standard potassium D-gluconate; under the conditions employed, this standard had a retention time of 22.0 minutes.

Preparation of Ion Exchange Resins

Dowex resins used as ion exchangers for purposes of desalting or column chromatography were prepared by preconditioning with either acid or base; the desired ion form was then prepared by treatment of the resin with the ion solution of choice through either a batch-wise or a column method. Cation exchange resins, Dowex-50, were treated first with 2.0 N NaOH then washed to neutrality with distilled water and then treated with 2.0 N HCl to give the acid form. Anion exchange resins, Dowex-1, were treated alternately with 2.0 N HCl, distilled water, 2.0 N NaOH, distilled water, and then either ammonium bicarbonate in the preparation of Dowex-1 (bicarbonate) or 2.0 N formic acid, distilled water, sodium formate in the preparation of Dowex-1 (formate). All resins were washed with distilled water in the final form and air dried or used in packed form in a column of known dimensions.

Colorimetric Assays

A Gilford spectrophotometer, model 2400, was used to measure absorbance units of sugar chromogens at their specific wavelengths. Cuvettes with a 1.0-cm path length were used in all cases. In cases where standard curves were developed, commercial-grade carbohydrates used were dried to constant weight over phosphorous pentoxide before

preparing the stock standard in solution. Blanks for all assays were made by substituting distilled water for the carbohydrate solution.

Reducing Sugar Assay. The Phenol/H_2SO_4 assay of Dubois et al. (80) was used to quantitate reducing sugar solutions. Standard curves were prepared using between 10 to 70 μg of carbohydrate in the standard assay. D-Glucose was used as standard for hexoses of which chromogens were read at 490 nm. Pentoses and uronic acids were quantitated using D-ribose and D-glucuronic acid, respectively, as standards which were read at 480 nm.

Aldonic Acid Assay. Aldonic acids were quantitated by the alkaline hydroxylamine assay of Hestrin (81) after conversion to their corresponding lactones. Lactonization was effected by heating an aqueous solution of the aldonic acid, 2 to 8 μmol, in 2.0-ml volume, with 2.0 N HCl for 15 min at 90°C. Excess HCl, which interfers with the hydroxylamine formation, was completely removed from the samples by use of a Rotomix evaporator, temperature-regulated at 90°C. D-Galactono-γ-lactone and potassium D-galactonate gave identical standard curves in this procedure. The purple-brown chromogen produced was read at 540 nm.

Aldehyde Colorimetric Assays. D-Lactaldehyde was determined as the tetraazopentamethine cyanine dye by the method of Paz et al. (82). Acetaldehyde, 0.05 to 0.10 μmol, was used to develop the standard curve. The blue chromogen which developed in the assay was read at 670 nm. The molar extinction coefficient for acetaldehyde of 60,000 (82) was used to quantitate the aldehyde solution.

Alpha-Keto Acid Determination. All α-keto acids were determined as their semicarbazones by the procedure of MacGee and Doudoroff (15).

Semicarbazones of α-keto acids have characteristic molar extinction coefficients of 10,200 at 250 nm (15). Standard curves were developed using sodium pyruvate as the standard.

2-Keto-3-Deoxy Sugar Acid Determination. The procedure of Weissbach and Hurwitz (83) was used to determine 2-keto-3-deoxy sugar acids. Carbohydrates were subjected to periodate oxidation in this assay which yields β-formyl pyruvate as reported (83), giving a characteristic chromogen with thiobarbituric acid absorbing maximally at 551 nm. Molar extinction coefficients for various sugar acids quantitated by this assay were determined using the semicarbazone assay to quantitate the α-keto acid content. As latter sections of this thesis will show, the following compounds have molar extinction coefficients at 551 nm in this assay of: 3,6-dideoxy-threo-D-hexulosonic acid (2-keto-3-deoxy-D-fuconate), 50,060; 3,6-dideoxy-erythro-D-hexulosonic acid (2-keto-3-deoxy-D-quinovonic acid), 10,930; 2-keto-3-deoxy-D-galactonate, 60,000.

Preparation of Substrates

Aldonic Acid Synthesis. Aldonic acids were prepared from respective aldoses by the hypoiodate oxidation procedure of Moore and Link (84). The aldose (2.0 g) was initially dissolved in distilled water (4.0 ml) to which was added 25.0 ml absolute methyl alcohol. D-Galactose has to be heated into solution before the addition of the alcohol. The aldose solution was then added to a stirring solution of 5.7 g resublimed iodine in 80.0-ml acetone-free methanol (absolute) temperature regulated at 40°C. A three-neck flask (500 ml) equipped with

ground-glass joints, a separatory funnel, $CaSO_4$ drying tube, and thermometer heated in a water bath-magnetic stirrer/heater apparatus. Heat was removed once the aldose was added to the reaction mixture and a solution of 40% KOH in methanol (115 ml) was gradually added over a period of 30 minutes. The endpoint of the reaction was detected upon the development of a straw-yellow color, indicating that the iodine was consumed, and was stirred 20 additional minutes to assure complete salt formation of the aldonic acid.

Recovery of the aldonic acids from the reaction mixture is dependent on their solubility in methanol/water medium. L-Arabonic, D-galactonic, and D-gluconic acids precipitated out of the reaction mixture as their potassium salts. These salts could be collected by suction filtration, and washed with methanol and ether to give dry products easily weighed and handled. D-Fuconate was also obtained as a potassium salt, however the reaction mixture had to be kept at $4^{o}C$ in order to initiate cyrstallization. After one day at this temperature, all of the salt could be collected by suction filtration and washed with methanol and ether. D-Lyxonic, D-mannonic, and D-xylonic acids were obtained as their barium salts by treating the reaction mixture with a suspension of 20% barium iodide in methanol (20 ml). The salts were collected by centrifugation and washed several times with methanol and ether before drying. The potassium salts could be obtained by treatment of the barium salts first with Dowex-50W (H^+) and then with Dowex-50W (K^+). In all cases, no less than an 80% molar yield was obtained, and all salt products were shown to be homogeneous by gas-liquid chromatography. Retention times for D-glucose, D-galactose, and D-fucose aldose, aldonic

acids, and acid-generated lactones as prepared by the this procedure are given (Table 1). In each case, only one peak is seen with the aldonic acid generated from the commercial aldose which form characteristically one lactone peak when prepared as in the hydroxylamine assay (see above).

Preparation of Oxaloacetic Acid. Oxaloacetic acid used in the synthesis of 2-keto-3-deoxy sugar acids was prepared from commercial grade sodium diethyloxaloacetic acid (Sigma Chem. Co.) as described by Cornforth et al. (85). The solid oxaloacetic acid diethyl ester (200 g) was initially washed by resuspension in ether (400 ml) and filtered by suction filtration through fritted glass until a light yellow powder was obtained. The collected powder, 158.0 g, was then acidified by dissolving into a stirring solution of 2.0 N H_2SO_4 (2.0 liter) and ether (500 ml) at 4°C. A large erylenmeyer flask equipped with a rod stirrer was used to mix the solution. After the solid had completely dissolved, about 20 minutes, the ether and aqueous layers were separated and the aqueous layer was extracted with three (250 ml) portions of ice-cold sodium bicarbonate (5-10% w/v) until the washings no longer were colored. The ether extracts were then dried over anhydrous $MgSO_4$ and dried to a syrup on a Spinco evaporator at 25°C under reduced pressure.

The syrup was then distilled at 135-140°C at 15 microns Hg to a clear oil using Bantum-ware micro-distillation equiptment, a thermostatically controled oil bath, and a HiVac diffusion pump. The resultant distillate, ethyl oxaloacetate, could be stored indefinitely

Table 1. Retention times for carbohydrates determined by gas-liquid chromatography. The following aldoses, aldonic acids, and aldonic acid lactones were prepared as trimethylsilyl derivatives and subjected to gas-liquid chromatography as described in Materials and Methods. Reported aldoses were commercial grade; aldonic acids (K^+ salts) were prepared as described in the text. Lactones of respective aldonic acids were prepared by heating in acid and evaporating to rid of excess HCl as described for the hydroxylamine assay. Retention times were all normalized to the retention time of D-gluconate, having a retention time of 22.0 minutes under the conditions as described in Materials and Methods.

CARBOHYDRATE	SUGAR FORM	RELATIVE RETENTION TIME (min/22.0 min)
D-FUCOSE	ALDOSE	0.587, 0.618, 0.676
	ALDONIC ACID	0.825
	LACTONE	0.726
D-GALACTOSE	ALDOSE	0.823, 0.858, 0.906
	ALDONIC ACID	1.00
	LACTONE	0.965
D-GLUCOSE	ALDOSE	0.830, 0.850, 0.892
	ALDONIC ACID	1.00
	LACTONE	0.972

at -20°C, later to be hydrolyzed to the free acid. The yield of ethyl oxaloacetate from the diethyl ester (200 g) was 55.0 g; the oil density was 1.108 g/ml. Free oxaloacetic acid was prepared by hydrolyzing aliquots of the ethyl oxaloacetate oil in concentrated HCl; 27.0 ml of the oil (30.0 g) was dissolved in 125.0 ml of HCl and left to stand for 2-3 days. The free acid that crystallized out upon standing was collected by filtration through fritted glass giving a yield of 14.8 g of free oxaloacetic acid per 30 g of the oil or a 50% weight yield. The collected acid was then dried over KOH in a desiccator and stored at -20°C until needed. Quantitation of the synthetic product by either the semicarbazone assay (15) or with malate dehydrogenase gave identical results.

Synthesis of D-Lactaldehyde. D-Lactaldehyde was prepared from L-threonine by the ninhydrin oxidative deamination procedure of Zagalak et al. (86). Commercial grade L-threonine (3.0 g) was rapidly added to a vigorously stirred solution of 9.1 g ninhydrin in 0.05 M sodium citrate buffer (pH 5.4) of 600-ml volume. Carbon dioxide was liberated in the course of the reaction so a large mixing vessel was used which permits maximal surface area. After 15 min stirring at 100°C, the resultant purple mixture was allowed to cool to room temperature. Once cooled, the solution was filtered through Whatman #1 filter paper and collected by suction filtration. The filtrate was then treated alternately with Dowex-50W (H^+) and Dowex-1-X8 (bicarbonate) as described by Zagalak et al. (86). The final solution was shown to be clear at neutral pH and when quantitated for aldehyde by either the colorimetric assay (82) or with alcohol dehydrogenase a yield of 14.8 μmol

product was found.

Chemical Synthesis of 2-Keto-3-Deoxy-D-Fuconate.

(i) Portsmouth Synthesis. 2-Keto-3-deoxy-D-fuconate was prepared by the alkaline aldol-condensation reaction of oxaloacetic acid and D-lactaldehyde as described by Portsmouth (87). This reaction is predicted to form two isomers, 3,6-dideoxy-threo-D-hexulosonic acid (2-keto-3-deoxy-D-fuconic acid; KDF) and 3,6-dideoxy-erythro-D-hexulosonic acid (2-keto-3-deoxy-D-quinovonic acid; KDQ), in a ratio of 1.0 to 2.0, respectively. In addition, the procedure is capable of completely resolving the two isomers by ion exchange chromatography.

A solution of 8.0-ml volume containing D-lactaldehyde (2.8 mmol) was added to 20.0 ml of 0.05 M potassium phosphate buffer (pH 7.5) at 25^{o}C and adjusted to the pH of the buffer with the aid of a Sargent pH Stat with an 8.0 N KOH reservoir. Oxaloacetic acid (5.7 mmol) was then added gradually as the pH of the solution was maintained at 7.5. Once all of the oxaloacetic acid was dissolved, the reaction mixture was left to stand for a total of 16 hrs with continuous stirring. Assays of the reaction mixture at the end of this incubation by the procedure of Weissbach and Hurwitz (83) showed no further production of 2-keto-3-deoxy sugar acid compounds and thus the reaction was judged to be complete.

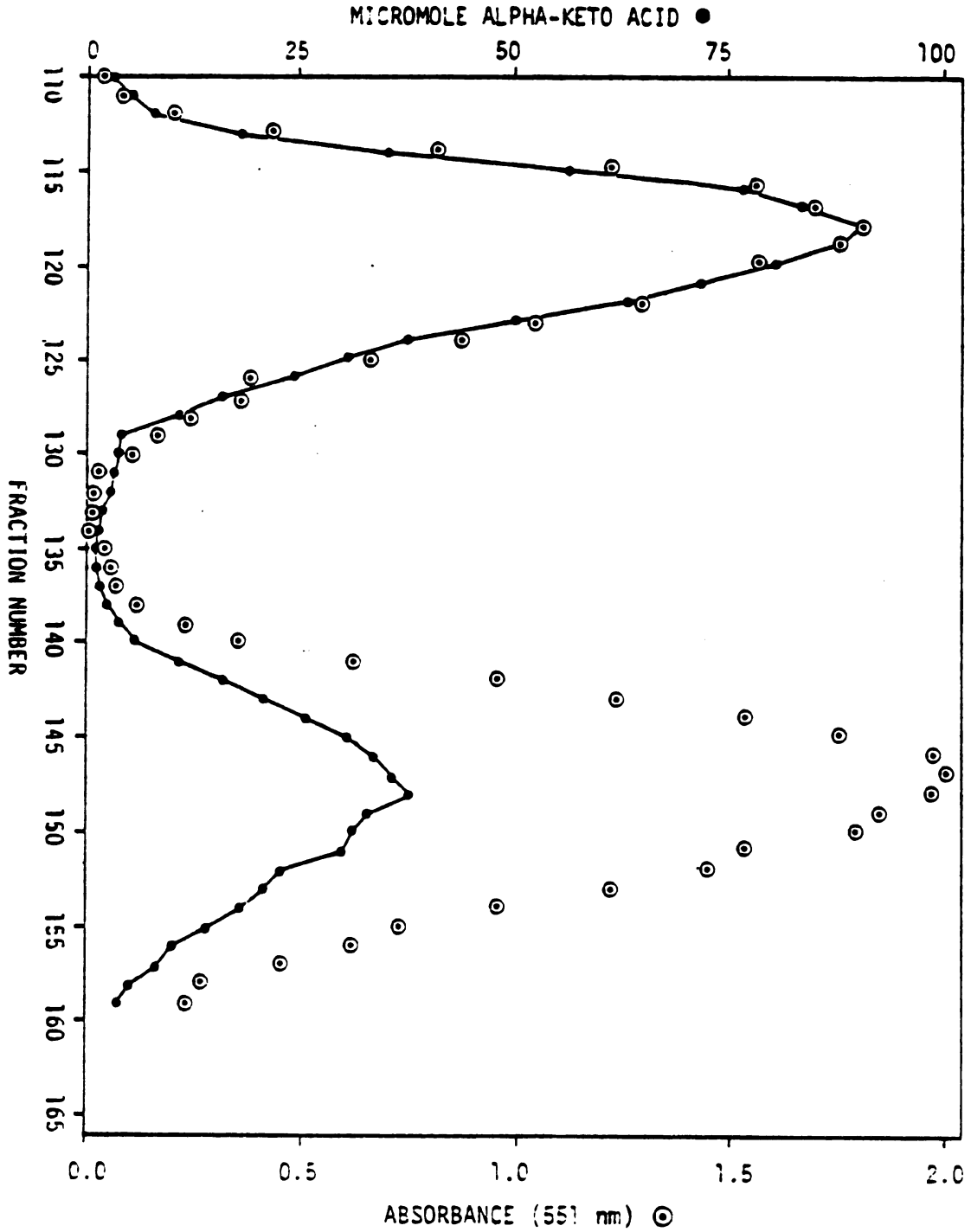
To prepare the reaction mixture for ion exchange chromatography, the pH of the solution was adjusted to pH 4.0 with formic acid and degassed by gentle aspiration over steam. The sample in 70.0-ml volume was applied to a 3.8 X 50.0 cm column of Dowex-1-X8 (formate), 200-400 mesh, at a rate of 140 ml per hour by gravity. Once the reaction

mixture was loaded, the column was washed in the following manner: (a) 2.0 liters of distilled water was passed through the column to wash products that did not stick to the resin from the ion exchanger, (b) 2.2 liters of a 0.23 M formic acid solution was passed over the resin to remove loosely bound products, and (c) 3.5 liters of 0.46 M formic acid solution was passed over the resin to elute the keto-deoxy sugar acids.

Unreacted D-lactaldehyde was detected by the aldehyde assay of Paz et al. (82) in approximately 252 to 420 ml of distilled water wash; a recovery of 223 µmol aldehyde was determined upon pooling and concentration of the peak fractions. Assays of the eluate fractions from the 0.23 M formic acid washings failed to show any additional aldehyde or semicarbazone-positive material. However, two well resolved peaks were found in eluate fractions subsequent to the 0.46 M formic acid wash; both peaks were found to give positive semicarbazone derivatives characteristic of α-keto acids and reacted as keto-deoxy sugar acids in the thiobarbituric acid assay (83).

The elution profile of the 2-keto-3-deoxy sugar acids (Fig. 1) following the 0.46 M formic acid washing was similar to that found by Portsmouth (87). The first peak, defined by Portsmouth as the erythro isomer (KDQ), and second peak, defined as the threo isomer (KDF), were pooled separately and concentrated to syrups under reduced pressure at 40°C several times with distilled water to remove formic acid. These syrups were taken up in known volumes of distilled water, neutralized with KOH, and characterized by known colorimetric assays. Semicarbazone assays of each isomer gve 873 µmol α-keto acid for KDQ

Figure 1. Elution profile of the 3,6-dideoxy-D-hexulosonic acids from the Portsmouth synthesis. The reaction mixture was prepared and treated as described in the text. Fractions of 21.0-ml volume were assayed for α-keto acid content by the semicarbazone assay (15) and for 2-keto-3-deoxy sugar acid content by the thiobarbituric acid assay (83) in eluate fractions of the 0.46 M formic acid wash.


MICROMOLE ALPHA-KETO ACID ●
0
25
50
75
100
110
115
120
125
130
135
140
145
150
155
160
165
FRACTION NUMBER
0.0
0.5
1.0
1.5
2.0
ABSORBANCE (551 nm) ⊙

(fractions 110 to 130) and 425 μmol α-keto acid for KDF (fractions 140 to 160), based on the molar extinction coefficient of 10,200 at 250 nm (15). Molar yields of these products were 67.2% and 32.8% for KDQ and KDF, respectively. The ratio of KDQ to KDF, observed as 2.0 to 1.0, is comparable to that value reported by Portsmouth (87). In addition, quantitation of each product by the semicarbazone assay facilitated the assignment of molar extinction coefficients for the thiobarbituric acid assay (TBA) (83); values for KDF and KDQ, at 551 nm, were determined as 50,060 and 10,930, respectively.

(ii) Dahms Synthesis. In contrast to the findings of Portsmouth, Dahms (33) reported that one isomer, KDF, was produced by the aldol-condensation between oxaloacetic acid and D-lactaldehyde. This fact contradicts chemical theory, as the asymmetric carbonyl group of D-lactladehyde should permit both syn- and anti-facial attack by the oxaloacetic acid carbanion generated in the chemical reaction (87). The result of such an attack must be the formation of two isomers, unless steric hindrance prevented one from being formed; as the reaction does yield an isomeric mixture in the Portsmouth synthesis, steric hindrance is ruled out.

After examining the protocol by which Dahms synthesized the compound KDF two possible postulates were drawn to explain why he failed to detect two isomers: (a) the alkaline conditions (pH 11) underwhich the synthesis was conducted resulted in the loss of KDQ, or (b) the method of chromatography failed to separate KDQ from KDF. When the Dahms' protocol was tried, a yield of thiobarbituric acid-positive material, comparable to that found by Portsmouth, was detected in the

reaction mixture and which did not diminish over time at pH 11 (see below). However, elution of the product with a gradient of 0.0-0.46 M formic acid from a Dowex-1-X8 (formate) column (0.5 X 15.0 cm) did not result in two TBA-positive peaks. These results suggested that the column dimensions used in the Dahms procedure could not resolve the two isomers formed, rather than indicate that only one isomer was generated. To prove this was the case, a reaction mixture was prepared according to the Dahms' protocol and was chromatographed by the procedure of Portsmouth.

The reaction mixture was prepared using a similar protocol as described for the Portsmouth synthesis; the main difference between the two procedures is the pH of the medium and the time of reaction. Oxaloacetic acid (3.4 mmol) was initially dissolved in 4.0 ml of 0.05 M carbonate-bicarbonate buffer (pH 10.7) to which was added 9.2 mmol of D-lactaldehyde (9.2 ml); the pH of the reaction mixture was adjusted to 11.0 with 8.0 N NaOH. The reaction was monitored for products releasing β-formyl pyruvate by the thiobarbituric acid assay and was judged complete when further production was negligible (about 20-40 min at 25^{o}C). The reaction mixture was treated with formic acid and degassed as done in the Portsmouth protocol and loaded onto another Dowex-1-X8 (formate) column of the same dimensions as that used before.

Approximately 1.16 mmol of unreacted D-lactaldehyde was recovered from the water wash. Elution of the column with 0.46 M formic acid revealed similar results as seen in the Portsmouth synthesis; two well resolved peaks were detected by both the semicarbazone and thiobarbituric acid assay (Fig. 2). The peak fractions were pooled separately

Figure 2. Elution profile of the 3,6-dideoxy-D-hexulosonic acids from the Dahms' synthesis. The reaction mixture was prepared and treated as described in the text. Fractions of 21.0-ml volume were assayed for α-keto acid content by the semicarbazone assay (15) and 2-keto-3-deoxy acid content by the thiobarbituric acid assay (83) in eluate fractions of the 0.46 M formic acid wash.

MICROMOLE ALPHA-KETO ACID ●
0
10
20
30
40
50
60
70
110
115
120
125
130
135
140
145
150
155
160
FRACTION NUMBER
ABSORBANCE (551 nm) ⊙
0.0
0.5
1.0
1.5

and treated as before. Assays of the pooled neutralized fractions gave 621 μmol α-keto acid for the first peak, and 420 μmol α-keto acid for the second peak by the semicarbazone assay. As defined by the Portsmouth procedure, yields of 59.7% for KDQ and 40.4% for KDF were observed. These results show that the mechanism of the reaction was the same in both procedures of synthesis as the products were produced in the same overall yield.

As noted in the Portsmouth synthesis, molar extinction coefficients for KDQ and KDF were determined to be approximately 11,000 and 50,000, respectively, at 551 nm. As these isomers were not separated by the Dahms procedure of chromatography, the reported molar extinction coefficient of 27,900 (33) should reflect that for the isomeric mixture. In fact, if the molar yields of each isomer (0.6 M for KDQ and 0.4 M for KDF) are used to calculate the molar extinction coefficient for the mixture, a value approximating that reported by Dahms is obtained:

$$\text{MIXTURE EXT. COEF.} = 11{,}000^{551\text{nm}}_{\text{KDQ}}\ (0.6\ \text{M}) + 50{,}000^{551\text{nm}}_{\text{KDF}}\ (0.4\ \text{M})$$

$$= 26{,}600$$

$$\text{DAHMS' EXT. COEF.} = 27{,}900$$

(iii) Summary of the Synthetic Methods. These results show that either synthesis (Portsmouth or Dahms) can be used to prepare isomeric mixtures of 3,6-dideoxy-D-hexulosonic acids which can be separated by methods of column chromatography. The procedure of Portsmouth should be used in purification of the isomers KDQ and KDF as the Dahms procedure fails to effect separation. This latter point is critical

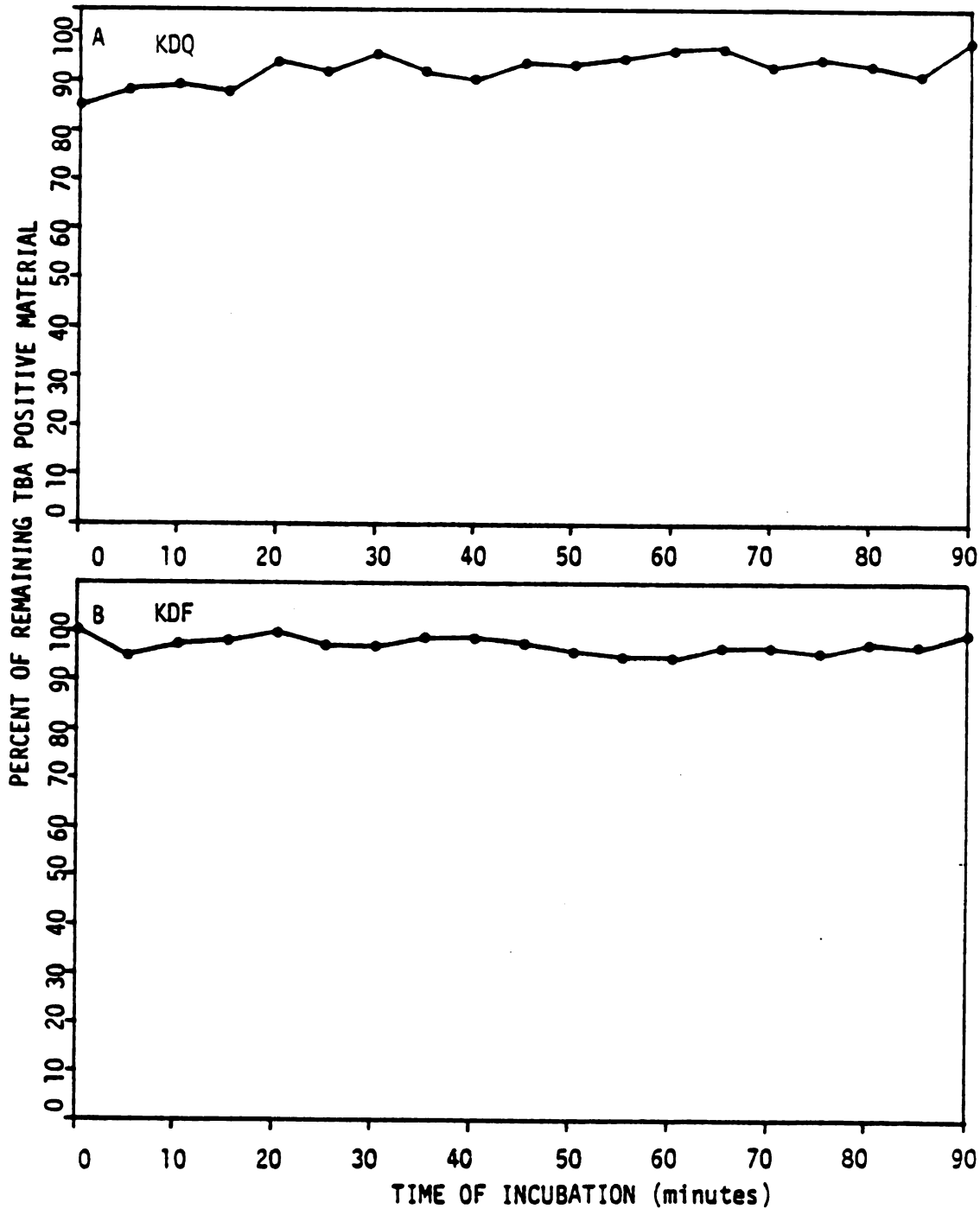
in the preparation of synthetic standards to be used as substrates in enzymatic reactions. With regard to the studies conducted by Dahms on the pseudomonad D-fuconate dehydratase and the KDF aldolase, misleading results may have been obtained. Although Dahms showed that his product was cleaved by periodate at a rate similar to that of 2-keto-3-deoxy-D-galactonate and slower than 2-keto-3-deoxy-D-gluconate (33) this does not prove the threo configuration for this compound in light of the fact that his substrate contained an isomeric mixture. To determine the configuration of the chemically synthesized product Dahms conducted a periodate rate study using the thiobarbituric acid assay to measure the formation of β-formyl pyruvate (88). This assay has been found to be inhibited to a limited degree by acetaldehyde (89), a product of the periodate oxidation of 3,6-dideoxy-D-hexulosonic acids. It is likely that when rates were measured by Dahms using the isomeric mixture as reactant, the rate of color development fortuitously matched that of 2-keto-3-deoxy-D-galactonate and pure (enzymatic) 2-keto-3-deoxy-D-fuconate. In addition, substrate specificity studies on the pseudomonad KDF aldolase using the reaction mixture prepared as described by Dahms gave yields of one mole of D-lactaldehyde and pyruvate per mole of substrate (34); this indicates that each isomer is used by this enzyme. If this is the case, both reported substrate specificity and kinetic constants for the KDF aldolase are inaccurate.

(iv) Alkaline Lability of the Portsmouth Synthetic Products. Dahms (88) suggested that the failure to produce two isomers in his synthesis was a result of the pH used in the synthesis rather than the method of column chromatography. To dispute this premise, the isomers from the Portsmouth synthesis were subjected to similar conditions of pH as found in the Dahms procedure. Results (Fig. 3) show that within the restraints of the Dahms synthesis no loss of either isomer occurs over a 90 min period of incubation; therefore, losses of either isomer would not be expected to occur once they were synthesized as a result of the high pH used in the Dahms synthesis.

Preparation of other 2-Keto-3-Deoxy Sugar Acids. 2-Keto-3-deoxy-D-galactonate (as well as 2-keto-3-deoxy-D-fuconate from D-fuconate) was prepared enzymatically from D-galactonate, using the purified D-galactonate (D-fuconate) dehydratase as described in Section II, Results. Purification of these products from the reaction mixture was accomplished using the chromatographic procedures as described in the Portsmouth synthesis (87). Quantitation of the α-keto acid content by the semicarbazone assay gave molar extinction coefficients of 50,060 for KDF and 60,000 for KDGal at 551 nm in the TBA assay.

2-Keto-3-deoxy-D,L-arabonate (D,L-KDA) and 4-hydroxy-2-keto-D,L-glutarate (KHG) were prepared by the aldol-condensation between oxaloacetic acid and glycolaldehyde and glyoxylate, respectively. The procedure of Stoolmiller and Abeles (57) was used in the synthesis and purification of KDA. The product of the reaction was detected using the TBA assay (83) both in the synthesis and subsequent

Figure 3. Alkaline lability of the chemically synthesized isomers of the Portsmouth synthesis. Stock isomers prepared and purified according to the Portsmouth procedure (87) were diluted in 0.05 M carbonate-bicarbonate buffer (25 ml) at a pH of 10.7; 2-keto-3-deoxy-D-quinovonic acid (KDQ) was used at 5.2 mM concentration and 2-keto-3-deoxy-D-fuconic acid (KDF) was used at 1.9 mM concentration. The pH of the mixture was titrated to a pH of 11.0 with the aid of a Sargent pH stat and an 8.0 N KOH resevoir upon mixing of the components. Samples were taken every 5.0 min and assayed immediately by the thiobarbituric acid assay (83) to determine losses over time of the TBA-positive material. (A) Time of incubation study of 2-keto-3-deoxy-D-quinovonic acid at pH 11.0, and (B) time of incubation study of 2-keto-3-deoxy-D-fuconic acid at pH 11.0.


A
KDQ
B
KDF
PERCENT OF REMAINING TBA POSITIVE MATERIAL
0 10 20 30 40 50 60 70 80 90 100
0 10 20 30 40 50 60 70 80 90
TIME OF INCUBATION (minutes)

purification steps. Quantitation of the purified product by the semicarbazone assay gave a molar extinction coefficient of 60,000 for the TBA assay at 551 nm.

KHG was prepared by the procedure of Aronson et al. (90), using the colorimetric assay of Kramer et al. (91), to detect the disappearance of glyoxylic acid in the reaction mixture. In the column chromatography, eluate fractions collected were assayed for glyoxylate as mentioned, for pyruvate and oxaloacetic acid by lactate dehydrogenase and malic acid dehydrogenase, respectively, and the reaction product (KHG) by the semicarbazone assay. Quantitation of the purified product as the semicarbazone gave reported yields; this product cannot be cleaved by periodate to give β-formyl pyruvate in the TBA assay and therefore no extinction coefficient is reported for this product.

2-Keto-3-deoxy-6-phospho-D-galactonate (barium salt) was a gift from the laboratory of W.A. Wood. The barium salt was taken in the solid state and converted to the corresponding potassium salt by treatment with Dowex-50W (K^+) in solution of known volume; quantitation of this phospho-ester was by the TBA assay and correlated to the weight amount for accurate concentration determination.

Enzymatic Assays

One unit of enzymatic activity is defined as the amount of enzyme that catalyzes the conversion of one micromole of substrate to product per minute at 30°C. Assays involving the oxidation or the reduction of pyridine nucleotides were monitored in microcuvettes with a 1.0-cm path length at 340 nm, using a thermostated Gilford spectrophotometer,

model 2400. Other assays that involved incubations were done in a temperature-controlled Precision Scientific Company water bath. The concentrations of the substrate added to an assay were determined on a weight basis of the added substrate, except in the case of carbohydrate derivatives, which were assayed by specific colorimetric assays as described above.

Dehydrogenase Assays (using D-Galactose, D-Galactonate, D-Fucose, or D-Fuconate as substrate). Assays of 0.2-ml volume consisted of 50 mM Hepes buffer (pH 7.0), 10.0 mM $MgCl_2$, 5.0 mM NAD^+ or $NADP^+$, 40.0 mM D-galactose, D-galactonate, D-fucose, or D-fuconate, and rate-limiting amounts of cell extract.

D-Galactonate (D-Fuconate) Dehydratase. Assays of 0.3-ml volume were composed of 50.0 mM Pipes buffer (pH 7.0), 10.0 mM $MgCl_2$, 1.0 mM EDTA (pH 7.0), 20.0 mM D-galactonate (K^+) or 30.0 mM D-fuconate (K^+), plus rate-limiting amounts of the dehydratase. The reaction mixture in 1.3 X 10.0 cm culture tubes were preincubated at 30°C before adding the enzyme to initiate the enzymatic assay. At appropriate time periods, the assay was stopped by the addition of 1.0 ml of semicarbazone reagent (1.0% semicarbazide·HCl plus 1.5% sodium acetate, in distilled water) directly to the sample. Once added, the samples were incubated at 30°C for an additional 20 min to form semicarbazone products of the α-keto acids produced by the enzyme. Samples were then diluted to 5.0-ml volume with distilled water and centrifuged with the aid of an International clinical centrifuge to remove protein precipitates. Controls to correct for protein were minus substrate (D-fuconate or D-galactonate).

Semicarbazones for α-keto acids were quantitated using the molar extinction coefficient of 10,200 at 250 nm (15). Under the conditions of the assay, an absorbance of 2.04 was equal to 1.0 μmol of 2-keto-3-deoxy-aldonate. The reaction was linear in the range of 5.0 nmol to 5.0 μmol product. One unit of dehydratase was defined as the amount of enzyme that converted 1.0 μmol of aldonic acid to α-keto acid per minute.

2-Keto-3-Deoxy-D-Galactonate Kinase (KDGal kinase). The reaction mixture of 0.2-ml volume consisted of 50.0 mM Hepes buffer (pH 7.0), 10.0 mM $MgCl_2$, 0.25 mM NADH, 2.5 mM PEP, 2.5 mM ATP, 11.0 mM 2-keto-3-deoxy-D-galactonate (enzymatic), excess pyruvate kinase and lactate dehydrogenase, and rate-limiting amounts of the enzyme. Controls to correct for ATPase and NADH oxidase were minus KDGal as substrate.

2-Keto-3-Deoxy-6-Phospho-D-Galactonate Aldolase (KDPGal aldolase). Assays of 0.2-ml volume consisted of 50.0 mM Hepes buffer (pH 8.0), 10.0 mM $MgCl_2$, 0.25 mM NADH, 2.5 mM 2-keto-3-deoxy-6-phospho-D-galactonate (enzymatic), excess lactate dehydrogenase, and rate-limiting amounts of the enzyme. Controls to correct for NADH oxidase were minus KDPGal as substrate.

2-Keto-3-Deoxy-D-Fuconate Aldolase (KDF aldolase). The reaction assay of 0.2-ml volume consisted of 50.0 mM Hepes buffer (pH 8.0), 10.0 mM $MgCl_2$, 0.25 mM NADH, 1.0 mM EDTA (pH 7.0), 10.0 mM 2-keto-3-deoxy-D-fuconate (chemically synthesized or enzymatic), excess coupling enzyme and rate-limiting amounts of the aldolase. Lactate dehydrogenase was used as the coupling enzyme when determining pyruvate production

by the aldolase. Alcohol dehydrogenase (horse liver) was used as the coupling enzyme when determining aldehyde (D-lactaldehyde) production by the aldolase; this coupling enzyme shows a broad substrate specificity for aldehydes and has been shown to act very well on D-lactaldehyde (86). Controls were run without KDF to correct for NADH oxidase when crude extracts were assayed by either of these assays.

End-Point Assays for the Determination of Pyruvate, D-Lactaldehyde and Oxaloacetic Acid. The reaction mixture of 0.2-ml volume consisted of 50.0 mM Hepes buffer (pH 8.0), 10.0 mM $MgCl_2$, 0.25 mM NADH, 0.025 to 0.10 mM substrate, and excess coupling enzyme. A substrate blank was used to correct for dilution when the coupling enzyme was added to initiate the assay. The end-point of the assay was judged complete when no further NADH was oxidized, as measured at 340 nm. Pyruvate was assayed with beef liver lactate dehydrogenase (92), D-lactaldehyde with horse liver alcohol dehydrogenase (86), and oxaloacetic acid with malic acid dehydrogenase from yeast (93). Quantitation of the oxidation of NADH in the assay was done assuming an extinction coefficient of 62,200 at 340 nm.

Selection of D-Fuconate- and D-Galactonate-Negative Mutants

An overnight culture of K. pneumoniae PRL-R3,U^-, strain CH-101 (see Results, this section), which is constitutive for growth on D-fuconate, was grown in nutrient broth and was harvested in a sterile centrifuge tube. The cell pellet was washed once in mineral-salts medium and resuspended in 5.0 ml of mineral medium containing 0.2 M ethylmethane sulfonate (EMS) (94). After incubating the EMS-treated culture for

2.0 hrs at 30°C on a reciprocal shaker the cells were harvested and suspended in 100 ml of mineral-medium broth which contained 0.5% D-glucose and 0.005% uracil. The culture was then incubated overnight, allowing for a ten-fold increase in cell number. Once grown, the culture was harvested and washed with mineral-medium. Washed cells were then suspended in 0.5% D-fuconate (K^+)-mineral-medium at a cell concentration of 2.0 X 10^8 cell/ml. Cultures were then incubated until a doubling of the cell number was observed, at which time the culture was made 10.0 mM in D-cycloserine, and penicillin-G was added at a concentration of 2000 units/ml (100). The cultures were then incubated for 6.0 hrs, allowing for 2-3 times the generation time on D-fuconate to occur. Cells which were unlysed by this treatment were enhanced in number by suspending them in 7.0 ml of mineral-medium plus 0.5% D-glucose and 0.005% uracil, after washing, and incubated for an overnight period of growth at 30°C.

The D-glucose-grown cultures were then plated onto mineral-media agar containing 0.5% D-fuconate, 0.005% D-glucose, and 0.005% uracil. Plates were inoculated with about 200 viable cells per plate, incubated at 30°C, and checked for the appearance of colonies after 24 and 48 hrs of incubation. Pin-point colonies were taken from the plates with a culture loop and transfered to D-glucose-mineral-medium (7.0 ml) where they were grown overnight. Colonies were screened for growth on D-fuconate and D-galactonate by streaking respective mineral-agar plates from these cultures. Growth was monitored visually upon incubation of the plates; positive growth showed a definite lawn of transluscent

cells, whereas negative or sparse growth showed no lawn or at best isolated colonies which most likely represented revertant colonies. Suspect mutants for either D-galactonate or D-fuconate negativity or both were then screened for normal growth on various carbohydrates by streaking onto appropriate plates.

Enzymatic characterization of the mutants was done using standard assay procedures for the dehydratase and aldolase of the D-fuconate pathway as described in this section. Nutrient broth cultures were used for crude extracts when testing for the dehydratase. Crude extracts for the KDF aldolase were prepared from nutrient broth cultures supplemented with 0.5% D-fuconate; D-fuconate was added at half-maximal growth on nutrient broth and incubated for 3.0 hr in the presence of the aldonic acid. In all cases, a control for both the normal levels of the dehydratase and the aldolase were run using a culture of the strain CH-101 grown under the same conditions.

Revertants for the Dehydratase-Negative Mutant

Dehydratase-negative mutant (CH-103) was grown in nutrient broth overnight and plated onto 0.5% D-galactonate, 0.005% D-glucose, and 0.005% uracil mineral-medium-agar plates. Plates were then incubated at 30°C and checked for growth at 24 and 48 hrs. Both pin-point and large colonies were picked and grown overnight in nutrient broth. Colonies were screened as described, for growth on D-galactonate and D-fuconate-mineral-medium plates. Crude extracts were prepared from nutrient broth cultures of colonies screened and assayed for the dehydratase by standard procedures.

IMViC Tests for the Bacteriological Classification of all Strains

Standard procedures for IMViC tests used in this section were adapted from a published source (96). As controls, the parental strain, Klebsiella pneumoniae PRL-R3,U^- (grown at 30°C) and Escherichia coli, strain B/r ara-2 (grown at 37°C) were used in all tests.

Indole production was determined using the Ehrlich Rosendale reagent with bacterial strains having grown 2.0 days in peptone water. A positive test gave a rose pink color upon the addition of the reagent to the ether extracted culture. A negative test gave no color change upon the addition of the reagent. Indole, at a concentration of 0.05 M in peptone water, was used as the control. Quantitation of the color yield was not made.

Acid production from 3-day-old glucose-phosphate-broth cultures of the strains was determined by the addition of a few drops of methyl red directly to the culture. Positive acid production in this test gave a definite red color. Negative test left a pale yellow color upon the addition of the dye. No quantitation of the color yield was made. A broth culture titrated to a pH of 3.0 was used as control.

Voges-Proskauer tests, for the detection of acetoin or acetylmethylcarbinol, were performed on 2-day-old glucose-phosphate-broth cultures of all strains tested. A positive test gave a pink color which developed upon standing after the addition of KOH directly to the culture. Acetoin at 0.05 M in the medium was used as the control. A negative test gave no color change. No attempt was made to quantitate the color yield.

Growth on citrate was checked using Simon's citrate medium with

and without 0.005% uracil. Inoculations were made from peptone water cultures grown overnight. Cultures in the test, were incubated at 30°C or at 37°C on a reciprocal shaker and turbidity measurements were made at 24 hrs of growth. Positive growth gave an absorbance of 0.3 at 600 nm or better; a 0.5% D-glucose-mineral-medium-broth culture inoculated with the strain CH-101 was used as a positive control.

Sources of Materials

All reagents (including carbohydrates, enzymes, and assay co-factors) included in this section were obtained from the Sigma Chemical Company, unless noted otherwise. Special notice is given to the commercial sources of the following materials:

Bacto-agar and nutrient broth, used for mineral-medium plates and for broth cultures were obtained in the dry state from Difco Laboratories. Glass beads used in the preparation of crude extracts were obtained as Microspheres (125-80 microns) from La Pine Scientific Company. Bovine serum albumin, used as the Lowry protein standard, was obtained from Sigma Chemical Company. N,O-Bis-(Trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from the Pierce Chemical Company. Packing material, 3% OV-17 on Gas-Chrom Q, was obtained from Applied Science Laboratories Incorporated as a dry powder. Dowex resins were obtained from the Sigma Chemical Company as dry beads, which were washed with HCl, NaOH, and methanol to extract impurities of the commerical preparations before use. 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) used in the colorimetric assay of Paz et al. (82) and acetaldehyde were obtained from the Aldrich Chemical Company. Thiobarbituric

acid (TBA) used in colorimetric assays for the detection of 2-keto-3-deoxy sugar acids, and semicarbazide·HCl used in the determination of α-keto acids were obtained from Sigma Chemical Company; TBA was recrystallized from hot ethanol after passing through aluminum oxide (BDH Laboratories) before use, as the commerical preparation has colored impurities which are exhibited in aqueous solution. Ninhydrin was obtained from the Pierce Chemical Company. Glycolaldehyde and glyoxylic acid were obtained from Sigma Chemical Company. Ethylmethane sulfonate was obtained from Eastman-Kodak. Indole and acetoin, used as standards in the IMViC tests were obtained from Sigma Chemical Company and Aldrich Chemical Company, respectively. Horse liver alcohol dehydrogenase was obtained from the Worthington Biochemical Corporation.

RESULTS

Selection of Mutant Strain CH-101 (D-Fuconate-Positive) from Klebsiella Pneumoniae PRL-R3,U⁻, and Gross Characterization

When cells of Klebsiella pneumoniae PRL-R3,U⁻ from either nutrient broth or D-glucose-mineral-medium-broth cultures were inoculated into 0.5% D-fuconate-mineral medium plus uracil, initiation of growth took approximately 60 hrs at 30°C on a reciprocal shaker. If such cultures were then plated onto mineral-media-agar plates containing 0.5% D-fuconate plus 0.005% D-glucose and 0.005% uracil, and incubated at 30°C for 48 hrs, two main colony types developed: pin-point and large-size colonies. The large colonies could grow to an absorbance of 0.3 at 600 nm in approximately 8 to 10 hrs of incubation when transferred to mineral-medium broth supplemented with D-fuconate and uracil, as above. However, when pin-point colonies were transferred to D-fuconate-mineral medium plus uracil growth took the normal 60-hr incubation as seen with the parental strain. Moreover, normal (i.e., parental strain) growth patterns were seen with both colony types on a variety of carbohydrates other than D-fuconate. These results indicated that a mutant strain with the augmented ability to use D-fuconate as the sole source of carbon for growth was selected from the parental strain. The large colonies on D-fuconate-mineral-agar plates were then designated D-fuconate-positive mutants. Selection of the strain CH-101 was performed in a similar manner as follows.

The parental strain, K. pneumoniae PRL-R3,U^-, was inoculated into fresh 0.5% D-fuconate-mineral broth, plus 0.005% uracil, and incubated at 30°C on a reciprocal shaker for approximately three days. Upon development of a turbid culture, aliquots of the culture were diluted into mineral-salts medium and used to inoculate several 0.5% D-fuconate-mineral-medium-agar plates, plus 0.005% uracil, at about 50 cells per plate. These plates were then incubated at 30°C and one colony which developed after about 48 hrs was then picked and grown overnight in nutrient broth. The nutrient-broth culture was then preserved by transferring cells to nutrient-agar slants which could be stored at 4°C after the normal overnight incubation. These slant cultures were then used for inoculants of the strain CH-101 in all subsequent studies noted in this thesis.

To show gross growth characteristics of both the parental strain and the D-fuconate-positive mutant strain, CH-101, these strains were grown on the aldonic acid D-fuconate and its analogue D-galactonate using standard mineral-salts medium. D-Glucose was used as control for positive growth with both bacterial strains. As the growth curve for the parental strain shows (Fig. 4), both D-glucose and D-galactonate were used readily as carbon sources, with a short lag period, whereas growth on D-fuconate began long after the other two carbohydrates were exhausted. Growth of the mutant strain, CH-101, on either D-glucose- or D-galactonate-mineral medium showed similar patterns as seen with the parental strain (Fig. 5), but growth on D-fuconate occured much sooner; approximately 3.5 hrs were required to induce this strain to use D-fuconate. From the growth curves, generation times for the

Figure 4. Growth of the Klebsiella pneumoniae PRL-R3,U^- (parental) strain on D-glucose, D-galactonate, and D-fuconate. An overnight culture of the parental strain in nutrient broth was used to inoculate three 7.0-ml cultures containing 0.5% D-glucose-, D-galactonate-, and D-fuconate-mineral broth (plus 0.005% uracil). Cultures were incubated at 30°C on a reciprocal shaker and turbidity measurements were taken at various times by sampling with sterile pasteur pipetes and reading the resultant absorbance at 600 nm. Plots were made of corrected absorbance versus the incubation time. Generation times were estimated as the time required for the culture to double from an absorbance of 0.15 to 0.30 at 600 nm.

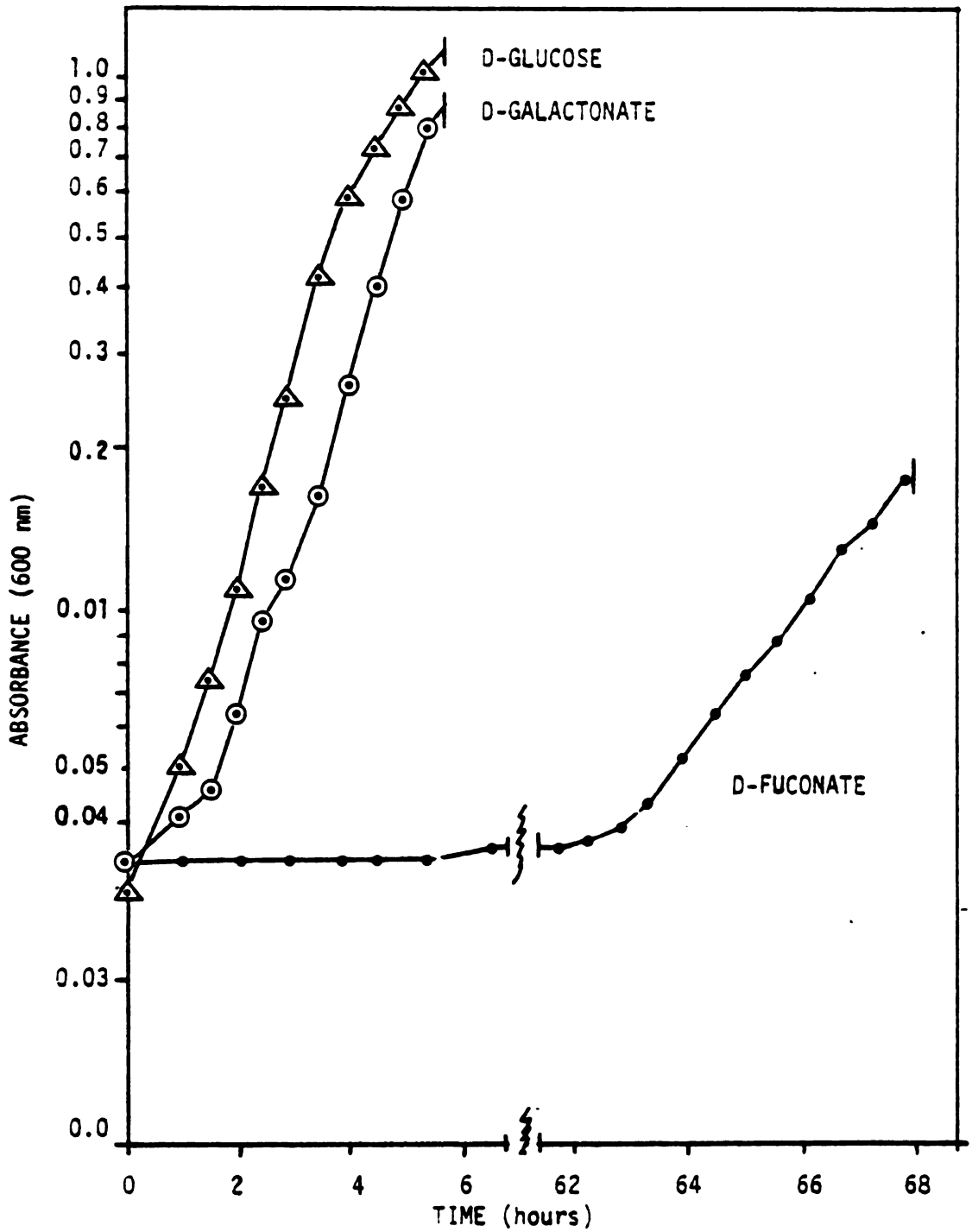
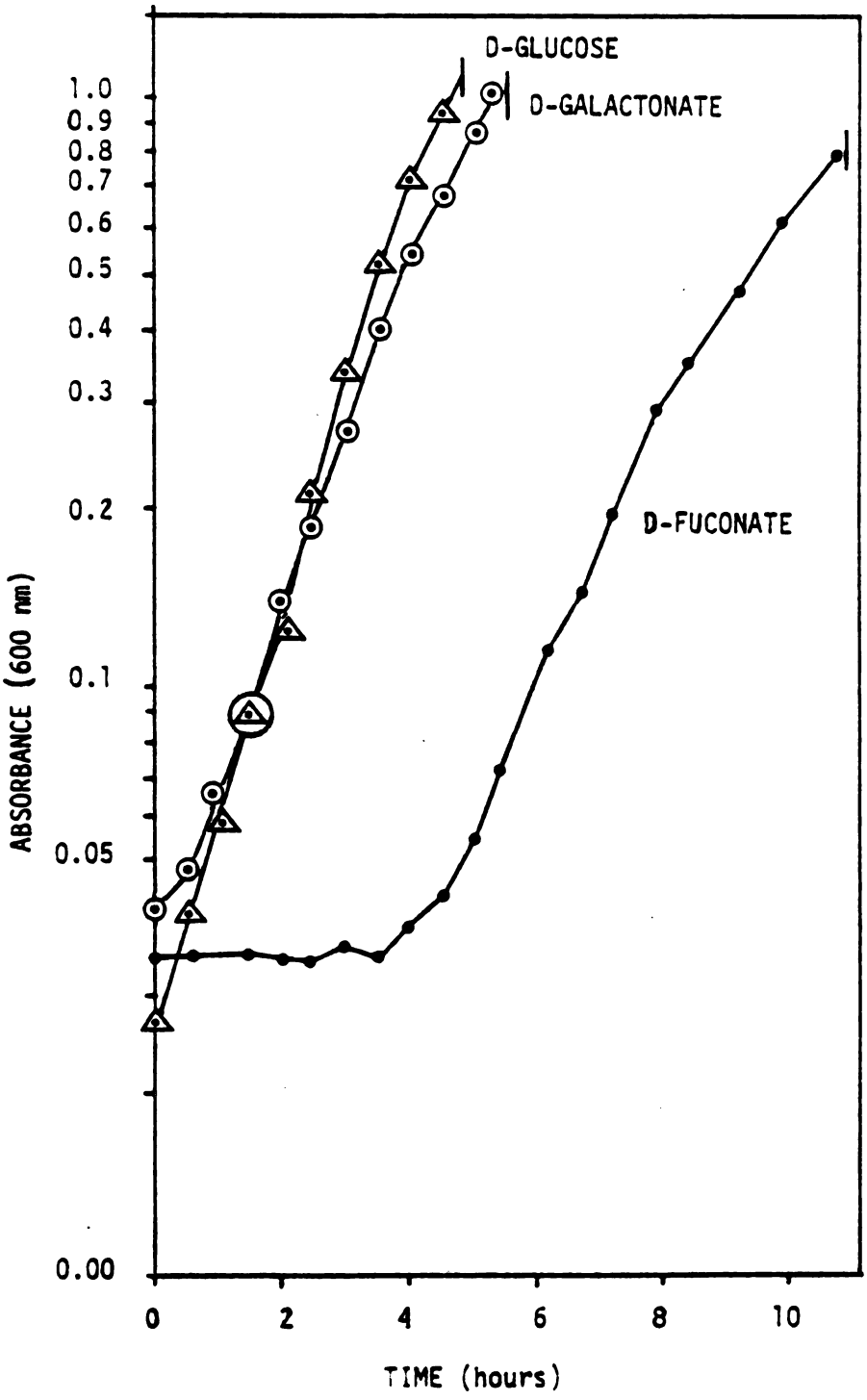

D-GLUCOSE
D-GALACTONATE
D-FUCONATE
ABSORBANCE (600 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.01
0.05
0.04
0.03
0.0
0
2
4
6
62
64
66
68
TIME (hours)

Figure 5. Growth of the D-fuconate-positive mutant strain, CH-101, on D-glucose, D-galactonate, and D-fuconate. Conditions are described in figure 4.


D-GLUCOSE
D-GALACTONATE
D-FUCONATE
ABSORBANCE (600 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.00
0
2
4
6
8
10
TIME (hours)

parental strain on D-glucose (0.72 hr), D-galactonate (0.89 hr), and D-fuconate (2.20 hr) and the strain CH-101 on D-glucose (0.78 hr), D-galactonate (1.06 hr), and D-fuconate (1.22 hr) were obtained. In addition, when strain CH-101 was grown on equimolar concentrations of D-glucose, D-galactonate, or D-fuconate in mineral broth, equal total growth was observed (Table 2). These results show that the D-fuconate-positive strain can readily assimilate the aldonic acid D-fuconate as the sole carbon and energy source for growth.

In summary, the process of obtaining a strain capable of using D-fuconate as a growth substrate apparently involved the selection of a D-fuconate-positive mutant in the normal bacterial population. As seen from the growth curves, the parental strain required a longer pre-incubation before using D-fuconate, but once growth had begun, the generation time was similar to (about one-half) that of the D-fuconate-positive strain. In this regard, serial transfer of the mutant strain on D-fuconate-mineral broth, as done by Mayo and Anderson (12) with Aerobacter aerogenes on L-mannose, failed to produce subsequent strains with shorter generation times on D-fuconate. If strains with shorter generation times were found in the case where K. pneumoniae acquires the ability to use D-fuconate for growth, then factors other than the single-step selection of a D-fuconate-positive mutant would have been considered.

Table 2. Growth yield of strain CH-101 on D-glucose, D-galactonate, and D-fuconate. Broth cultures of the mutant strain CH-101 were grown at 30°C for 48 hrs (see Materials and Methods for details) to exhaust the carbon sources. Corrected optical density readings (absorbance) were taken for each carbohydrate at the indicated concentrations at the end of this incubation.

CARBOHYDRATE	CARBOHYDRATE CONCENTRATION mM	CORRECTED ABSORBANCE 600 nm
D-GLUCOSE	2.8	0.37
	5.6	0.73
D-GALACTONATE	2.8	0.36
	5.6	0.72
D-FUCONATE	2.8	0.35
	5.6	0.69

Elucidation of the Enzymatic Reactions Involved in the Catabolism of D-Fuconate

Enzymatic Reactivity of D-Fuconate. Several possible avenues were considered as candidates for the catabolic pathway of D-fuconate. Those involving initial dehydration or dehydrogenation of D-fuconate were considered the most plausible and were examined first. In addition, dehydrogenases for D-galactose and D-fucose were looked for with the idea that their absence would indicate that the D-fuconate pathway was unique rather than part of the pathway for the metabolism of an aldose. Other possible reactions, such as D-fuconate epimerization, would be examined only if D-fuconate dehydration or dehydrogenation could not be detected.

For the following studies, extracts were prepared from freshly harvested cells.

The postulated dehydrogenase reactions were tested using either NAD^+ or $NADP^+$ as cofactor at both neutral and basic pH ranges. I was not able to detect (less than 0.001 μmol product X min^{-1}X mg $protein^{-1}$) such activities on the substrates D-fucose, D-galactose, D-fuconate, or D-galactonate (see Materials and Methods for conditions) using cell extracts from D-galactose-, D-galactonate-, or D-fuconate-grown K. pneumoniae, strain CH-101. However, D-fuconate dehydratase activity (0.07 to 0.22 μmol product X min^{-1}X mg $protein^{-1}$) was detected in all of the above extracts. This activity was dependent on the carbon source used for growth as determined by the semicarbazone assay for α-keto acid production (15). The formation of α-keto acid was found to be linear with time (Fig. 6) and proportional to protein concentration (Fig. 7)

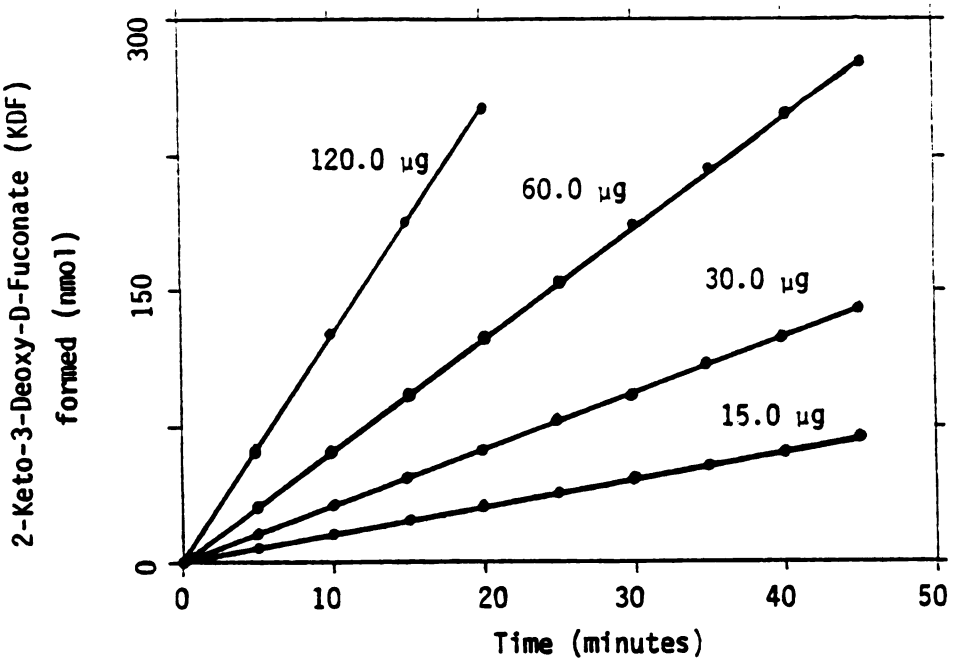


Figure 6. Linearity of the D-fuconate dehydratase assay with respect to time. The µg amounts for each curve indicate the amount of protein (crude extract) used in the assay.

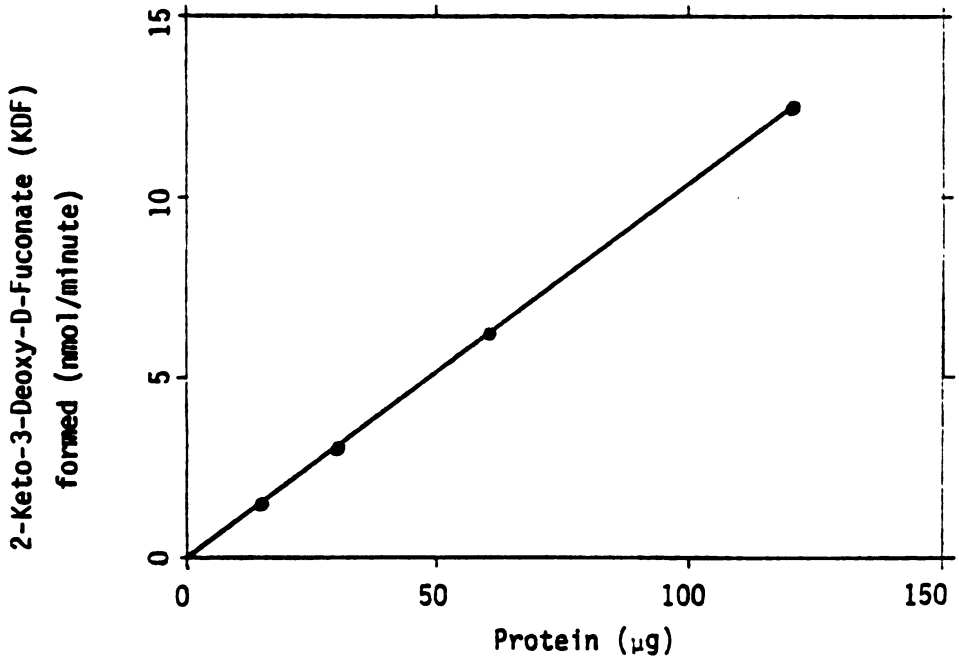


Figure 7. Proportionality of the D-fuconate dehydratase activity with protein concentration. The data are taken from Fig. 6.

when crude extracts of the strain CH-101, grown on D-fuconate, were assayed using 30.0 mM D-fuconate and 1.0 mM EDTA in the reaction mixture. Dehydratase activity was also detected with D-galactonate as substrate (0.19 to 0.64 μmol product X min^{-1}X mg $protein^{-1}$); this activity was also dependent on the carbon source used for growth and was linear with time and proportional to protein concentration (data not shown).

The inital reaction involving D-fuconate was therefore a dehydration leading presumably to 2-keto-3-deoxy-D-fuconate (KDF). A detailed study of this dehydratase is presented in Section II of this thesis.

Enzymatic Reactivity of 2-Keto-3-Deoxy-D-Fuconate. The enzymatic breakdown of 2-keto-3-deoxy-D-fuconate in crude extracts by either an aldol-cleavage or an oxido-reductase reaction was next examined. 2-Keto-3-deoxy-D-fuconate aldolase activity was detected in extracts of strain CH-101 grown on D-fuconate (0.11 μmol product X min^{-1}X mg $protein^{-1}$) but not on D-glucose or D-galactonate (less than 0.001 μmol product X min^{-1}X mg $protein^{-1}$). Assays of crude extracts prepared from cells grown on any of these three substrates with either NAD^+ or $NADP^+$ showed no oxido-reductase activity on KDF (less than 0.001 μmol product X min^{-1} X mg $protein^{-1}$). To clearly demonstrate the existence of a 2-keto-3-deoxy-D-fuconate aldolase in extracts of the D-fuconate-positive strain, the products of the aldolase-catalyzed cleavage were quantitated enzymatically.

The production of pyruvate was measured using the standard reaction mixture and beef heart lactate dehydrogenase (LDH) as the coupling

enzyme; LDH prefers pyruvate as substrate having a rate of reduction over ten-fold higher than other α-keto and α,γ-diketo acids (92). D-Lactaldehyde production was measured using horse liver alcohol dehydrogenase (HLADH) as the coupling enzyme in the reaction mixture; HLADH possesses a broad substrate specificity for aldehydes, among which are acetaldehyde, glycolaldehyde, formaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, isobutyraldehyde, glyceraldehyde, and lactaldehyde (86). Results of a study using either chemically synthesized KDF or enzymatically prepared KDF (see Section II, Results) with these coupling enzymes (Table 3) show that not only are both pyruvate and an aldehyde (presumably D-lactaldehyde from the chemical structure of KDF) produced from the substrate 2-keto-3-deoxy-D-fuconate, but that both products are produced at the same rate. These data confirm that an aldolytic activity for KDF is present in crude extracts of the strain CH-101 when grown on D-fuconate.

Further investigation of the KDF aldolase from extracts of the strain CH-101 grown on D-fuconate revealed a pH optimum of 8.0 (Fig. 8). In addition, a divalent metal was absolutely required for activity as demonstrated by complete inactivation of the enzyme with 1.0 mM EDTA and restoration of maximal activity with 10.0 mM $MgCl_2$ (Fig. 9). Examination of the effect of substrate concentration on the velocity of the reaction revealed that the Km of the enzyme for 2-keto-3-deoxy-D-fuconate was approximately 0.25 mM. Using 2.5 mM substrate, the assay for the aldolase was found to be constant with time (Fig. 10) and proportional to protein concentration (Fig. 11). Crude extracts from D-fuconate grown cells of this strain were found to possess additional

Table 3. KDF aldolase reaction products: measurement of the reaction products with appropriate coupling enzymes. The aldolase was assayed as described in the text with lactate dehydrogenase and alcohol dehydrogenase (HLADH) as coupling enzymes to measure the production of pyruvate and D-lactaldehyde, respectively. A crude extract of the strain CH-101 grown on D-fuconate was used. Standard assays for the KDF aldolase were followed according to established protocols using either chemically or enzymatically prepared 2-keto-3-deoxy-D-fuconate (KDF) as substrate. Assays of the chemically synthesized KDF contained 1.59 munits of activity and assays of enzymatic KDF contained 1.12 munits of activity.

COUPLING ASSAY USED	SPECIFIC ACTIVITY OF THE ASSAY ON	
	SYNTHETIC KDF	ENZYMATIC KDF
	nmol NAD^+/min[a]	nmol NAD^+/min[a]
LACTATE DEHYDROGENASE ONLY	1.64 ± 0.08	1.10 ± 0.08
ALCOHOL DEHYDROGENASE ONLY	1.53 ± 0.11	1.13 ± 0.03
LACTATE DEHYDROGENASE & ALCOHOL DEHYDROGENASE	3.42 ± 0.06	2.37 ± 0.08

[a] these values are averages from three determinations ± S.D.

Figure 8. pH optimum of the KDF aldolase. Crude extracts of the strain CH-101 were grown on 0.5% D-fuconate-mineral-medium broth and prepared as described in Materials and Methods. Maleic acid was used below pH 7.0; Tris-maleate buffer between pH 7.0 and 8.0; and glycine buffer above pH 8.0 at concentrations of 50.0 mM in the assay. Maximal activity was 0.40 μmol product X min^{-1} in the assay at pH 8.0. Hepes buffer at pH 8.0 gave the same velocity as Tris-maleate (not plotted).

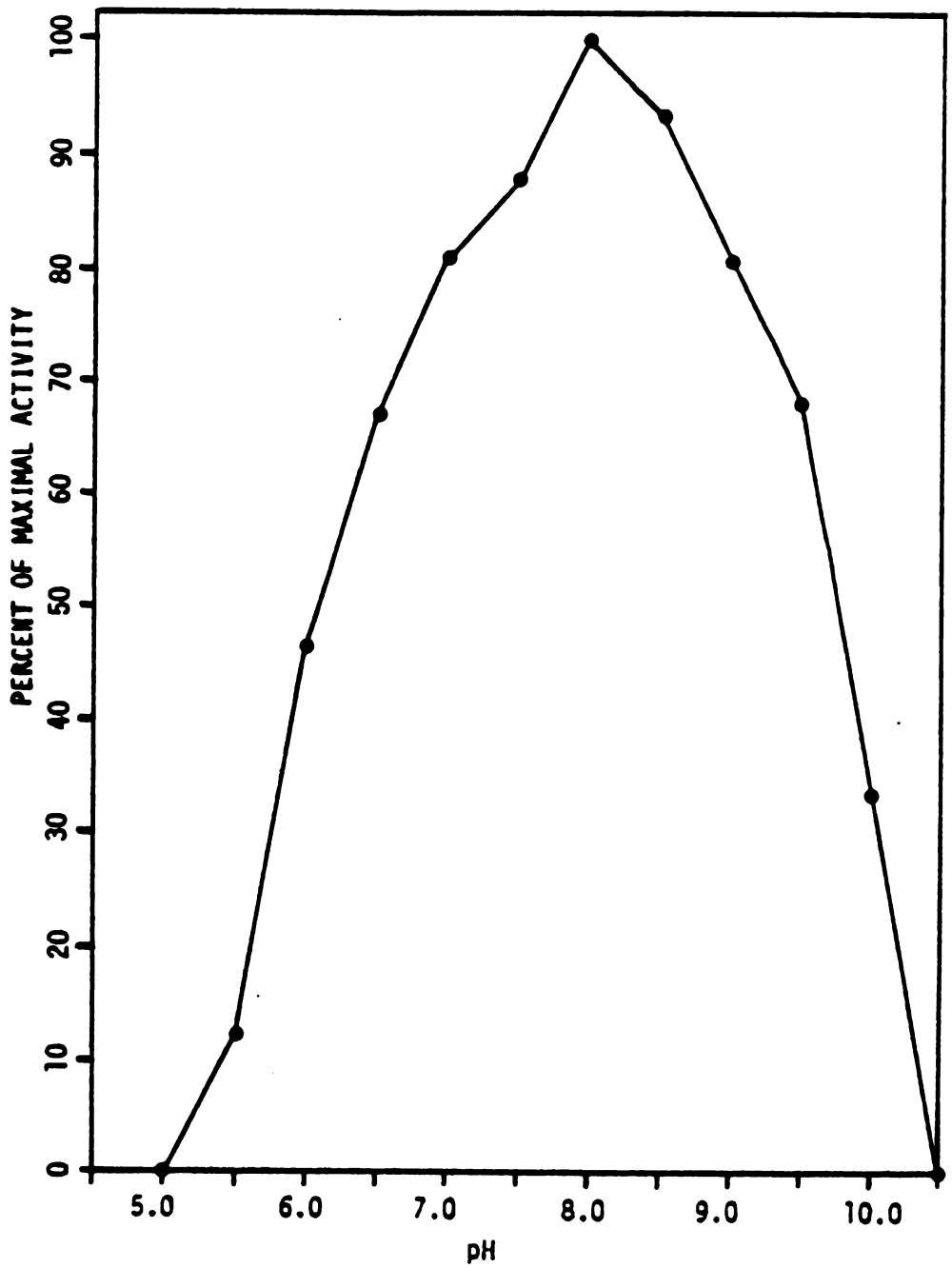
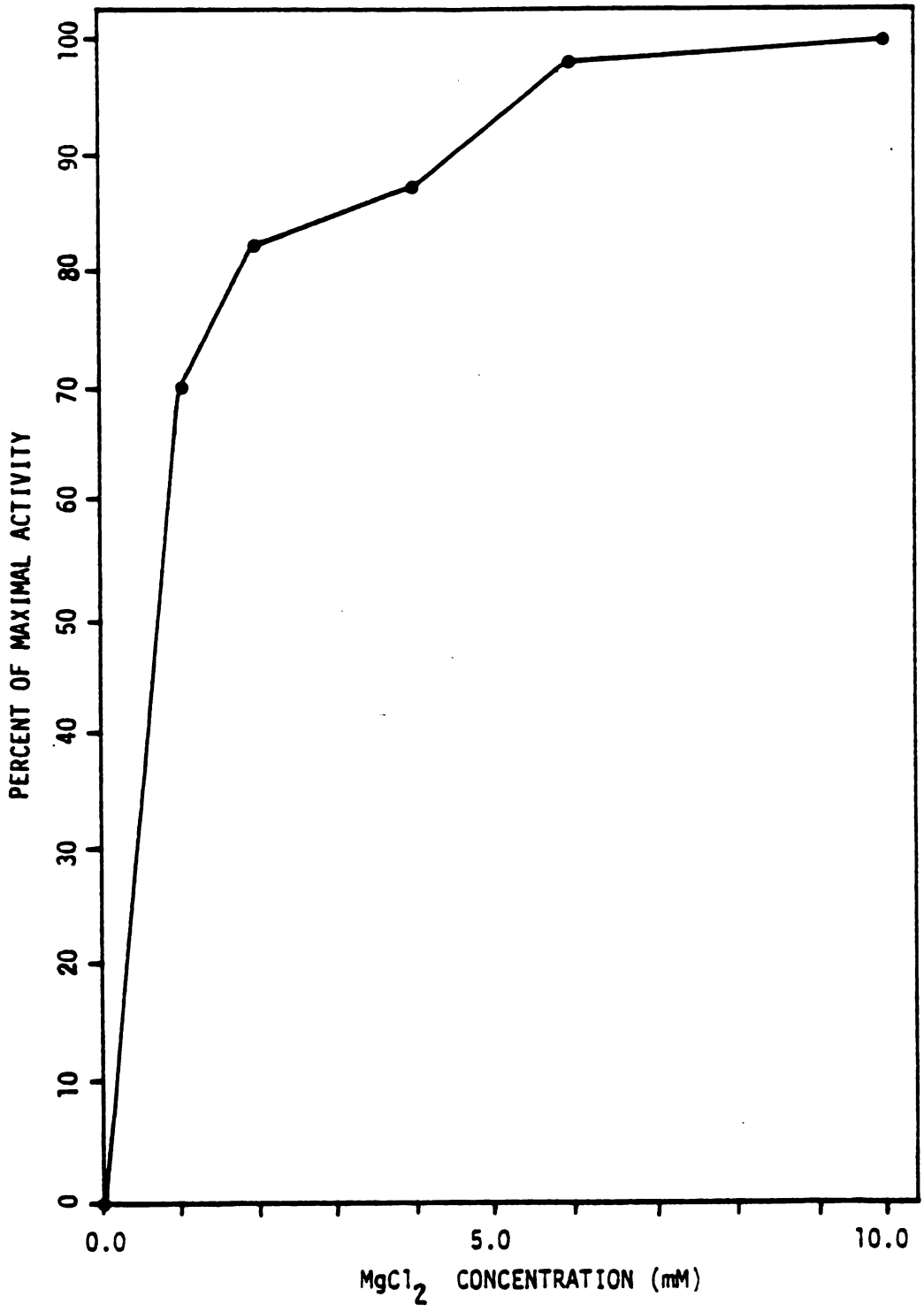

PERCENT OF MAXIMAL ACTIVITY
0
10
20
30
40
50
60
70
80
90
100
5.0
6.0
7.0
8.0
9.0
10.0
pH

Figure 9. Metal ion requirement of the KDF aldolase. Crude extracts of the strain CH-101 grown on 0.5% D-fuconate-mineral broth were prepared as described in Materials and Methods. Crude extracts were then treated with 1.0 mM EDTA and chromatographed on a column of Sephadex G-25 (0.5 X 10.0 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). KDF aldolase activity was assayed using 2-keto-3-deoxy-D-fuconate as substrate in the lactate dehydrogenase coupled assay with the $MgCl_2$ concentration varied. In all trials, reaction mixtures were made 10.0 mM in KCl to assure activity of the coupling enzyme. Maximal activity was 0.40 μmol product X $minute^{-1}$.


PERCENT OF MAXIMAL ACTIVITY
0
10
20
30
40
50
60
70
80
90
100
0.0
5.0
10.0
MgCl2 CONCENTRATION (mM)

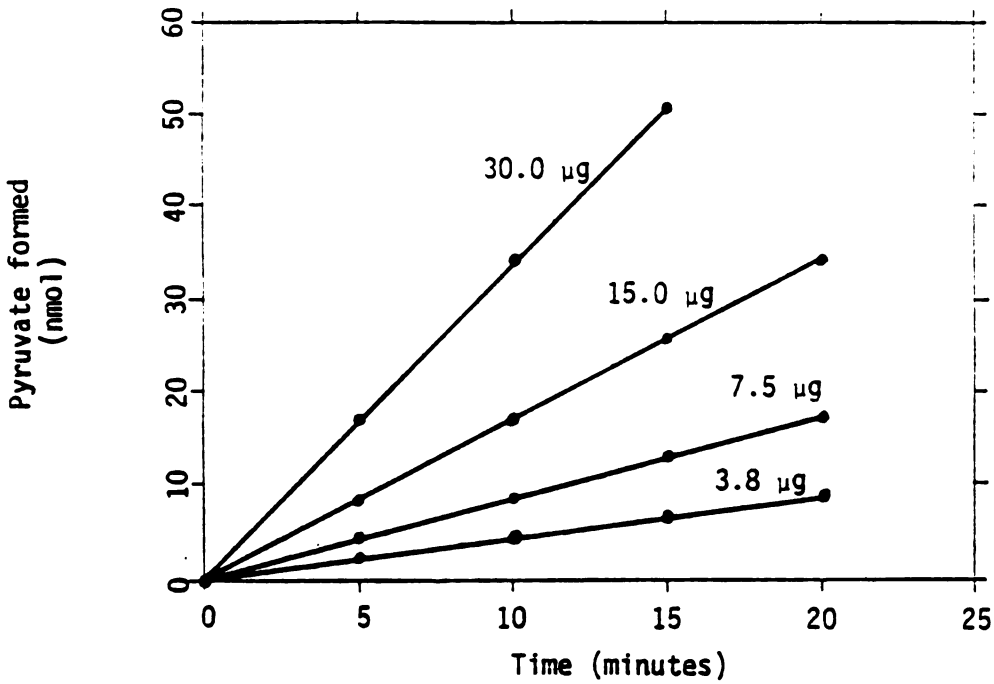


Figure 10. Linearity of the 2-keto-3-deoxy-D-fuconate assay with respect to time. The µg amounts for each curve indicate the amount of protein (crude extract) used in that assay.

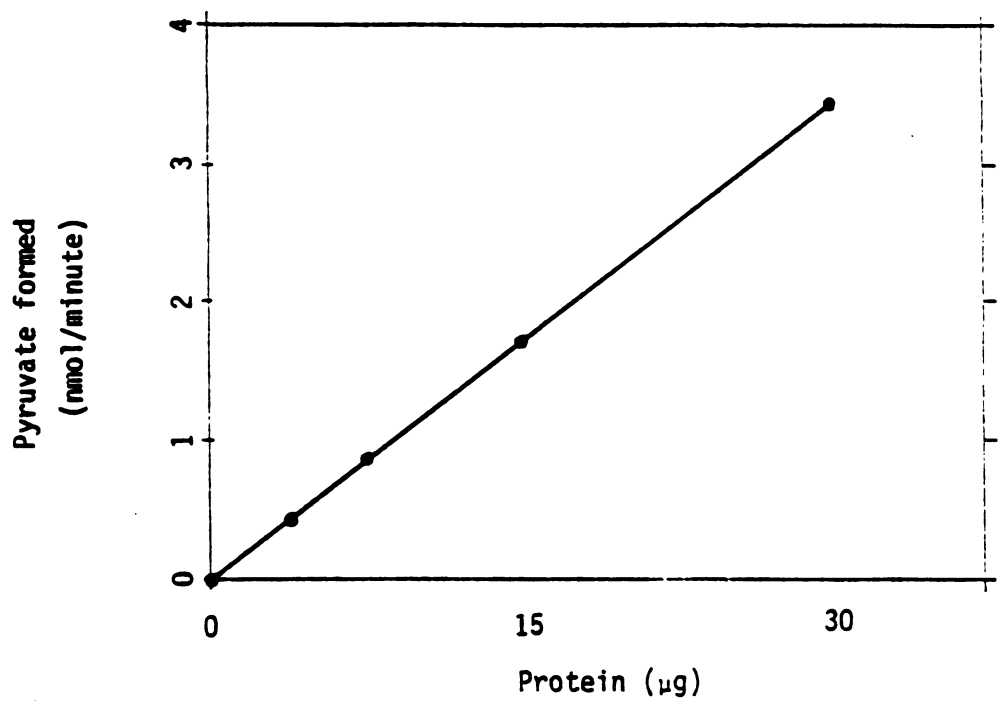


Figure 11. Proportionality of the 2-keto-3-deoxy-D-fuconate aldolase activity with protein concentration. These data are taken from Fig. 10.

activities on the substrates 2-keto-3-deoxy-D-quinovonic acid, 2-keto-3-deoxy-D-galactonate, and 2-keto-3-deoxy-D,L-arabonate when tested in the aldolase assay. These activities showed a similar inactivation by EDTA and divalent metal requirement (Table 4) as that found with the substrate 2-keto-3-deoxy-D-fuconate suggesting that the aldolase of the D-fuconate pathway had a varied substrate specificity. When mixtures of these substrates were assayed, rates were found to be competitive rather than additive (Table 5) suggesting that one enzyme acted on all four substrates.

Inducibility of the D-Fuconate and D-Galactonate Pathway Enzymes on Various Carbohydrates

Subsequent to the initiation of studies on the catabolic pathway of D-fuconate, a pathway for the catabolism of D-galactonate was discovered in both the parental strain and the D-fuconate-positive strain, CH-101, of K. pneumoniae. In much the same manner as described for the D-fuconate pathway, activities for a D-galactonate dehydratase, a 2-keto-3-deoxy-D-galactonate (KDGal) kinase, and a 2-keto-3-deoxy-6-phospho-D-galactonate (KDPGal) aldolase (but not a 2-keto-3-deoxy-D-galactonate aldolase) were found in crude extracts of the strain CH-101 grown on D-galactonate. Due to the analogous nature of these two aldonic acids, it was of interest to determine how closely related their pathways were. Investigations of the inducibility of the pathway enzymes proved to be useful in this regard.

When crude extracts prepared from both the parental strain and strain CH-101 grown on various carbohydrates were examined for pathway

Table 4. Effect of $MgCl_2$ on aldolase activities in EDTA- treated extracts. The following substrates were assayed using standard protocol for the KDF aldolase assay and EDTA-treated crude extracts from D-fuconate-grown cells (see figure 9, for conditions). The reaction mixture consisted of 0.2-ml volume and the following components: Hepes buffer, 50.0 mM, pH 8.0; $MgCl_2$, 10.0 mM; NADH, 0.25 mM; excess lactate dehydrogenase; EDTA, 1.0 mM; and the following keto-deoxy sugar acids: 2-keto-3-deoxy-D-fuconate (KDF), 10.0 mM; 2-keto-3-deoxy-D-galactonate (KDGal), 5.0 mM; 2-keto-3-deoxy-D,L-arabonate (KDA), 5.0 mM; 2-keto-3-deoxy-D-quinovonic acid (KDQ), 10.0 mM; and 4-hydroxy-2-keto-D,L-glutarate (HKG), 10.0 mM.

SUBSTRATE	ACTIVITY	
	MINUS $MgCl_2$ units/ml	PLUS $MgCl_2$ units/ml
KDF	< 0.02	0.378
KDGal	< 0.02	0.113
KDA	< 0.02	0.251
KDQ	< 0.02	0.175
HKG	< 0.02	< 0.020

Table 5. Additive enzymatic activity studies on keto-deoxy sugar mixtures with KDF aldolase. Limiting amounts of D-fuconate-grown strain CH-101 crude extract were assayed for aldolase activity using the following substrates and established assays. The components of the assay were: Hepes buffer, 50.0 mM, pH 8.0; $MgCl_2$, 10.0 mM; EDTA, 1.0 mM; NADH, 0.25 mM; excess lactate dehydrogenase; and KDF (10.0 mM), KDGal (5.0 mM), KDA (5.0 mM), and KDQ (10.0 mM).

MIXTURE TRIAL	SUBSTRATE	RESULTANT ACTIVITY units/ml[a]
KDF & KDA	KDF	0.378 ± 0.004
	KDA	0.251 ± 0.004
	KDF & KDA	0.346 ± 0.050
KDF & KDQ	KDF	0.378 ± 0.004
	KDQ	0.175 ± 0.005
	KDF & KDQ	0.183 ± 0.016
KDF & KDGal	KDF	0.378 ± 0.004
	KDGal	0.113 ± 0.011
	KDF & KDGal	0.298 ± 0.005

[a] these values are averages from three determinations ± S.D.

enzymes involved in the catabolism of D-fuconate and D-galactonate the following trends were observed (Table 6): (i) Both the parental strain and strain CH-101 possessed D-galactonate dehydratase, 2-keto-3-deoxy-D-galactonate kinase, 2-keto-3-deoxy-6-phospho-D-galactonate aldolase, and D-fuconate dehydratase activities on D-galactonate-mineral broth. However, these activities were elevated in the strain CH-101 over the parental strain when grown on this substrate. (ii) The parental strain showed absolute inducibility of all the D-galactonate pathway enzymes and the D-fuconate dehydratase by D-galactonate and the KDF aldolase by a D-galactonate/D-fuconate mixture. Whereas, the strain CH-101 exhibited constitutive production of all enzymatic activities noted above except for the KDF aldolase; this latter enzyme apparently required D-fuconate as an inducer. (iii) The D-fuconate dehydratase appeared in both Klebsiella strains at about one-third the activity found for the D-galactonate dehydratase regardless of the carbon source used for growth. Both the D-fuconate and D-galactonate dehydratase activities showed similar patterns of induction in both strains. (iv) The KDF aldolase inducibility suggested that the dehydratase product from D-fuconate is the actual inducer of this enzyme as the parental strain required a mixture of both D-fuconate and D-galactonate to exhibit such activity. (The KDF aldolase activity was not detected on any other carbon source but D-fuconate; testing for this activity on the sugar acids D-glucarate, D-galactarate, D-glucuronic acid, and D-galacturonic acid did not reveal specific activities higher than that found for either strain on D-galactonate.)

Table 6. Inducibilities for enzymes of the D-fuconate and D-galactonate pathways. Crude extracts were prepared from cultures grown overnight in 0.5% carbohydrate-mineral-medium broth (plus 0.005% uracil) at 30°C and 7.0-ml volume, as described in Materials and Methods.

BACTERIAL STRAIN	INDUCER	SPECIFIC ACTIVITY				
		DEHYDRATASE		KINASE	ALDOLASE	
		D-Galactonate	D-Fuconate	KD-D-Galactonate	KDPGalactonate	KDFuconate
K. pneumoniae PRL-R3,U⁻ PARENTAL STRAIN	D-GALACTONATE	0.326	0.110	0.193	0.067	NEG
	NUTRIENT BROTH	NEG	NEG	NEG	NEG	NEG
	D-Galactonate & D-Fuconate mix	0.163	0.050	0.111	0.034	0.046
K. pneumoniae PRL-R3,U⁻ STRAIN CH-101	D-GALACTONATE	0.644	0.220	0.275	0.101	NEG
	D-FUCONATE	0.371	0.125	0.311	0.123	0.114
	D-GLUCONATE	0.412	0.140	0.224	0.100	NEG
	D-GLUCOSE	0.146	0.045	0.099	0.044	NEG
	D-GALACTOSE	0.194	0.065	0.178	0.073	NEG
	NUTRIENT BROTH	0.406	0.155	0.339	0.350	NEG
	D-Galactonate & D-Fuconate mix	0.508	0.132	0.293	0.112	0.102
E. coli STRAIN K-10 (28)	D-GALACTONATE	0.060	unknown	0.240	0.300	unknown
Pseudomonad (33)	L-ARABINOSE or D-FUCOSE	0.03 to 0.107 (97)	0.077	unknown	unknown	0.032

NEG = a value of specific activity less than 0.001 μmol product X $minute^{-1}$ X mg $protein^{-1}$.

These data are consistent with the following suppositions: Firstly, that the acquired ability of K. pneumoniae to utilize D-fuconate as a sole carbon source for growth is involved with the constitutive production of the D-fuconate dehydratase. Secondly, that the dehydratase activities on D-fuconate and D-galactonate, in both the parental strain and the strain CH-101, were the result of one enzyme. Lastly, that the D-fuconate pathway is independent of the D-galactonate pathway beyond the initial dehydration reaction. Support for these suppositions was provided by an analysis of D-fuconate- and D-galactonate-negative mutants derived from the strain CH-101.

Mutant Analysis and Verification of Enzyme Deficiency

K. pneumoniae, strain CH-101, was subjected to mutagenesis with ethylmethane sulfonate, screened for cells impaired for growth on D-fuconate by treating with a mixture of penicillin-G/D-cycloserine, enhanced by growing in D-glucose-mineral broth, and then plated onto mineral-medium supplemented with 0.5% D-fuconate, 0.005% D-glucose, and 0.005% uracil (see Materials and Methods, this section). Of five plates inoculated (about 200 colonies per plate), 11 pin-point colonies were found. Screening of cultures from these pin-point colonies on D-fuconate- and D-galactonate-mineral-agar plates revealed that one exhibited impaired growth on both D-fuconate and D-galactonate, 5 showed impaired growth on D-fuconate, and 10 showed normal growth on D-galactonate (Table 7). All strains showing impaired growth on D-fuconate and/or D-galactonate were screened for growth on a variety of carbohydrates to determine if other pathways were affected by the mutagenesis. Strain CH-102, exemplifying mutant strains normal on

Table 7. Screening of mutagenized cultures for growth on D-fuconate and D-galactonate. The strain CH-101 was treated as in the text. Pin-point colonies, numbered arbitrarily, which developed from D-fuconate plates supplemented with growth-limiting amounts of D-glucose were grown overnight in 0.5% D-glucose-mineral-medium broth. Plates were checked for positive growth using an untreated culture of the strain CH-101 on D-glucose as control after 48 hrs of incubation at 30°C.

BACTERIAL STRAIN	GROWTH*		
	D-GLUCOSE	D-GALACTONATE	D-FUCONATE
CH-101	+	+	+
CH-102	+	+	-
CH-103	+	-	-
CH-104	+	+	+
CH-105	+	+	-
CH-106	+	+	+
CH-107	+	+	-
CH-108	+	+	+
CH-109	+	+	-
CH-110	+	+	+
CH-111	+	+	-
CH-112	+	+	+

* (+) is positive growth; noticeable bacterial lawn.

(-) is negative growth; no bacterial lawn, at best isolated colonies (revertants).

D-galactonate but impaired for growth on D-fuconate, and strain CH-103, impaired for growth on both aldonic acids, showed normal growth patterns on all carbohydrates but D-fuconate and/or D-galactonate when compared with the parental strain and strain CH-101 (Table 8). Strains CH-104, CH-106, CH-108, CH-110, and CH-112 were no different than the parental strain CH-101 when checked for growth on D-fuconate or D-galactonate and therefore were not studied further.

To check mutant strains for deficiences in enzymes of the D-fuconate pathway, crude extracts were prepared form cultures grown under conditions which normally exhibited these activities in the strain CH-101 (Table 9). Enzymatic assays confirmed that the strain (CH-103) which did not exhibit growth on either D-fuconate or D-galactonate was in fact deficient in the dehydratase for either of these substrates. This strain was classified dehydratase-negative. All other strains having normal growth on D-galactonate (CH-102, CH-105, CH-107, CH-109, and CH-111) were found to have normal levels of both the D-fuconate and D-galactonate dehydratase (specific activities of 0.40 and 0.13 μmol product X min^{-1}X mg $protein^{-1}$, respectively).

Of the strains showing no growth on D-fuconate only one strain (CH-102) was shown to be deficient in the KDF aldolase (specific activity less than 0.001 μmol product X min^{-1}X mg $protein^{-1}$). This strain was classified as KDF aldolase-negative. Stains CH-105, CH-107, CH-109, and CH-111 were found to possess normal levels of the aldolase despite their negative growth on D-fuconate; these strains must have reverted to being D-fuconate-positive. A summary of the geneology and phenotype of these bacterial strains is given (Table 10).

Table 8. Mutant screening on various carbohydrates. Cultures were grown in 0.5% (w/v) carbohydrate-medium broth at 30°C on a reciprocal shaker for 48 hrs. Turbidity measurements at 600 nm (Coleman Jr. spectrophotometer) were made at the end of this incubation. Strain CH-102 and CH-103 are D-fuconate and D-galactonate/D-fuconate growth impaired mutants, respectively. Strain CH-113 is the revertant obtained from strain CH-103.

CARBOHYDRATE	MEDIUM	TURBIDITY MEASUREMENTS (aborbance, 600 nm)			
		STRAIN CH-101	STRAIN CH-103	STRAIN CH -102	STRAIN CH-113
D-GLUCOSE	BROTH	0.88	1.00	1.25	0.93
D-GALACTOSE	"	1.50	1.30	1.25	1.30
D-FRUCTOSE	"	0.95	1.35	1.25	1.02
D-MANNITOL	"	0.90	1.05	1.20	1.10
L-ARABINOSE	"	0.95	0.95	1.05	1.20
D-GLUCONATE	"	0.90	0.93	1.05	1.10
D-FUCONATE	"	0.64	0.07	0.07	0.70
D-GALACTONATE	"	1.00	0.07	1.05	1.00
D-FUCONATE	AGAR	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE
D-GALACTONATE	"	POSITIVE	NEGATIVE	POSITIVE	POSITIVE

Table 9. Enzyme activities in mutant and revertant strains. Crude extracts were prepared from strains grown overnight in broth when determining D-fuconate dehydratase, D-galactonate dehydratase, KDGal kinase, and KDPGal aldolase activity deficiencies. Crude extracts were prepared from cultures grown to half-maximal absorbances (0.3 O.D.,600nm) on nutrient broth and then made 0.5% in D-fuconate, allowing 3.0 hr to induce, when determining KDF aldolase activity deficiencies. Strains CH-101, CH-102, CH-103, and CH-113 are defined in Table 10.

GROWTH MEDIUM	PATHWAY ENZYME	SPECIFIC ACITIVITY (units/mg)			
		STRAIN CH-101	STRAIN CH-103	STRAIN CH-102	STRAIN CH-113
NUTRIENT BROTH	DEHYDRATASE				
	D-GALACTONATE	0.400	<0.001	0.423	0.380
	D-FUCONATE	0.130	<0.001	0.140	0.130
	KDGal KINASE	0.320	0.295	0.350	0.315
	ALDOLASE				
	KDPGal	0.300	0.330	0.310	0.290
	KDF	<0.001	<0.001	<0.001	<0.001
NUTRIENT BROTH & D-FUCONATE	DEHYDRATASE				
	D-GALACTONATE	0.389	<0.001	0.385	0.390
	D-FUCONATE	0.130	<0.001	0.130	0.130
	KDGal KINASE	0.330	0.325	0.340	0.350
	ALDOLASE				
	KDPGal	0.250	0.237	0.240	0.250
	KDF	0.112	<0.001	<0.001	0.102

Table 10. Genealogy and phenotype of the Klebsiella pneumoniae strains

STRAIN	PARENT	GENOTYPE	PHENOTYPE
PRL-R3,U^-	PRL-R3	Uracil auxotroph of the wild-type strain.	Gat^+, Fnt^-
CH-101	PRL-R3,U^-	Derepressed for D-galactonate (D-fuconate) dehydratase and other D-galactonate pathway enzymes.	Gat^+, Fnt^+
CH-102	CH-101	KDF aldolase-negative mutant.	Gat^+, Fnt^-
CH-103	CH-101	D-galactonate (D-fuconate) dehydratase-negative mutant.	Gat^-, Fnt^-
CH-113	CH-103	D-galactonate (D-fuconate) dehydratase revertant.	Gat^+, Fnt^+

Abbreviations: Gat = D-galactonate, Fnt = D-fuconate.

Further analysis of the enzymatic deficiencies of the mutant strains showed that the dehydratase-negative mutant had neither D-fuconate or D-galactonate dehydratase or KDF aldolase activities, yet the KDF aldolase-negative mutant was found to have normal levels of both D-fuconate and D-galactonate dehydratase activities. These results further supported the premises that there is one enzyme for both the dehydration of D-fuconate and D-galactonate, and that the dehydration product from D-fuconate may be the true inducer of the KDF aldolase.

Procurement of Revertants for Growth on D-Galactonate from Strain CH-103

Revertants of the strain CH-103 were prepared as described in Materials and Methods by plating cells onto D-galactonate-mineral-agar plates supplemented with growth-limiting amounts of D-glucose. Visual observation of the plates after 2.0 days of growth showed two main colony types, small pin-point colonies of about 0.5 mm in diameter, and large colonies of about 2.0 mm in diameter. Counting of the colony type showed a near equal distribution of pin-point (59.2%) to large (40.8%) variety. One of the large-type colonies was taken as strain CH-113 and further characterized.

Nutrient broth cultures of the strain CH-113 plated onto D-galactonate- and D-fuconate-mineral-agar plates were found to have normal distributions of colony size (2.0 mm) and number (about 200 colonies per plate) after 48 hrs of incubation. Inoculates from these cultures into liquid broth showed normal (strain CH-101) growth patterns (Table 8). In addition, when crude extracts were prepared from these cultures

and assayed for the dehydratase activities on both D-fuconate and D-galactonate, normal levels were found (specific activities of 0.38 and 0.14 μmol product X min^{-1}X mg $protein^{-1}$ for D-galactonate and D-fuconate, respectively) (Table 9). These data support the premise that there is one dehydratase activity common to the catabolism of both D-fuconate and D-galactonate, although the possibility that two enzymes controlled by a single regulatory gene has not been ruled out. Section II of this thesis will confirm that both activities reside on a single protein.

Classification of the Bacterial Strains

To confirm that all mutants used in this investigation were derived from the parental strain, K. pneumoniae PRL-R3,U^-, comparative growth patterns on a variety of carbon sources and standard genus type (IMViC) tests were performed.

Nutrient broth cultures, 7.0 ml, of the parental strain (K. pneumoniae PRL-R3,U^-), the D-fuconate-positive mutant strain (CH-101), and all subsequentially derived mutant strains (CH-102, CH-103, and CH-113) were tested for growth on thirty different carbohydrates. Mineral-medium cultures supplemented with 0.5% carbohydrate were inoculated from these cultures as to give a reasonable initial turbidity (0.05 O.D. at 600 nm) and were then incubated at 30°C on a reciprocal shaker. Uracil was added to all mineral-broth cultures in trials testing for positive growth on the given carbon source. Omission of uracil from the growth medium allowed testing for the parental trait of auxotrophicity in the given strain. At the end of 30.0 hrs of incubation, cultures were

checked for growth by making turbidity measurements using a Coleman Jr. spectrophotometer (Table 11).

All strains showed good growth on the aldoses D-glucose, D-galactose, D-mannose, L-arabinose, D-ribose, D-xylose, and the 6-deoxy aldose L-rhamnose, the ketoses L-sorbose and D-fructose, the hexitols D-glucitol and D-mannitol, and the sugar acids D-gluconate, D-glucuronate, D-galacturonate, D-glucarate, and D-mannonate. No growth for any strain was seen on the aldoses D-arabinose, D-lyxose, or D-glucose without uracil, the 6-deoxy aldose D-fucose, or the sugar acids D-xylonate, L-arabonate, or D-lyxonate. In addition, all strains showed positive growth on glycerol, citrate, maltose, melibiose, cellobiose, and lactose. Expected variations were seen on D-galactonate and D-fuconate. The parental strain, D-fuconate-positive mutant (CH-101), KDF aldolase-negative mutant (CH-102), and the revertant on D-galactonate (CH-113), grew well on D-galactonate while only the strain CH-101 and the revertant were able to use D-fuconate. The dehydratase-negative mutant (CH-103) did not use either D-fuconate or D-galactonate as sole carbon sources for growth.

All strains gave identical IMViC test results (Table 12). Despite the fact that Klebsiella species do not normally produce indole, the strains tested were all found to be positive for this test. Simkins (98) has also reported the production of indole from the parental strain of this bacterial species. The methyl red test failed to show acid from any of the Klebsiella strains, but was positive for E. coli. All Klebsiella were found to give positive Voges-Proshauer test whereas E. coli was found to be negative. Citrate was utilized by the Klebsiella

Table 11. Growth studies of the Klebsiella pneumoniae PRL-R3,U$^-$ and derived mutant strains. Details are given in the text. Compounds showing differences are underlined.

CARBOHYDRATE	OPTICAL DENSITY (600 nm)				
	PRL-R3,U$^-$ [a]	CH-101[b]	CH-103[c]	CH-102[d]	CH-113[e]
D-Glucose (-uracil)	NEG	NEG	NEG	NEG	NEG
D-Glucose (+uracil)	0.90	0.85	0.85	0.81	0.84
D-Galactose	1.10	1.00	1.00	1.00	1.00
D-Mannose	0.93	0.90	0.95	0.87	0.94
L-Sorbose	0.76	0.67	0.74	0.73	0.70
L-Arabinose	0.88	0.86	0.85	0.82	0.85
D-Ribose	0.90	0.80	0.90	0.75	0.85
D-Xylose	1.00	0.98	1.00	0.95	0.95
D-Arabinose	NEG	NEG	NEG	NEG	NEG
D-Lyxose	NEG	NEG	NEG	NEG	NEG
D-Fructose	0.77	0.83	0.72	0.85	0.70
L-Rhamnose	0.77	0.74	0.83	0.75	0.75
D-Glucitol	0.70	0.73	0.70	0.75	0.70
D-Mannitol	0.69	0.77	0.68	0.70	0.70
D-Fucose	NEG	NEG	NEG	NEG	NEG
D-Gluconate	0.80	0.83	0.81	0.75	0.85
D-Galactonate	0.71	0.74	NEG	0.75	0.72
D-Fuconate	NEG	0.52	NEG	NEG	0.50
D-Glucuronate	0.65	0.80	0.75	0.72	0.70
D-Galacturonate	0.55	0.65	0.55	0.50	0.60
D-Glucarate	0.65	0.59	0.54	0.62	0.65
D-Xylonate	NEG	NEG	NEG	NEG	NEG
L-Arabonate	NEG	NEG	NEG	NEG	NEG
D-Mannonate	0.30	0.34	0.27	0.22	0.38
D-Lyxonate	NEG	NEG	NEG	NEG	NEG
Maltose	0.68	0.83	0.85	0.83	0.80
Melibiose	0.72	0.78	0.88	0.85	0.75
Cellobiose	0.70	0.85	0.90	0.83	0.85
Lactose	0.57	0.58	0.60	0.60	0.65
Glycerol	0.55	0.62	0.65	0.60	0.60
Citrate	0.72	0.80	0.78	0.78	0.75

NEG = < 0.06 O.D. (600 nm).

[a] uracil auxotroph of wild type.

[b] D-fuconate-positive strain.

[c] D-galactonate (D-fuconate) dehydratase-negative mutant.

[d] KDF aldolase-negative mutant.

[e] D-galactonate (D-fuconate) dehydratase revertant.

Table 12. Standard IMViC tests for the Klebsiella pneumoniae PRL-R3,U$^-$ strain and derived strains.

BACTERIAL STRAIN	METHYL RED	VOGES-PROSKAUER	INDOLE	CITRATE UTILIZATION	
				URACIL +	URACIL -
E. coli B/r ara-2	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE
K. pneumoniae PRL-R3,U$^-$ (PARENTAL STRAIN)	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE
K. pneumoniae CH-101 (D-FUC$^+$ STRAIN)	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE
K. pneumoniae CH-103 (DEHYDRATASE-NEGATIVE MUTANT STRAIN)	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE
K. pneumoniae CH-102 (ALDOLASE-NEGATIVE MUTANT STRAIN)	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE
K. pneumoniae CH-113 (DEHYDRATASE REVERTANT)	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE

strains only when the medium was supplemented with 0.005% uracil; *E. coli* did not use citrate in the presence or absence of uracil. Kauffman (99) reports *Klebsiella* species as not forming indole, give positive Voges-Proskauer tests, give a negative methyl red test, and usually grow on ammonium citrate; *Escherichia* species usually form indole, give a negative Voges-Proskauer test, give a positive methyl red test, and do not use ammonium citrate. Variations from the class norm, in the IMViC tests for the *Klebsiella* strains derived from *K. pneumoniae* PRL-R3,U^-, must be indigenous to the strain.

Thus, the only significant differences in the strains was in their ability to grow on D-fuconate or D-galactonate.

DISCUSSION

The results of this section showed the presence of a pathway involving the dehydration of D-fuconate to 2-keto-3-deoxy-D-fuconate, which is then cleaved to pyruvate and D-lactaldehyde (Fig. 12) in the D-fuconate-positive mutant strain (CH-101). (The fate of D-lactaldehyde was not pursued, but is presumed to be oxidized thru lactate to pyruvate.) D-Fuconate and D-galactonate dehydratase activities as well as a 2-keto-3-deoxy-D-fuconate aldolase activity were detectable in crude extracts prepared from this strain grown on D-fuconate. The absence of aldose dehydrogenase activities leading to D-fuconate or D-galactonate suggested that these aldonic acids were only used directly as carbon sources rather than as intermediates of aldose pathways. In addition, the gain mutation which facilitates growth of K. pneumoniae on D-fuconate has been determined, by enzyme induction and genetic studies, to be the constitutive production of a dehydratase that acts on both D-fuconate and D-galactonate.

Comparision of the dehydratase levels in the parental strain and the D-fuconate-positive strain grown on D-galactonate, the inducer of the D-galactonate pathway (found to be present in both strains), revealed a D-galactonate to D-fuconate activity ratio of 3:1. Other enzymes of the D-galactonate pathway were found in these strains at about the same level as the dehydratase except for the KDF aldolase, which is lacking in both strains grown on this substrate. Growth of these strains on non-inducers of the D-galactonate pathway (i.e., nutrient broth,

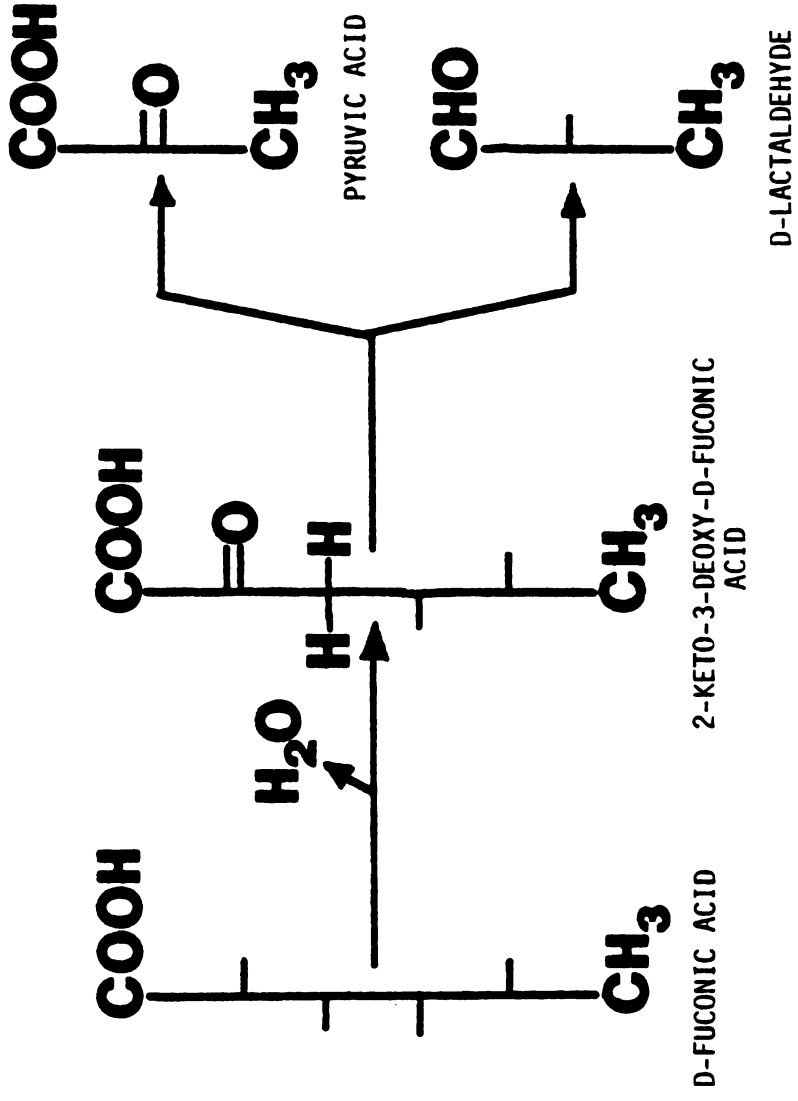


Figure 12. The D-fuconate pathway as elucidated in this study.

D-glucose, D-gluconate, etc.) did not result in the production of the D-galactonate pathway enzymes in the parental strain; however, such activities were found in the D-fuconate-positive mutant, when grown on these same carbon sources, in approximately the same level as seen for the parental strain grown on D-galactonate. As shown by comparative growth studies, the parental strain would not use D-fuconate as a growth substrate immediately, whereas the D-fuconate-positive strain readily uses D-fuconate for growth. Levels of the D-galactonate pathway enzymes, the D-fuconate dehydratase, and KDF aldolase in the D-fuconate-positive strain grown on D-fuconate were found to be comparable to those found in cells grown on the various non-inducers of the D-galactonate pathway. The parental strain, however, exhibited the KDF aldolase activity only when grown on a mixture of D-galactonate and D-fuconate.

Analyses of mutants negative for the D-fuconate pathway, i.e., D-fuconate dehydratase-negative and KDF aldolase-negative mutants, revealed that both lost the ability to grow on D-fuconate with an accompanying loss of the respective enzymatic activities. KDF aldolase-negative mutants retained the ability to use D-galactonate whereas the D-fuconate dehydratase-negative mutants did not. In addition, normal levels (strain CH-101) of both the D-galactonate and D-fuconate dehydratase activities were found in the KDF aldolase-negative mutants whereas both activities were lost in the dehydratase-negative mutants. Examination of a revertant of the dehydratase-negative mutant on D-galactonate revealed that both the D-fuconate and D-galactonate dehydratase activities were restored.

These results are consistent with the following conclusion: D-fuconate serves as a fortuitous substrate for the dehydratase of the D-galactonate pathway which is constitutively produced in the D-fuconate-positive strain (constitutivity results in the production of D-galactonate dehydratase, D-fuconate dehydratase, KDGal kinase, and KDPGal aldolase activities in the strain CH-101 without the aid of D-galactonate as inducer; in the parental strain these enzymes are apparently induced coordinately (100)). This constitutive production of the dehydratase allows D-fuconate to be assimilated for growth, as the aldonic acid does not induce the D-galactonate pathway. The further catabolism of the dehydratase product from D-fuconate employs an aldolytic activity independent of the D-galactonate pathway. The origin of this enzyme is unresolved, but some postulates have been considered, the testing of which was outside the scope of this thesis, and may be expounded.

Many bacterial aldolases have vary limited substrate specificities (101). The KDF aldolase, however, seems to possess a broad substrate specificity for keto-deoxy sugar acids; of five keto-deoxy sugar acids tested, 2-keto-3-deoxy-D-fuconate, 2-keto-3-deoxy-D-quinovonate, 2-keto-3-deoxy-D-galactonate, and 2-keto-3-deoxy-D,L-arabonate were found to serve as suitable substrates. Such varied substrate specificities have been shown with aldolases of meta-cleavage pathways in catechol-metabolizing pseudomonads (102). Pseudomonads are known to possess catabolic pathways for the breakdown of phenol and cresols to hydroxy-phenolic compounds which can then serve as substrates for subsequent reactions of a meta-cleavage pathway. One such compound, para-hydroxy phenol

(hydroquinone), if formed, could give rise to the product 2-keto-3-deoxy-D,L-arabonate if acted on by a hydrolyase. As noted, this compound seems to serve as a good substrate for the KDF aldolase. It is possible that the aldolytic activity found in K. pneumoniae on KDF is one in unision with a meta-cleavage pathway for catechol as seen in pseudomonads.

Although Klebsiella species are not normally associated with the ability to metabolize catechol, the fact that pseudomonads possess the enzymatic meta-cleavage pathway enzymes on transmissible plasmids (103 a,b,c) suggests the possibility that by transduction K. pneumoniae could acquire this trait. Testing for a catechol pathway, using induction of the KDF aldolase as a gauge when growing K. pneumoniae on substances (phenol, 2.5 mM, and sodium benzoate, 5.0 mM, in 0.5% sodium citrate) known to induce meta-cleavage pathway enzymes in pseudomonads (102), failed to show such activity. However, when procedures of plasmid isolation (104) were followed, the presence of high molecular weight DNA in crude extracts of both the parental strain and the D-fuconate-positive strain grown on nutrient broth was observed (preliminary data not included in this thesis). If not found as artifactual, this nucleic acid material may represent a plasmid (105 a,b) possessing the genetic information responsible for the noted KDF aldolase activity. Such a finding would be one of the few known cases where a catabolic pathway for a carbohydrate is located on a plasmid.

SECTION II

Purification and Characterization of D-Galactonate (D-Fuconate) Dehydratase

INTRODUCTION

In section I of this thesis, the constitutive production of D-fuconate dehydratase was shown to be the key event in the mutational acquisition of the ability of K. pneumoniae to use D-fuconate as a carbon source. This enzyme initiates D-fuconate catabolism, and the product of the reaction induces the next enzyme in the pathway, KDF aldolase. Mutant analysis and other studies suggested that the dehydratase was also instrumental in initiating the catabolism of D-galactonate. Such a dual specificity for a dehydratase has not been previously demonstrated.

Literature surveys of aldonic acid dehydrases as complilled by Dahms (106) and Wood (107), reveal that (i) dehydration results in the formation of an α-keto acid, (ii) the configuration of the aldonic acid substrate at the dehydration site is trans, (iii) a divalent metal ion is commonly required for best activity, (iv) thiols are required if a metal ion is not, (v) the optimal pH range is from 7.0 to 8.0, (vi) Km values range from 0.1 to 8.0 mM, and (vii) the dehydration reaction is essentially irreversible.

Further characterization, as far as physical studies are concerned, has been lacking in the literature. Recently, however, a specific D-galactonate dehydratase from a pseudomonad was purified and characterized more fully (97). Although most dehydratases exhibit mono-substrate specificities, exceptions are the D-fuconate dehydratase in a pseudomonad which acts both on L-arabonate and D-fuconate (33), and the

D-glucarate dehydratase of E. coli which acts on both D-glucarate and D-idarate (108); neither of these enzymes have been characterized by physical means.

This section describes the isolation and characterization of the D-galactonate (D-fuconate) dehydratase.

MATERIALS AND METHODS

All materials and methods not described in this section have been described in Section I.

Bacterial Strain and Culture Conditions

Bacterial Strain. A nutrient broth agar slant of *Klebsiella pneumoniae* PRL-R3,U^-, strain CH-101, a mutant which is derepressed for the D-galactonate (D-fuconate) dehydratase (see Section I, Results), was used for the preparation of the dehydratase.

Medium. The bacteria were grown in 0.65 % K^+ D-galactonate-mineral medium plus 0.005% uracil; this carbon source was found to produce the highest specific activity of the enzyme (Section I, Results). Mineral medium was prepared directly in the fermenter chamber (10.0-liter capacity) and sealed before autoclaving at 20 psi and 121°C for 75 min; measures were taken to prevent back pressure in the chamber due to autoclaving and possible contamination of the fermenter contents. D-Galactonate and uracil were autoclaved separately and added aseptically to the sterile contents of the fermenter.

Starter Culture Preparation. A 1.0-liter exponentially growing culture of the strain CH-101 in D-galactonate-mineral medium was prepared for inoculation of the 10-liter culture. To prepare this inoculum, a 7.0-ml culture, grown overnight in nutrient broth, was transferred to a flask containing 250 ml of fresh 0.65% D-galactonate-mineral medium plus uracil. The resultant culture was then incubated on a

rotary shaker until a turbidity of 0.60 O.D. at 600 nm was reached. Serial transfer of this culture (250 ml to 500 ml, and 500 ml to 1.0 liter) in fresh D-galactonate-mineral medium was performed to prepare the final 1.0-liter starter culture.

Cell Growth. D-Galactonate-mineral medium in a New Brunswick Scientific Co., Inc., Microferm fermenter was temperature-equilibrated at 30°C. Starter culture was then added, aseptically, with aeration and stirring turned off, to the sterile fermenter contents. Vigorous aeration (10.0 lb/in^2) and mixing (200 rpm) were resumed to promote optimal growth. Growth was monitored by withdrawing 2.0-ml samples with sterile pasteur pipetes and making absorbance readings on a Gilford, model 2400, spectrophotometer at 600 nm. Samples were diluted to obtain corrected absorbance values and plotted versus the time of growth. Cells were harvested when the corrected absorbance value no longer changed with time; usually, a final absorbance of 2.0 at 600 nm was reached after 5.0 hrs of growth.

Harvesting Cell Cultures. Bacterial cultures grown in the microfermenter were harvested with the aid of a Sharples air-driven centrifuge cooled to 4°C with Freon and run at a speed of 40,000 rpm. The cells were weighed in tared plastic beakers and stored at -20°C until needed.

Protein Determination.

Protein concentration was determined either by the procedure of Lowry et al. (79) using bovine serum albumin as the standard or by the aid of a nomograph using absorbance values of the protein solution at 280 nm and 260 nm above the buffer absorbance. The Lowry chromogen

was measured on a Gilford 300 N colorimeter equipped with a red filter at 600 nm. A Gilford spectrophotometer, model 2400, was used to measure in the ultraviolet range.

D-Galactonate (D-Fuconate) Dehydratase Assay

The assay for the D-galactonate (D-fuconate) dehydratase was performed as described in Section I, Materials and Methods, unless otherwise indicated. D-Galactonate was used as the substrate at a concentration of 20.0 mM in the assay when assaying column fractions during a given purification step and when reporting activities of this enzyme under various assay conditions, unless noted differently.

Enzyme Purification Procedures

DEAE-Cellulose Chromatography. DEAE-cellulose was prepared by washing it sequentially with 1.0 N NaOH, 1.0 N HCL, and 1.0 N NaOH as described by Peterson and Sober (109). Washed cellulose ion exchanger was de-fined by decantation. When not in use, the exchanger was stored at 4°C in 0.02% sodium azide and 1.0 mM EDTA to prevent bacterial growth. Packed columns were judged equilibrated when both the pH and conductance of the eluate matched that of the buffer. Protein loading was done by passing the protein sample through the column and washing with the appropriate buffer until the protein absorbance was no longer observed in the eluate. Adsorbed protein was eluted with a linear gradient of KCl. Once the resin was used in an enzyme purification step, the cellulose column was washed with 1.0 M KCl to remove all protein and was unpacked to store as above.

Sepharose A-5M Chromatography. Commercial Bio-Gel Sepharose A-5M (Bio-Rad Laboratories), which has an exclusion limit of 5.0-million-molecular weight, was swollen in 0.05 M potassium phosphate buffer (pH 7.0) at $4^{o}C$ for 24 hours. Columns were packed under a 16-cm pressure head to establish linear flow rates of 5.0 ml per hr with the aid of a Gilson Multiplus-2 peristatic pump. Packed columns were equilibrated by washing with at least four bed-volumes of the same buffer. After use, the columns were washed with 0.02% sodium azide to prevent bacterial growth. The volume of the protein sample applied to the columns were kept to 2.0% of the column bed-volume. Calibration of the column for void volume (V_o) and inclusion volume (V_t) was done using Blue Dextran (Sigma Chem. Co.) and $K_3Fe(CN)_6$, respectively (110).

Hydroxyapatite Chromatography. Bio-Gel HT hydroxyapatite (Bio-Rad Laboratories), pre-swollen in 1.0 mM sodium phosphate buffer, pH 6.8, was used to prepare chromatographic columns. Pre-swollen hydroxyapatite was specified to contain 0.175 mg hydroxyapatite material per ml of well mixed slurry. To determine the capacity of the material for the dehydratase, at given purification steps, several mini-columns were used. Mini-columns of hydroxyapatite were prepared using standard 17.8 cm pasteur pipetes. They were packed with 2.0 mm of glass wool, upon which a 2.0-mm bed of celite and various amounts of the hydroxyapatite material were layered. Columns ranging from 44 to 175 mg of hydroxyapatite were packed under gravity and equilibrated with 1.0 mM potassium phosphate buffer (pH 6.8). Protein was applied to each column, washed with the equilibration buffer, and eluate fractions were assayed for dehydratase activity. Adsorbed

dehydratase was eluted with 0.4 M potassium phosphate buffer (pH 6.8). The amount of dehydratase bound to a given amount of hydroxyapatite was determined by plotting the percent of bound dehydratase versus mg protein per mg hydroxyapatite. This graph (not shown) was used to calculate the optimal amount of protein to add to the column. (Similar preliminary studies were conducted in determining the capacity of the DEAE-cellulose ion exchanger resin noted previously.)

The hydroxyapatite slurry was first diluted five-fold with 1.0 mM potassium phosphate buffer (pH 6.8) and loaded onto a 0.5-cm bed of celite by gravity flow. Before application of the sample, protein was diluted to give a potassium phosphate concentration approximating 1.0 mM and a pH of 6.8; these conditions were found to be optimal for the binding of the dehydratase protein. The column was washed with the equilibration buffer until no protein could be detected in eluate fractions. The column was then eluted with a linear gradient of potassium phosphate buffer (pH 6.8). Sequential washes of the column with 1.0 M KCl, 1.0 M potassium phosphate, and 0.02% sodium azide were done to condition the column for storage.

Sephadex G-200 and Bio-Gel P-300 Chromatography. Materials obtained in the dry state were swollen in the appropriate buffer for 2.0 days at 4^{o}C. Conditions of packing, equilibration, sample loading, and calibration were followed as described for the Sepharose A-5M gel filtration material.

Pressure Dialysis Concentration of Protein

Pressure dialysis was performed using an Amicon Diaflo apparatus, 50-ml capacity, equipped with a 1-liter pressure bell jar and a PM-30

membrane filter, having a 30,000-molecular-weight range cut-off. Concentration of the protein was performed under nitrogen pressure (50 psi) as to establish flow rates no less than 20.0 ml per hour. When not in use, the filter was stored at $4^{o}C$ in 95% ethyl alcohol after first washing it with distilled water.

Conductivity Measurements

Column fractions from purification steps were assayed for salt concentration using a standard conductivity meter with variable conductance control. Samples of 0.05-ml volume were diluted 100-fold with de-ionized water and read against a buffer blank. The instrument was calibrated using the highest salt concentration of the gradient in the appropriate buffer.

Polyacrylamide Gel Electrophoresis

General Procedures. Slab gels were prepared using a Bio-Rad vertical slab gel apparatus, model 220, equipped with rectangular glass plates and appropriate spacers and combs. All slab gels prepared had dimensions of 100 X 140 mm; thickness, as determined by the spacer used, was either 1.5 or 3.0 mm. Plates were prepared by cleaning with chromic acid solution, neutralizing with sodium thiosulfate, and then rinsing with distilled water before air-drying before use. Components of the gel mixture were degassed over ice prior to mixing. Plates were sealed at the sides using appropriate spacers or with 2.0% Bacto-agar. Electrophoresis was performed using an ISCO, model 492, Electrophoresis Power Supply (150 mamp, 1000 volt range) at constant current. After electrophoresis, slab gels were removed by first removing the spacers, followed by lubricating the gel-glass space with a stream of

water, and then by prying the plates apart with the aid of a spatula. All slab gels prepared consisted of a 9.0-cm high running gel and a 2.0-cm high stacking gel. The stacking gel was used to make 1.0-cm deep wells for convenient application of the protein sample.

Preparation of Native Gels. Slab gels of 10.0 cm X 15.0 cm X 3.0 mm were poured. Running gels were filled to a height of 9.0 cm with a solution containing 5.0% acrylamide, 0.13% bisacrylamide (N,N'-methylenebisacrylamide), 0.025% TEMED (N,N,N',N'-tetramethylethylenediamine), 0.045% ammonium persulfate (prepared fresh each time and added just prior to pouring the gel), and 0.375 M Tris-HCl buffer (pH 8.8). Once hardened, a stacking gel containing 5.0% acrylamide, 0.13% bisacrylamide, 0.025% TEMED, 0.045% ammonium persulfate, and 0.063 M Tris-HCl buffer at a pH of 6.8 was poured over the running gel, with a three-channel comb in place. The poured stacking gel was overlaid with a thin layer of 1-butanol to generate a level surface, and allowed to harden over a period of 20 min (as was done with the running gel). The final slab gel was attached to the electrophoresis apparatus by the use of clamps and was then pre-electrophoresed at a constant current of 20 milliamps at $4^{o}C$ for 8.0 hrs to remove unreacted ammonium persulfate and to condition the gel for use at this temperature.

The electrophoresis buffer contained 0.2 M glycine, 0.05 M Tris-(hydroxymethyl)aminomethane (Tris), 0.06% thioglycolic acid with a final pH of 8.8. Prior to the application of the protein sample, 0.2 ml of 0.05% bromophenol blue dye in 10% glycerol was applied to each channel and run into the stacking gel at 5.0 milliamps current. The current

was stopped before application of the protein samples at 0.2-ml volume to each channel. Once protein samples were applied, the current was restored to 5.0 mamps until the dye marker entered the running gel, then increased to 10.0 mamps for an interval of 20 minutes. At the end of this time, the current was increased to 20.0 mamps, and allowed to run at this current for a total of 4-6 hrs at $4^{o}C$; the dye marker at this time was within 0.5 cm of the bottom of the slab gel.

Gels were stained for protein by placing into a solution of 0.1% Coomassie Brilliant Blue R, 10% trichloroacetic acid (TCA), and 27% (v/v) isopropanol and allowed to stain overnight at room temperature. Dye bands were marked by the insertion of a small piece of wire before destaining, as the latter process removes all traces of the dye band. Destaining was achieved by immersion in 10% acetic acid with three changes of destaining solution or until a faint blue background was obtained. Slab gels, once destained, could be dried for photographic purposes by transferring the gel to a sheet of filter paper (treated initially by soaking in 1% glycerol and 10% acetic acid for 30-45 min) and drying with the aid of a Model 224, Gel Slab Drier (Bio-Rad Laboratories) under 733 mm Hg vaccum for 45 minutes. The dried gel shows no shrinkage once treated as described and is easily preserved in a notebook.

Protein profiles of the stained channels were made by scanning the gel with a Gilford Gel Scanner at 600 nm and plotted on graph paper with a Sargent recorder. Slab gels were laid length-wise on their edges in quartz boat cuvettes such to allow measurements to be made at the center of the gel; distilled water was used to cover the gels while

scanning as to prevent dehydration. Traces prepared in this manner could be calibrated in terms of true gel length by taking into consideration the speed of the gel scanner and the recorder.

Preparation of SDS Gels. Slab gels of 10.0 cm X 15.0 cm X 1.5 mm were prepared as described above. Running gels of 9.0 cm heigth were comprised of 10.0% acrylamide, 0.26% bisacrylamide, 0.025% TEMED, 0.045% ammonium persulfate, 0.1% SDS, and 0.375 M Tris-HCl buffer at a pH of 8.8. The stacking gel consisted of 5.0% acrylamide, 0.13% bisacrylamide, 0.025% TEMED, 0.045% ammonium persulfate, 0.1% SDS, and 0.0625 M Tris-HCl buffer (pH 6.8). Both gels were allowed 20 min for polymerization to occur at room temperature. Appropriate combs were used to give the desired number of channels in the stacking gel. A layer of 1% SDS was used to generate a flat surface for both the running gel and the stacking gel. The electrophoresis buffer was comprised of 0.2 M glycine, 0.05 M Tris(hydroxymethyl)aminomethane (Tris), and 0.1% SDS at a final pH of 8.8. Pre-electrophoresis at 5.0 mamps preceded sample application at room temperature to rid the gels of excess ammonium persulfate.

Samples were prepared by diluting 0.05 mg of standard protein or dehydratase preparation in 0.2 ml of 1.2% SDS, 0.29 M Tris-HCl buffer (pH 6.9), and 10% glycerol. Immediately prior to electrophoresis, the sample solutions were made 2.0% in 2-mercaptoethanol and then heated at 100^{o}C for 5.0 minutes. After heating, the samples were made 0.05% in bromophenol blue dye, and 0.04 ml sample was added to each channel. Slab gels were subjected to electrophoresis at 5.0 mamps through the stacking gel and 10 to 20 mamps through the running gel. Electrophoresis

under these conditions required about 4.0 hrs at room temperature. Gels were stained and destained, scanned for protein stained areas, and dried as described for native gels.

Isoelectric Focusing

Isoelectric focusing of the D-galactonate (D-fuconate) dehydratase was performed in a 110-ml capacity LKB-Produckter (Stockholm, Sweden) vertical column equipped with a Lauda-water bath cooled to $4^{o}C$ with a 10% ethylene glycol solution. The procedure of Vesterberg (111) was used as rationale for the preparation of the electrophoretic medium. Solutions were loaded and eluted by the aid of a Gilson Multiplus-2 peristaltic pump and gradients prepared using a 110-ml-capacity conical gradient maker equipped with a tapered rod stirrer for proper mixing. The electrophoresis medium, comprise mainly of sucrose (high concentrations at the anode end, bottom; and low concentrations at the cathode end, top), Pharmolyte ampholytes (Pharmacia, pH 4.0 to 6.5), and protein sample was generated by the following solutions, loaded in this order: (i) dense electrolyte solution, 23.54 ml, comprised of 58.94% sucrose and 0.043 M sulfuric acid; (ii) dense electrolyte junction buffer, 5.0 ml, comprised of 55.24% sucrose and 1.07% Pharmolyte ampholytes (pH 4.0 to 6.5); (iii) an 80.0-ml linear gradient of 51.53 to 4.99% sucrose (negative gradient) in approximately 1.0% Pharmolyte ampholytes (pH 4.0 to 6.5), plus about 2.0 mg of protein sample; (iv) a light electrolyte junction buffer, 5.0 ml, of 2.5% sucrose and 1.0% Pharmolyte ampholytes (pH 4.0 to 6.5); and (v) a light electrolyte solution of 0.18 M NaOH.

Protein was subjected to isoelectric focusing at 4°C for a period of several days at constant voltage using an ISCO Electrophoretic Power Supply, model 492, which has an upper limit of 1000 volts capacity. Eluate fractions were collected from the base of the column and assayed for both enzyme activity by the standard dehydratase assay (above) and pH with a Beckman pH meter equipped with microelectrodes.

Enzymatic Assays for Molecular Weight Standards

Calibration Standards. Ferritin, blue dextran, and $K_3Fe(CN)_6$, used to calibrate gel filtration columns, were determined by their characteristic absorbances at 230 or 280 nm, 630 nm, and 420 nm, respectively.

Lactate Dehydrogenase. Beef heart lactate dehydrogenase was assayed using the coupled assay procedure described in Section I following the oxidation of NADH with time in the presence of sodium pyruvate at 340 nm.

Pyruvate Kinase. Rabbit muscle pyruvate kinase was assayed for the production of pyruvate from phosphoenol pyruvate (PEP). The reaction mixture (0.2 ml) contained 7.5 mM $MgCl_2$, 0.3 mM ADP, 0.25 mM PEP, 50.0 mM potassium phosphate buffer (pH 7.5) and non-rate-limiting amounts of lactate dehydrogenase; the temperature was 30°C and the rate of aborbance change was measured at 340 nm.

Alkaline Phosphatase. *E. coli* alkaline phosphatase was assayed in accordance with standard procedures following the production of *p*-nitrophenol from the substrate *p*-nitrophenyl phosphate (112). Aliquots of the enzyme sample (0.4 ml) were inoculated in a 1.0-ml solution consisting of 50.0 mM glycine buffer (pH 10.5) and 5.5 mM

p-nitrophenyl phosphate, for 30 min at 37°C. At the end of this time, 10.0 ml of 0.02 N NaOH was added to the samples, the solution was mixed, and absorbance readings were made at 405 nm against a reagent blank.

Catalase. Bovine liver catalase was assayed using a 1.5-ml solution of freshly prepared 0.5% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.5). Initial velocities were determined after blanking the instrument against the buffer alone at 240 nm. Rates were measured as the absorbance drop from 0.45 to 0.40 O.D. at this wavelength (113). Quantitation of the amount of hydrogen peroxide lost was made using the absorbance values reflected by various stock solutions of the substrate (114).

Fumarase. Fumarase was assayed according to the procedure of Racker (115) following the conversion of L-malate to fumarate at 240 nm and 25°C. A reaction mixture of 1.0 ml was used with various aliquots of the enzyme sample.

Methods for the Determination of Molecular Weight

Sucrose Density Gradient Centrifugation. Sucrose density gradient centrifugation was run in 1.3 X 5.0 cm cellulose nitrate centrifuge tubes containing 4.6-ml volume of 5.0 to 20.0% sucrose in 0.05 M Tris-HCl buffer (pH 7.5). Gradients were poured at room temperature and cooled to 4°C over a period of several hours before use. Centrifugation was performed in a model L-2 preparative Beckman centrifuge with an SW-39 rotor for 15.0 hrs at 35,000 rpm at 4°C. Following the centrifugation, tubes were punctured and fractions were collected from the bottom.

Sedimentation Velocity and Sedimentation Equilibrium Analysis. Sedimentation velocity and equilibrium studies were performed using a Beckman Model E Ultracentrifuge equipped with ultraviolet absorption optics as described by Lamers et al. (116). Protein concentration was observed as a moving boundary of absorbance above the buffer and was quantitated by Beers' law, as this absorbance is linear within the confines of the experiment.

(i) Sedimentation Velocity. For sedimentation velocity analysis, centrifugation was performed in an aluminum An-G six-cell-capacity rotor at 20°C and at 32,000 rpm. Sedimentation of each cell was determined by making scans of the moving boundary at regular time intervals, determining the half height of the boundary (therefore, the distance from the center of rotation), and using these values in appropriate equations (see Results, this section) to calculate the sedimentation constant.

(ii) Sedimentation Equilibrium. Sedimentation equilibrium was performed using the meniscus depletion method of Yphantis (117) using a four-sector AnF titanium rotor with standard double-sector cells. Protein sample (0.12 ml) was layered onto 0.01 ml of FC-43 silicon oil to prevent convection disturbances; the total sample volume was restricted to one-fourth the cell capacity to facilitate ease of equilibrium measurements. The centrifugation was done at 20°C and 11,000 rpm allowing 24 hrs for equilibration between trials. Measurements of absorbance and migration distance at the given absorbance, above background, were made at equilibrium for various distances from the center of rotation to determine the concentration/radius factor

needed for the molecular weight calculation at equilibrium (118) (see Results, this section, for calculations).

Amino Acid Composition Determination

All protein samples prepared as described in the Results, this section, were analyzed by Doris Bauer on an automated Beckman, model 121-B, Amino Acid Analyzer. Known standard amino acids were used to calibrate the column systems used to make the analyses. Quantitation of the amino acids was done by determination of the peak area as plotted on a multipoint recorder and by dividing by a predetermined color constant for that amino acid. All amino acids were converted automatically to their ninhydrin derivatives in the course of the analysis. Norleucine was used as the internal standard for each amino acid determination, unless noted otherwise.

Sources of Materials

D-Galactonate was prepared from D-galactono-γ-lactone (Sigma Chemical Company) by neutralization with stock 1.0 N NaOH. Membrane filters (PM-10 and PM-30) used in pressure dialysis concentration of protein samples, were obtained from the Amicon Corporation. Phenol reagent (Folin and Ciocalteu) used in Lowry protein determinations was obtained from the Harleco Corporation. DEAE-cellulose as Cellex-D (anion exchange cellulose) in the hydroxide form, and Bio-Gel P-300 (100-200 mesh) were obtained from Bio-Rad Laboratories. Sephadex G-200 (medium grade) and Blue Dextran were obtained from Pharmacia Fine Chemicals. Dowex-1-X8 (200-400 mesh) was obtained from the Sigma Chemical Company. "Enzyme-grade" ammonium sulfate was obtained from the Mallinckrodt Company. Acrylamide was used without recrystallization from the

Ames Company, bisacrylamide from Miles Laboratories, and sodium dodecyl sulfate (SDS) from the Pierce Chemical Company. Norleucine and other amino acid standards were obtained from Calbiochem and the Pierce Chemical Companies. Specially prepared hydrolysis tubes were obtained from the MSU Glass-blowing Laboratory for acid hydrolysis treatment of protein samples for amino acid composition determinations. Cellulose nitrate centrifuge tubes for sucrose density gradient sedimentation studies were obtained from the Beckman Company. All chemicals and enzymes not mentioned were obtained from the Sigma Chemical Company or of better commercial grade.

RESULTS

Purification of D-Galactonate (D-Fuconate) Dehydratase

During the course of the purification of the D-galactonate (D-fuconate) dehydratase, some techniques or conditions were found to be ineffective or counterproductive. Substantial losses of enzymatic activity resulted from dialysis against 0.05 M potassium phosphate buffer (pH 7.0), despite the fact that the enzyme was stable in this buffer upon storage at $4^{o}C$ or by freezing. Losses of the enzyme activity occurred at pH ranges below 5.0 and above 9.0; therefore, pH precipitation was eliminated as a viable method. Bentonite treatment at 60 mg dry material per ml crude extract (15.0 mg/ml protein concentration), as used in other enzyme purification procedures, did not adsorb the dehydratase. Carboxymethyl-cellulose did not bind the enzyme at pH ranges required for enzyme stability (pH 5.0 to 9.0). The following scheme was reproducible and yielded an electrophoretically homogeneous enzyme preparation.

Cell Extract. Frozen packed cell, 111.5 g wet weight, from two consecutive 10.0-liter D-galactonate-mineral-medium cultures of the strain CH-101 were thawed in cold 0.05 M potassium phosphate buffer (pH 7.0) to give a final cell slurry of 400 ml. The cell extract was prepared by sonic treatment in the presence of glass beads (125 to 88 microns). All purification steps were carried out at 0^{o} to $4^{o}C$. A summary of the purification is given (Table 13).

Table 13. Purification of D-galactonate (D-fuconate) dehydratase.

FRACTION	VOLUME	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	RECOVERY
	ml	mg	units[a]	units/mg protein	%
Crude extract	485	7830	2470	0.315	100
Protamine sulfate supernatant	908	6670	2380	0.357	96
Ammonium sulfate ppt. (40 to 70%)	800	3910	2120	0.542	86
DEAE-Cellulose I	835	668	1340	2.01	54
Sepharose A-5M	224	243	993	4.09	40
Hydroxyapatite	470	155	950	6.13	39
DEAE-Cellulose II	75	98	782	7.98	32
Sephadex G-200	23	84	657	7.82	27

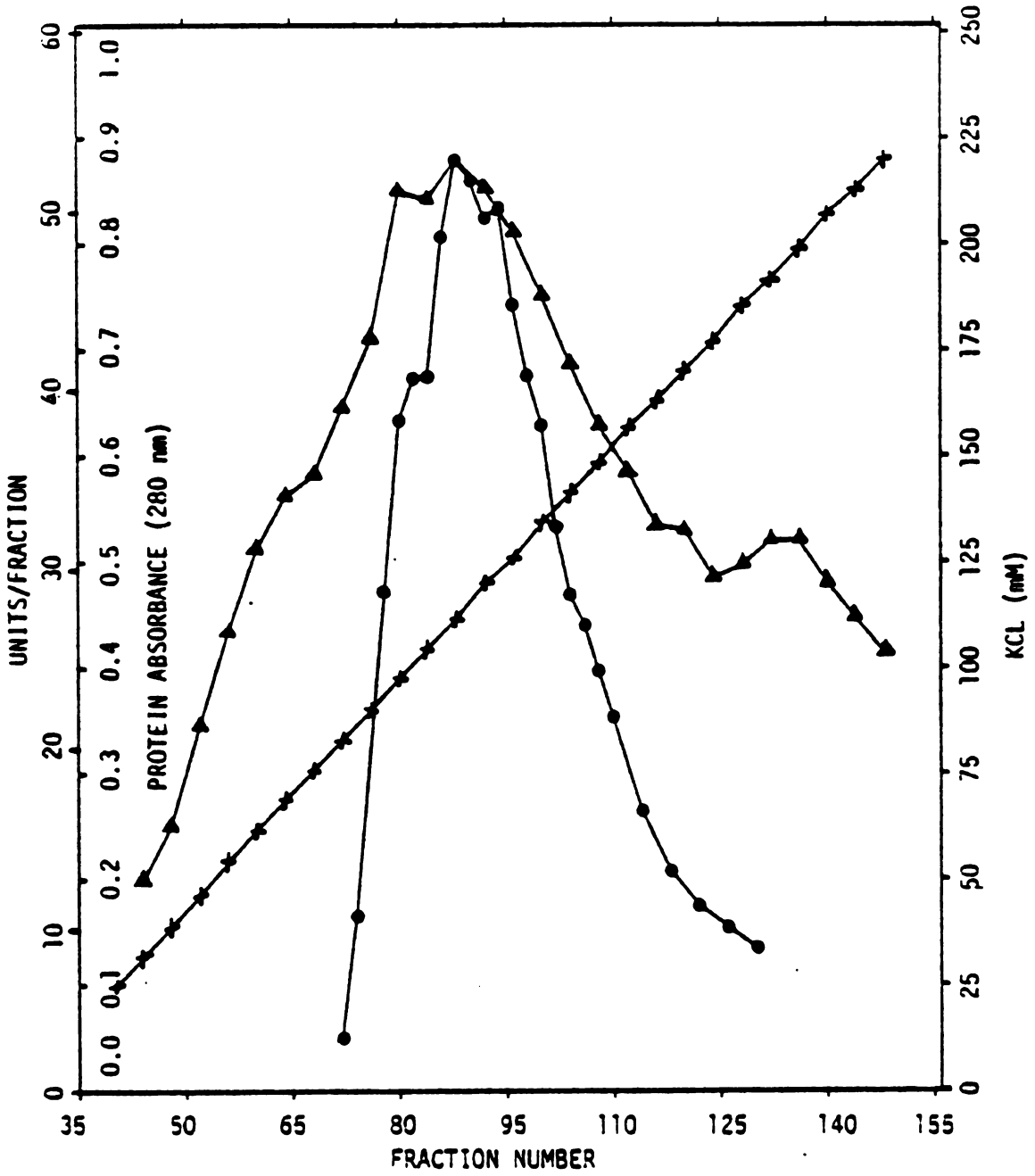
[a] one unit equals the amount of enzyme that converts 1.0 μmol of D-galactonate to product per min at 30°C

Protamine Sulfate Fractionation. The crude extract was diluted to 780 ml (to 10.0 mg/ml protein concentration) with the sonication buffer, to which was added 20.3 g of well ground ammonium sulfate to make a 2.6% solution. To the solution was added 156 ml of 2.0% protamine sulfate (pH 5.0) slowly while stirring. After 30 min stirring, the solution was centrifuged at 12,000 X g to sediment the precipitate. The supernatant was collected by decantation.

Ammonium Sulfate Precipitation. To 908 ml of supernatant from the protamine sulfate step was added slowly 182.7 g of ammonium sulfate to bring the concentration to 40% of saturation. The solution was stirred for 30 min, and then centrifuged at 12,000 X g for 10 minutes. The 955 ml of supernatant was decanted, and 180.1 g more ammonium sulfate was added to bring the concentration to 70% of saturation. After 30 min, the solution was centrifuged once again. The supernatant was discarded, and the pelleted material was suspended in 200 ml of 0.05 M potassium phosphate buffer (pH 7.0) for further purification.

DEAE-Cellulose Chromatography I. The pooled 40 to 70% ammonium sulfate fraction was diluted to 800 ml with 0.05 M potassium phosphate buffer (pH 7.0) and loaded onto a 4.0 X 18.5 cm DEAE-cellulose column, pre-equilibrated with the same buffer at pH 7.0. A 3.6-liter linear gradient of 0.0 to 0.35 M KCl in the same buffer was used to elute the protein from the column (Fig. 13). Fractions 74 to 115, containing the peak of activity, were pooled for further purification. Pooled fractions showed no activity (less than 0.001 unit/mg) for the KDGal kinase or the KDPGal aldolase.

Figure 13. DEAE-Cellulose chromatography I. Fractions of 20.0-ml volume were collected at a flow rate of 2.0 ml per minute. All other details are given in the text. Symbols: ● D-galactonate (D-fuconate) dehydratase, ▲protein absorbance (280 nm), + salt gradient concentration.


UNITS/FRACTION
PROTEIN ABSORBANCE (280 nm)
KCL (mM)
FRACTION NUMBER
0
10
20
30
40
50
60
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
25
50
75
100
125
150
175
200
225
250
35
50
65
80
95
110
125
140
155

Sepharose A-5M Chromatography. The pooled fractions from the DEAE-cellulose column were concentrated to 34.0 ml by pressure dialysis as described in Materials and Methods, and loaded onto a 2.6 X 80.0 cm column of Sepharose A-5M equilibrated with 0.05 M potassium phosphate buffer (pH 7.0); the sample was divided into two portions, 17.0 ml each, and loaded sequentially with buffer washes in between runs. Protein was eluted from the column using the same buffer. Elution profiles of each run, as exemplified by one such run (Fig. 14), showed peak dehydratase activity in fractions 52 to 76; these fractions were pooled from each run and combined for further purification.

Hydroxyapatite Chromatography. The combined sample from the Sepharose A-5M chromatography step, 224 ml, was diluted to 0.5 unit per ml with 1.0 mM potassium phosphate buffer (pH 6.8) and loaded onto a 7.9 X 2.0 cm hydroxyapatite column equilibrated with the same buffer. (Capacity studies with the enzyme at this stage of purification gave a loading capacity ratio of 2.4 mg protein per ml packed hydroxyapatite, resulting in better than 95% of the dehydratase bound.) Dehydratase activity was eluted with a 2.4-liter linear gradient of 4.0 to 400 mM potassium phosphate buffer (pH 6.8). Fractions 21 to 42, containing the peak activity, were pooled for further purification (Fig. 15).

DEAE-Cellulose Chromatography II. The pooled hydroxyapatite fractions were loaded directly onto a 1.5 X 15.5 cm DEAE-cellulose column equilibrated with 0.05 M potassium phosphate buffer (pH 6.0). Elution of the enzyme was done employing an 400-ml linear gradient of 0.0 to 0.35 M KCl in the above buffer. Fractions 52 to 72, containing the

Figure 14. Sepharose A-5M chromatography of the pooled DEAE-cellulose I fractions. Fractions of 4.0-ml volume were collected. All other details are given in the text. Symbols: ● D-galactonate (D-fuconate) dehydratase, ▲ protein absorbance (280 nm).

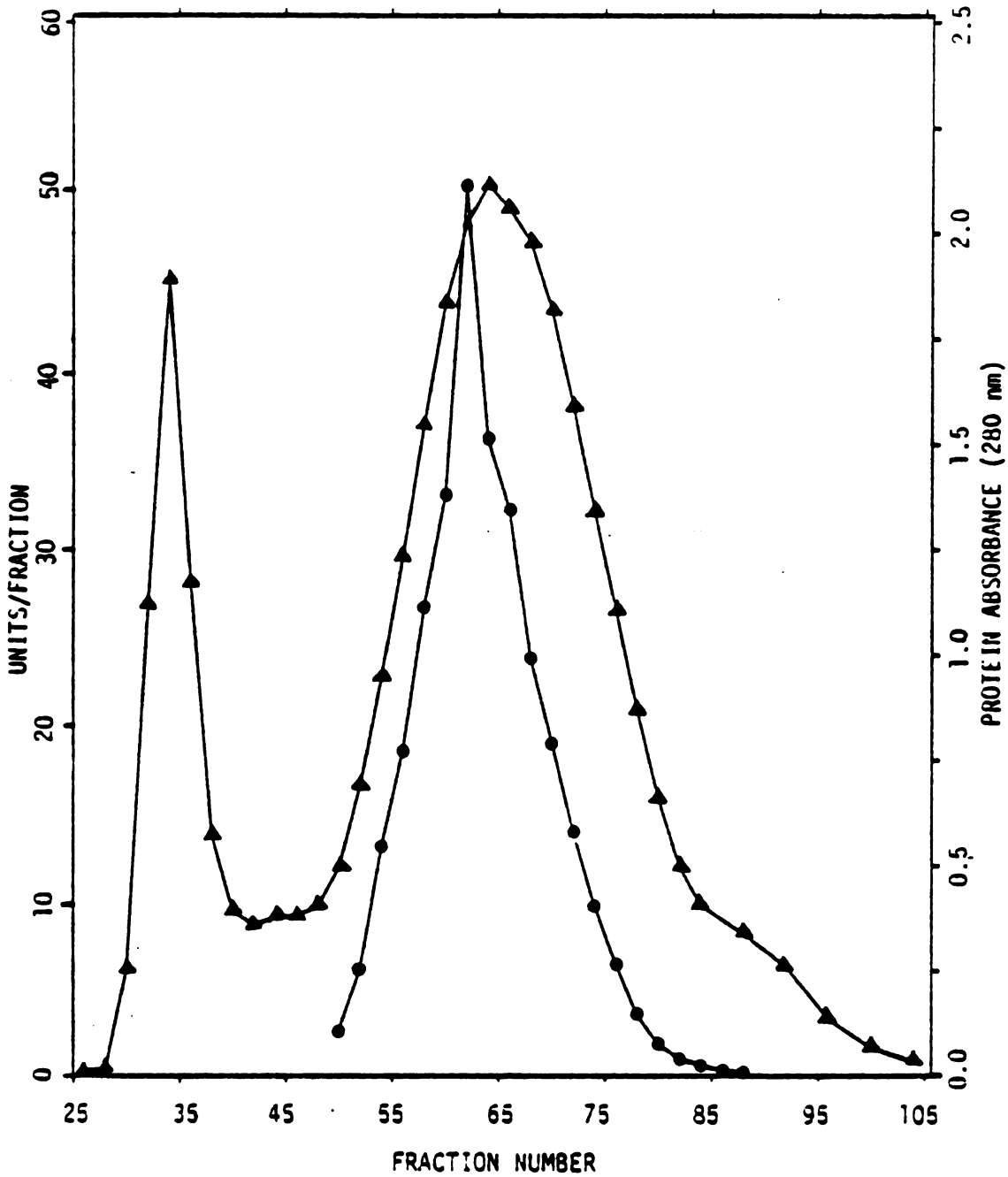
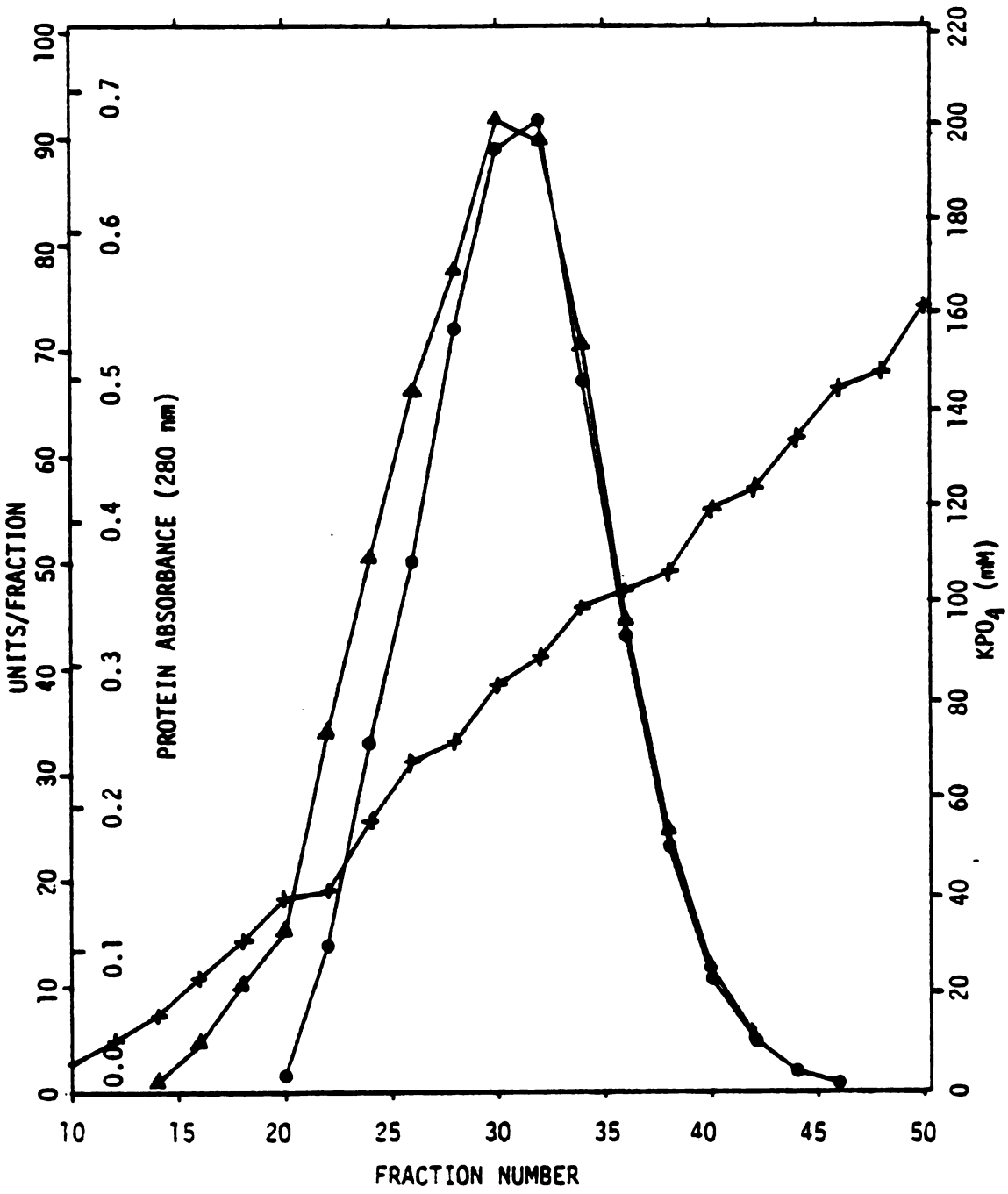

UNITS/FRACTION
PROTEIN ABSORBANCE (280 nm)
FRACTION NUMBER
0
10
20
30
40
50
60
0.0
0.5
1.0
1.5
2.0
2.5
25
35
45
55
65
75
85
95
105

Figure 15. Hydroxyapatite chromatography of the combined Sepharose A-5M pooled fractions. Fractions of 22.0-ml volume were collected at a flow rate of 200 ml per hour. All other details are given in the text. Symbols: ● D-galactonate (D-fuconate) dehydratase, ▲ protein absorbance, ✚ salt gradient concentration.


UNITS/FRACTION
PROTEIN ABSORBANCE (280 nm)
KPO4 (mM)
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
100
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
20
40
60
80
100
120
140
160
180
200
220
10
15
20
25
30
35
40
45
50

peak activity, were pooled for further purification (Fig. 16).

Sephadex G-200 Chromatography. Fractions pooled from the second DEAE-cellulose column were concentrated to 26.0 ml by pressure dialysis and loaded onto a 4.0 X 95.0 cm column of Sephadex G-200 equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). Protein was eluted from the column using the same buffer. Fractions 52 to 70 (Fig. 17), containing the peak activity, were pooled and concentrated by pressure dialysis to a final volume of 23.0 ml to constitute the purified dehydratase protein used for all kinetic and physical studies in this section.

Determination of Purity of the Dehydratase Preparation

As criteria for homogeneity both native and denaturing polyacrylamide gels were used to judge the purity of the final purification step used in the dehydratase preparation.

SDS Polyacrylamide Gel Electrophoresis. Slab gels comprised of 10% acrylamide were prepared as described in Materials and Methods. Two such gels were run. One gel shows the dehydratase electrophoresed in the presence of known molecular weight standards (Fig. 18). From the molecular weights of catalase (60,000 MW), malate dehydrogenase (35,000 MW), tryptophanase (55,000 MW), aldolase (40,000 MW), and ovalbumin (45,000 MW), a subunit molecular weight for the dehydratase of approximately 45,000 was determined. The second gel (Fig. 19) shows that only one protein band can be observed visually or with the aid of a gel scanner. When crude extracts of this strain were run on the same SDS gels one observed a protein band corresponding to the band observed in the purified dehydratase preparation; this band of protein was observed throughout the purification.

Figure 16. DEAE-Cellulose II chromatography of the hydroxyapatite pooled fractions. Fractions of 4.0-ml volume were collected at a flow rate of 90.0 ml per hour. All other details are given in the text. Symbols: ● D-galactonate (D-fuconate) dehydratase, ▲ protein absorbance (280 nm), ✚ salt gradient concentration.

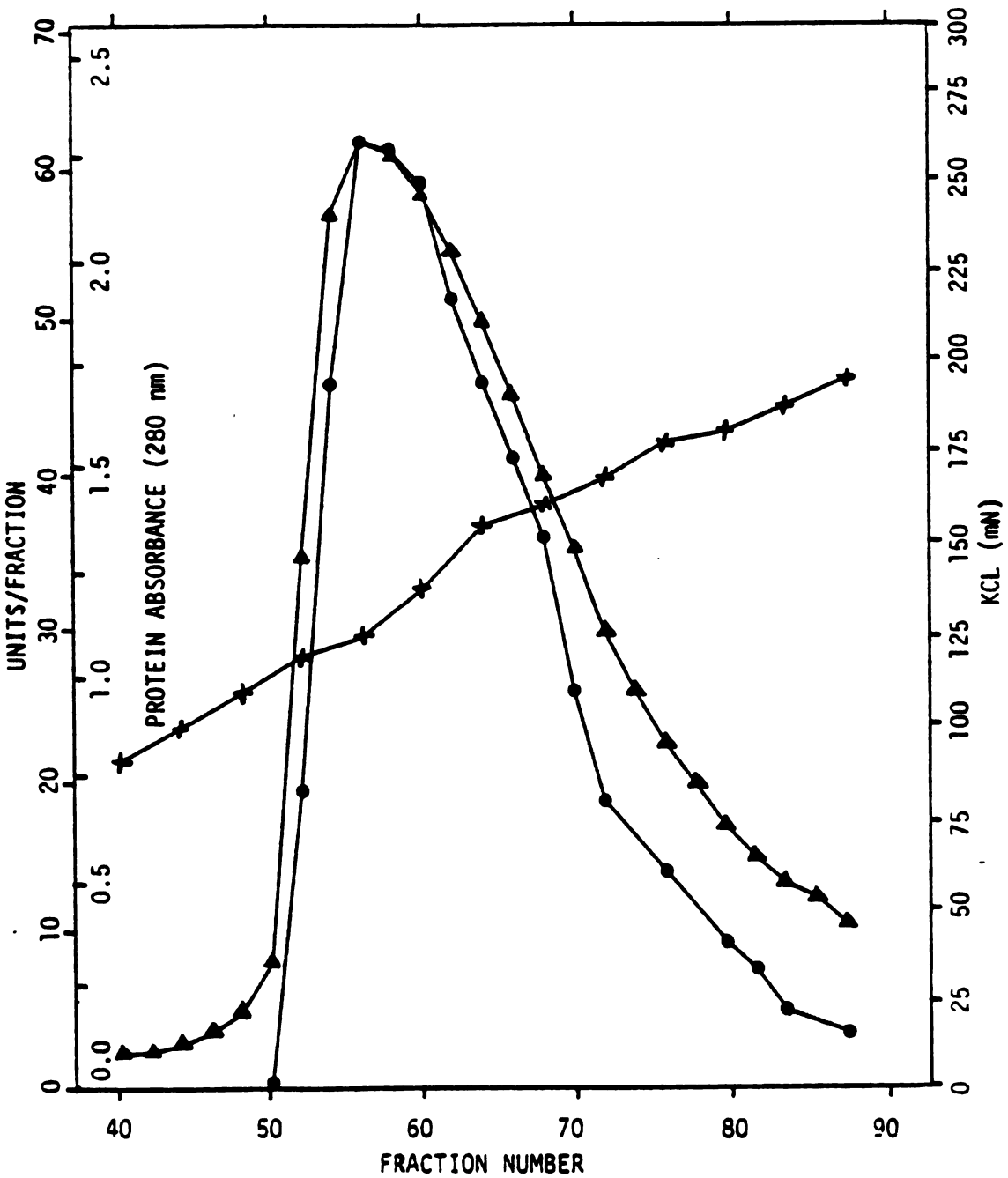

UNITS/FRACTION
PROTEIN ABSORBANCE (280 nm)
KCL (mM)
FRACTION NUMBER
0
10
20
30
40
50
60
70
0.0
0.5
1.0
1.5
2.0
2.5
0
25
50
75
100
125
150
175
200
225
250
275
300
40
50
60
70
80
90

Figure 17. Sephadex G-200 chromatography of the pooled DEAE-cellulose II step fractions. Fractions of 8.0-ml volume were collected at a flow rate of 50.0 ml per hour. Symbols: ● D-galactonate (D-fuconate) dehydratase, ▲ protein absorbance (280 nm).

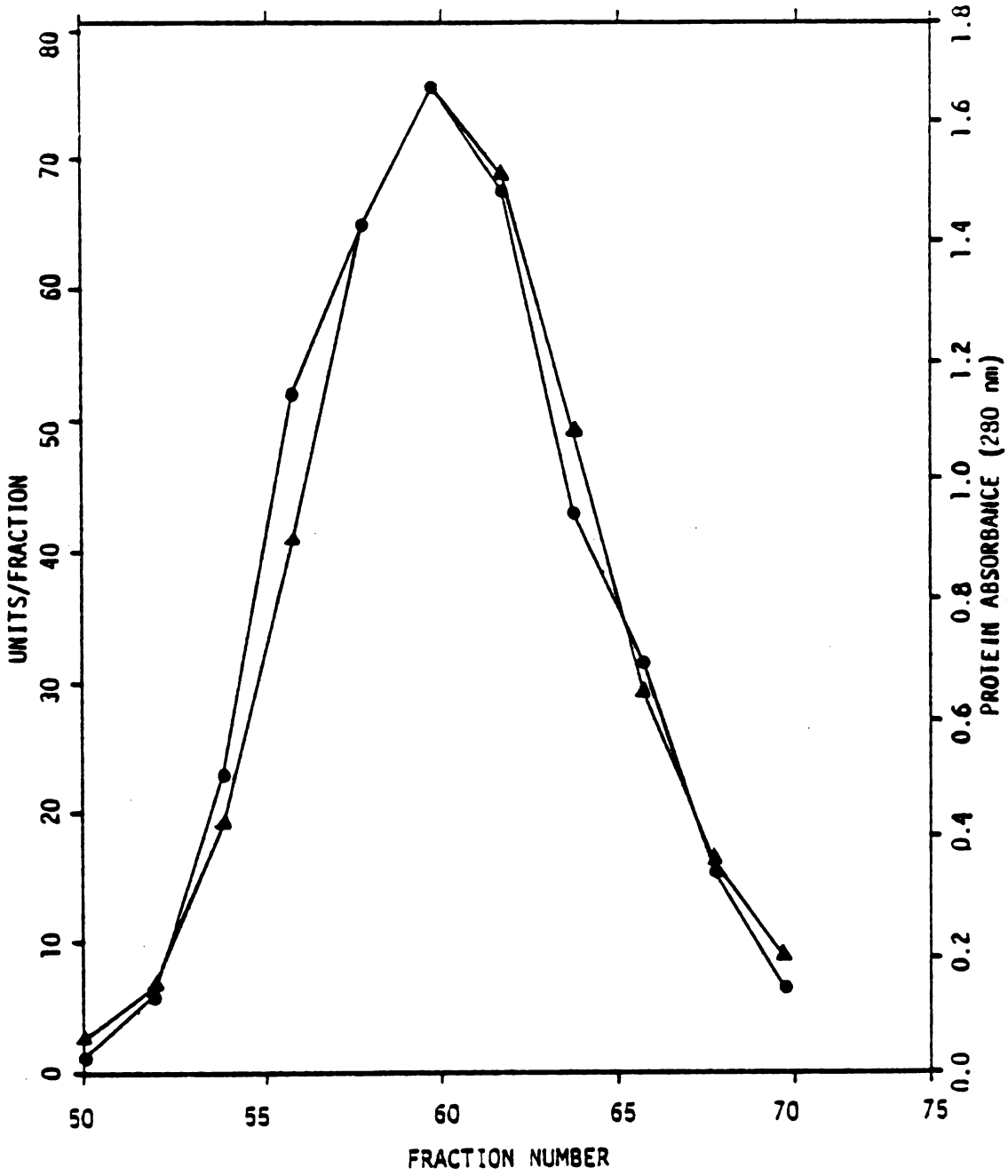

UNITS/FRACTION
PROTEIN ABSORBANCE (280 nm)
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
50
55
60
65
70
75

Figure 18. SDS polyacrylamide gel electrophoresis of D-galactonate (D-fuconate) dehydratase in the presence of molecular weight standards. Protein samples, containing mixtures of known molecular weight standards, standard proteins and D-galactonate (D-fuconate) dehydratase, or the dehydratase alone, were electrophoresed, after treatment with SDS and 2-mercaptoethanol, on SDS polyacrylamide slab gel containing 10% acrylamide; methods of gel preparation and electrophoresis, and protein sample preparation are detailed in Materials and Methods, this section. Protein samples, containing no more than 50 µg protein total, were subjected to electrophoresis in six separate channels, one channel apart. Following electrophoresis, the gel was stained for protein, destained, and then dried for photographic purposes. Protein mixtures of the gel (channels arbitrarily numbered) are: (1) catalase (15µg), ovalbumin (15 µg), and malate dehydrogenase (15 µg); (2 and 5) D-galactonate (D-fuconate) dehydratase (30 µg); (3) catalase (15 µg), D-galactonate (D-fuconate) dehydratase (15 µg), and malate dehydrogenase (15 µg); (4) tryptophanase (15 µg), D-galactonate (D-fuconate) dehydratase (15µg), and aldolase (15 µg); (6) tryptophanase (15 µg), ovalbumin (15 µg), and aldolase (15 µg). (The order of listing the proteins of each channel corresponds to their position on the gel from top to bottom.)

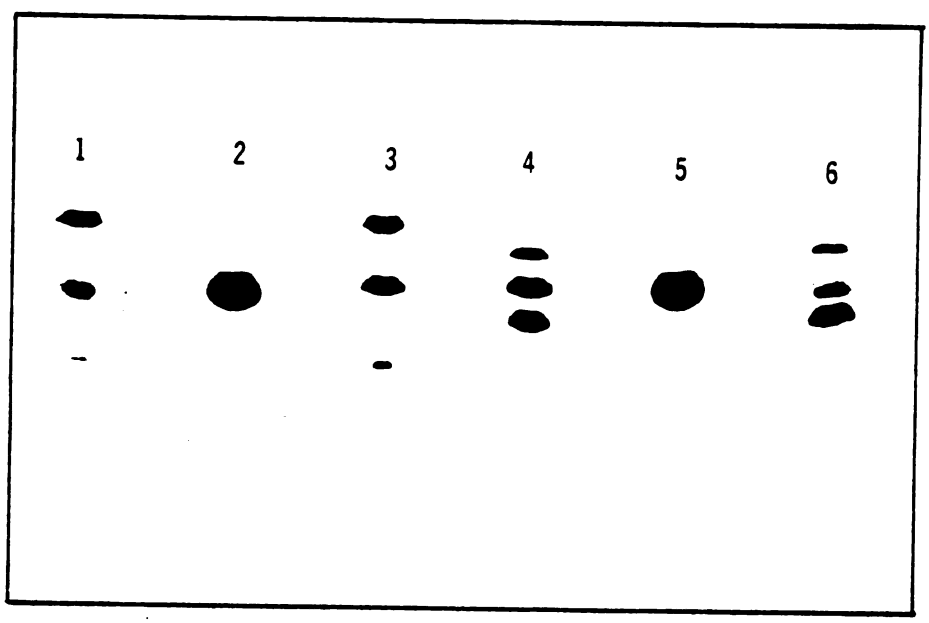
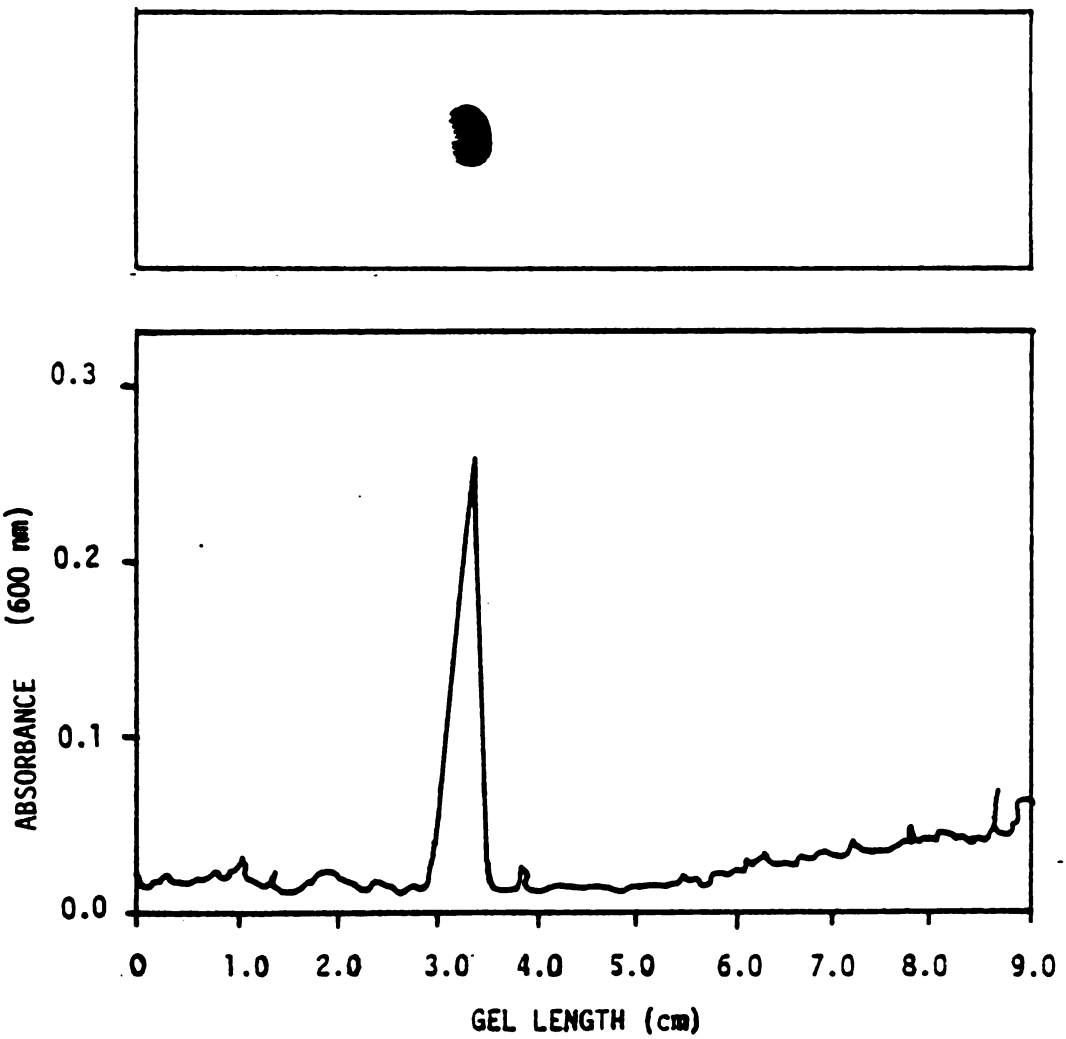

1
2
3
4
5
6

Figure 19. SDS polyacrylamide gel electrophoresis of D-galactonate dehydratase for determination of purity. A 10% acrylamide SDS polyacrylamide gel was prepared for electrophoresis of a 30 μg protein sample of the Sephadex G-200 purified D-galactonate (D-fuconate) dehydratase; methods of gel and sample preparation are given in Materials and Methods, this section. Following electrophoresis, the gel was stained for protein and destained. A scan of the gel was made prior to drying for purposes of photography. The figure shows the actual protein scan of the gel with photograph superposed.


0.3
0.2
0.1
0.0
ABSORBANCE (600 nm)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
GEL LENGTH (cm)

Native Gel Electrophoresis to show Co-Migration of Protein with Dehydratase Activity. When the Sephadex G-200 purified dehydratase was run under native conditions on a 5.0% acrylamide polyacrylamide gel, a single major protein band was observed as shown by the accompanying gel photograph and protein scan (Fig. 20). To confirm that the major band on native gels was actually the dehydratase, and not a contaminant in the preparation, the Sephadex G-200 protein fraction was run again on 5.0% acrylamide in the three-channel gel apparatus described in Material and Methods. The channels were excised lengthwise and treated as follows: (i) the middle channel was cut into slices along the width of the gel starting at the stacking gel/running gel junction and transferred to individual culture tubes containing 1.0-ml volume of 0.05 M potassium phosphate buffer (pH 7.0) and left at $4^{o}C$ to extract the enzyme in an overnight period of incubation; (ii) gel channels from the two outer edges of the gel were stained for protein and scanned at 600 nm.

A plot of protein absorbance versus slice number (Fig. 21), after correcting for gel swelling (measurements of the stained gels showed that some swelling had occurred in the destaining process, increasing the running gel from 92 to 93 mm in length), showed the activity band of slices 27 and 28 as corresponding to 42.3 to 45.6 mm actual gel length. The protein profile indicated that the major protein band fell within this range; i.e., 41.3 to 45.0 mm of gel length. Given that protein samples running on the outer channels run slightly slower than the inner channels, these data show good agreement of co-migration of protein with dehydratase activity; therefore, the major band on

Figure 20. Native polyacrylamide gel electrophoresis of D-galactonate (D-fuconate) dehydratase for determination of purity. A 5.0% acrylamide polyacrylamide slab gel was prepared, 10.0 cm X 15.0 cm X 1.5 cm, for the electrophoresis of a 30 μg protein sample of the Sephadex G-200 purified D-galactonate (D-fuconate) dehydratase; methods for gel and sample preparation are given in Materials and Methods, this section. Following the electrophoresis, the gel was stained for protein and was destained. A scan of the gel was made prior to drying for purposes of photography. The figure shows the actual protein scan of the gel with photograph superposed.

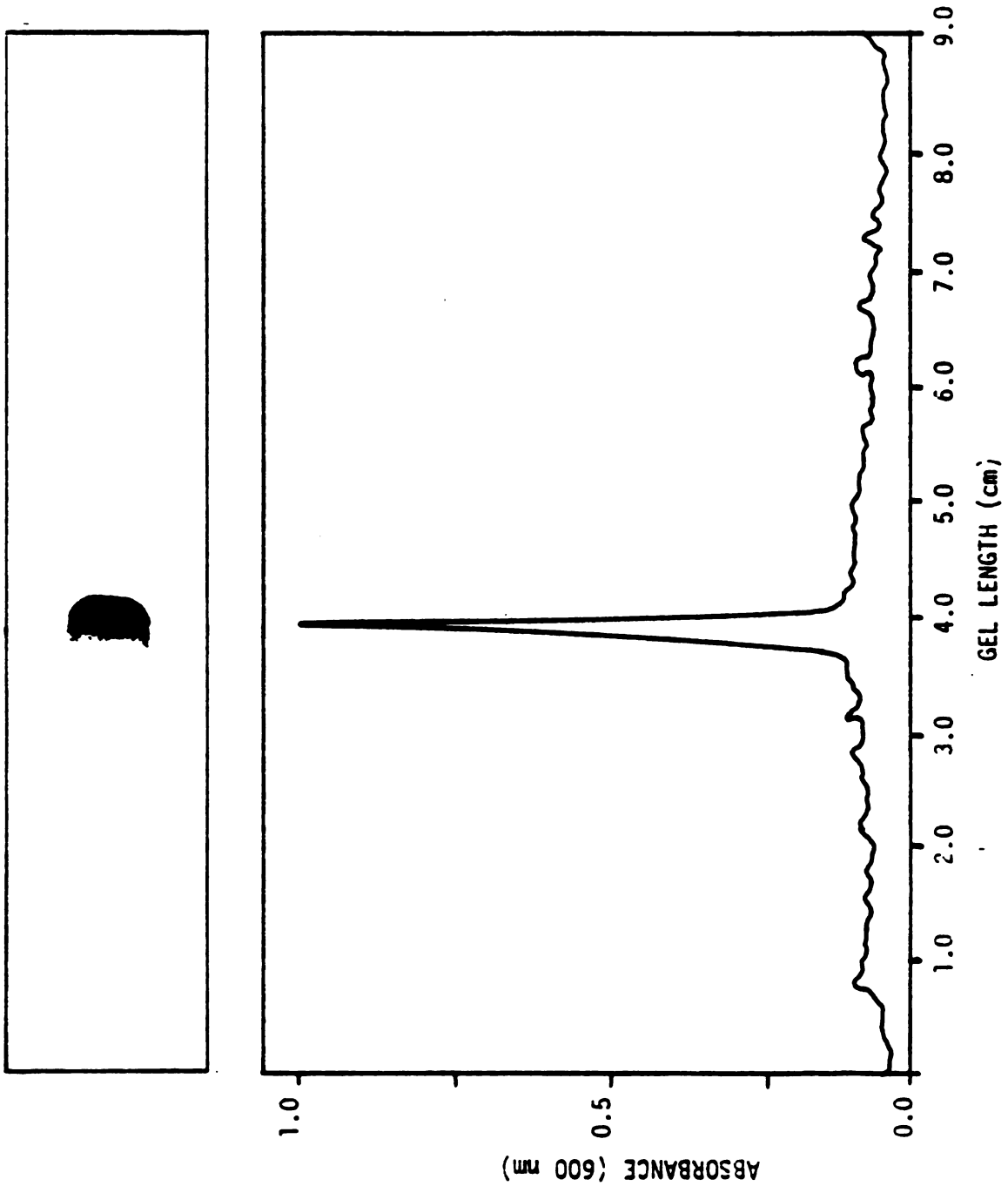
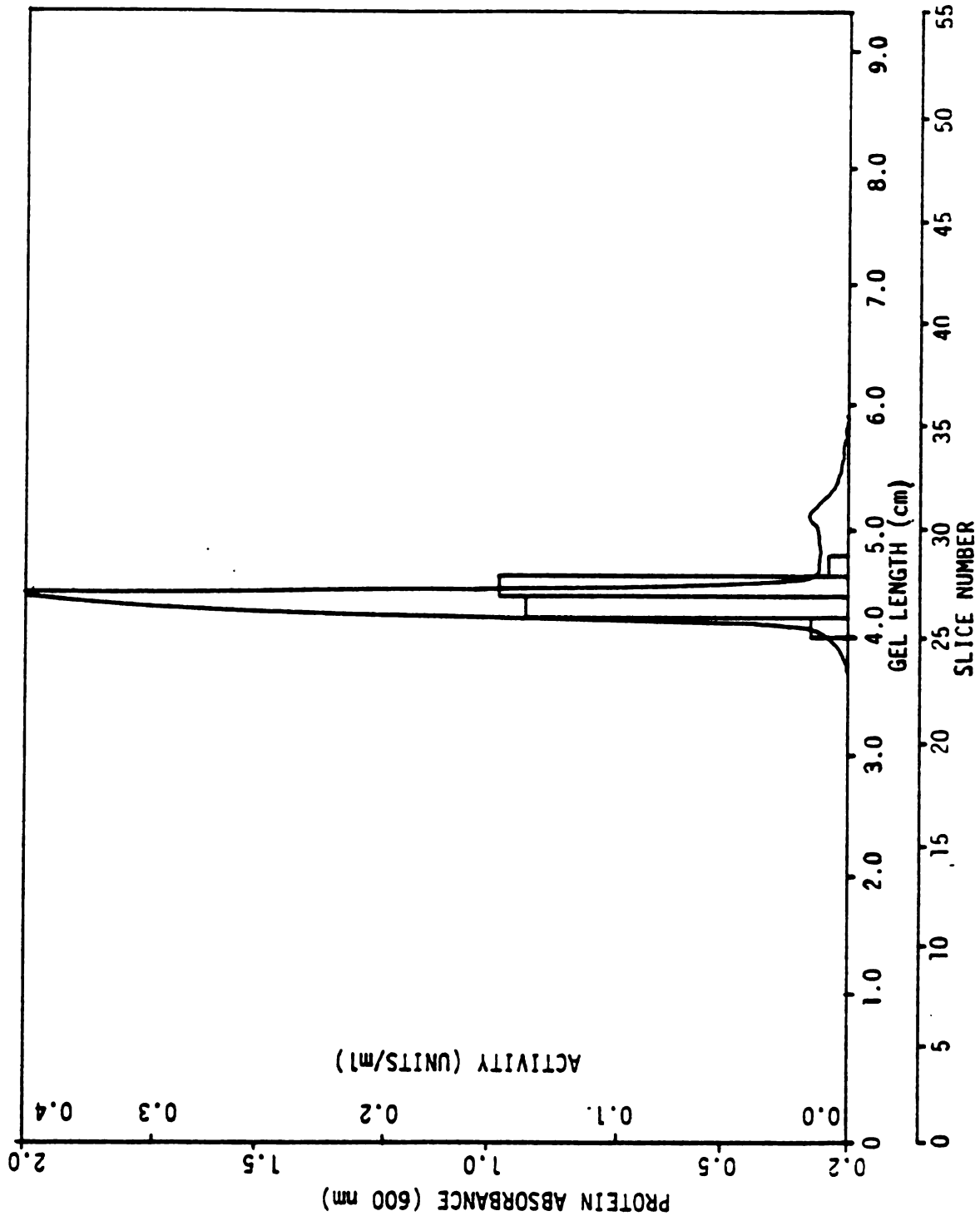

ABSORBANCE (600 nm)
0.0
0.5
1.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
GEL LENGTH (cm)

Figure 21. Plot of dyed protein absorbance and dehydratase activity versus gel length and slice number on native polyacrylamide gels. Slab gels were prepared as described in Materials and Methods. To each channel was added 0.2 ml sample mixture comprised of 0.37 mg dehydratase protein, 10.0% glycerol, and 0.19 M Tris-HCl buffer (pH 6.8). The tracking dye, in 0.2-ml volume, was run into the stacking gel before application of the protein sample as described. Electrophoresis was performed at 4°C and 20 milliamps constant current. The middle channel was excised lengthwise and cut into millimeter slices (1.61 mm per slice; 57 slices total) to extract the dehydratase while the other two channels were excised and stained for protein. D-Galactonate was used as the substrate for the dehydratase. Coomassie blue was used to stain for protein. Scans of the dyed protein bands to obtain the protein profile were plotted using a scanner speed of 2.0 cm per min and recorder speed of 0.5 cm per inch, thereby allowing true gel length in centimeter distance to be plotted directly on the actual gel trace. Symbols: bars reflect the D-galactonate (D-fuconate) dehydratase activity, solid lines reflect the dyed protein absorbance (600 nm).


PROTEIN ABSORBANCE (600 nm)
0.2
0.5
1.0
1.5
2.0
ACTIVITY (UNITS/ml)
0.0
0.1
0.2
0.3
0.4
GEL LENGTH (cm)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
SLICE NUMBER
0
5
10
15
20
25
30
35
40
45
50
55

native gels was shown to be the dehydratase protein.

That both SDS and native polyacrylamide gels showed only one major protein band for the dehydratase preparation the purity of the enzyme was estimated as over 95%, or near homogeneity.

Characterization of D-Galactonate (D-Fuconate) Dehydratase

Stability. The dehydratase was found to retain most of its activity (greater than 90%) for at least a month when stored unfrozen ($4^{o}C$) or frozen in 0.05 M potassium phosphate buffer (pH 7.0). When stored in the unfrozen state, 0.02% sodium azide was added to prevent bacterial growth; the presence of azide did not affect the enzyme activity. Attempts to improve the stability of the enzyme by using glycerol, dithiothreitol, or 2-mercaptoethanol, did not prove to be any better than the buffer alone.

pH Optimum. The pH optimum for the dehydratase reaction using either K^{+} D-galactonate or K^{+} D-fuconate as substrate showed a pH range from 6.5 to 7.5, depending on the buffer type (Fig. 22). Pipes (Na^{+}, pH 6.5), Hepes (Na^{+}, pH 7.0), and Tris-HCl (pH 7.5) buffers gave maximal dehydratase activities on both substrates.

Substrate Specificity and Kinetic Constants. Of nine sugar acids tested at a concentration of 40.0 mM, only D-fuconate and D-galactonate served as substrates for the dehydratase. Compounds that did not serve as substrates (less than 0.05 μmol product X min^{-1}X mg $protein^{-1}$) were: D-arabonate, L-arabonate, D-gluconate, D-glucarate, D-lyxonate, D-mannonate and D-xylonate.

From Lineweaver-Burk plots, the Km values for D-fuconate (Fig. 23) and D-galactonate (Fig. 24) were determined to be 1.27 and 0.90 mM,

Figure 22. Effect of pH and buffer composition on D-galactonate (D-fuconate) dehydratase activity. The reaction mixture of 0.3-ml volume consisted of 10.0 mM $MgCl_2$, 1.0 mM EDTA (pH 7.0), 20.0 mM K^+ D-galactonate, rate limiting amounts of the dehydratase, and 50.0 mM buffer. Relative activity (percent) on D-galactonate refers to a specific activity of 8.5 units per mg protein. Symbols: ◆ acetate/acetic acid buffer, ▲ sodium phosphate buffer, ◉ Hepes buffer, ● Pipes buffer, ✚ Tris-glycine buffer.

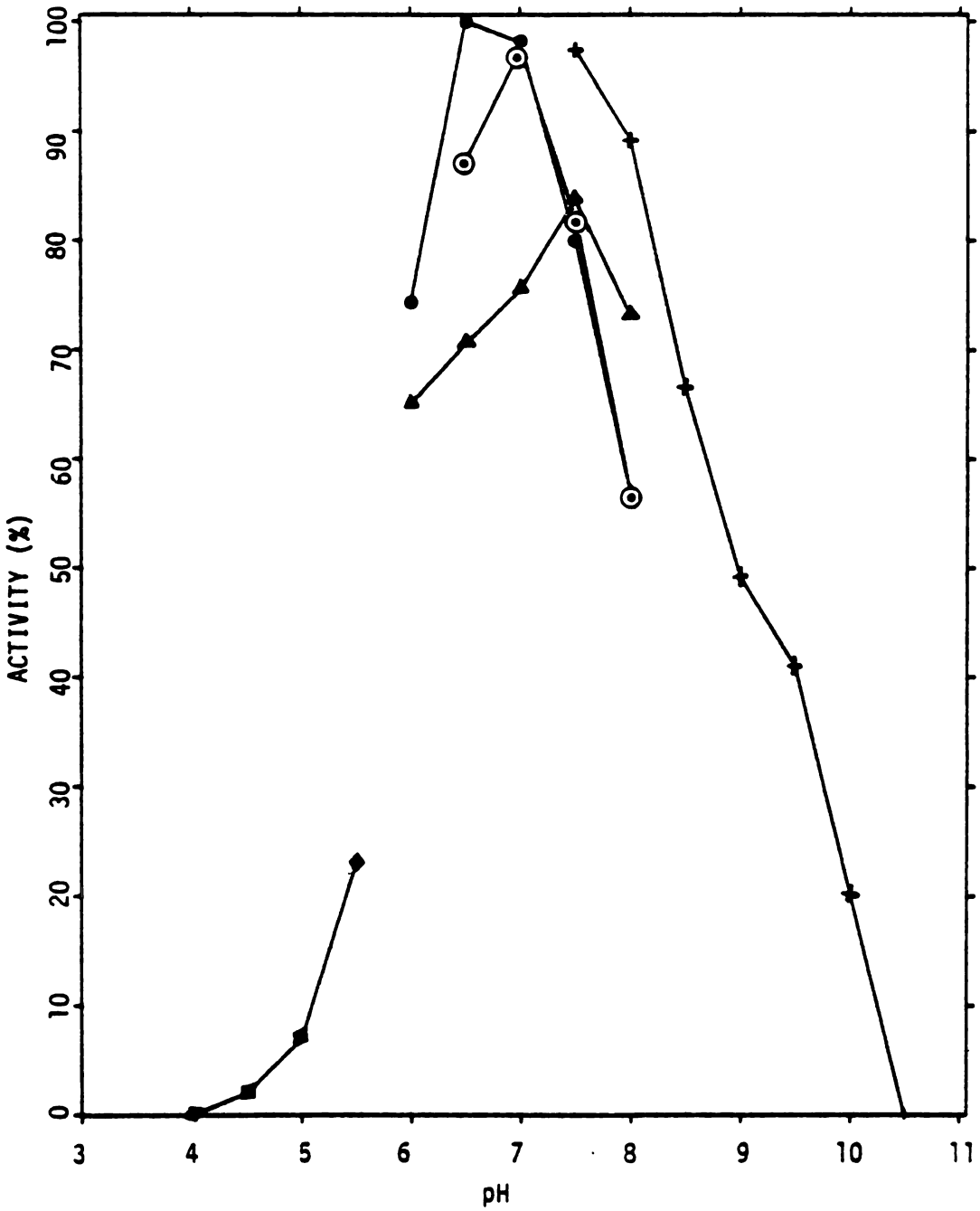

ACTIVITY (%)
0
10
20
30
40
50
60
70
80
90
100
3
4
5
6
7
8
9
10
11
pH

Figure 23. Lineweaver -Burk plot using D-fuconate as substrate in the dehydratase reaction mixture. Purified dehydratase (Sephadex G-200) was assayed in 0.3-ml volume reaction mixture comprised of 10.0 mM $MgCl_2$, 1.0 mM EDTA (pH 7.0), 50.0 mM Pipes buffer (pH 7.0), and varying amounts of K^+ D-fuconate. Standard procedures for the formation of semicarbazone derivatives of the dehydratase product was used in measuring enzymatic activity. Initial velocity (V) is in micromole product formed per minute, and substrate concentration (S) is in millimolar D-fuconate concentration in the reaction mixture.

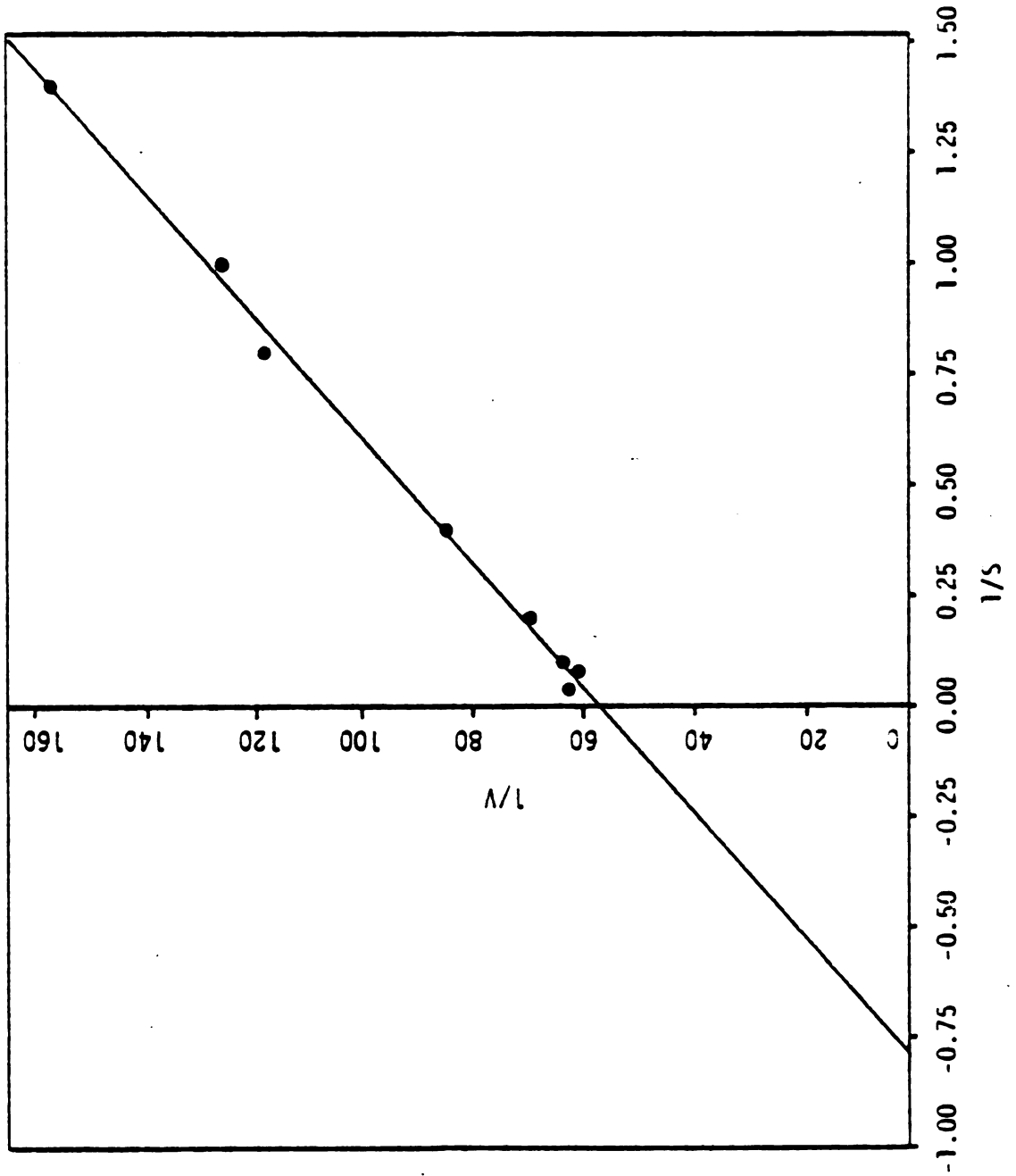
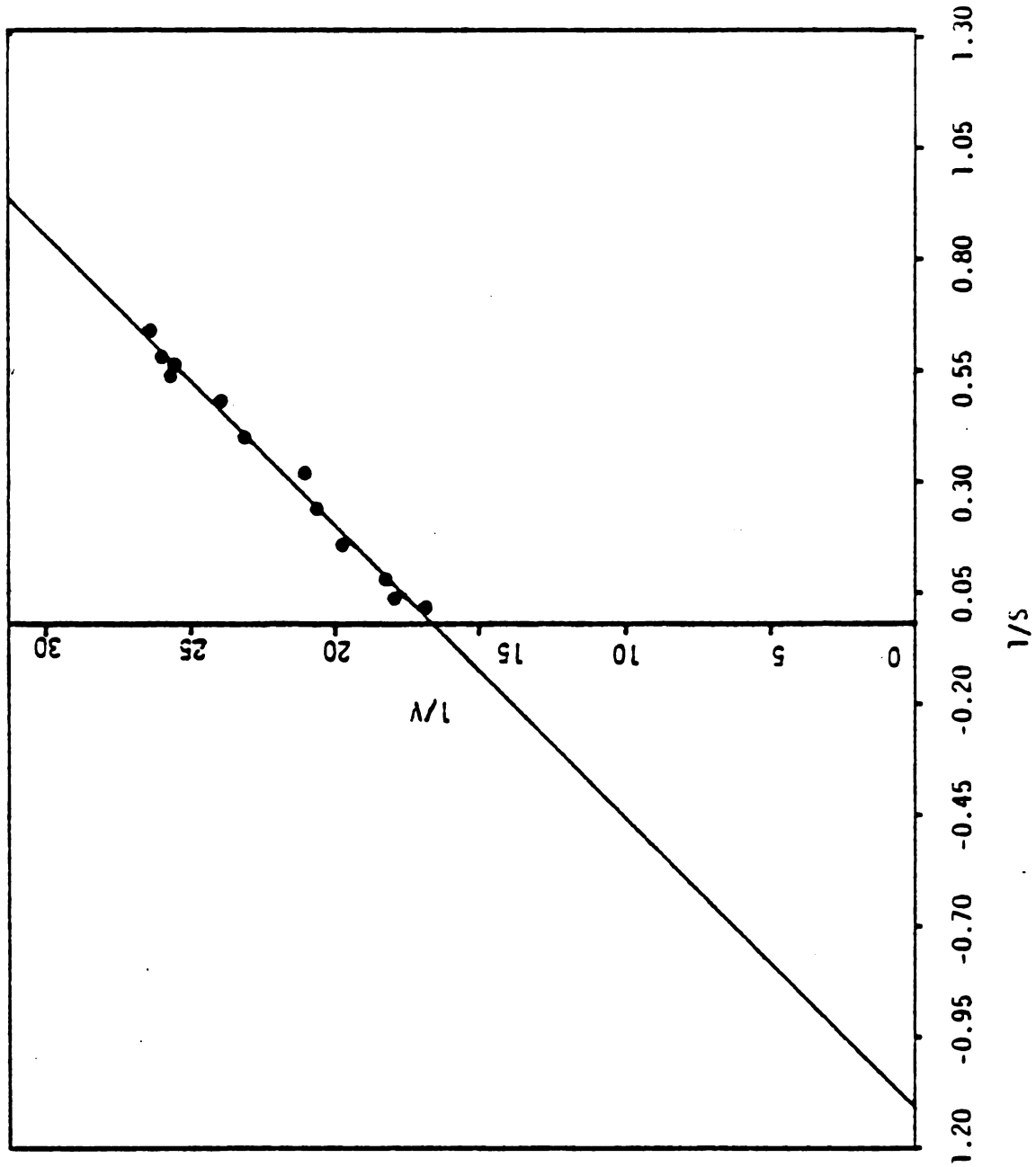

1/V
0
20
40
60
80
100
120
140
160
1/S
-1.00
-0.75
-0.50
-0.25
0.00
0.25
0.50
0.75
1.00
1.25
1.50

Figure 24. Lineweaver-Burk plot using D-galactonate as substrate in the dehydratase reaction mixture. Purified dehydratase (Sephadex G-200) was assayed in 0.3-ml volume reaction mixture comprised of 10.0 mM $MgCl_2$, 1.0 mM EDTA (pH 7.0), 50.0 mM Pipes buffer (pH 7.0), and varying amounts of K^+ D-galactonate. Standard procedures for the formation of semicarbazone derivatives of the dehydratase product were used in measuring enzymatic activity. Initial velocity (V) is in units of micromole product formed per minute, and substrate concentration (S) is in millimolar D-galactonate concentration in the reaction mixture.


1/V
0
5
10
15
20
25
30
1/S
-1.20
-0.95
-0.70
-0.45
-0.20
0.05
0.30
0.55
0.80
1.05
1.30

respectively. The relative Vmax value for D-galactonate was 3.33-fold higher than for D-fuconate.

Divalent Cation Activation in the Presence of EDTA. Preliminary studies of the effect of divalent metals on the dehydratase-catalyzed reaction showed that a rate existed in the absence of metal added to a reaction mixture comprised of 20.0 mM D-galactonate and 170 mM Tris-HCl buffer (pH 8.0). This rate increased with time and was completely inhibited by 1.0 mM EDTA (Fig. 25). Addition of $MgCl_2$ (10.0 mM) to the EDTA-treated mixture not only restored activity but resulted in a rate that was constant with time (data not shown) and proportional to enzyme concentration (Fig. 26). These data suggested that the presence of EDTA in the assay function to remove deleterious metal(s) that inhibit the enzyme at lower protein concentrations. Examination of the trials without EDTA (Fig. 26) show that proportionality of activity with protein concentration is found only at the higher protein concentrations, suggesting that the unknown metal(s) are depleted from the reaction mixture if enough protein is added. Support for the presence of such deleterious metal(s) in the enzyme preparation is found when the dehydratase was dialyzed against 1.0 mM EDTA (Table 14); although considerable enzyme activity was irreversibly lost by this dialysis, complete removal of the activity that can be at least partially restored by $MgCl_2$ was the result of this treatment.

To test the effectiveness of various divalent metals to reverse the effect of EDTA, the dehydratase was pre-incubated in a reaction mixture comprised of D-galactonate, EDTA, and Tris-HCl buffer. Metals were then added to the reaction mixture and the course of the reaction was

Figure 25. Effect of EDTA on the dehydratase in the absence of metal in the enzymatic reaction mixture. The enzymatic assay was scaled up to 1.0-ml volume consisting of 20.0 mM D-galactonate, 167 mM Tris-HCl buffer (pH 8.0), and dehydratase. In one trail, EDTA was added to the above reaction mixture to make a 1.0 mM solution; in the other trial, components were left as above. Reaction mixtures were incubated at 30°C for 10 minutes to temperature equilibrate. Protein was added to initiate the enzymatic reaction in both trials; 51.0 μg protein was added to the EDTA-minus trial, and 510 μg of protein was added to the EDTA-plus trial. Samples of 0.05-ml volume were taken from each reaction mixture at regular time intervals and added to 0.2 ml of semicarbazone reagent (1.0% semicarbazide·HCl and 1.5% sodium acetate) and incubated at 30°C for 15 min as to terminate the reaction and to form semicarbazone derivatives of the dehydratase product. Absorbance readings were taken at 250 nm and converted to micromole α-keto acid as to plot the micromole amount of product formed versus the time of incubation.

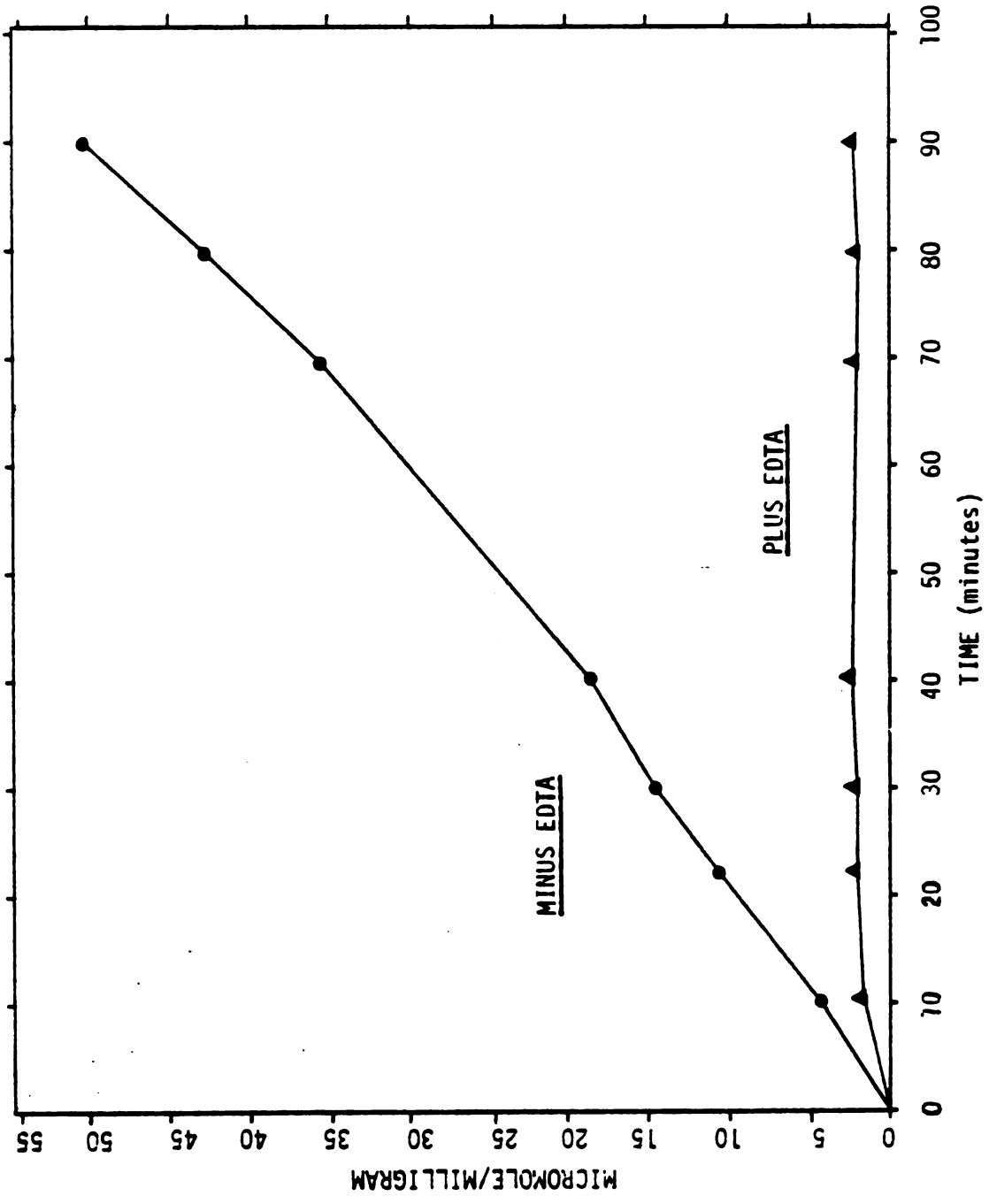

MICROMOLE/MILLIGRAM
0
5
10
15
20
25
30
35
40
45
50
55
TIME (minutes)
0
10
20
30
40
50
60
70
80
90
100
MINUS EDTA
PLUS EDTA

Figure 26. Effect of EDTA on the dehydratase rate parameters in the presence of $MgCl_2$. The dehydratase was assayed in 1.0-ml volume comprised of 20.0 mM D-galactonate , 167 mM Tris-HCl buffer (pH 8.0), with or without $MgCl_2$ (10.0 mM) or EDTA (1.0 mM). Protein was added to initiate the enzymatic reaction and assayed using the sampling technique described (Fig. 25). (In these trials, only the +EDTA/+$MgCl_2$ showed constant time rates at any one particular protein concentration; rates for other trials were taken within 30 min after starting the reaction where plots of product formed per unit of time were more linear.) Symbols: ▲ reaction mixture and dehydratase only; ● reaction mixture, dehydratase, and EDTA; + reaction mixture, dehydratase, and $MgCl_2$; ◆ reaction mixture, dehydratase, EDTA, and $MgCl_2$.

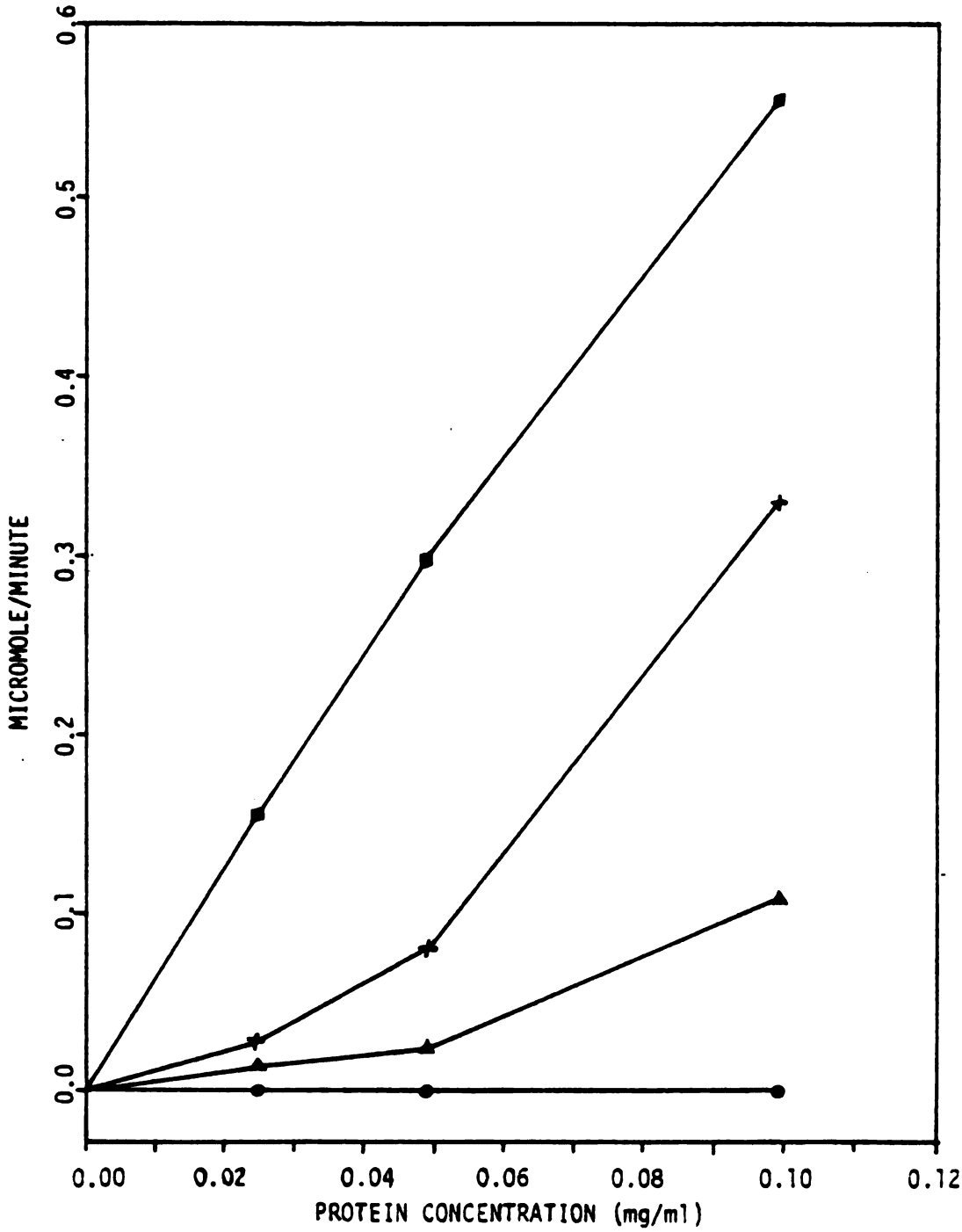

MICROMOLE/MINUTE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.00
0.02
0.04
0.06
0.08
0.10
0.12
PROTEIN CONCENTRATION (mg/ml)

Table 14. Effect of dialysis of the dehydratase against EDTA. Dehydratase (2.0 ml) was first dialyzed against 200.0-ml volume of 1.0 mM EDTA in 0.05 M potassium phosphate buffer (pH 7.0) with three changes of buffer, then dialyzed against 0.05 M potassium phosphate buffer alone with three changes of buffer. The dehydratase was assayed before and after dialysis in 1.0-ml volume comprised of 20.0 mM K^+ D-galactonate, 0.05 M Tris-HCl buffer (pH 8.0), at 30°C with or without $MgCl_2$ (10.0 mM) or EDTA (1.0 mM). At regular time intervals, 0.05 ml samples were taken and added to 0.2 ml of semicarbazone reagent followed by incubation at the same temperature for 15.0 min additional. Trials, diluted to 1.0-ml volume with distilled water, were then read at 250 nm and rates determined by plotting the micromole amount of α-keto acid produced versus time using the molar extinction coeficient of 10,200 as reported by MacGee and Doudoroff (15).

TREATMENT	CONDITION	ACTIVITY
		Nmol product per mg protein
Before dialysis	$-MgCl_2$/-EDTA	1.41
	$+MgCl_2$/+EDTA	7.40
	$+MgCl_2$/-EDTA	4.39
After dialysis	$-MgCl_2$/-EDTA	0.00
	$+MgCl_2$/+EDTA	0.71
	$+MgCl_2$/-EDTA	0.80

followed. Of the metals tested, only $MgCl_2$ (100%), $MnCl_2$ (42%), and $FeSO_4$ (53%) reversed the effect of EDTA inactivation (Table 15). Both $ZnCl_2$ and $CoCl_2$ showed no gain in activity over the control minus metal.

Isoelectric Point. Purified dehydratase (Sephadex G-200) was subjected to isoelectric focusing at 600 volts for 2.5 days in the electrophoretic medium described in Materials and Methods. Assays of the eluate fractions collected at the end of this time period (Fig. 27) show a pH gradient ranging from 4.0 to 6.5 had been established with better than 90% of the dehydratase activity focused in fractions 27 and 28 resulting in a recovery of 100% of the initial activity. The isoelectric point was pH 4.7 to 4.8.

Molecular Weight Determination. The following methods were used to determine the molecular weight of the native D-galactonate (D-fuconate) dehydratase: (i) analytical gel filtration; (ii) sucrose density gradient sedimentation; (iii) sedimentation velocity using a Beckman Model E Ultracentrifuge; and (iv) sedimentation equilibrium using a Beckman Model E Ultracentrifuge.

(i) Analytical Gel Filtration. A sample mixture containing the dehydratase plus molecular weight standards *E. coli* alkaline phosphatase (80,000 MW) (119), beef heart lactate dehydrogenase (142,000 MW) (92), pig heart fumarase (194,000 MW) (120), and rabbit muscle pyruvate kinase (237,000 MW) (121,122), was chromatographed using a column of Bio-Gel P-300 equilibrated with 0.05 M potassium phosphate buffer (pH 7.0).

A plot of the peak fraction for each molecular weight standard from the elution profile (Fig. 28) versus corresponding molecular weight gave

Table 15. Metal ion activation of the dehydratase in the presence of EDTA. Dehydratase was pre-incubated in 0.3-ml volume comprised of 20.0 mM K^+ D-galactonate, 1.0 mM EDTA (pH 7.0), 50.0 mM Tris-HCl buffer (pH 8.0), and 0.74 unit of the dehydratase. After 10.0 min at 30°C, metals were added to a concentration of 10.0 mM in each trial. Relative activity observed were recorded as percent of $MgCl_2$-activated dehydratase (100% reflects a specific activity of 7.5 units per mg protein in the assay).

METAL ION	RELATIVE ACTIVITY
	%
EDTA alone (minus metal)	0.0
$MgCl_2$	100.0
$MnCl_2$	41.6
$FeSO_4$	53.1
$ZnCl_2$	0.0
$CoCl_2$	0.0

Figure 27. Elution profile of pH and dehydratase activity from a 110.0-ml volume isoelectric focusing column. An 80.0-ml electrophoretic medium comprised of 51.5 to 5.0% sucrose, 1.0% Pharmolyte ampholytes (pH 4.0 to 6.5), plus dehydratase was prepared as described in the Materials and Methods section, and used for focusing. Dehydratase, 3.63 mg, was subjected to 2.5 days of focusing at 4°C and 600 volts. At the end of this time period, fractions of 1.8-ml volume were collected at a rate of 2.0 ml per min. Both pH and dehydratase activity units were measured directly for each fraction collected. D-Galactonate was used as substrate for the D-galactonate (D-fuconate) dehydratase. Symbols: ⦿ D-galactonate (D-fuconate) dehydratase, ▲ pH gradient.

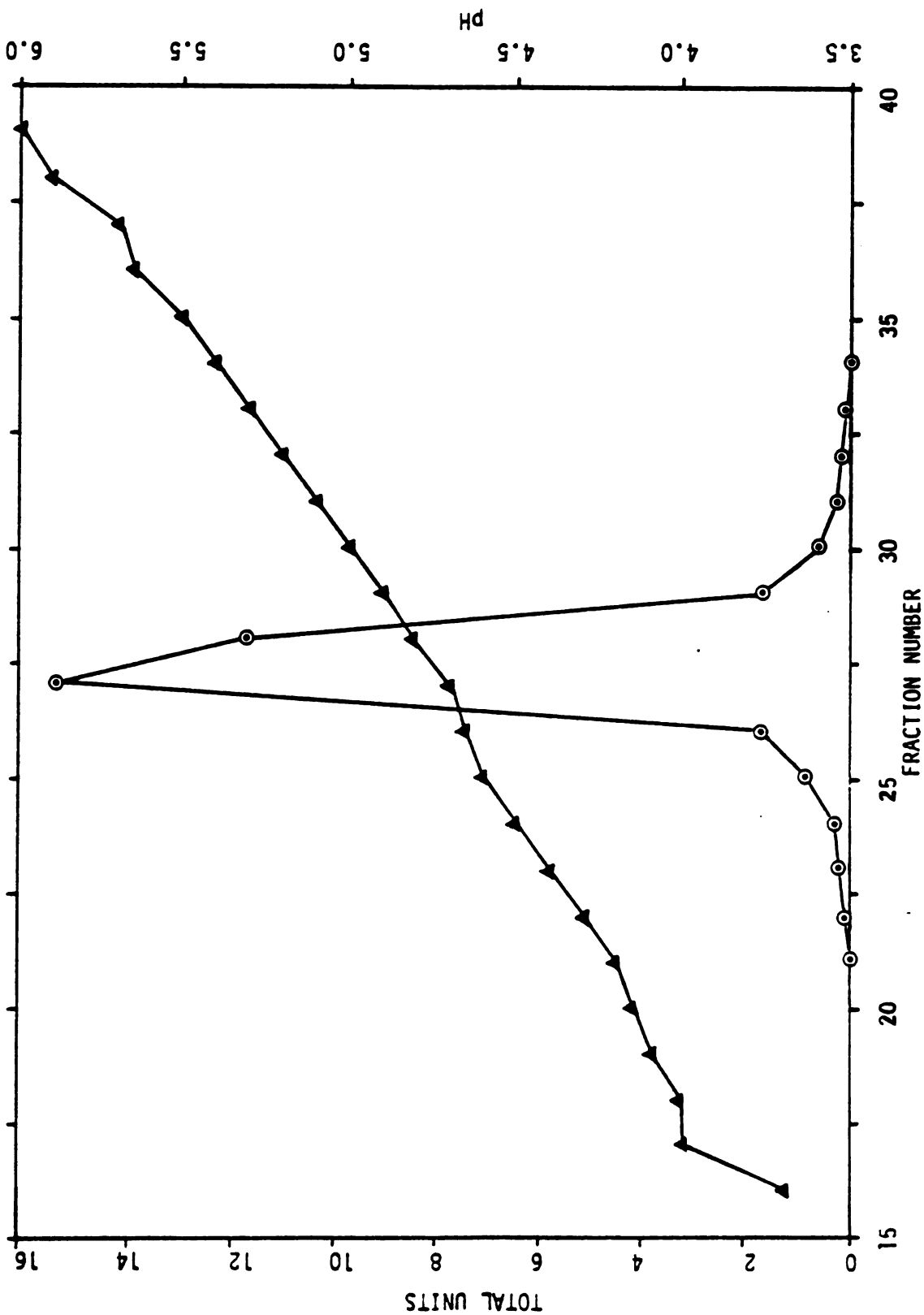
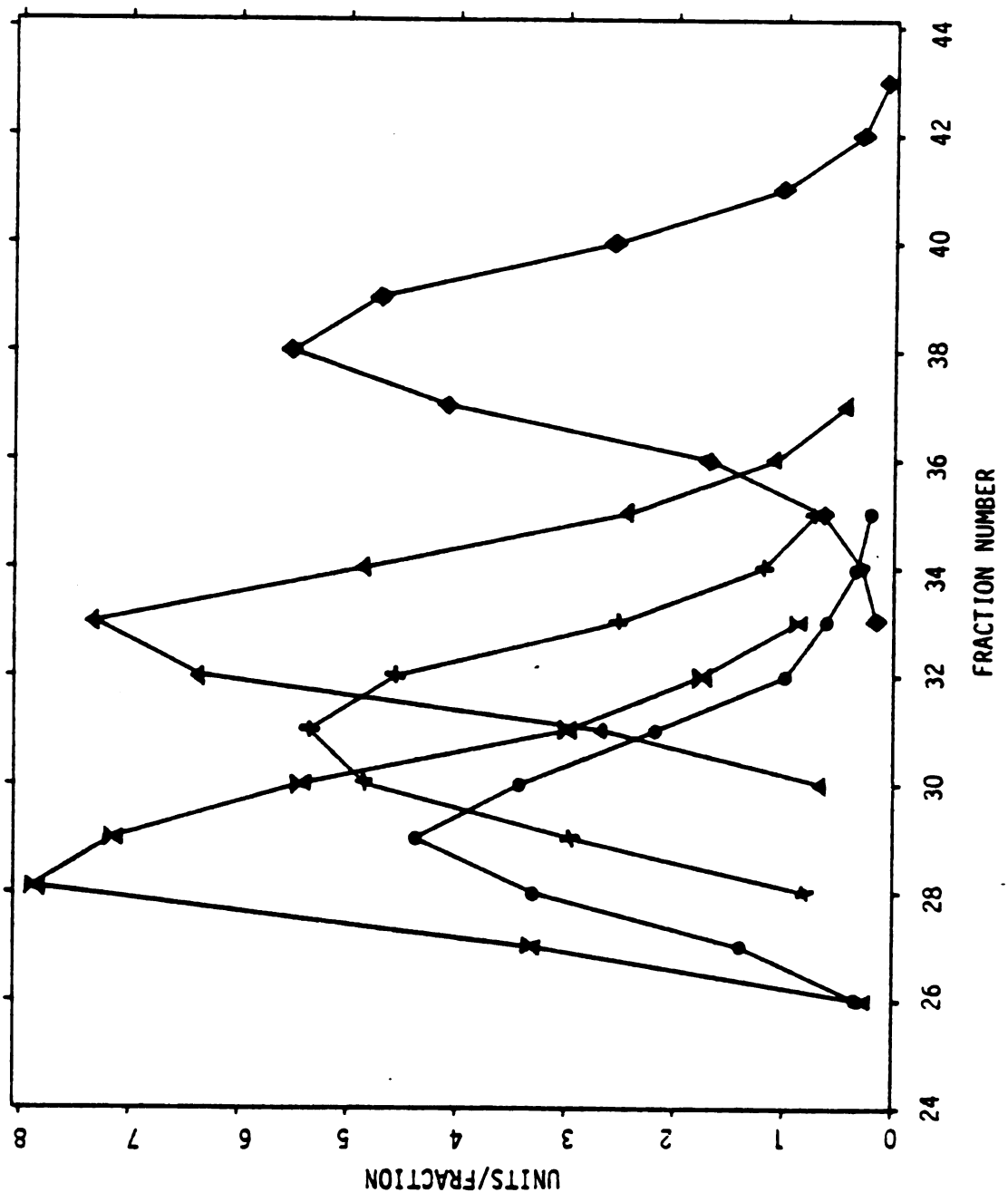

TOTAL UNITS
0
2
4
6
8
10
12
14
16
FRACTION NUMBER
15
20
25
30
35
40
pH
3.5
4.0
4.5
5.0
5.5
6.0

Figure 28. Elution profile of molecular weight standards and D-galactonate (D-fuconate) dehydratase as chromatographed on Bio-Gel P-300. A 2.0-ml volume sample containing 4.0 mg dehydratase, 0.2 mg E. coli alkaline phosphatase, 0.1 mg beef heart lactate dehydrogenase, 0.2 mg fumarase, and 0.25 mg pyruvate kinase, and 10.0% glycerol, was chromatographed using a 1.5 X 81.0 cm column of Bio-Gel P-300 equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). Elution of the protein sample was done using the same buffer collecting 4.0-ml volume per fraction at a rate of 4.0 ml per hr. A stock solution of horse spleen ferritin (450,000 MW)and $K_3Fe(CN)_6$ in potassium phosphate buffer in 10.0% glycerol was run separately to determine void volume (V_o) and total inclusion volume (V_t), respectively. Symbols and peak tube fractions from the plot: ⧗ D-galactonate (D-fuconate) dehydratase, 28.2 (Kav 0.08): ● pyruvate kinase, 28.8 (Kav 0.11); + fumarase, 30.7 (Kav 0.20); ▲ lactate dehydrogenase, 32.6 (Kav 0.29); ◆ alkaline phosphatase, 38.2 (Kav 0.56); Ferritin (26.5) and $K_3Fe(CN)_6$ (42.5) were considered to have Kav values of 0.0 and 1.0, respectively in calculations of the stokes radius (Table 16). Peak fractions were obtained by extrapolation along peaks of the elution profile. (Kav refers to the ratio $[(V_t-V_e)/(V_t-V_o)]$ where V_e is the elution volume for that particular molecular weight standard.)


UNITS/FRACTION
0
1
2
3
4
5
6
7
8
24
26
28
30
32
34
36
38
40
42
44
FRACTION NUMBER

a molecular weight of 255,000 for the D-galactonate (D-fuconate) dehydratase (Fig. 29).

(ii) Sucrose Density Gradient Sedimentation. The D-galactonate (D-fuconate) dehydratase was centrifuged as described in Materials and Methods using bovine liver catalase (247,500 MW: 11.3 S) and beef heart lactate dehydrogenase (142,000 MW: 7.4 S) as marker proteins. Following the procedure of Martin and Ames (123), the elution position of the dehydratase and marker proteins (Fig. 30) were used to determine a molecular weight and a Svedberg constant for the dehydratase; an average molecular weight of 286,300 $\pm$ 6900, and a Svedberg constant of 12.13 $\pm$ 0.12 were obtained.

(iii) Sedimentation Velocity. Sephadex G-200 purified D-galactonate (D-fuconate) dehydratase was equilibrate against 0.05 M potassium phosphate buffer (pH 7.0) by gel filtration on Sepharose A-5M before subjecting to ultracentrifugation as described in Materials and Methods. Calculation of the sedimentation constant for the various protein concentrations used was performed for each time point using the following equation (124):

$$S = \left[\frac{1}{\omega^2}\right] X \left[\frac{\text{Ln}\ (r_2 / r_1)}{(t_2 - t_1)}\right]$$

where: S is the sedimentation constant, ω is the angular velocity in radians per second (ω = 2 RPM/60), r is the distance of the boundary from its axis of rotation in centimeters, and t is the time in

Figure 29. Bio-Gel P-300 chromatograhy of D-galactonate (D-fuconate) dehydratase with molecular weight standards: plot of molecular weight versus corresponding peak fraction number. As described in the text, molecular weight standards were chromatographed with the D-galactonate (D-fuconate) dehydratase. From the elution profile of these proteins (Fig. 28) peak tube fractions were approximated. Semilog plots of the molecular weight of a standard protein versus the peak tube fraction, from the elution profile on P-300 gel, were prepared. The D-galactonate (D-fuconate) dehydratase molecular weight was extrapolated from the plot at the proteins' elution volume.

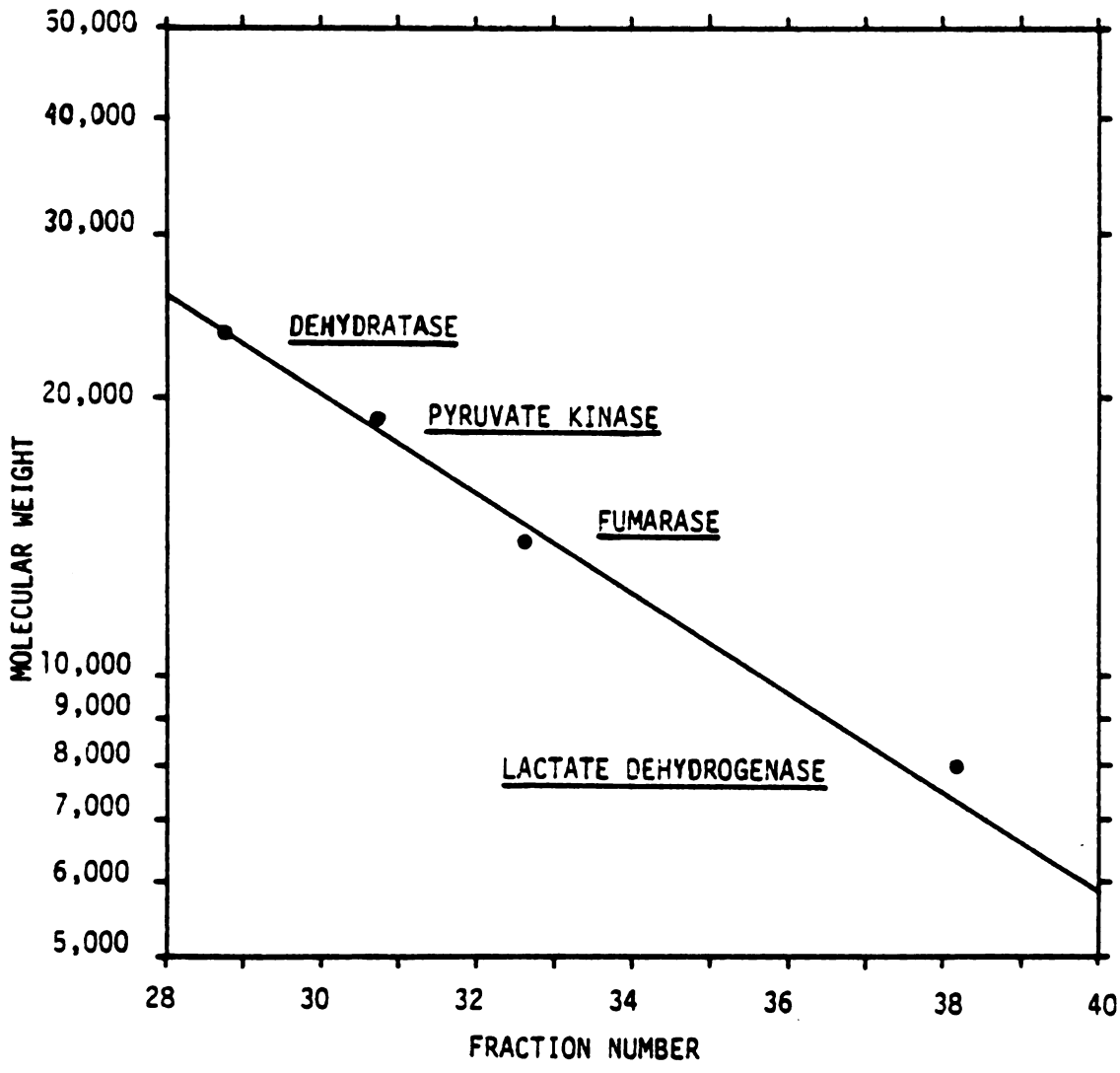
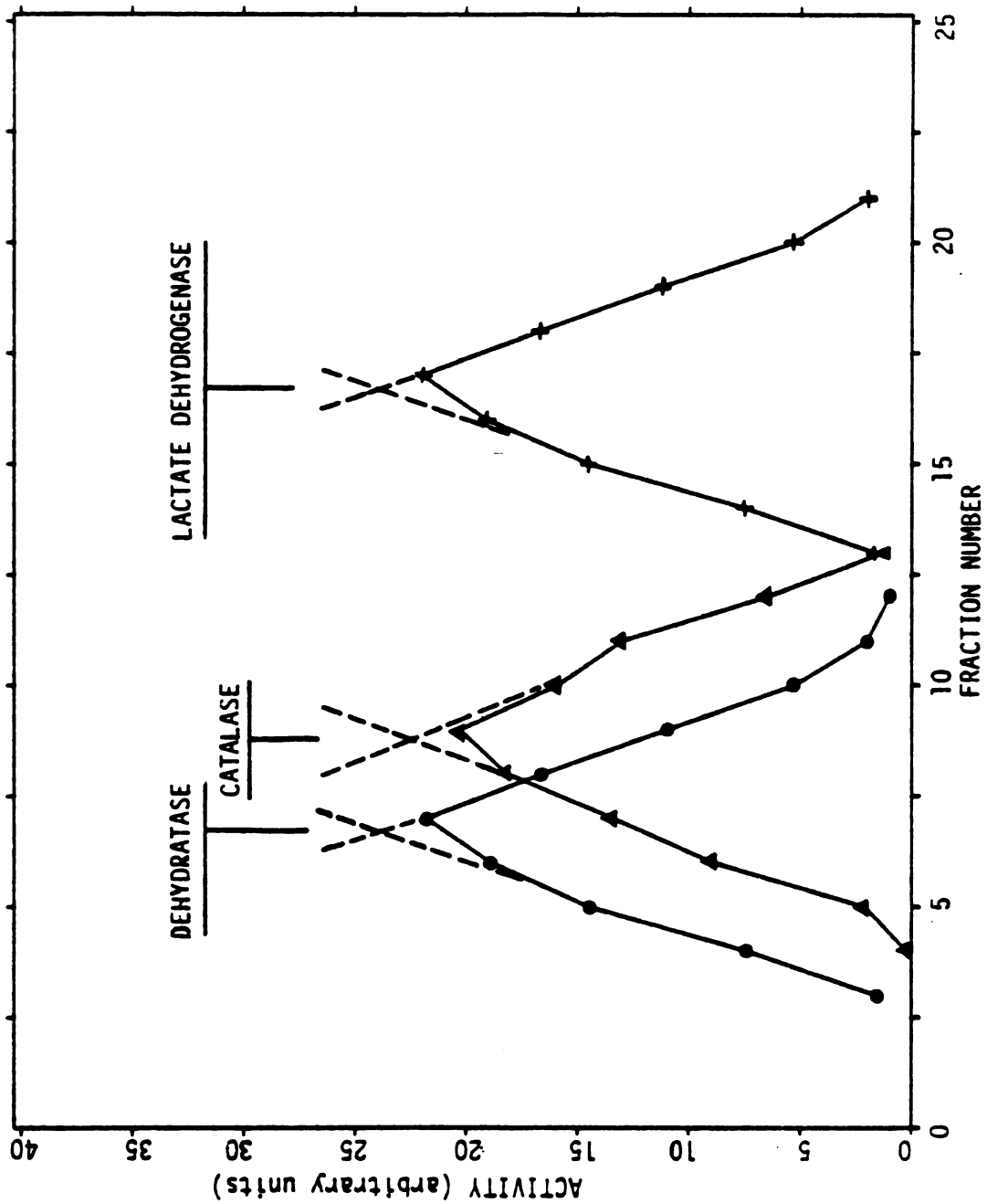

50,000
40,000
30,000
20,000
10,000
9,000
8,000
7,000
6,000
5,000
MOLECULAR WEIGHT
DEHYDRATASE
PYRUVATE KINASE
FUMARASE
LACTATE DEHYDROGENASE
28
30
32
34
36
38
40
FRACTION NUMBER

Figure 30. Sucrose density gradient sedimentation of the D-galactonate (D-fuconate) dehydratase in presence of marker proteins. Sucrose gradients were prepared as described in Materials and Methods. The protein sample of 0.1-ml volume contained 0.787 unit (0.363 mg) Sephadex G-200 purified dehydratase, 4375 units (0.125 mg) bovine liver catalase, and 1276 units (0.110 mg) beef heart lactate dehydrogenase in 0.05 M Tris-HCl buffer (pH 7.5) and 5.0% sucrose.

Following centrifugation, as described in text, 10-drop volume fractions were collected and assayed for dehydratase and marker protein. Migration distance was calculated as described by Martin and Ames (123), using the peak tube number for each protein obtained by extrapolation as shown. Symbols and migration distances from the center of rotation (cm): ● D-galactonate (D-fuconate) dehydratase, 2.86 cm; ▲ catalase, 2.63 cm; + lactate dehydrogenase, 1.76 cm.


DEHYDRATASE
CATALASE
LACTATE DEHYDROGENASE
ACTIVITY (arbitrary units)
FRACTION NUMBER
0
5
10
15
20
25
30
35
40

seconds.

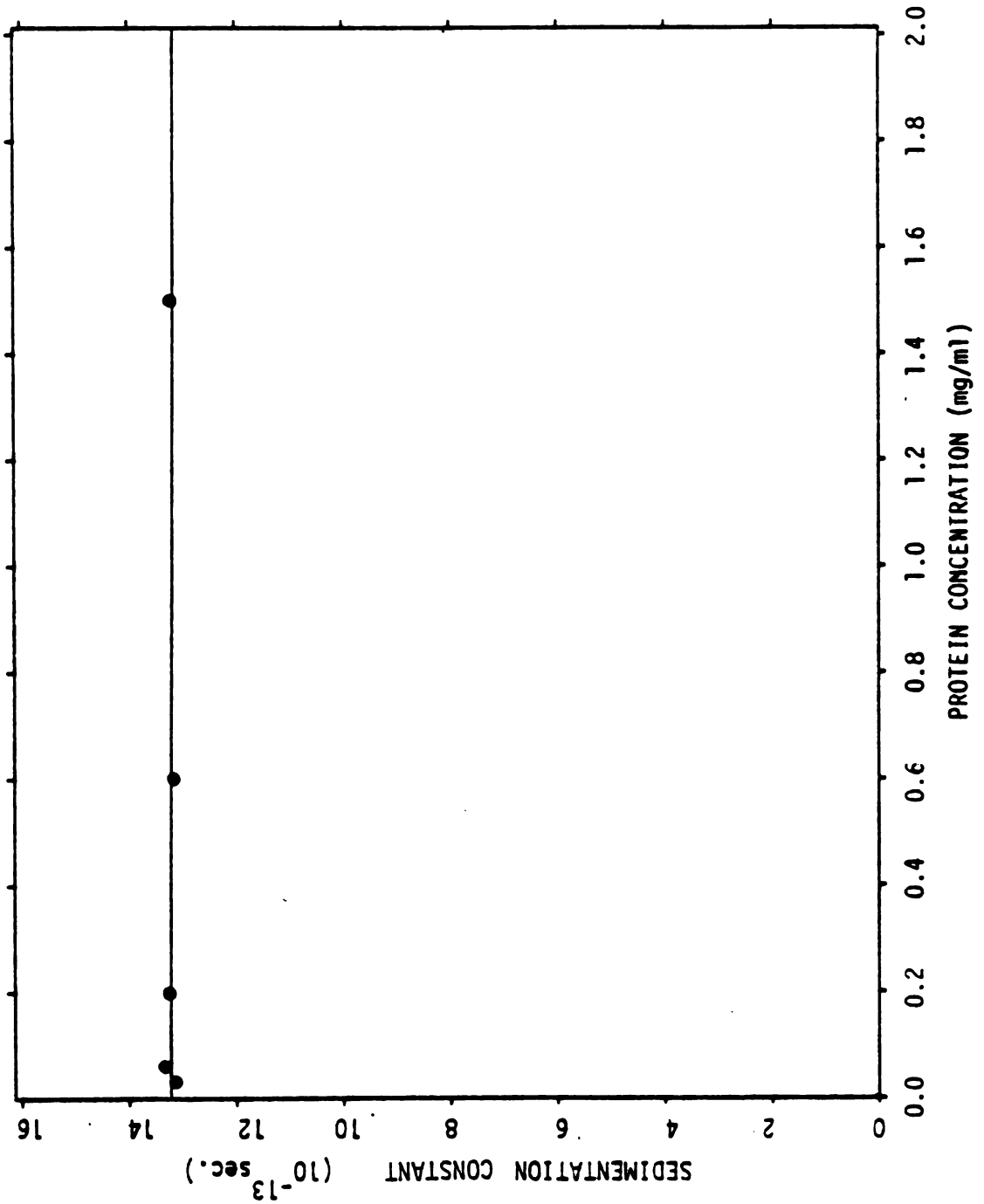
A plot of the average sedimentation constant determined for each protein concentration used (Fig. 31) show an extrapolated zero protein concentration value of 13.22 $\pm$ 0.09 X 10^{-13} seconds for S. Little deviation from this value was found for any protein concentration used. Using both the derived sedimentation constant and Stokes radius from the gel filtration experiment (see Table 16, for calculations) a molecular weight of 317,300 was obtained.

(iv) Sedimentation Equilibrium. The same protein sample of the D-galactonate (D-fuconate) dehydratase used in the sedimenation velocity experiment was used for sedimentation equilibrium. Dehydratase was diluted with 0.05 M potassium phosphate buffer (pH 7.0) to a protein concentration giving a low absorbance (less than 0.1) at 280 nm and was subjected to ultracentrifugation as described in Materials and Methods. Calculation of the molecular weight was performed using the following equation:

$$MW = \left[\frac{2\,RT}{(1-\bar{\nu}\rho)\,\omega^2}\right] \times 2.303 \times \left[\frac{d\,(\text{Log } C)}{d\,(r^2)}\right]$$

where: MW is the molecular weight, R is the gas constant (8.314 X 10^7 ergs,$^oC^{-1}$, $mole^{-1}$), T is the absolute temperature of the centrifuge run (293.2^oK), ρ is the density of the solvent at the given temperature (0.999 g/ml), ω is the angular velocity in radians per second (ω = 2RPM/60), $\bar{\nu}$ is the partial specific volume of the protein, assumed to be 0.733 g/ml^3 (that for an "average" protein), r is the

Figure 31. Sedimentation velocity determination of the sedimentation constant for the D-galactonate (D-fuconate) dehydratase. The Sephadex G-200 purified D-galactonate (D-fuconate) dehydratase, equilibrated against 0.05 M potassium phosphate buffer (pH 7.0), was subjected to ultracentrifugation as described in Materials and Methods. Five concentrations of the protein, 0.03 mg/ml, 0.06 mg/ml, 0.20 mg/ml, 0.60 mg/ml, and 1.50 mg/ml, were run synchronously and absorbance traces were made for each at regular time intervals using a heat-sensitive plotter. Values for the half height of the absorbance boundary and corresponding migration distances from the meniscus were measured for each trace. An average of seven such measurements were made for each protein concentration. Calculations of the sedimentation constant were as described in the text. For each protein concentration, calculations were made varying the r_1 and t_1 values to give a good statistical average for the sedimentation constant (S). Measurements of r are made knowing that the first reference hole of the double sector cell marks both the beginning of the absorbance trace and a distance of 5.7 cm from the center of rotation; while the second reference hole marks the end of the absorbance trace, a distance of 1.6 cm from the first reference hole.


SEDIMENTATION CONSTANT (10^{-13} sec.)
0
2
4
6
8
10
12
14
16
PROTEIN CONCENTRATION (mg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0

distance from the center of rotation in centimeters, and C is the protein concentration at the given r value.

Determination of the $\left[d\ (\text{Log C})\ /\ d\ (r^2)\right]$ factor (the slope) from a plot of the Log C versus r^2 (Fig. 32) from three separate centrifuge runs, gave an average molecular weight of 293,200 $\pm$ 3900 for the dehydratase.

Subunit Molecular Weight. SDS polyacrylamide gels, 10%, were prepared as described in Materials and Methods. D-Galactonate (D-fuconate) dehydratase, Sephadex G-200 purified protein, was electrophoresed with known molecular weight standards catalase (60,000 MW), trypsin (23,000 MW), and lysozyme (14,300 MW). From a plot of the Log (MW) versus mobility (Fig. 33) a subunit molecular weight of approximately 46,000 was interpolated for the dehydratase.

Summary of Both Native and Subunit Molecular Weight Determinations. Through either single-step experiments or by combination of molecular weight parameters, an average molecular weight of 289,100 was determined for the dehydratase native enzyme (Table 16). The dehydratase subunit molecular weight, of about 46,000, suggested that the native enzyme was comprised of six identical subunits.

Amino Acid Composition of the Dehydratase

Sephadex G-200 purified protein was used for all studies involving the amino acid composition and dry weight analysis of the D-galactonate (D-fuconate) dehydratase. For amino acid composition, the protein was equilibrated against 0.05 M potassium phosphate buffer (pH 7.0) by passing over a Sepharose A-5M gel filtration column of appropriate

Figure 32. Sedimentation equilibrium Log C versus r^2 plot. The Sephadex G-200 purified D-galactonate (D-fuconate) dehydratase, equilibrated against 0.05 M potassium phosphate buffer (pH 7.0), was subjected to ultracentrifugation to establish equilibrium as described in Materials and Methods. A protein concentration of 0.09 mg/ml in the above buffer was run against a buffer blank and absorbance traces were made using a heat sensitive plotter. Values for both the absorbance at 280 nm and corresponding distances from the center of rotation were made as described for the sedimentation velocity run. Baseline absorbances for these distances were subtracted from absorbance measurements as they originated from buffer absorbances only, not protein concentration. Baseline absorbances were established by pelleting the sample to the bottom of the cell, running at 24,000 rpm for 24 hrs, and recording absorbance values at regular distances from the center of rotation. Additional equilibrium measurements, not shown, were obtained by setting the speed back to 11,000 rpm after pelleting and allowing for a 24 hr re-equilibration. (The process of pelleting and restoration of equilibrium was repeated several times accounting for the three separate trials mentioned in the text). Simplification of the molecular weight calculation was made by substituting corrected absorbance (280 nm) values for the concentration factor (C), as the absorption optical system used followed Beers' law at protein concentrations used in these ultracentrifuge runs.

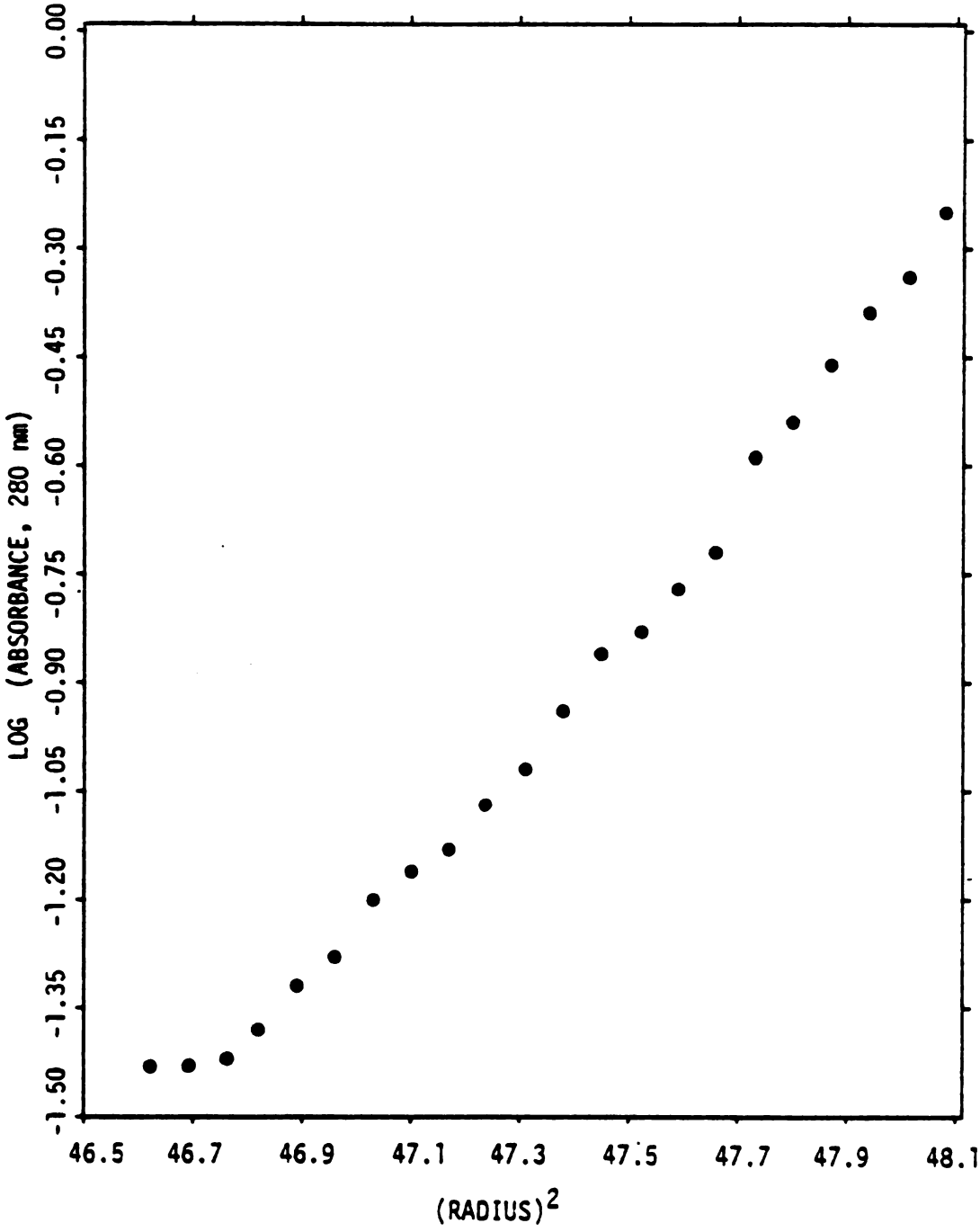

LOG (ABSORBANCE, 280 nm)
0.00
-0.15
-0.30
-0.45
-0.60
-0.75
-0.90
-1.05
-1.20
-1.35
-1.50
46.5
46.7
46.9
47.1
47.3
47.5
47.7
47.9
48.1
(RADIUS)2

Figure 33. Determination of the subunit molecular weight of D-galactonate (D-fuconate) dehydratase by SDS polyacrylamide gel electrophoresis. A 10% SDS polyacrylamide slab gel, 12-channel capacity, was prepared as described in Materials and Methods, this section. D-Galactonate (D-fuconate) dehydratase (Sephadex G-200) and appropriate molecular weight standards were loaded using stock samples of 0.5 mg per ml at 0.04-ml per channel; all protein samples were run in different channels, spaced one channel apart. Gels were run for 4.0 hrs at 20 milliamp current at room temperature. Gels were stained for protein with Coomassie blue. Upon destaining, migration distances for the dye marker and the protein stained bands were taken as measured from the stacking gel/running gel boundary. Mobility is the ratio of the protein migration distance to the dye marker migration distance (dye marker was added to each protein sample). Values of mobility plotted for catalase (0.342), D-galactonate (D-fuconate) dehydratase (0.445), trypsin (0.708), and lysozyme (0.895) were determined in the above manner. Linear regression was used to interpret the slope of the plot relating Log (MW) versus mobility in order to extrapolate a molecular weight for the dehydratase.

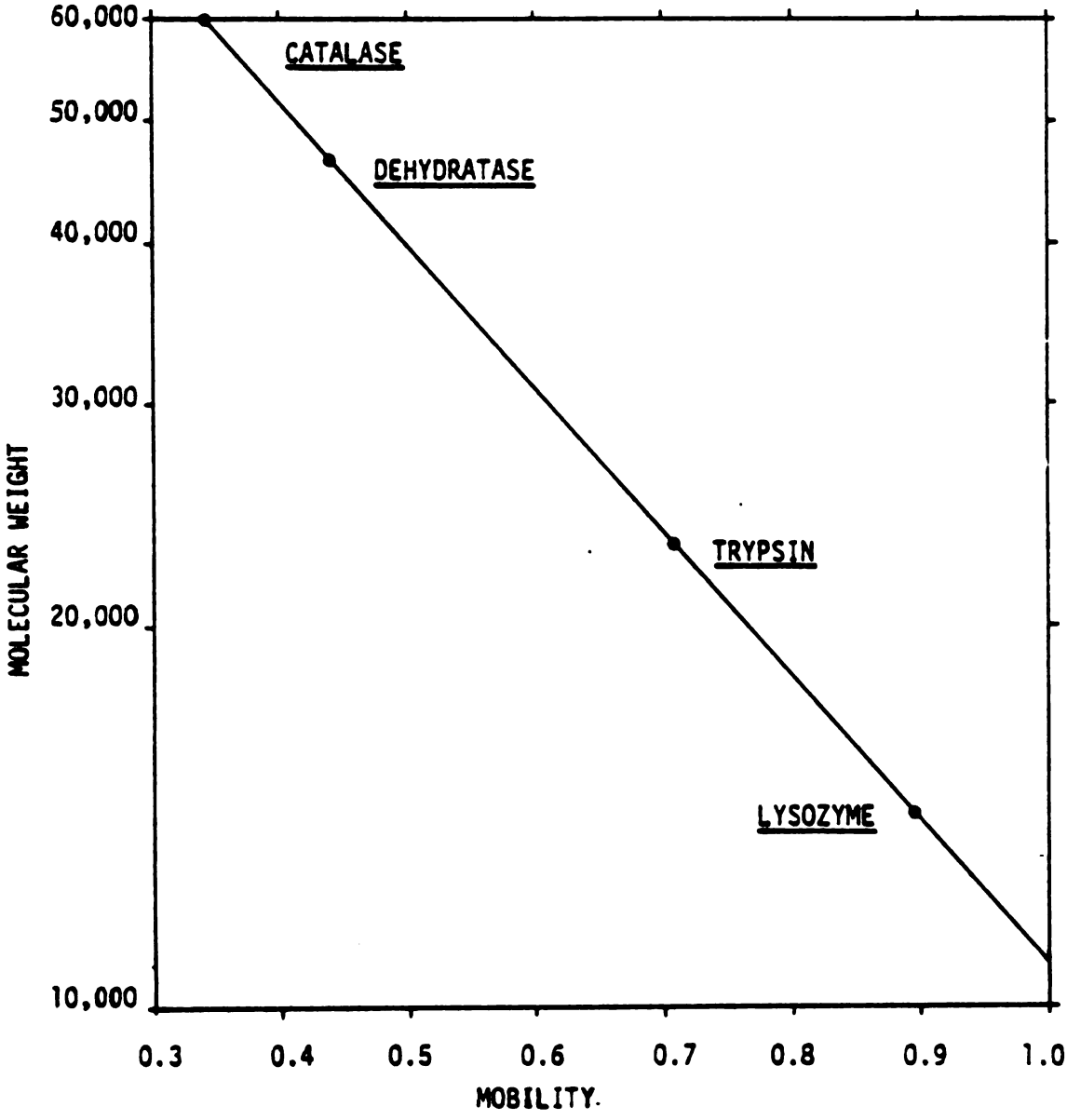

60,000
50,000
40,000
30,000
20,000
10,000
MOLECULAR WEIGHT
CATALASE
DEHYDRATASE
TRYPSIN
LYSOZYME
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
MOBILITY.

Table 16. Summary data for the molecular weight of D-galactonate (D-fuconate) dehydratase. The various methods used to determine the molecular weight and their respective molecular parameters are listed along with determined molecular weight. Parameters are defined as follows: C is the protein concentration, r is the radius from the center of rotation, N is the number of particles per mole (Avagodros number; 6.023×10^{23}), $\bar{v}$ is the partial specific volume (0.773 g/ml^3), ρ is the density of the solvent at 20°C (0.999 g/ml^3), η is the viscosity of the medium at 20°C of the solvent at 20°C (1.0), π is a constant (3.14). The Stokes radius, a, for the dehydratase was determined as 55.39×10^{-8} cm (Å) using fumarase (51.0 Å) and pyruvate kinase (59.3 Å) from gel filtration data and linear regression of the plot of Stokes radius versus Kav (data not shown).

Table 16. Summary data for the molecular weight of the D-galactonate (D-fuconate) dehydratase.

METHOD	PARAMETER	MOLECULAR WEIGHT	
		Native Enzyme	Subunit
Gel filtration	Log (MW) versus eluate volume	255,000	--
Sucrose density gradient sedimentation	$(MW)_1 = (MW)_2 \times \left[\frac{(S_1)}{(S_2)}\right]^{3/2}$	286,000	--
Sedimentation equilibrium	Log (C) versus r^2	293,200	--
Gel filtration and Sucrose density gradient sedimentation	$MW = \left[\frac{N \cdot 6 \cdot \pi \cdot \eta \cdot a \cdot s}{(1 - \bar{\nu}\rho)}\right]$	293,500	
Gel filtration and sedimentation velocity	$MW = \left[\frac{N \cdot 6 \cdot \pi \cdot \eta \cdot a \cdot s}{(1 - \bar{\nu}\rho)}\right]$	317,300	
SDS polyacrylamide gels	Log (MW) versus mobility	--	46,000
Average		289,100	46,000

dimensions. Dry weight analysis required exhaustive dialysis of the protein sample against double-distilled water to remove all salts, followed by drying at 110°C to a constant weight over phosphorous pentoxide. Protein concentration was determined by the Lowry procedure using bovine serum albumin as the standard.

Acid hydrolysis treatment of the protein was used to determine all amino acids except tryptophan and cysteine (Table 17). Extrapolation to zero time, correcting for losses in threonine and serine due to acid hydrolysis, showed that the following amino acids were found in the dehydratase: AspX, gluX, threonine, serine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine.

Tryptophan was determined by the procedure of Penke et al. (125) using 100 µg of dried dehydratase in 1.0-ml volume of 3.0 N mercaptoethane sulfonic acid at 110°C for 24 hours. The sample was dried, suspended in citrate buffer, and 0.5 ml loaded onto the short column of the amino acid analyzer. Quantitation using 5-β(4-pyridyl-ethyl-L-cysteine) (PEC) standard at 20 nmol in the sample as the internal standard was done in three successive trials on the amino acid analyzer.

Cysteine was determined as the S-carboxymethyl derivative by the procedure of Crestfield (126). Using a concentrated protein sample, a reaction mixture (0.6 ml) comprised of 1.0 mg protein, 8.0 M urea, 0.2% EDTA, and 0.6 M Tris-HCl buffer (pH 8.6) was prepared. The reaction mixture was stirred for several hours to totally dissolve the enzyme. After flushing the sample with nitrogen, 2-mercaptoethanol was added to make a 2.0% solution to reduce disulfide bonds. The reduction

Table 17. Amino acid composition of the dehydratase following acid hydrolysis. Protein samples from a liquid solution of the Sephadex G-200 purified D-galactonate (D-fuconate) dehydratase (0.30-ml volume, 0.33 gm/ml protein concentration) were transferred to acid-washed hydrolysis tubes, taken to dryness under reduced pressure, and then treated with 0.5 ml of 6.0 N constant-boiling HCl and 10.0 mg of crystalline phenol. Samples were then degassed under vacuum, sealed, and hydrolyzed by heating at 110^{o}C for various lengths of time. Trials were run at 24, 52, and 72 hours. Each sample contained 100 μg of protein and were separately hydrolyzed; six trials total were made. Once hydrolyzed, each sample was dried, suspended in 2.0-ml volume of 0.2 M citrate buffer (pH 2.0) with 10.0 nmol norleucine, as the internal standard, and a total volume of 0.5 ml was injected into the amino acid analyzer. Each amino acid was determined and normalized to the internal standard. Extrapolation to zero time corrects for losses of threonine and serine due to the acid hydrolysis; these values were reported in the text. Total acid content for aspartic and glutamic acids are reported, as asparagine and glutamine are converted to respective acids by this treatment.

Table 17. Amino acid composition of the dehydratase after acid hydrolysis treatment. See adjoining page for details.

AMINO ACID	NANOMOLE AMINO ACID DETERMINED			
	24 hr	52 hr	72 hr	zero time
AspX	18.26	18.72	19.09	17.87
GluX	23.55	23.65	23.74	23.46
Threonine	6.07	5.54	5.10	6.53
Serine	6.91	6.33	5.86	7.40
Proline	0.99	1.02	1.04	0.97
Glycine	20.51	20.96	21.32	20.13
Alanine	25.35	25.24	25.14	25.45
Valine	15.68	15.72	15.76	15.64
Methionine	5.95	5.82	5.71	6.06
Isoleucine	13.94	14.13	14.27	13.79
Leucine	21.75	21.97	22.14	21.57
Tyrosine	9.45	9.23	9.06	9.63
Phenylalanine	10.71	10.64	10.58	10.77
Histidine	3.37	3.39	3.40	3.36
Lysine	10.57	10.54	10.52	10.59
Arginine	11.51	12.09	12.56	11.02

reaction was carried out in the dark at room temperature for four hours. At the end of this incubation, 22.0 mg of iodoacetate in 0.08-ml volume of 1.0 N NaOH was added to carboxymethylate the free sulfhydryl groups of the enzyme. After 20 min stirring, the reaction mixture was exhaustively dialyzed against ammonium bicarbonate buffer (50.0 mM, pH 8.1) at $4^{o}C$ in the dark to remove excess reagents. The sample was dried under reduced pressure, with several distilled water washes to drive off the buffer, and then subjected to acid hydrolysis as above. Norleucine was used as the internal standard in the amino acid analysis.

When the complete amino acid composition of the D-galactonate (D-fuconate) dehydratase is considered (Table 18) the following equation can be used to calculate the protein weight of the dehydratase injected into the analyzer with each trial:

PROTEIN WEIGHT = (SUM TOTAL of the AMINO ACID RESIDUE combined weight) - (SUM TOTAL of the AMINO ACID RESIDUE mole number) - 1 X (the molecular weight of H_2O; i.e., 18.0 g/mole)

Performing the calculations gives a value of 24.31 µg dehydratase protein used per each amino acid analysis run. Assuming a molecular weight of 289,100 g/mole enzyme, as determined by the various methods (Table 16), this weight corresponds to 8.41×10^{-2} nmol protein. Using the latter value, for calculation of the mole ratio of amino acid to protein, gives the number of residues per dehydratase molecule.

According to the dry weight analysis of the dehydratase protein, a value of 1.020 mg protein was obtained from a 4.0-ml volume sample determined as 0.260 mg/ml by the Lowry method. These data show a dry

Table 18. Total amino acid composition of the D-galactonate (D-fuconate) dehydratase. Details are described in the text.

AMINO ACID	AMOUNT		MOLES/MOLE ENZYME	MOLE RATIO	MOLE PERCENT
	nmole	µg			
Cysteine	5.27	0.64	62.7	5.5	2.5
AspX	17.87	2.37	212.5	18.5	8.3
GluX	23.46	3.44	279.0	24.3	11.0
Threonine	6.53	0.78	77.7	6.8	3.1
Serine	7.40	0.78	88.0	7.7	3.5
Proline	0.97	1.11	11.5	1.0	0.5
Glycine	20.13	1.51	239.4	20.8	9.4
Alanine	25.45	1.83	302.6	26.3	11.9
Valine	15.64	1.83	186.0	16.2	7.3
Methionine	6.06	0.90	72.1	6.3	2.8
Isoleucine	13.79	1.81	164.0	14.3	6.4
Leucine	21.57	2.83	256.5	22.3	10.1
Tyrosine	9.63	1.75	114.5	10.0	4.5
Phenylalanine	10.77	1.78	128.1	11.1	5.0
Histidine	3.36	0.52	40.0	3.5	1.6
Lysine	10.59	1.55	126.0	11.0	4.9
Arginine	11.02	1.92	131.0	11.4	5.1
Tryptophan	4.68	0.81	55.7	4.8	2.2

weight/Lowry ratio of 0.981, meaning that the Lowry procedure gives a slightly higher than expected protein concentration. When protein used in the amino acid analysis was assayed by the Lowry procedure a value of 0.333 mg/ml was determined. In all analyses made, a 0.30-ml sample of this protein stock solution was used for the protein aliquot and dried. In comparision then, this aliquot should only reflect a 0.327 mg/ml stock protein sample by dry weight and 0.30 ml would give only 98.0 μg protein for the analysis. After suspending in 2.0-ml volume and taking a 0.5-ml aliquot to inject into the amino acid analyzer, only 24.5 μg protein should be found in each amino acid run. This value shows a 99.0% correlation to the weight of protein found in the summarized amino acid composition data noted (Table 18 and text above). These data show that there is good agreement between the protein determination by dry weight, Lowry procedure, or by amino acid composition analysis.

Preparation and Identification of the Products of the Dehydratase-Catalyzed Reaction

Enzymatic Preparation of the Dehydratase Products. The respective dehydratase products of D-fuconate and D-galactonate were prepared enzymatically. The dehydratase was partially purified from a D-galactonate-grown culture of the strain CH-101, derepressed for the D-galactonate (D-fuconate) dehydratase, through the first DEAE-cellulose chromatographic step. This preparation had 7.8 mg protein per ml with specific activities for D-fuconate and D-galactonate of 0.89 and 2.67 units/mg, respectively; no KDGal kinase or KDPGal aldolase activity

were detected in the pooled dehydratase fractions at this stage of purification.

Reaction mixture (25.0 ml) to prepare 2-keto-3-deoxy-D-fuconate (KDF) and 2-keto-3-deoxy-D-galactonate (KDGal) contained 10.0 mM $MgCl_2$, 1.0 mM EDTA (pH 7.0), 50.0 mM Pipes buffer (pH 7.0), 80.0 mM K^+ D-fuconate or K^+ D-galactonate, and the dehydratase preparation (7-10 mg of protein). The time course for the production of α-keto acid in both cases was determined using the semicarbazone assay of MacGee and Doudoroff (15). The reaction for the enzymatic synthesis of KDF was judged to be complete in 4.0 hrs and for KDGal in 2.0 hrs (Fig. 34). The reaction mixture was then deproteinated by centrifugation through CF-25 Centri-flo membrane cones (Amicon Corp.) of 10.0-ml capacity at room temperature with the aid of a clinical centrifuge. Filtrates were collected, taken to pH 4.0 with formic acid and degassed over steam.

Purification of both the D-fuconate and D-galactonate dehydratase product was effected by ion exchange chromatography on Dowex-1-X8 (formate) resin. The dehydratase product from D-fuconate was eluted by washing with 0.46 M formic acid, whereas the D-galactonate product was eluted with a linear gradient of 0.23 to 0.46 M formic acid. Chromatography of the D-fuconate product (Fig. 35) showed one symmetrical peak; fractions 129 to 157, containing most of the thiobarbituric acid (TBA)-positive material were pooled. Likewise, chromatography of the D-galactonate product (Fig. 36) showed one symmetrical peak; fractions 218 to 247, containing most of the TBA-positive material, were pooled. Syrups of the pooled fractions were prepared for both products by reduced pressure evaporation, which were then suspended in distilled water

Figure 34. Time course for the enzymatic preparation of the dehydratase products from D-fuconate and D-galactonate. The time course of each reaction mixture, using either D-fuconate or D-galactonate as substrate, as described in the text, was followed by withdrawing 0.05-ml samples at regular time intervals and reacting with 0.2 ml of the semicarbazone reagent (1.0% semicarbazone·HCl and 1.5% sodium acetate). These samples wre then incubated at 30°C for 15 min, after which they were diluted to 1.0-ml volume. The protein precipitates, which developed upon reacting this reagent with protein, was centrifuged out and the clear solutions were then read at 250 nm against a reagent blank. The α-keto acid content of each time trial, for both substrates, was determined using the extinction coefficient of 10,200 (15). Symbols: ● dehydratase product from D-galactonate, ▲ dehydratase product from D-fuconate.

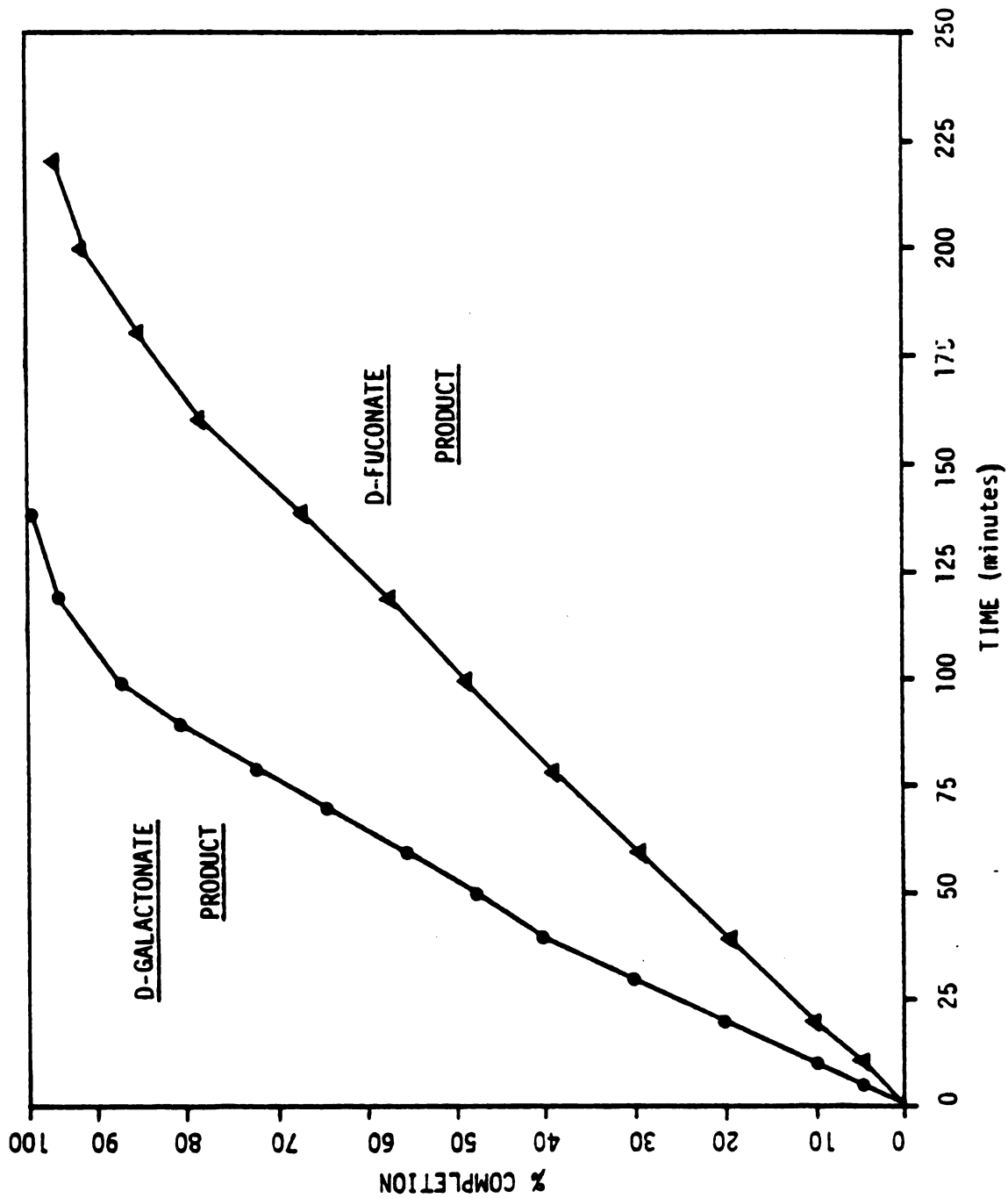

% COMPLETION
TIME (minutes)
D-GALACTONATE
PRODUCT
D-FUCONATE
PRODUCT

Figure 35. Purification of the D-fuconate dehydratase product. The reaction mixture, described in the text, was adjusted to pH 4.0 with formic acid and degassed before loading onto a 3.8 X 50.0 cm Dowex-1-X8 (formate) column, 200-400 mesh. The sample, 25.0 ml, was loaded at a flow rate of 140 ml per hr and collected at 19.0 ml per fraction. The column was then washed with 2.0-liter volumes of distilled water and 0.23 M formic acid. Eluate fractions were collected as above and were assayed for 2-keto-3-deoxy sugar acids by the thiobarbituric acid assay (TBA) of Weissbach and Hurwitz (83). No TBA-positive material was found in these washings. To elute the product a resevoir of 3.5-liter volume comprised of 0.46 M formic acid was used. Eluate fractions were assayed as above. Quantitation of the TBA chromogen was based on the derived molar extinction coefficient for 2-keto-3-deoxy-D-fuconate of 50,060 at 551 nm.

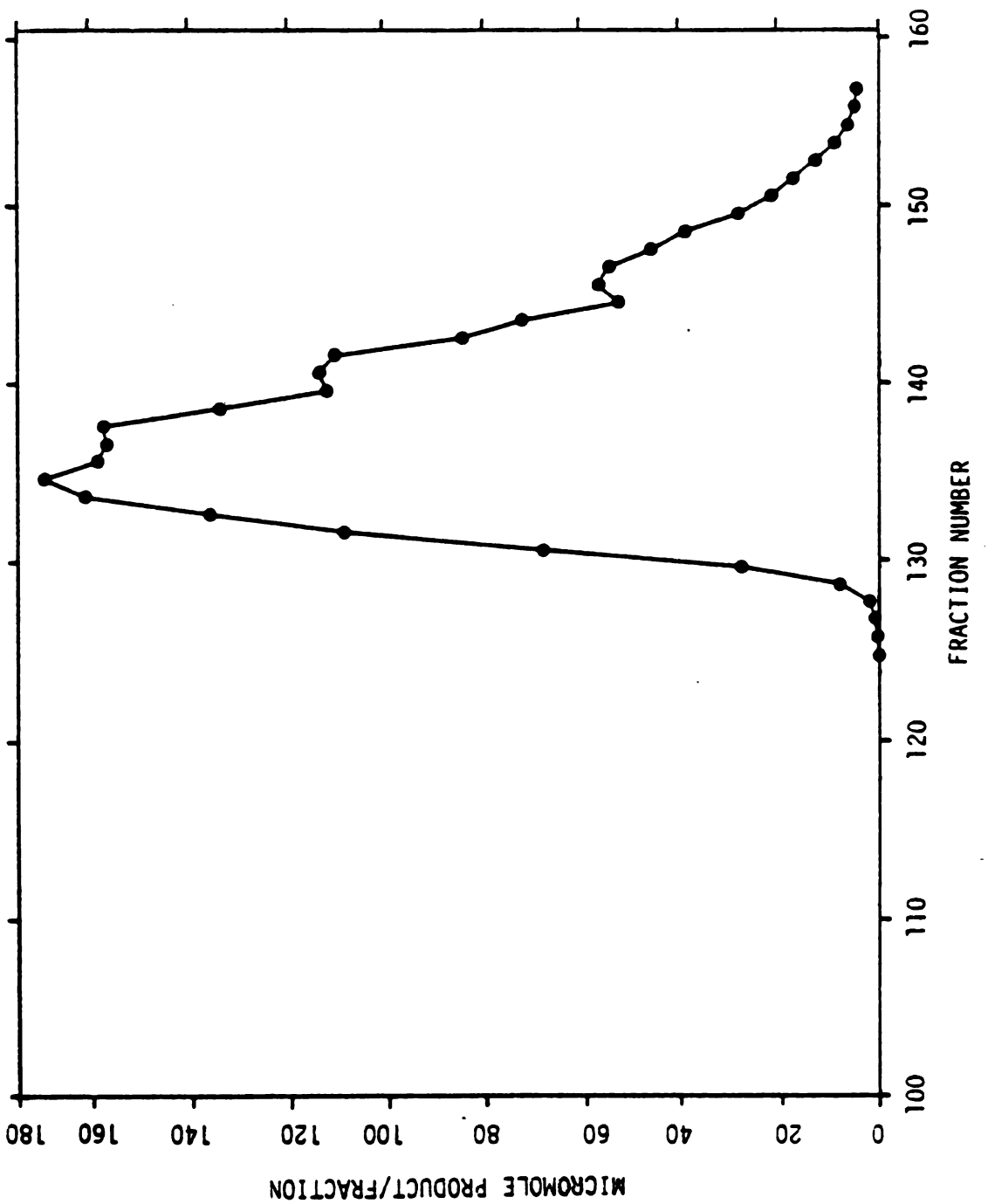
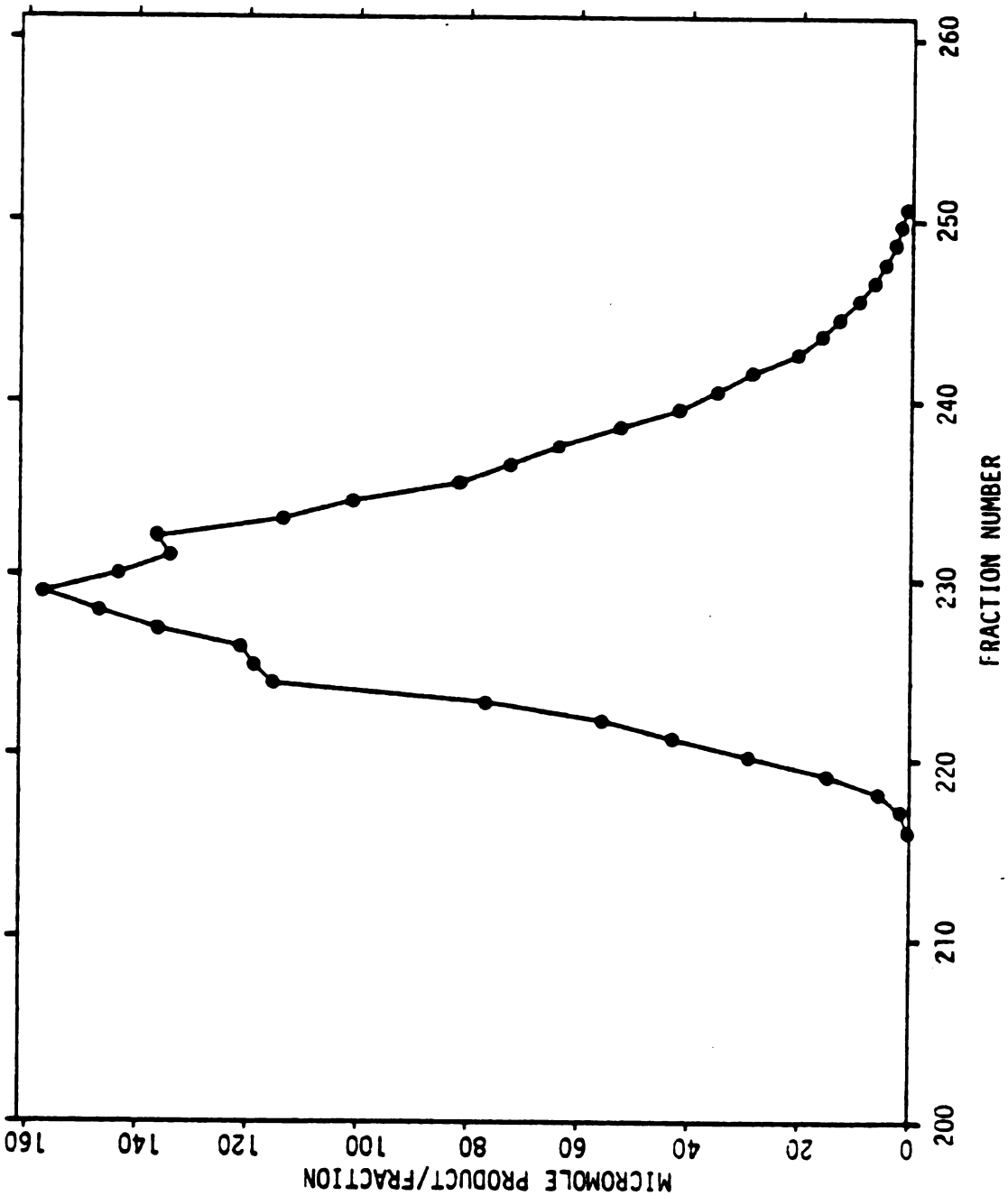

MICROMOLE PRODUCT/FRACTION
0
20
40
60
80
100
120
140
160
180
FRACTION NUMBER
100
110
120
130
140
150
160

Figure 36. Purification of the D-galactonate dehydratase product. The reaction mixture, as described in the text, was adjusted to pH 4.0 with formic acid and degassed before loading onto a 3.8 X 50.0 cm Dowex-1-8X (formate) column, 200-400 mesh. The sample, 25.0 ml, was loaded at a flow rate of 140 ml per hr and colleted at 19.0 ml per fraction. The column was then washed with 2.0-liter volumes of distilled water. Eluate fractions were collected as above and were assayed for 2-keto-3-deoxy sugar acids by the thiobarbituric acid assay (TBA) of Weissbach and Hurwitz (83). No TBA-positive material was found in the wash fractions. To elute the product a 5.0-liter linear gradient of 0.23 to 0.46 M formic acid was used as described by Portsmouth (87). Eluate fractions were assayed as above. Quantitation of the chromogen was based on the derived molar extinction coefficient for 2-keto-3-deoxy-D-galactonate of 60,000 at 551 nm.


MICROMOLE PRODUCT/FRACTION
0
20
40
60
80
100
120
140
160
FRACTION NUMBER
200
210
220
230
240
250
260

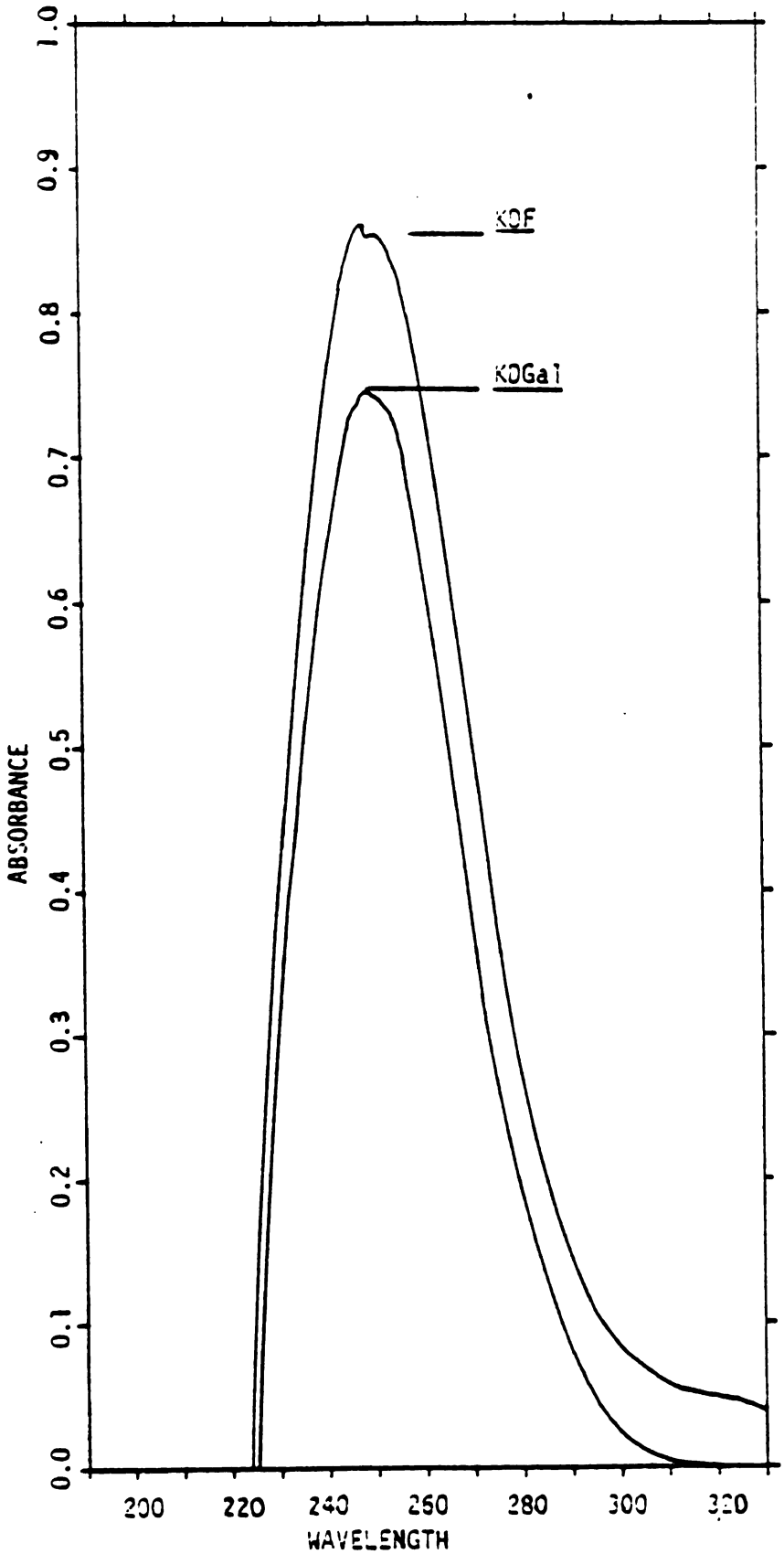
prior to neutralization to pH 7.0 with KOH. Semicarbazone assays of the potassium salt form of these products gave yields of 2.22 mmol and 2.10 mmol for D-fuconate and D-galactonate, respectively. In accord with defined molar extinction coefficients determined for the chemically synthesized isomers KDQ and KDF (Section 1, Materials and Methods) for the thiobarbituric acid assay, values of 50,060 and 60,000 for the D-fuconate and D-galactonate product, repectively, at 551 nm were determined.

Identification of the Products of the Dehydratase-Catalyzed Reaction. To prove the structure of the dehydratase product on D-fuconate as 2-keto-3-deoxy-D-fuconate, a four-part analysis was devised to fully characterize the product. The steps taken consisted of: (i) formation of characteristic chromogens for 3,6-dideoxy-D-hexulosonic acids; (ii) co-chromatography of the enzymatic product with chemically synthesized standards on Dowex-1-X8 (formate) resin; (iii) treatment with hydrogen peroxide to form mono-cyclic pentonic acids (lactones) and determination of the molar uptake of periodate and the rates of periodate oxidation; (iv) confirmation that the enzymatic and chemically synthesized products are both biologically active; i.e., that each serve as suitable substrates for the next enzyme of the pathway.

(i) Formation of Characteristic Chromogens. 2-Keto-3-deoxy sugar acids form characteristic semicarbazone chromogens which absorb maximally at 250 nm (87). Respective semicarbazone derivatives of both D-fuconate and D-galactonate products were prepared. Absorption spectra of both semicarbazone derivatives (Fig. 37) show maximal values at 250 nm wavelength.

Figure 37. Absorption spectra of semicarbazone derivatives of the purified dehydratase products. Semicarbazone derivatives for both the D-fuconate and the D-galactonate dehydratase products were prepared as described by MacGee and Doudoroff (15). To approximately 1.0-ml volume of sample, 0.5 μmol product, was added 1.0 ml of the semicarbazone reagent (1.0% semicarbazone·HCl and 1.5% sodium acetate). Derivatives were allowed to form over a 15 min period at 30°C. A reagent blank, using distilled water in place of carbohydrate, was used as control.

Scans of both semicarbazone derivatives of the products and the reagent blank were made using a Cary, model 15, spectrophotometer. Tracings were made at a recorder speed of 10 nm wavelength per division (8.0 mm per division) beginning at 330 nm and proceeding to 200 nm in the ultraviolet region. In this figure, the upper peak is the D-fuconate dehydratase product and the lower peak is the D-galactonate dehydratase product.


KDF
KDGal
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
200
220
240
260
280
300
320
WAVELENGTH

Weissbach and Hurwitz (83) described an absorption maximum peaking between 545 and 550 nm for compounds yielding β-formyl pyruvate upon periodate oxidation in the thiobarbituric acid assay. Absorption spectra of the TBA chromogens formed for both D-fuconate and D-galactonate products showed absorption maxima at 551 nm wavelength (Fig. 38). If periodate were deleted from the reaction mixture, no absorbance above that of the reagent blank was found for either dehydratase product using the assay. These data show that the dehydratase products both have α-keto acid character and yield β-formyl pyruvate upon periodate oxidation. Thus, the structure of the compounds, limited to the first five carbons can be characterized as: R-CHOH-CHOH-CH_2-CO-COOH.

(ii) Co-Chromatography of Isomeric Mixtures. Mixtures of the dehydratase product from D-fuconate and chemically synthesized isomers were chromatographed on identical Dowex-1-X8 (formate) columns using co-migration after elution with formic acid as a gauge to structural similarity. Isomers were distinquished in eluate fractions by their characteristic molar extinction coefficients for the TBA assay; i.e., values of 10,930 and 50,060 for KDQ and KDF, respectively (see Section 1, Materials and Methods). Elution profiles of the KDQ and KDF mixture (Fig. 39, A) show that the chromatography was effective in separating the isomers. In addition, recovery of each isomer from eluate fractions was better than 80% (Table 19); a recovery of 80% or better was found in all trial mixtures run. Chromatography of the D-fuconate dehydratase product with KDQ (Fig. 39, B) revealed two well resolved peaks, one migrating as KDQ and the other as KDF. Chromatography of KDF with the D-fuconate dehydratase product (Fig. 39, C)

Figure 38. Absorption spectra of the thiobarbituric acid assay (TBA) chromogen of the purified dehydratase products. The assay procedure of Weissbach and Hurwitz (83) was used to prepare the TBA chromogens. The D-fuconate dehydratase product (1.6×10^{-7} mole) and the D-galactonate dehydratase product (4.3×10^{-7} mole) in 0.25 ml of distilled water were treated with 0.25 ml of 0.025 $NaHIO_4$ in 0.125 N H_2SO_4 and incubated at room temperature for 20 min. At the end of this time, 0.5 ml of a solution of 2.0% sodium arsenite in 0.5 N HCl was added, and the solution was mixed. To this solution was added 2.0 ml of a solution of 0.3% thiobarbituric acid (pH 2.0), the samples were mixed, and then heated for 20 min at 100°C. A reagent blank, with all reagents above except that distilled water was used in place of carbohydrate, was used as a control. Once samples were cooled to room temperature, the red chromogen which had developed was read against the reagent blank and were then scanned using a Cary, model 15, spectrophotometer.

Traces were made at a recorder speed of 10 nm wavelength per division (8.0 nm per division) beginning at 610 nm and proceeding to 350 nm in the visual region. In the figure, the upper peak is the D-galactonate product and the lower peak is the D-fuconate product.

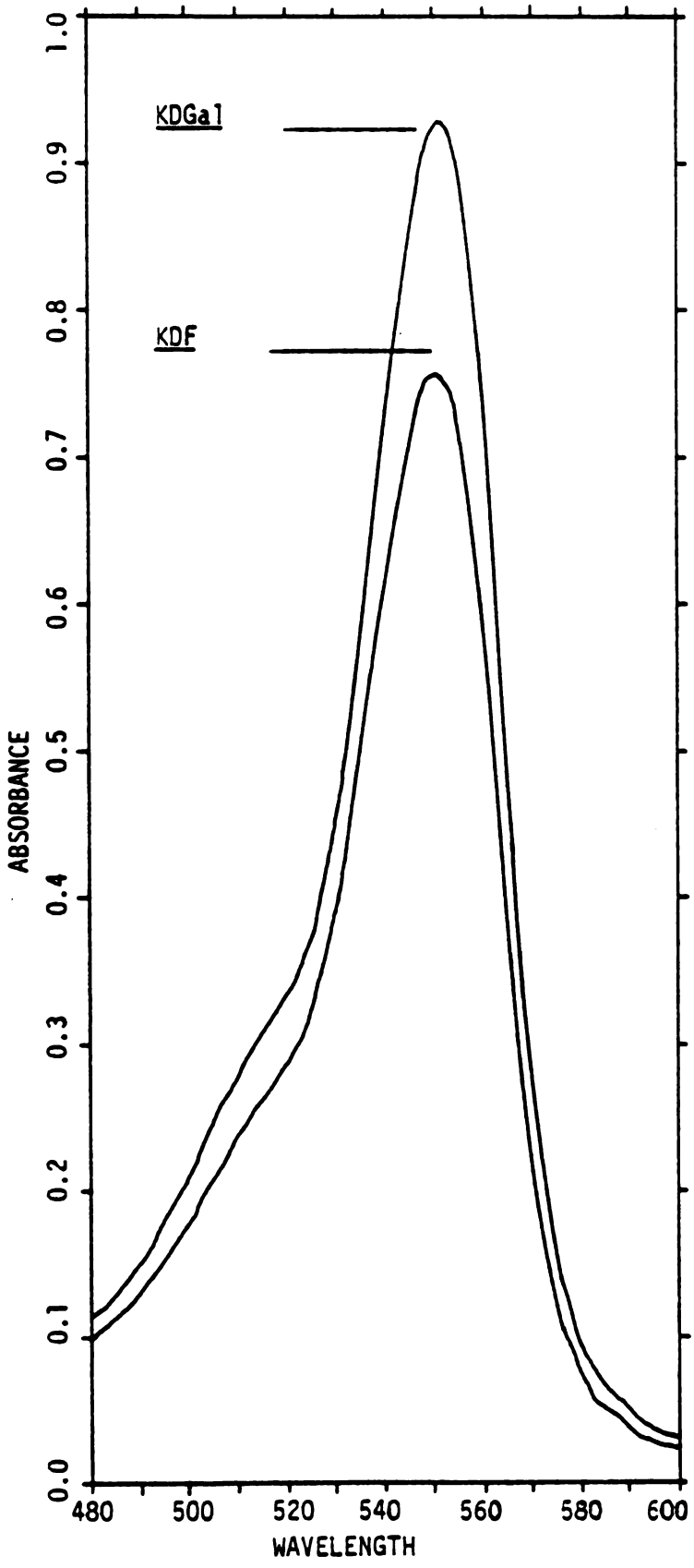

KDGal
KDF
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
480
500
520
540
560
580
600
WAVELENGTH

Figure 39. Chromatographic identification of the D-fuconate dehydratase product. Mixtures of the D-fuconate dehydratase product and sythetic isomers, 3,6-dideoxy-threo-D-hexulosonic acid (KDF) and 3,6-dideoxy-erythro-D-hexulosonic acid (KDQ), were chromatographed on separate 1.7 X 50.0 cm Dowex-1-X8 (formate) columns, 200-400 mesh. All mixtures were diluted to 50.0-ml volume, adjusted to pH 4.0 with formic acid, and degassed before loading onto the columns. After loading, columns were washed with distilled water and 0.23 M formic acid, 1.0-liter volume each. Eluate fractions, 4.0-ml volume, of the water washes showed no thiobarbituric acid (TBA)-positive material using the assay of Weissbach and Hurwitz (83). Elution of the products was effected with 0.46 M formic acid; three bed volumes were required. KDF and KDQ were distinquished by their respective molar extinction coefficients at 551 nm in the TBA assay as 50,060 and 10,930.

Figures: (A) chromatographic mixture of KDQ (25 μmol) and KDF (25 μmol); (B) chromatographic mixture of KDQ (25 μmol) and D-fuconate dehydratase product (10 μmol); (C) chromatographic mixture of KDF (9 μmol) and D-fuconate dehydratase product (9 μmol).

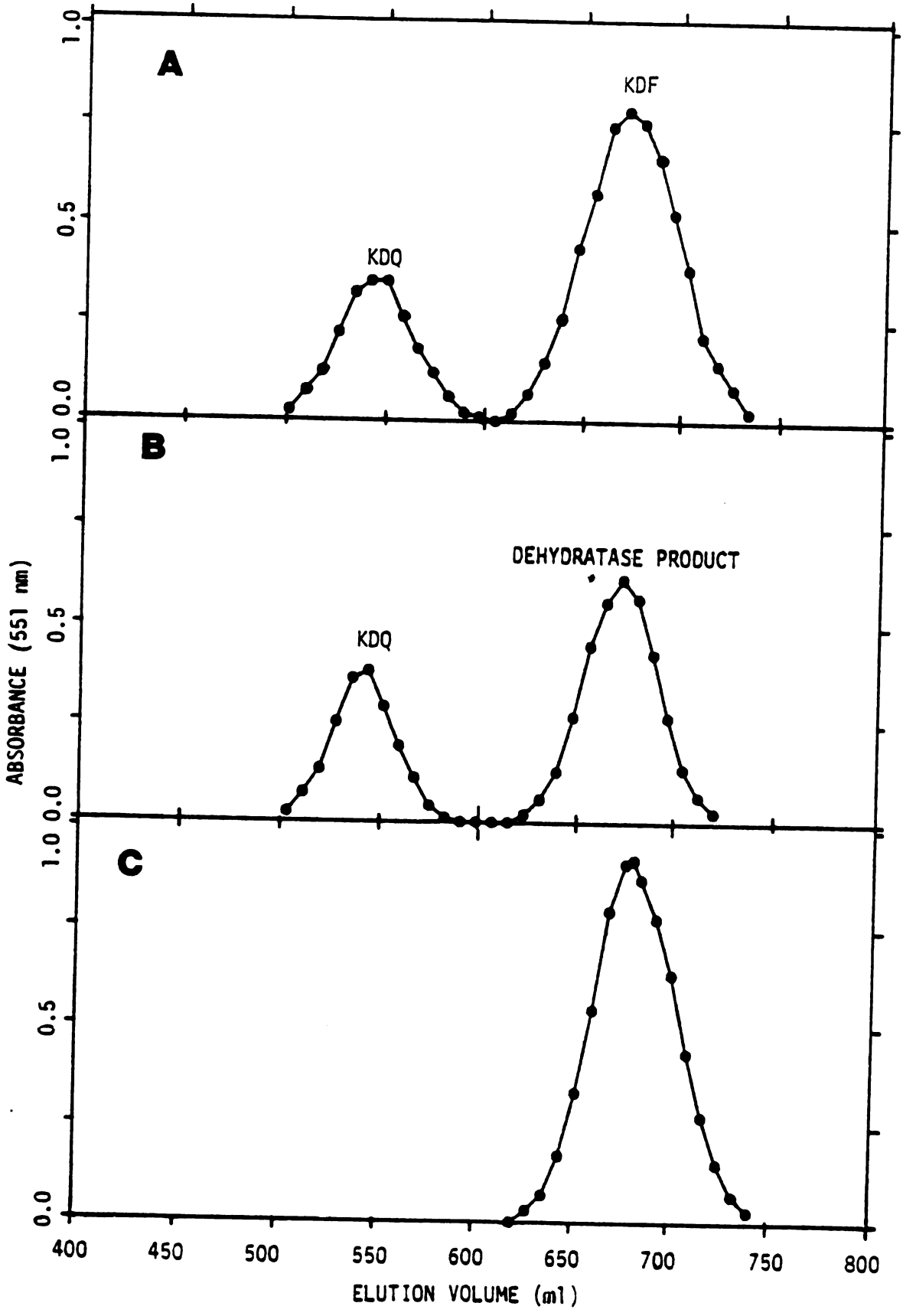

A
KDF
KDQ
B
DEHYDRATASE PRODUCT
KDQ
C
ABSORBANCE (551 nm)
1.0
0.5
0.0
400
450
500
550
600
650
700
750
800
ELUTION VOLUME (ml)

Table 19. Recoveries of chromatographic mixtures used to identify the D-fuconate dehydratase product. Mixtures of the D-fuconate dehydratase product, KDF, and KDQ, in combinations described in the text, after having been chromatographed on Dowex-1-X8 (formate) columns were treated as follows: (a) resolved peak fractions were pooled and concentrated to syrups under reduced pressure; (b) syrups were suspended in distilled water and adjusted to pH 7.0 with KOH to constitute stock solutions for the analysis. Stock solutions were then assayed for α-keto acid content by the procedure of MacGee and Doudoroff (15) to determine the recovery of a given isomer and by the procedure of Weissbach and Hurwitz (83) to obtain the molar extinction coefficient at 551 nm for the thiobarbituric acid assay; the latter was used to distinquish the two isomers.

MIXTURE	POOLED ELUATE FRACTIONS ml	RECOVERY %	EXTINCTION COEF. 551 nm
KDQ & KDF	510 to 580	100	10,930
	620 to 720	88	50,060
KDQ & DEHYDRATASE PRODUCT	510 to 580	80	10,930
	620 to 720	90	50,060
KDF & DEHYDRATASE PRODUCT	620 to 720	89	50,060

revealed a single symmetrical peak, migrating the same as KDF. Quantitation of the pooled fractions (Table 19) showed an additive recovery of thiobarbituric-acid-positive material, in this latter instance, corresponding to 90% of the material loaded, suggesting that both products co-migrated. These data show that when D-fuconate is used as substrate for the dehydratase, a product suggestive of 2-keto-3-deoxy-D-fuconate (KDF) is found. Thus, no epimerization of D-fuconate occurred; the threo configuration of the substrate was retained in the product.

(iii) Periodate Consumption and the Rates of Periodate Oxidation. To further verify the stereochemistry of the C_4-C_5 hydroxyls of the D-fuconate product as threo, the periodate rate study of Portsmouth (87) was adopted. According to chemical laws of periodate oxidation of vicinyl hydroxyl groups, cis hydroxyl groups are cleaved at a faster rate than are trans hydroxyl groups. In addition, compounds which differ only in the stereochemistry about the vicinyl hydroxyl groups have the same periodate uptake. With these facts in mind, the following procedures were used.

The Portsmouth chemically synthesized products, 2-keto-3-deoxy-D-fuconate (KDF; threo) and 2-keto-3-deoxy-D-quinovonic acid (KDQ; erythro), and the enzymatic product from D-fuconate were quantitated as semicarbazones (15). Aliquots equivalent to 0.05 mmol of product were taken to a syrup by evaporation under reduced pressure at 40°C and suspended in 1.2-ml of distilled water. To each sample, secured in 0.5-dram vials equipped with stir bars and teflon caps, was added 0.5 mmol of hydrogen peroxide (fresh). The samples were stirred for 24 hrs at 4°C, after-which, α-keto acid was undetectable, thus demonstrating the completeness

of the reaction.

Catalase, 1 to 2 mg, was then added to remove unreacted hydrogen peroxide. After 24 hrs of constant stirring, the samples were heated at 100°C for 10 min to precipitate the enzyme and were centrifuged at 12,000 X g for 10 minutes. The supernatant was then passed over a 0.25 X 15.0 cm Dowex-50W-X8 (H^+) ion-exchange column to yield catalase-free pentonic acid. These eluate fractions were then taken to syrups and suspended in distilled-water. Quantitation of these solutions by use of alkaline hydroxylamine reagent (81), using D-galactono-γ-lactone as the standard, showed the following concentrations for each trial: the D-fuconate enzymatic product (50.0 mM), KDQ (49.4 mM), and KDF (51.3 mM). Periodate consumption was then performed on these stock solutions to further verify the concentrations of the peroxide-treated carbohydrates, as the periodate-rate study is strongly influenced by this factor.

To determine the periodate uptake, the pentonic acids were converted to corresponding acid salts. Aliquots of the above stock pentonic acids were neutralized to pH 7.0 with 1.0 N NaOH and diluted to exactly ten times the initial sample volume to prepare the stock solutions needed for this study. Samples from these solutions were then treated with periodate, and periodate loss was determined (the oxidation reaction was judged complete in 2.5 hrs with all carbohydrate solutions). These results (Table 20) confirm the dehydratase product concentration as 50.0 mM, chemically synthesized isomers KDQ as 49.4 mM and KDF as 51.3 mM. Thus, the concentrations of all carbohydrate solutions were confirmed by two independent methods of analysis; in addition,

Table 20. Periodate uptake of the pentonic acids produced by hydrogen peroxide treatment of the D-fuconate dehydratase product and chemically synthesized isomers. Stock solutions of the pentonic acids (5.0 mM) were prepared and quantitated as described in the text. Aliquots were reacted with 1.0-ml volume of a reaction mixture consisting of 0.1 mM sodium periodate and 50.0 mM sodium formate (pH 4.0). Reaction mixtures were incubated in the dark at 30°C. A reagent blank, without carbohydrate, was used as control. At regular time intervals, 0.25-ml samples were taken from the reaction mixture and read at 223 nm against the reagent blank using a Gilford 2400 spectrophotometer and microcuvettes. Final absorbance readings were recorded and subtracted from the control to measure periodate loss. Quantitation of the periodate loss was based on a molar extinction coefficient of the periodate ion of 10,000 at 223 nm (114).

SAMPLE SOLUTION	AMOUNT OF CARBOHYDRATE	ABSORBANCE	ΔA	PERIODATE LOSS
	nmol	223 nm		nmol
CONTROL	0	1.650	0.000	0.0
DEHYDRATASE PRODUCT	20	1.450	0.200	20.0
	40	1.250	0.400	40.0
	80	0.850	0.800	80.0
KDF	20	1.445	0.205	21.0
	40	1.240	0.410	41.0
	80	0.829	0.821	82.0
KDQ	20	1.452	0.198	20.0
	40	1.255	0.395	40.0
	80	0.860	0.790	80.0

the pentonic acid character is confirmed by the ability to form lactones and the uptake of one mole of periodate per mole of carbohydrate.

As the periodate oxidation rate is second order, for meaningful comparisons to be made, both the initial periodate ion concentration and the intial substrate concentration must be determined and constant for all trials in the rate studies. Relative rates of periodate oxidations were done using the known stock pentonic acid solutions (Table 21). Analysis of these data showed that the rate for KDQ was twice that for KDF, which is in concert with theory as the former isomer is cis and the latter is trans. In addition, the dehydratase product had the same rate as that of KDF.

Based on the data published by Portsmouth (87), a ratio of KDF to KDQ in such studies as above range from 1.00:1.90 to 1.00:2.66 depending on the pH and the periodate ion concentration used. Experimental data reported here show a ratio of KDF to KDQ as 1.00:2.88 and a ratio of the dehydratase product to KDQ as 1.00:2.58; thus, the trans configuration of the C_4-C_5 hydroxyls of the dehydratase product is confirmed. No epimerization of the substrate was expected to have occurred in the enzymatic reaction as a result of this experiment.

(iv) Confirmation of the Biological Activity of the Purified Dehydratase Product. As shown in Section 1, Results, both the enzymatic product from D-fuconate and the chemically synthesized threo product are enzymatically cleaved to yield one mole of pyruvate and one mole of D-lactaldehyde per mole of substrate when incubated with the KDF aldolase from extracts of D-fuconate-grown K. pneumoniae, strain CH-101. These results indicate a strong similarity in structure

Table 21. Relative rates of periodate oxidation of the dehydratase product and the synthetic isomers. Stock pentonic acid solutions, approximately 50.0 mM, which were adjusted to pH 7.0 and diluted to appropriate concentrations for the experiment with 50.0 mM sodium formate buffer (pH 4.0). Reaction solutions in 0.20-ml volume consisted of 1.2×10^{-2} µmol of carbohydrate, 2.0×10^{-2} µmol of sodium periodate, and 10.0 µmol of sodium formate buffer (pH 4.0). Continuous rates were measured in microcuvettes at 30°C on a Gilford spectrophotometer at 223 nm. Initial rates were measured over 12.0 minutes and rates recorded in arbitrary units.

CARBOHYDRATE STOCK SOLUTION	RATE (absorbance change/12.0 min)
DEHYDRATASE PRODUCT	0.124
SYNTHETIC KDQ	0.320
SYNTHETIC KDF	0.120

as well as confirm the biological activity of the products.

Summary of the Product Identification Studies. The following facts concerning the configuration of the dehydratase product from D-fuconate are used to confirm the product from D-fuconate as being 3,6-dideoxy-threo-D-hexulosonic acid (2-keto-3-deoxy-D-fuconate):

(a) The dehydratase product from both D-fuconate and D-galactonate form semicarbazone derivatives and yield β-formyl pyruvate upon periodate treatment characteristic of α-keto acids and 2-keto-3-deoxy sugar acids, respectively.

(b) The enzymatically prepared product from D-fuconate co-migrates with chemically synthesized 3,6-dideoxy-threo-D-hexulosonic acid on Dowex-1-X8 (formate) resins.

(c) Treatment of the enzymatically prepared product from D-fuconate with hydrogen peroxide yields a pentonic acid which consumes one mole of periodate per mole of carbohydrate.

(d) Periodate rate studies confirm that the D-fuconate product is cleaved no faster than the trans isomer (KDF) and slower than the cis isomer (KDQ); the configuration of the enzymatic product at the C_4-C_5 hydroxyls is therefore trans.

(e) Both the enzymatically prepared product from D-fuconate and the chemically prepared threo isomer (KDF) are cleaved by KDF aldolase by an aldol-cleavage to yield simutaneously one mole of pyruvate and one mole of D-lactaldehyde per mole of substrate.

DISCUSSION

D-Galactonate (D-fuconate) dehydratase, a key enzyme in the catabolism of D-fuconate, converts D-fuconate to 2-keto-3-deoxy-D-fuconate. The enzymatic product was identified by characteristic semicarbazone derivatives, reaction with thiobarbituric acid-periodate, by comparative periodate oxidation rate studies, and comparison of its chromatographic properties with chemically synthesized material.

The dehydratase protein has been purified essentially to homogeneity based upon both native and denaturing polyacrylamide gel electrophoresis. Homogenous preparations of the dehydratase reflect a maximum 25-fold purification from the crude extract and an 27% overall recovery. Based on the amount of pure enzyme recovered, 84 mg, the percent recovery, and the amount of protein in the crude extract, 7830 mg, the dehydratase comprises about 4.0% of the cell-free extract.

The average molecular weight of the enzyme was determined to be 289,100 by gel filtration, sucrose density gradient sedimentation, sedimentation velocity, and sedimentation equilibrium methods. A subunit molecular weight of approximately 46,000 suggest that the enzyme is made up of six identical subunits. The native molecular weight is greater than that reported for the specific D-galactonate dehydratase isolated from a pseudomonad of about 250,000 MW (97).

The physical characterization of the enzyme has shown an isoelectric point of 4.7 to 4.8, a value which is slightly lower than those reported for other water-soluble proteins (an average pI of 5.44 ± 1.1) from bacterial sources (127). The amino acid composition of the enzyme shows

normal distributions of the common amino acids except for proline, 0.5 mole %, which is lower than seen with other proteins (4.81 ± 2.1, averaged from 208 proteins) (128).

The specificity of the enzyme for D-fuconate and D-galactonate has been shown. This is in contrast to the D-galactonate dehydratase from a pseudomonad which has absolute specificity for D-galactonate (97). In addition, the specificity of this enzyme contrasts with that found for the pseudomonad L-arabonate, D-fuconate dehydratase which acts on both L-arabonate and D-fuconate but not on D-galactonate (33). The induction of the D-galactonate (D-fuconate) dehydratase is solely dependent on D-galactonate in the parental strain while being constitutively produced in the strain CH-101 used for the purification of the enzyme (see Section 1, Results).

The lack of thiol cofactor requirement for enzymatic activity of D-galactonate (D-fuconate) dehydratase is similar to that found for the D-galactonate dehydratase from a pseudomonad (97), D-glucarate dehydratase from Pseudomonas acidovours (56), and a purified D-xylo-aldonate dehydratase (129). Divalent metals ($Mg^{2+} > Fe^{2+} > Mn^{2+}$) are an absolute requirement for activity as demonstrated by complete inactivation of the dehydratase with EDTA and restoration of the activity by the addition of the above metals. Such specificities for divalent metals have been shown with other aldonate dehydratases (25,33,56,130,131).

D-Galactonate (D-fuconate) dehydratase does not process detectable KDGal kinase, KDF aldolase, or KDPGal aldolase activities in the purified enzyme preparation. The further metabolism of KDF is dependent on aldolytic cleavage, whereas that of KDGal is dependent on phosphorylation and cleavage, as described in the preceding section.

REFERENCES

REFERENCES

1. Mortlock, R.P., Fossitt, P.P., and Wood, W.A., Proc. Nat. Acad. Sci. (U.S.A.) 54, 572 (1965).

2. Camyre, K.P., and Mortlock, R.P., J. Bacteriol. 90, 1157 (1965).

3. Tanaka, S., Lerner, S.A., and Lin, E.C.C., J. Bacteriol. 93, 642 (1967).

4. Torriani, A., and Rothman, P., J. Bacteriol. 81, 835 (1961).

5. Lerner, S.A., Wu, T.T., and Lin, E.C.C., Science 146, 1313 (1964).

6. Wu, T.T., Lin, E.C.C., and Tanaka, S., J. Bacteriol. 96, 447 (1964).

7. Rigby, P.W.J., Burleigh, Jr., B.D., and Hartley, B.S., Nature 251, 200 (1974).

8. Rigby, P.W.J., Gething, M.J., and Hartley, B.S., J. Bacteriol. 125, 728 (1976).

9. Clarke, P.H. (1974) in Evolution in the Microbial World (Carlile, M.J. and Skekel, J.J., editors), pp 183-217, Cambridge Univeristy Press, London.

10. Hacking, A.J., Aquilar, J., and Lin, E.C.C., J. Bacteriol. 136, 522 (1978).

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

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

13. Mayo, J.W., and Anderson, R.L., J. Bacteriol. 100, 948 (1969).

14. Hegeman, G.V., and Rosenberg, S.L., Ann. Review of Microbiol. 24, 429 (1970).

15. MacGee, J., and Doudoroff, M., J. Biol. Chem. 210, 617 (1954).

16. Entner, N. and Doudoroff, M., J. Biol. Chem. 196, 852 (1952).

17. Ashwell, G., Methods. Enzymol. 3, 73 (1957).

18. Stouthamer, A.H., Biochem. Biophys. Acta 48, 484 (1961).

19. DeLey, J., and Doudoroff, M., J. Biol. Chem. 227, 745 (1957).

20. Pallerone, N.J. and Doudoroff, M., J. Biol. Chem. 223, 499 (1956).

21. Szymona, M., and Doudoroff, M., J. Gen. Micro. 22, 167 (1960).

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

23. Dahms, A.S., and Anderson, R.L., Biochem. Biophys. Res. Commun. 36, 809 (1969).

24. Elzaing, T.A., Hassan, M.M., and Allan, A.M., J. Bacteriol. 114, 457 (1973).

25. Andreesen, J.R., and Gottschalk, G., Arch. Mikrobiol. 69, 160 (1969).

26. Narrod, S.A. and Wood, W.A., J. Biol. Chem. 220, 45 (1966).

27. Gunsalus, T.C., Horecker, B.C., and Wood, W.A., Bact. Rev. 19, 79 (1955).

28. Deacon, J., and Cooper, R.A., FEBS Letters 77, 201 (1977).

29. Cooper, R.A., Arch. Microbiol. 118, 199 (1978).

30. Cooper, R.A., FEBS Letters 103, 216 (1979).

31. Dahms, A.S., and Anderson, R.L., J. Biol. Chem. 247, 2222 (1972).

32. Dahms, A.S., and Anderson, R.L., J. Biol. Chem. 247, 2228 (1972).

33. Dahms, A.S., and Anderson, R.L., J. Biol. Chem. 247, 2233 (1972).

34. Dahms, A.S., and Anderson, R.L., J. Biol. Chem. 247, 2238 (1972).

35. Yuen, R., and Schachter, H., Can. J. Biochem. 50, 798 (1972).

36. Nwokoro, N.A., and Schachter, H., J. Biol. Chem. 250, 6185 (1975).

37. Nwokoro, N.A., and Schachter, H., J. Biol. Chem. 250, 6191 (1975).

38. Chan, J.Y., Nwokoro, N.A., and Schachter, H., J. Biol. Chem. 254, 7060 (1979).

39. Dagley, S., and Tridgell, P.W., Biochem. J. 95, 48 (1965).

40. Blumenthal, J., and Fish, D.C., Biochem. Biophys. Res. Commun. 11, 239 (1963).

41. Blumenthal, J., and Jepson, T., Biochem. Biophys. Res. Commun. 17, 282 (1964).

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

43. Ashwell, G., Wabba, A.J., and Hickman, J., J. Biol. Chem. 235, 1559 (1960).

44. Kerster, K., and DeLey, J., Antonie von Leeweenhoek 34, 293 (1968).

45. Eisenberg, R.C. and Dobrogosz, W.J., J. Bacteriol. 93, 941 (1967).

46. Fraenkel, D.G. and Horeaker, B.L., J. Biol. Chem. 239, 2765 (1964).

47. Frampton, E.W. and Wood, W.A., J. Biol. Chem. 236, 2571 (1961).

48. DeLey, J., Enzymologia 16, 99 (1957).

49. Frampton, E.W. and Wood, W.A., J. Biol. Chem. 236, 2578 (1961).

50. Neijssel, O.M. and Tempest, D.W., Arch. Microbiol. 105, 183 (1975).

51. Andreesen, J.R., and Gottscalk, G., J. Bacteriol. 107, 570 (1971).

52. Kersters, K., Khan-Matsubara, J., Nelen, L., and DeLey, J., Antonie von Leewenhoek 37, 233 (1971).

53. Palleroni, N.J., and Doudoroff, M., J. Bacteriol. 74, 180 (1957).

54. Allam, A.M., Hassan, M.M., and Elzaing, T.A., J. Bacteriol. 124, 1128 (1975).

55. Pedrosa, F.O., and Zancan, G.T., J. Bacteriol. 119, 336 (1974).

56. Jeffcoat, R., Hassel, H., and Dagley, S., Biochem. J. 115, 969 (1969).

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

58. Touster, O. (1969) in Comprehensive Biochemistry (Florkin, M., and Stotz, E.H., editors) Vol. 17, pp 219-240, Elsevier Publishing Company, New York.

59. LeBlanc, D.J., and Mortlock, R.P., J. Bacteriol. 106, 82 (1971).

60. LeBlanc, D.J., and Mortlock, R.P., J. Bacteriol. 106, 90 (1971).

61. Boulter, J., Gielow, B., McFarland, M., and Lee, N., J. Bacteriol. 117, 920 (1974).

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

63. Heath, E.C., and Ghalamor, M.A., J. Biol. Chem. 237, 2423 (1962).

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

65. Eagon, R.G., J. Bacteriol. 82, 548 (1961).

66. Hanson, T.E., and Anderson, R.L., Proc. Nat. Acad. Sci. (USA) 61, 269 (1968).

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

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

69. Egan, J.B., and Morse, M.C., Biochem. Biophys. Acta 97, 310 (1965).

70. Liss, M., Horwitz, S.B., and Kaplan, N.O., J. Biol. Chem. 237, 1342 (1962).

71. Markwell, J., Shimamoto, G.T., Bissett, D.L., and Anderson, R.L., Biochem. Biophys. Res. Commun. 71, 221 (1976).

72. Kelker, N.E., Simkins, R.A., and Anderson, R.L., J. Biol. Chem. 247, 1479 (1972).

73. Palmer, R.E., and Anderson, R.L., J. Bacteriol. 112, 1316 (1972).

74. Palmer, R.E., and Anderson, R.L., Biochem. Biophys. Res. Commun. 45, 125 (1971).

75. Palmer, R.E., and Anderson, R.L., J. Biol. Chem. 247, 3415 (1972).

76. Palmer, R.E., and Anderson, R.L., J. Biol. Chem. 247, 3420 (1972).

77. Kelker, N.E., Hanson, T.E., and Anderson, R.L., J. Biol. Chem. 245, 2060 (1970).

78. LeLoir, L.F., Biochem. J. 91, 1 (1964).

79. Lowry, O.H., Rosenbrough, N.J., Fair, A.L., and Randall, R.J., J. Biol. Chem. 193, 265 (1951).

80. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F., Anal. Chem. 28, 350 (1956).

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

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

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

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

85. Cornforth, J.W., Firth, M.E. and Gottschalk, G., Biochem. J. 68, 57 (1958).

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

87. Portsmouth, P., Carbohydrate Research 8, 193 (1968).

88. Dahms, A.S., Ph.D. Thesis (1969) Metabolism of D-Fucose and L-Arabinose in a Pseudomonad, pp 185-186.

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

90. Aronson, L.D., Rosso, R.G., and Adams, E., Biochim. Biophys. Acta. 132, 200 (1967).

91. Kramer, D.N., Klein, N., and Baselia, R.A., Anal. Chem. 31, 250 (1959).

92. Pese, A., McKay, R.H., Stolzenbach, F., Cohn, R.D., and Kaplan, N.O., J. Biol. Chem. 239, 1753 (1964).

93. Hohorst, H.S., and Reim, M. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H.U., editor), pp 335-339, Academic Press, New York and London.

94. Loveless, A., and Howarth, S., Nature 184, 1780 (1959).

95. Bissett, D.L., Ph.D. Thesis (1975), Lactose and D-Galactose Metabolism in Staphylococcus aureus. . ., p 179.

96. Baker, F.J. (1967) in Handbook of Bacteriological Technique (Baker, F.J., and Breach, M.R., editors) pp 259-262, Appleton-Century-Crofts Publishing Company, London.

97. Donald, A., Sibley, D., Lyons, D.E., and Dahms, A.S., J. Biol. Chem. 254, 2132 (1979).

98. Simkins, R.A., Ph.D. Thesis (1975), L-Sorbose-1-Phosphate Reductase from Aerobacter Aerogenes: Its Purification, Characterization and Role in the Metabolism of L-Sorbose and D-Fructose, pp 103-105.

99. Kauffman, M.D. (1966) in The Bacteriology of Enterobacteriaceae, pp 19-41 (Escherichia) and pp 311-330 (Klebsiella), Williams and Wilkins Company, Baltimore.

100. Hayes, W. (1968) in The Genetics of Bacteria and Their Viruses, pp 711-718, John Wiley and Sons, Inc., New York.

101. Rutter, W.J., Fed. Proced. 23, 1248 (1964).

102. Feist, C.F. and Hegemon, G.D., J. Bacteriol. 100, 869 (1969).

103. a.) Worsey, M.J., and Williams, P.A., J. Bacteriol. 124, 7 (1975).
b.) Williams, P.A., and Murray, K., J. Bacteriol. 120, 416 (1974).
c.) Wong, C.L., and Dunn, W.W., Genet. Res. 23, 227 (1974).

104. Revzin, A.R., Private Communication.

105. a.) Palachaudhun, S., and Chakrabarty, A., J. Bacteriol. 126, 410 (1976).
b.) Duggleby, C.J., Bayley, S.A., Worsey, M.J., Williams, P.A., and Broda, P., J. Bacteriol. 130, 1274 (1977).

106. Dahms, A.S., Ph.D. Thesis (1969) Metabolism of D-Fucose and L-Arabinose in a Pseudomonad, pp 258-262.

107. Wood, W.A. (1972) in The Enzymes (Boyer, P.D., editor), Vol VII, 3rd Edition, pp 281-301, Academic Press, New York and London.

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

109. Peterson, E.A., and Sober, H.A., Methods Enzymol. 5, 3 (1962).

110. McGuinness, E.T., J. Chemical Educ. 50, 826 (1973).

111. Vesterberg, O., Methods Enzymol. 22, 389 (1971).

112. Linhardt, K., and Walter, K. (1963) in Methods of Enzymatic Analysis, (Bergmeyer, H.U., editor), pp 779-787, Academic Press, New York and London.

113. Luck, H. (1962) in Methods of Enzymatic Analysis, (Bergmeyer, H.U., editor), pp 885-894, Academic Press, New York and London.

114. Crouthomil, C.E., Meek, H.V., Martin, D.S., and Banks, C.V., J. Amer. Chem. Soc. 71, 3031 (1949).

115. Racker, E., Biochim. Biophys. Acta 4, 211 (1950).

116. Lamers, K., Putney, F., Steinberg, J.Z., and Schochman, H.K., Arch. Biochem. Biophys. 103, 379 (1964).

117. Yphantis, D.A., Biochemistry 3, 297 (1964).

118. VanHolde, Fractions 1, (1967).

119. Garen, A., and Levinthal, C., Biochim. Biophys. Acta 38, 470 (1960).

120. Kanarek, L., Marler, E., Bradshaw, R.A., Fellows, R.E., and Hill, R.L., J. Biol. Chem. 239, 4207 (1964).

121. Morawiecki, A., Biochim. Biophys. Acta 44, 604 (1960).

122. Steinmetz, M. and Deal, Jr., W.C., Biochemistry 5, 1399 (1966).

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

124. Brewer, J.M., Pesce, A.J., and Ashworth, R.B. (1974) in Experimental Techniques in Biochemistry, pp 195-197, Prentice-Hall, Englewood, Cliffs, New Jersey.

125. Penke, B., Ferenczi, R. and Kovacs, K., Anal. Biochem. 60, 45 (1974).

126. Crestfield, A.M., Moore, S. and Stein, W.H., J. Biol. Chem. 238, 622 (1963).

127. Malamud, D., and Drysdale, J.W., Anal. Biochem. 86, 620 (1978).

128. Reeck, G., Handbook of Biochemistry and Molecular Biology (3rd edt.), (G.D. Fasman, ed.), "Proteins", Vol. III, pp 504-519 (1976), (CRC Press).

129. Dahms, A.S., Fed. Proc. 33, 1900 (1974).

130. Smilely, J.D., and Ashwell, G., J. Biol. Chem. 235, 1571 (1960).

131. Bender, R., and Gottschalk, G., Eur. J. Biochem. 40, 309 (1973).